

***Escherichia coli* aerobic respiratory
complexes as modulators of
aminoglycoside susceptibility and
targets for drug discovery**

PhD Thesis for the degree of PhD in Microbiology

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Declaration

I confirm that no part of this thesis has been submitted in support of an application for any degree or qualification at either the University of Kent, any other university or higher education learning institution.

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18th May 2023

Abstract

Antibiotic resistance is an increasing problem making the treatment of infectious bacterial diseases more difficult. This leads to higher mortality rates and a greater economic burden in the future. Consequently, there is a pressing demand for new antimicrobials. Bacterial aerobic respiration is linked to antibiotic susceptibility in Gram-negative pathogens. Susceptibility to aminoglycosides has previously been linked to antibiotic uptake related to the proton motive force (PMF) and reactive oxygen species production (ROS) but the relative contributions of each was poorly understood. A combination of ROS assays and PMF disruption via uncoupler were used to study the relative ROS vs. PMF contributions to gentamicin toxicity in a pathogenic *E. coli* clinical isolate. The data gathered supported the hypothesis that maintenance of the PMF rather than the production of ROS underpins the mechanism of how the aerobic respiratory chain potentiates the toxicity of aminoglycosides.

Cytochrome *bd-I* is a terminal oxidase of the *E. coli* aerobic respiratory chain and an interesting new target for the development of new antimicrobials. To investigate the structure and phylogenetics of cytochrome *bd-I* a hhsearch pipeline was used to infer phylogenetic relationships in cytochrome *bd-I* that could be linked to structural features. Phylogenetic evidence suggested that the presence/absence of amino acid residue 101 (in the *E. coli* sequence) provides information on the position of the haem *d* cofactor: absence of residue 101 supports a haem *d* cofactor close to the periplasm (as in the *Geobacillus thermodenitrificans* cytochrome *bd* structure), whereas the presence of residue 101 was consistent with the haem b595 in this position and the haem *d* closer to the cytoplasmic side (as in *E. coli* cytochrome *bd-I*). Potential drug targeting sites on *E. coli* cytochrome *bd-I* were also investigated, aside from the quinol binding pocket, with the oxygen entry tunnel being the most promising site.

A drug repurposing approach was then used to target *E. coli* cytochrome *bd-I* to search for new inhibitors as potential antimicrobials. An *in silico* molecular docking pathway was developed to screen FDA approved drugs for their ability to bind in the quinol binding site of cytochrome *bd-I*. Experimental methods were also developed to screen the top *in*

silico hits generating several interesting new potential inhibitors with the prodrug quineestrol being the most promising. Isolated *E. coli* membranes containing cytochrome *bd*-I as the sole respiratory oxidase were assayed for respiratory activity in the presence of various concentrations of Quineestrol, and an IC₅₀ in the nM range was obtained, which was consistent with subsequent respiratory experiments with whole cells. This work has paved the way for future structural, drug-derivatisation and mutagenesis studies, and the drug repurposing pipeline can readily be adapted for other target sites on a range of respiratory complexes and expanded drug libraries.

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Presentations and Publications

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- "Respiratory inhibition and antibiotic susceptibility in bacterial pathogens" - FIREBIO seminar University of Kent, November 2019.
- "Interplay between aerobic respiration, haem ligands, nitric oxide and antibiotic susceptibility" - Tetrapyrrole Discussion Group Meeting University of Bristol, September 2019.

Abbreviations

CFU = Colony-forming unit

EBSL = Extended-spectrum β -lactamase

E. coli = *Escherichia coli*

GSNO = S-Nitrosoglutathione

LB = Lysogeny broth

MSA = Multiple sequence alignment

NEB = New England BioLabs

NO = Nitric oxide

OD₆₀₀ = Optical density at 600nm

PBS = Phosphate-buffered saline

PMF = Proton motive force

ROS = Reactive oxygen species

SOB = Sugar Optimal Broth

RMSD = Root mean square deviation

TMH = Transmembrane helix

TTFA = 2-thenoyltrifluoroacetone

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Chapter 1

Introduction

1.1. Antibiotic Resistance

1.1.1. History of Antibiotics

Over the last 100 years antibiotics have had a huge impact on modern medicine contributing to the much-increased average human lifespans (Hutchings et al. 2019). Antibiotic discovery and use has enabled treatment of previously life threatening bacterial diseases, allowed invasive surgery to become commonplace and reduced the risk of immune compromising therapies for organ transplant (Hutchings et al. 2019). The use of antibiotic producing microbes to treat infection such as mould from bread stretches back thousands of years (Gould 2016). The first modern antimicrobial agent introduced in 1910 was arsphenamine (Salvarsan) used to treat syphilis (Gould 2016). The discovery of penicillin in 1929 by Sir Alexander Fleming (Fleming 1929) started a golden era for antibiotic discovery that lasted for the next four decades, peaking in the 1950's with new classes of antibiotics being discovered nearly every year (Hutchings et al. 2019). Most of the early antibiotics discovered during the golden era were natural products produced by bacteria or fungi (Hutchings et al. 2019). Actinomycetales such as *Streptomyces* were an important source of natural product antibiotics such as the aminoglycoside and tetracycline classes (Waksman et al. 2010). A few classes were also derived from chemical synthesis such as the quinolones (Andriole 2005). During the 1960s discovery of new natural product antibiotics slowed down with the focus switching more to the modification of the existing antibiotic scaffolds such as the β -lactamase resistance semi-synthetic penicillin's oxacillin, methicillin and cloxacillin (Zaffiri et al. 2012). However, only a limited number of effective alterations are possible from a single core (Coates et al. 2011). This, coupled to the slowdown in discovery of natural product antibiotics due to the restricted number of easy to culture bacteria that produce antibiotic compounds, lead to a decline in antibiotic discovery from the 1970s onwards (Coates et al. 2011; Hutchings et al. 2019). Since 1970 only a few new classes of antibiotics have been introduced such as carbapenems, oxazolidinone and cyclic lipopeptides along with some more analogues using existing cores such as cephalosporin (Fig. 1.1)(Coates et al. 2011).

1.1.2. Antibiotic Resistance Problem

The emergence of antibiotic resistance in bacteria is becoming an increasing threat to human health (Fig. 1.1). A resistance mechanism to penicillin was first observed in the 1940's due to β -lactamases in *E. coli* (Abraham and Chain 1940). Bacteria previously susceptible to penicillin such as strains of staphylococci soon started showing resistance to penicillin (Davies 1947). Usually, resistance is observed within 2 years of a new antibiotic entering use (Coates et al. 2011). Some bacterial strains have now evolved to be resistance to multiple classes of antibiotics such as carbapenem-resistant *Enterobacteriaceae* (CRE) resulting in difficulty of treatment and high death rates for systemic infections (Potter et al. 2016).

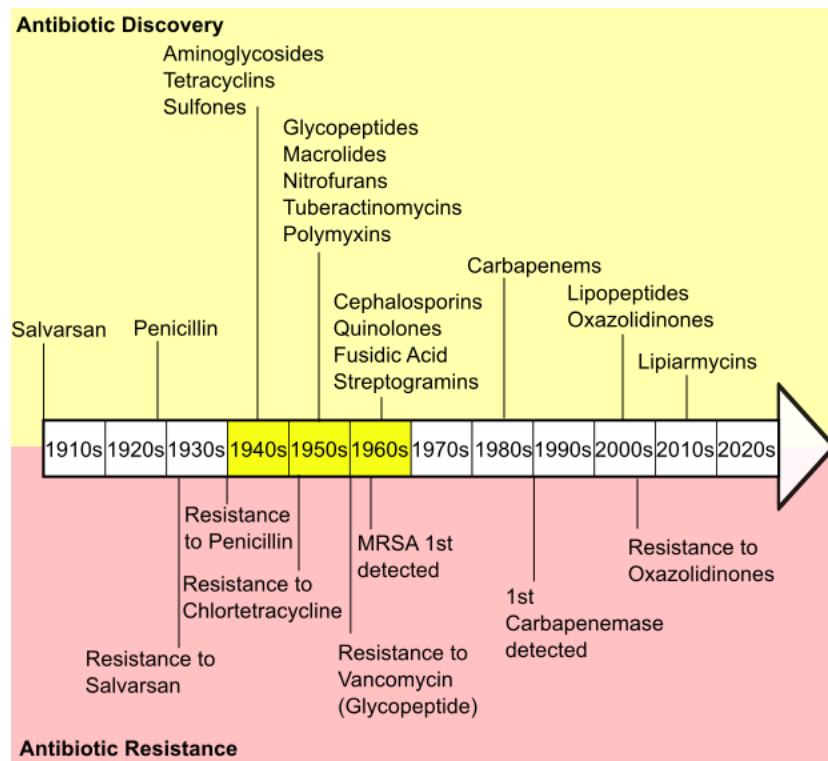


Figure 1.1 - Antibiotic discovery and resistance timeline. Decade of selected antibiotic classes being discovery shown on the top and selected dates of resistance emergence on the bottom. The golden era of antibiotic discovery is highlighted.

In 2009 the European Centre for Disease Prevention and Control reported 25,000 deaths per year and 2.5 million extra hospital days caused by multi-drug resistant bacteria (ECDC/EMA 2009). Lord Jim O'Neill's independent Review on Antimicrobial Resistance for

the UK government from July 2014 predicted that the annual global deaths from antimicrobial resistant pathogens would rise to 10 million by 2050 (Dodds 2017). Furthermore, the report predicted that the economic cost would be a loss of 7% of the global domestic product between 2015-2050 (Dodds 2017). Clearly a cost to both healthcare and the economy is present from antibiotic resistance.

Antibiotic resistance can arise via several mechanisms. The main resistance mechanism groups are: 1. Modification of antibiotic structure; 2. Modification of metabolic pathways to bypass the antibiotic effect; 3. Alteration of the target site to reduce antibiotic binding; and 4. Reduced intracellular antibiotic concentration due to either reduced permeability or increased efflux (Munita and Arias 2016). Bacteria evolve to become resistant to antibiotics by two main methods. Firstly, mutations may occur in genes associated with the specific antibiotic mechanism (Davies and Davies 2010; Munita and Arias 2016). Mutations leading to antibiotic resistance are preserved in the presence of the antibiotic as the non-resistant cells are killed (Munita and Arias 2016). Secondly, genes conferring antibiotic resistance can be transferred between species by horizontal gene transfer (Martinez 2014; Munita and Arias 2016). This may be from an intrinsically resistant species to a susceptible species via conjugation or transduction (Munita and Arias 2016). As antibiotic resistance is generally maintained only in the presence of antibiotics the use of antibiotics is the main cause of resistance. Hence, the misuse of antibiotics is a big problem for driving antimicrobial resistance (Dodds 2017). This can include patient misuse by not taking the prescribed dose, lack of rapid laboratory tests leading to the ineffective antibiotics being prescribed and antibiotics being over-prescribed (Llor and Bjerrum 2014). Furthermore, antibiotics are still used in huge quantities in agriculture given in animal feed with an estimate 80% of antibiotic consumption by mass in the USA in 2011 going into agriculture (Dodds 2017). This may give an economic advantage to food production but it also creates a large reservoir for antimicrobial resistance (McEwen and Collignon 2018).

Gram-negative pathogenic *E. coli* strains are common causes of urinary tract infections, enteritis, pyelonephritis and septicaemia (Allocati et al. 2013). *E. coli* causes around 40,000 cases of bacteraemia annually in England with 5,000 associated deaths and over a third of these cases is caused by drug-resistant strains (Public Health England). The

emergence of multi-drug resistance in *E. coli* is also becoming an increasing problem (Poirel et al. 2018). This has been linked partially to antibiotic use in farm animals (Poirel et al. 2018). One specific uropathogenic clonal group O25b:H4-ST131 which emerged in 2008 has spread worldwide (Nicolas-Chanoine et al. 2008) and is particularly associated with multi-drug resistance including extended-spectrum β -lactamase (ESBL) producing isolates (Nicolas-Chanoine et al. 2014). One specific isolate of the ST131 clonal group is *E. coli* EC958 which can cause both urinary tract infections and septicaemia (Forde et al. 2014). *E. coli* is intrinsically resistant to penicillin producing β -lactamases (Abraham and Chain 1940; Martinez 2014). Along with a variety of β -lactamases, *E. coli* EC958 also encodes the gene *bla*_{CTX-M-15} for the ESBL CTX-M-15 (Totsika et al. 2011). Other antibiotic resistance genes expressed by *E. coli* EC958 include *tet(A)* and *tet(R)* for tetracycline resistance, whilst *mph(R)*, *mrx* and *mph(A)* provide erythromycin resistance (Totsika et al. 2011). These antibiotic genes are all located on the large plasmid pEC958 (Totsika et al. 2011; Forde et al. 2014).

To deal with the increasing antimicrobial resistance problem antibiotics need to be used more wisely such as with more careful prescribing (Llor and Bjerrum 2014) or a reduction in agricultural use (McEwen and Collignon 2018). Also, there is a critical need for new antibiotics to be developed to combat resistance (Coates et al. 2011; Hutchings et al. 2019). This especially applies to new classes of antibiotics as very few have been developed recently (Coates et al. 2011; Hutchings et al. 2019).

1.2. *E. coli* aerobic respiration

1.2.1. Introduction to *E. coli* aerobic respiration

E. coli can respire both aerobically and anaerobically with aerobic respiration being the main focus in this thesis. The electron transport chain in *E. coli* is comprised of several different routes for electron entry (via dehydrogenase complexes) and routes for electron exit (via terminal oxidoreductases) (Uden and Bongaerts 1997). These are linked together by the quinone pool with ubiquinone, menaquinone and demethylmenaquinone being present in *E. coli* (Uden and Bongaerts 1997; Borisov and Verkhovsky 2015). During aerobic

growth the main quinone used is ubiquinone (Unden and Bongaerts 1997). The aerobic respiratory chain (Fig. 1.2) can start with the dehydrogenases NADH dehydrogenase I/II and succinate dehydrogenase that transfer electrons to ubiquinone reducing it to ubiquinol (Unden and Bongaerts 1997). Ubiquinol is then oxidised by the terminal oxidases with protons being translocated from the cytoplasm into the periplasm to generate the proton motive force (PMF) (Borisov and Verkhovsky 2015). The ATP synthase then uses the energy conserved in the proton gradient to generate ATP. There are 3 different terminal oxidases that are expressed by *E. coli*; cytochrome *bo'*, cytochrome *bd-I* and cytochrome *bd-II* (Borisov and Verkhovsky 2015).

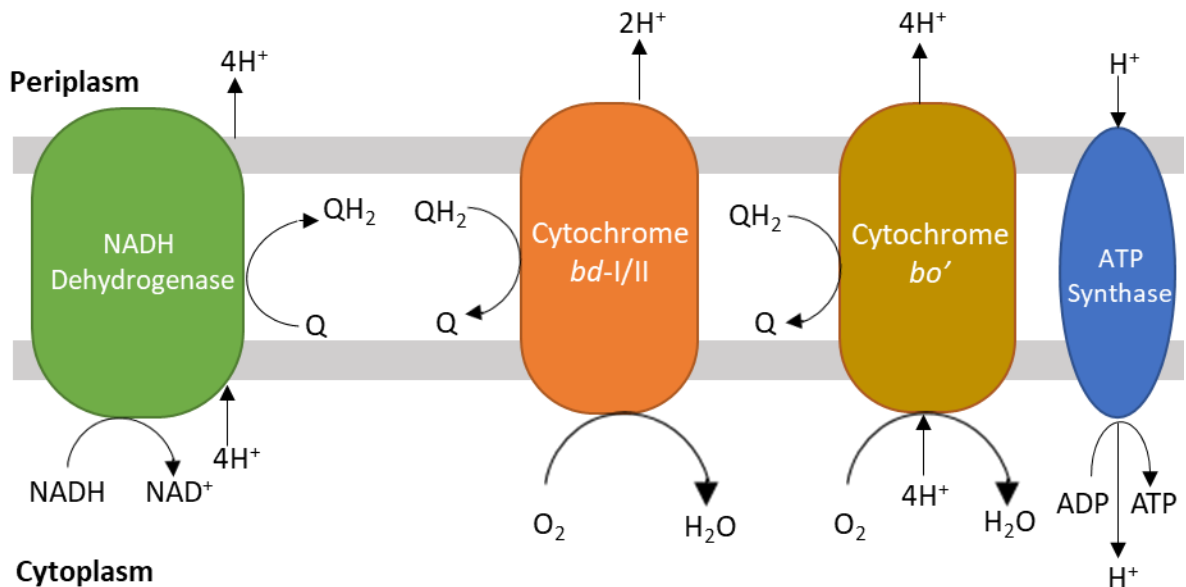


Figure 1.2 - A simplified diagram of aerobic respiration in *E. coli*

1.2.2. Cytochrome *bo'*

The haem-copper terminal oxidase cytochrome *bo'* uses the substrate ubiquinol-8 and catalyses the reduction of oxygen to water with 4 protons translocated from the cytoplasm to the periplasm for every O_2 molecule reduced ($H^+/e^- = 2$) (Choi et al. 2017c). Cytochrome *bo'* contains 4 distinct sub-units (I-IV) (Fig. 1.3) (Abramson et al. 2000). Homology to aa_3 type cytochrome *c* oxidases is seen for subunits I, II and III whilst subunit

IV of unknown function shows little sequence homology to other terminal oxidases (Abramson et al. 2000). Subunit I contains a haem *b* along with a bimetallic active site formed of Cu_B and haem *o*₃ (Abramson et al. 2000). Ubiquinol-8 is oxidised in two single electron steps transferring first to haem *b* and then onto haem *o*₃/Cu_B. Oxygen binds to haem *o*₃ to be reduced to water (Abramson et al. 2000).

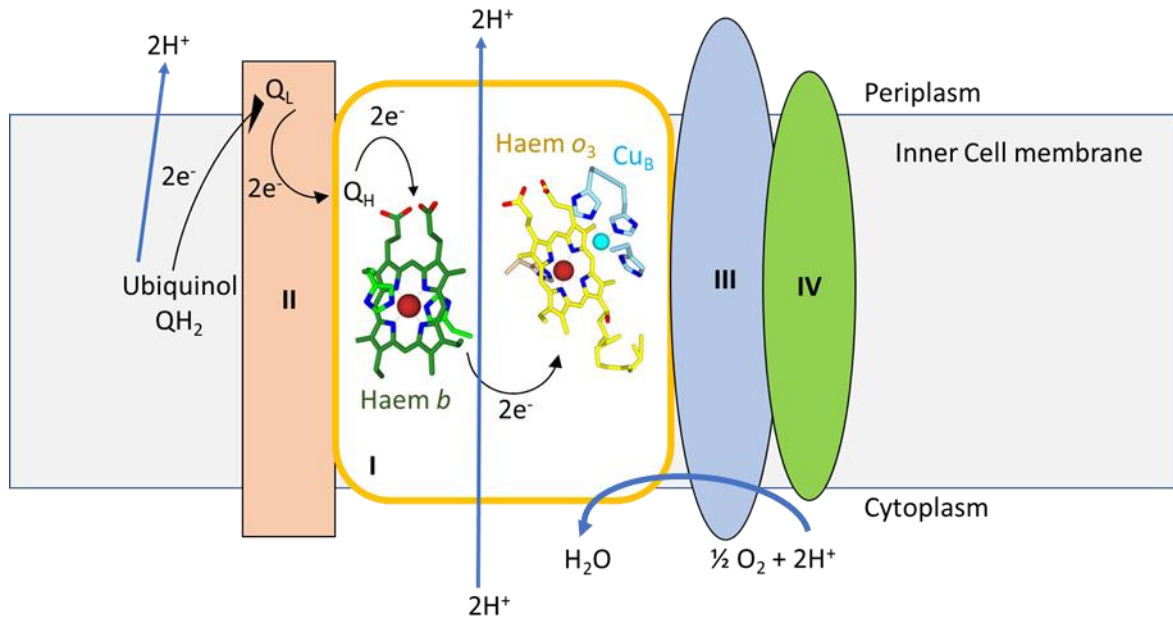


Figure 1.3 - Basic structure of cytochrome *bo'* showing the four transmembrane subunits. Electrons are transferred from ubiquinol through haem *b* and then haem *o*₃ to reduce oxygen to water in subunit I. Protons transferred into the periplasm come from both translocation through subunit I and ubiquinol.

The structure for *E. coli* cytochrome *bo'* oxidase was determined in 2000 and contains 25 transmembrane helices (Abramson et al. 2000). 15 transmembrane helices are located in subunit I with the first 12 forming a transmembrane barrel (Fig. 1.4) (Abramson et al. 2000). The ubiquinol binding site has been determined to be in a cleft formed from the α -helices 1-3 of subunit I (Abramson et al. 2000). This is exposed to the bilayer allowing binding of ubiquinol (Abramson et al. 2000)(Choi et al. 2017c). As 0, 1 or 2 ubiquinone's can be co-purified with cytochrome *bo'* this suggested the presence of multiple ubiquinone binding sites (Sato-watanabe et al. 1994)(Puustinen et al. 1996). Data from investigations into the steady state kinetics of cytochrome *bo'* using inhibitors proposed two ubiquinone

binding sites termed Q_L and Q_H (Musser et al. 1997). The low affinity binding site (Q_L) would be where bound quinone rapidly exchanges with the quinone pool in the inner membrane (Abramson et al. 2000)(Choi et al. 2017c). The Q_H site would contain a stably bound ubiquinone with electrons being passed from the ubiquinone in the Q_L site to the ubiquinone in the Q_H site and then onto haem b (Choi et al. 2017c). The Q_H site acts to stabilize the semiquinone whilst ubiquinone oxidation occurs at the Q_L site (Choi et al. 2017c). However, site-directed mutagenesis on predicted sequences for the Q_L site (Bossis et al. 2014) has failed to identify this site (Choi et al. 2017b). The current site-directed mutagenesis evidence suggests that there is only one ubiquinone binding site with substrate oxidation and ubisemiquinone stabilisation occurring at the same site in subunit I (Choi et al. 2017c).

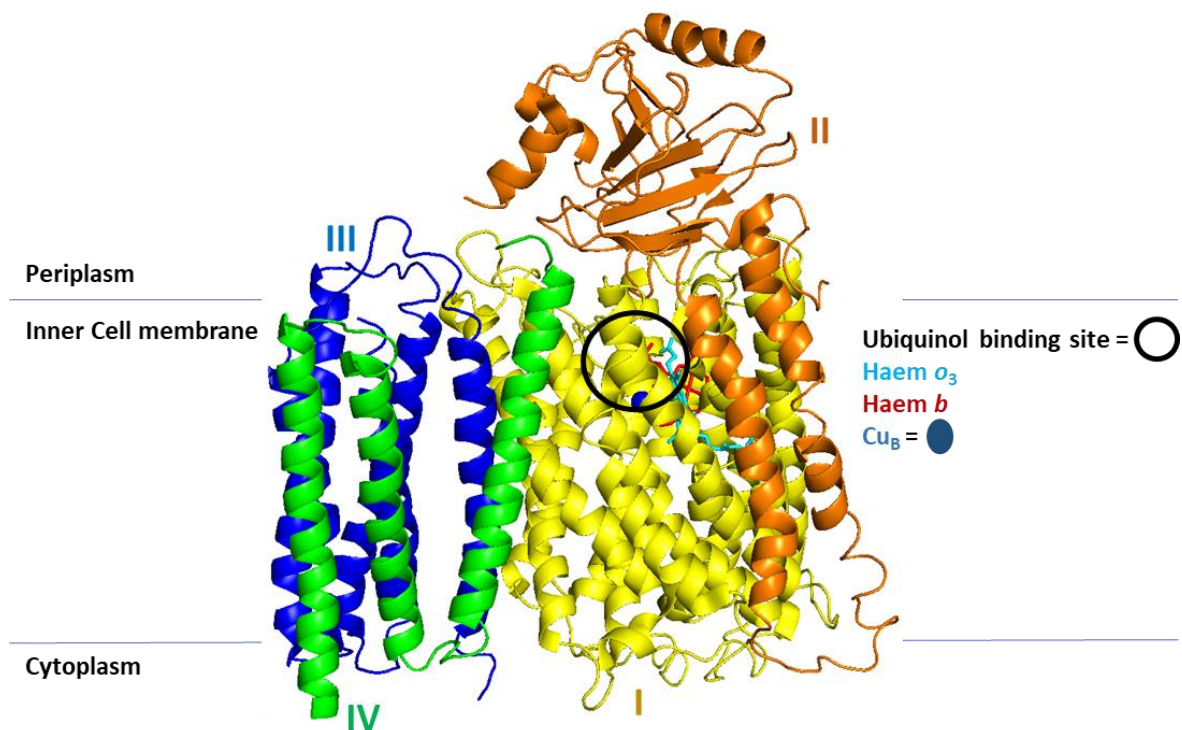


Figure 1.4 - X-ray crystal structure of cytochrome *bo'* from *E. coli* shown parallel to the inner membrane (PDB = 1FFT). Subunits I, II, III and IV are yellow, orange, blue and green respectively. Adapted from (Abramson et al. 2000).

1.2.3. Cytochrome *bd*-I

Cytochrome *bd*-I generates a proton motive force across the membrane by the vectorial transport mechanism involving the deposition of protons into the periplasm upon ubiquinol oxidation (Fig. 1.5)(Giuffrè et al., 2014). Unlike the haem copper oxidase cytochrome *bo'*, cytochrome *bd*-I contains no copper atoms and additional protons are not pumped across the membrane ($H^+/e^- = 1$) (Borisov et al. 2011a). Three haem groups are used as cofactors with two *b*-type haems (haems b_{558} and b_{595}) along with a haem *d* all located in proximity to the periplasmic space (Safarian et al. 2019; Theßeling et al. 2019). Haem b_{558} functions in quinol oxidation whilst haem *d* is where O_2 binds to be reduced to H_2O (Giuffrè et al., 2014). Cytochrome *bd*-I has two integral membrane polypeptides CydA (57kDa) and CydB (43kDa) (Giuffrè et al., 2014). The small protein CydX (4kDa) containing a single transmembrane helix (Hoeser et al., 2014) is also required for the enzymatic function of cytochrome *bd*-I oxidase (Vanorsdel et al., 2013). Once the *E. coli* structure was solved a

fourth subunit (CydH) was identified that is not encoded by the *cydABX* operon (Safarian et al. 2019; Theßeling et al. 2019).

Ubiquinol binds to a highly conserved Q loop in the extracellular hydrophobic region between helices 6 and 7 of CydA (Safarian et al. 2019). The quinol can then be oxidised with haem b_{558} accepting the electrons whilst 2 protons are released into the periplasm (Safarian et al. 2019). The haem b_{595} and haem d cofactors then receive the transferred electrons forming a high spin active site in which O_2 binding and reduction to water occurs (Safarian et al. 2019).

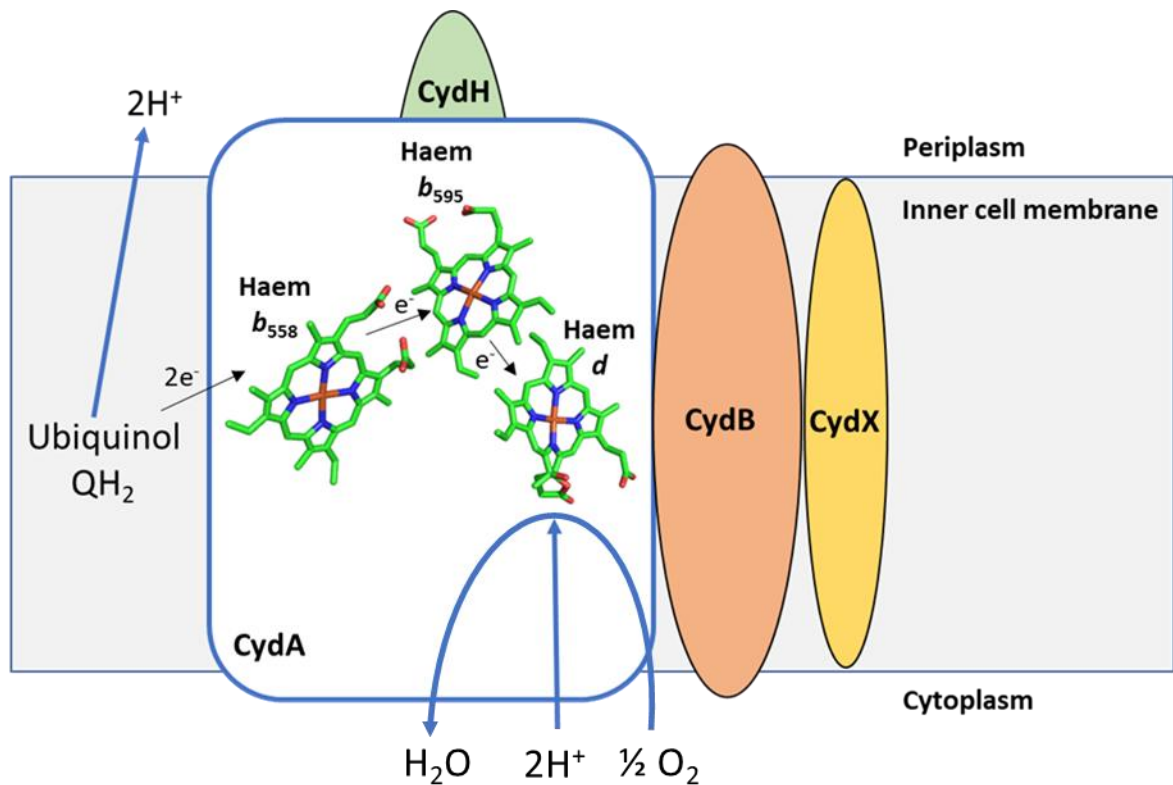


Figure 1.5 - Basic structure and electron flow in cytochrome *bd-I*.

Two further genes *cydC* and *cydD* are required for the functional activity of cytochrome *bd-I* (Georgiou et al. 1987; Poole et al. 1989). These encode a heterodimeric ATP-binding cassette (ABC-type) transporter (Cook, Membrillo-Hernandez and Poole, 1997)(Cook et al., 2002). CydC is involved in the export of cysteine (Pittman et al., 2002) and glutathione to help maintain periplasmic redox homeostasis (Pittman, Robinson and Poole, 2005). Mutants lacking in *cydC* or *cydD* show sensitivity to oxidative stress in the form

of hydrogen peroxide, temperature sensitivity and cannot exit stationary phase (Goldman, Gabbert and Kranz, 1996)(Cook, Membrillo-Hernandez and Poole, 1997). Mutations in the *cydDC* operon do not affect *cydAB* transcription or assembly of CydA and CydB in the membrane (Goldman, Gabbert and Kranz, 1996)(Cook, Membrillo-Hernandez and Poole, 1997). However, the assembled complexes lack the haem *d* resulting in loss of function (Georgiou, Fang and Gennis, 1987).

1.2.4. Cytochrome *bd*-II

Compared to cytochromes *bd*-I and *bo'*, far less work has been performed on cytochrome *bd*-II. Cytochrome *bd*-II is encoded by the genes *appC* and *appB* which show around 60% homology to CydA and CydB of cytochrome *bd*-I (Dassa et al., 1991). The small subunit AppX is also present (Grauel et al. 2021). The spectral properties of cytochrome *bd*-II are also very similar to cytochrome *bd*-I (Sturr, Krulwich and Hicks, 1996). Along with cytochrome *bd*-I maximal expression is seen under microaerobic conditions and so it appears to be oxygen-regulated (Atlung and Brondsted, 1994). Furthermore, a triple knockout of *cyo*, *cyd* and *appCB* genes was more sensitive to oxygen compared to a double *cyo* and *cyd* gene knockout suggesting an oxygen-detoxifying role (Dassa et al., 1991).

Originally cytochrome *bd*-II was believed not to contribute to the proton motive force and so function uncoupled with electron flux through the cytochrome (Bekker et al., 2009). However, subsequent studies used a combination of oxygen pulse, absorbance and fluorescent spectroscopy techniques to demonstrate that in the steady-state cytochrome *bd*-II generates a proton motive force (H^+/e^- ratio of 0.94 ± 0.18) (V. B. Borisov et al., 2011a)(Bloch et al., 2012). This evidence indicates that cytochrome *bd*-II generates a proton motive force by an identical mechanism to cytochrome *bd*-I (V. B. Borisov et al., 2011a). The structure of *E. coli* cytochrome *bd*-II has now been solved by cryo-EM (Grauel et al. 2021; Grund et al. 2021). The structure is very similar to cytochrome *bd*-I with the haem ligands in the same orientation leading to the same reaction mechanism being predicted (Grauel et al. 2021; Grund et al. 2021). One main difference is the lack of a fourth subunit (CydH in cytochrome *bd*-I) (Grauel et al. 2021; Grund et al. 2021)

1.2.5. Aminoglycosides and bacterial respiration

There is a complex relationship between aminoglycoside toxicity and bacterial aerobic respiration, so this section seeks to introduce the mechanism of action for this antibiotic class and the published evidence for how respiratory activity can influence lethality/toxicity. Aminoglycosides are one of the first classes of antibiotics discovered with the initial member streptomycin discovered by Waksman, Schatz, and Bugie in 1944 being isolated from *Streptomyces griseus* (Schatz et al. 1944). Most aminoglycosides are natural product antibiotics originating from the *Streptomyces* or *Micromonospora* genus (Jana and Deb 2006). *Streptomyces* derived aminoglycosides (suffix -mycin) include kanamycin, neomycin and tobramycin (Jana and Deb 2006). *Micromonospora* derived aminoglycosides (suffix -micin) include gentamicin and netilmicin (Jana and Deb 2006). Aminoglycosides are used to treat infections mainly from Gram-negative aerobic bacteria or in combination with other antibiotics to target Gram-positives (Jana and Deb 2006). Streptomycin was the first antibiotic used to treat tuberculosis infections (Daniel 2006). Several other aminoglycosides were soon discovered including gentamicin (Fig. 1.6) in 1963 (Weinstein 1963) which entered widespread clinical use. Semisynthetic derivatives including amikacin, isepamicin and netilmicin were then developed in the 1970s to try and improve the pharmacological profile and deal with antimicrobial resistance (Becker and Cooper 2013). The development of newer β -lactams combined with β -lactamase inhibitors, cephalosporins, carbapenems and fluoroquinolones in the 1970s with reduced toxicity compared to aminoglycosides lead to a reduction in their use and development (Becker and Cooper 2013). Recently, renewed interest in aminoglycosides is present due to the increasing antibiotic resistance problem (Becker and Cooper 2013).

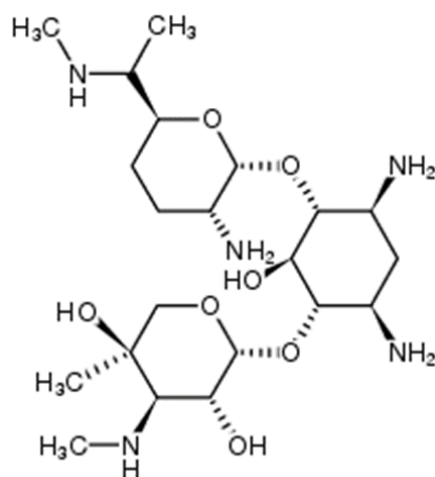


Figure 1.6 - Structure of gentamicin an example aminoglycoside. Structure from e-Drug3D database (Pihan et al. 2012).

The main target site for aminoglycosides is the ribosome and in particular the 30S subunit (Davis 1987; Jana and Deb 2006). Irreversible binding to the aminoacyl-tRNA recognition site (A-site) leads to misreading translation errors (Jana and Deb 2006). Several crystal structures have been published of aminoglycosides binding to the 30S subunit (Borovinskaya et al. 2007). Whilst the interactions are slightly different for each aminoglycoside the same translation errors result (Jana and Deb 2006). Truncated or mistranslated proteins are therefore synthesized (Davis et al. 1986). Aminoglycosides are bactericidal antibiotics with the mechanism of cell death still being debated. Continuous uptake of aminoglycosides could result in complete ribosomal inhibition and cell death (Becker and Cooper 2013). Mistranslated proteins may be inserted into the membrane leading to membrane destabilization and cell death (Davis et al. 1986). Thirdly, oxidative stress has been implicated in aminoglycoside lethality (Kohanski et al. 2007). A combination of the above methods may be occurring leading to cell death (Becker and Cooper 2013).

Resistance to aminoglycosides can occur via several mechanisms. These include: 1. aminoglycoside modifying enzymes (AME); 2. reduced intracellular concentration by increased efflux or reduced permeability; 3. target modifications in the 30S subunit that interfere with aminoglycoside binding (Jana and Deb 2006; Becker and Cooper 2013). AME's include aminoglycoside acetyltransferases, phosphotransferases and nucleotidyltransferases (Ramirez and Tolmasky 2010; Labby and Garneau-Tsodikova 2013).

Efflux pumps of the resistance nodulation division (RND) family are common Gram-negative resistance mechanisms for aminoglycosides (Anes et al. 2015). Post-transcriptional modification of the 16S ribosomal RNA using 16S ribosomal RNA methyltransferases is a common method of target modification (Doi et al. 2016).

The aminoglycoside class of antibiotics is particularly interesting as the mechanism of action is intertwined with aerobic respiration (Fig. 1.7). Loss of aerobic respiration has been shown to reduce the efficacy of several antibiotic classes including aminoglycosides in Gram-negative bacteria (Lobritz et al. 2015; Stokes et al. 2019). Gram-negative anaerobic bacteria are intrinsically resistant to aminoglycosides (Becker and Cooper 2013). Antibiotic treatment has previously been linked to increased bacterial respiration rate (Kohanski et al. 2007). This can lead to increased production of reactive oxygen species (ROS) such as hydrogen peroxide and superoxide and consequent oxidative stress damage (Kohanski et al. 2007, 2008; Dwyer et al. 2014). For example, superoxide can cause destabilisation of iron-sulphur complexes (Imlay and Fridovich 1991; Keyer and Imlay 1996) leading to iron dysregulation. Released free ferrous iron can react with hydrogen peroxide in the Fenton reaction to produce hydroxy radicals that can damage DNA, lipids and proteins (Dwyer et al. 2014). The role of ROS in cell lethality is debated with one study showing no increase in hydrogen peroxide levels or intracellular free iron upon antibiotic treatment in *E. coli* (Lio and Imlay 2013).

Misfolded proteins in the membrane due to aminoglycoside action can cause membrane damage (Davis et al. 1986; Kohanski et al. 2008). Previous work has suggested that aminoglycosides can activate the membrane-located Cpx stress response and Arc two-component redox responsive system due to the misfolded membrane proteins (Kohanski et al. 2008), which leads to metabolic and respiratory changes (Kohanski et al. 2008). Aminoglycoside-induced stimulation of the tricarboxylic acid (TCA) cycle then leads to enhanced electron donor (NADH) levels and the hyperactivation of the aerobic respiratory chain (Kohanski et al. 2007). ROS species are then thought to result from elevated respiratory rates (Kohanski et al. 2007).

Uptake of aminoglycosides is also related to aerobic respiration in Gram-negative bacteria (Fig 1.7) (Taber et al. 1987). The current model of aminoglycoside uptake starts

with the positively charged aminoglycosides binding to the negatively charged lipopolysaccharides of the outer membrane (Vanhoof et al. 1995). Then two energy dependant (EDP I/II) steps follow (Taber et al. 1987). EDPI is a slow uptake of aminoglycosides followed by EDPII which is a rapid energy dependant uptake with the exact mechanisms being unknown (Taber et al. 1987; Becker and Cooper 2013). There is also considerable evidence for a link between maintenance of the PMF and aminoglycoside toxicity not involving ROS formation. It is suggested that the electrical component of the PMF ($\Delta\psi$) provides the main driving force for the uptake of positively-charged aminoglycosides (Taber et al. 1987; Farha et al. 2013). Aminoglycoside uptake has been shown to be related to $\Delta\psi$ in *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis* (Taber et al. 1987). Use of uncouplers of the PMF such as 2,4-dinitrophenol (DNP) that dissipate the electric component of the PMF have been used to study aminoglycoside uptake (Taber et al. 1987; Gage and Neidhardt 1993; Lewis et al. 1994). EDP I and EDP II can be blocked using uncouplers (Taber et al. 1987). DNP has been shown to inhibit dihydrostreptomycin uptake and lethality in *E. coli* K-12 (Campbell and Kadner 1980). Nitric oxide (NO) as a respiratory inhibitor has shown to provide resistance to gentamicin in *E. coli* (Ribeiro et al. 2021) and *Salmonella enterica* (McCollister et al. 2011) and decrease gentamicin uptake in *Salmonella enterica* (McCollister et al. 2011).

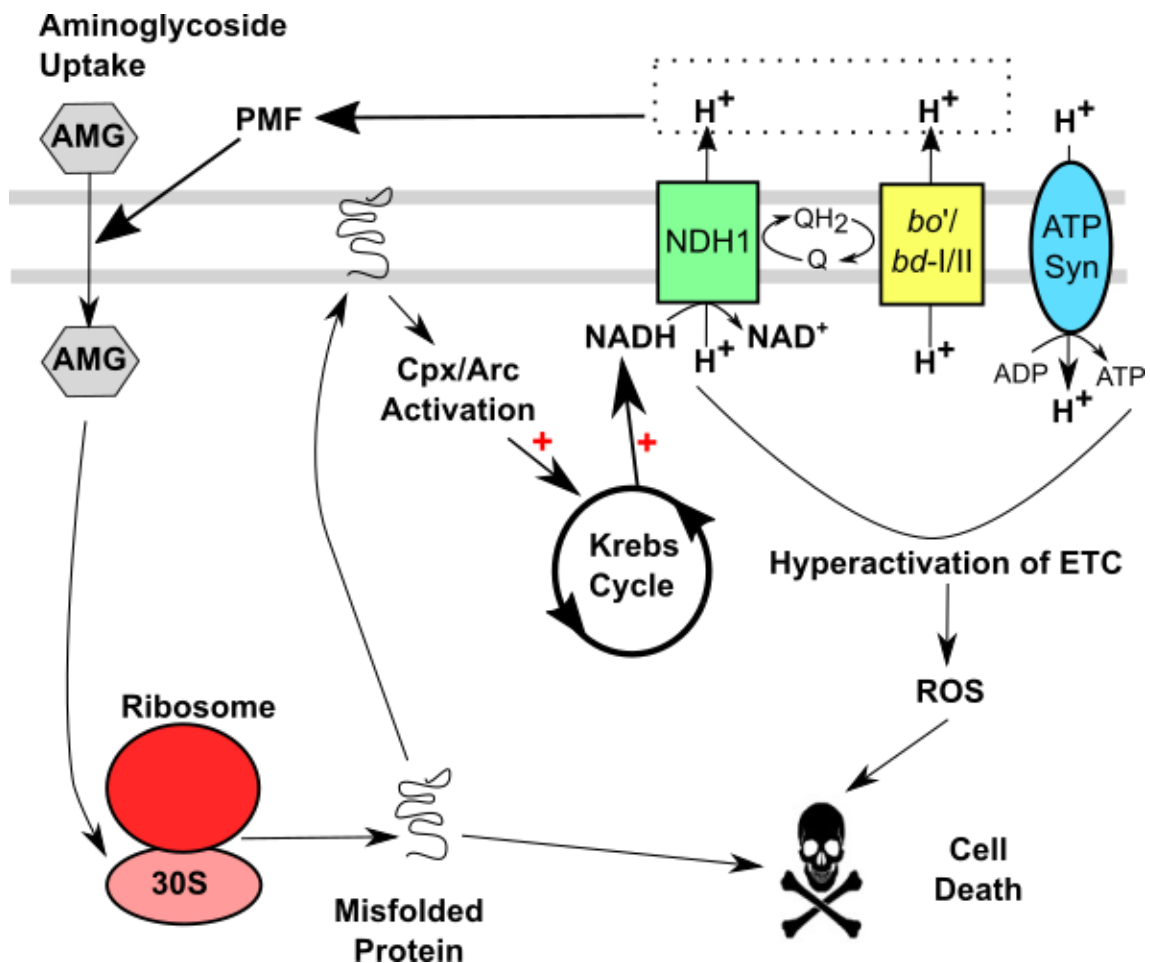


Figure 1.7 - Relationships between aminoglycoside toxicity and aerobic respiration in *E. coli*. Misfolded proteins from aminoglycoside action can lead to increased energy metabolism and respiration rates via Cpx/Arc system activation. Increased NADH levels produced lead to increased activities of NADH dehydrogenase (NDH1), terminal oxidases (cytochromes *bo'*, *bd-I* and *bd-II*) and ATP synthase (ATP Syn). Hyperactivation of the respiratory chain can provide increased PMF for the uptake of aminoglycosides and increased ROS both potentially contributing to cell death.

1.3. Cytochrome *bd* as a drug target

1.3.1. Cytochrome *bd* overview and phylogenetics

Cytochrome *bd* oxidases are widely distributed amongst bacterial phyla (Borisov et al. 2011a). They are also present in archaea but not in eukaryotes (Borisov et al. 2011a; Murali Ranjani et al. 2021). The evolutionary picture is complicated with horizontal gene

transfer leading to multiple copies of cytochrome *bd* oxidases being acquired independently by some phyla (Esposti et al. 2015). Horizontal gene transfer may also be the reason for some members of the archaea acquiring cytochrome *bd* (Hao and Golding 2006). Gene duplication also plays a major part in cytochrome *bd* evolution with cytochrome *bd*-II believed to have arisen from a gene duplication of cytochrome *bd*-I (Esposti et al. 2015; Murali Ranjani et al. 2021). Additionally, asymmetric evolution of the *cydA* and *cydB* genes has been reported with *cydB* evolving 1.2 times faster than *cydA* (Hao and Golding 2006).

Cytochrome *bd* oxidases can be divided into long and short Q-loop oxidases with the long Q-loop oxidases having an extended C-terminal portion of the Q-loop (Hao and Golding 2006). Some cytochrome *bd* oxidases also contain only *b*-type haem cofactors according to the spectroscopic signatures (apart from *Gluconobacter oxydans* (Miura et al. 2013)), usually displaying some resistance to cyanide and are thus termed cyanide insensitive oxidases (CIO) (Esposti et al. 2015). CIO have been identified in *B. subtilis* (Azarkina et al. 1999), *P. aeruginosa* (Cunningham et al. 1997) and *C. jejuni* (Jackson et al. 2007). The CIO from *Gluconobacter oxydans* has uniquely been reported to produce the spectral peaks for oxygenated and ferric haem *d* (Miura et al. 2013). No sequence signatures have been found to characterise CIO's (Murali Ranjani et al. 2021) with both long and short Q-loop CIO's present (Cunningham et al. 1997; Jackson et al. 2007)

Along with its role in aerobic respiration cytochrome *bd* is important for the pathogenesis and virulence of many pathogenic bacteria. These include strains of *E. coli* (Shepherd et al. 2016), *Salmonella* (Jones-Carson et al. 2016), *Brucella* (Endley et al. 2001), *Staphylococcus* (Götz and Mayer 2013) and *Mycobacterium* (Cai et al. 2021). Cytochrome *bd* is important in resistance to both environmental and host immune system stresses. These environmental stresses include antibiotics (Beebout et al. 2021), cyanide (Borisov et al. 2015a) and sulphide (Korshunov et al. 2016). Cytochrome *bd* has also been shown to promote resistance to reactive nitrogen and oxygen species (RNS and ROS) produced by the human immune system (Borisov et al. 2015a). These species include hydrogen peroxide (Al-Attar et al. 2016), peroxyxynitrite (Borisov et al. 2015b) and nitric oxide (Mason et al. 2009). Direct decomposition of hydrogen peroxide (Al-Attar et al. 2016) and peroxyxynitrite (Borisov et al. 2015b) has been shown for cytochrome *bd* in *E. coli*.

As cytochrome *bd* is important during infection and has a unique structure to prokaryotes it provides a promising target for new antimicrobials (Friedrich et al. 2022). Cytochrome *bd* is an especially promising target as the structure is known for several pathogenic bacteria (Friedrich et al. 2022).

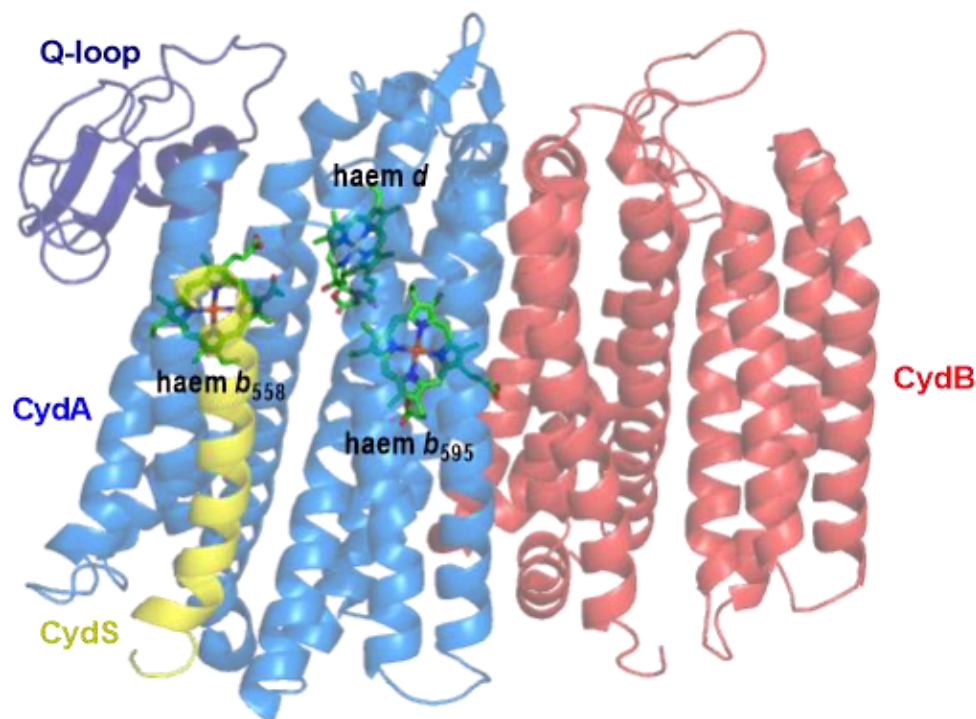
1.3.2. Cytochrome *bd* structure

Multiple cytochrome *bd* structures have been published. The first was a short Q-loop structure from *G. thermodenitrificans* (Safarian et al. 2016). Two structures for the long Q-loop *E. coli* cytochrome *bd*-I (Safarian et al. 2019; Theßeling et al. 2019) have been published along with two from *E. coli* cytochrome *bd*-II (Grauel et al. 2021; Grund et al. 2021). Other cytochrome *bd* structures include those from *Mycobacterium smegmatis* (Wang et al. 2021), *Mycobacterium tuberculosis* (Safarian et al. 2021) and *Corynebacterium glutamicum* (Grund et al. 2023).

The *G. thermodenitrificans* short Q-loop oxidases structure (Fig. 1.8) is an X-ray crystal structure to 3.05Å (Safarian et al. 2016). 19 transmembrane helices are present with CydA and CydB each comprising 9 transmembrane helices sharing the same overall fold (Safarian et al., 2016). Two 4-helix bundles are present with an additional peripheral helix for CydA and CydB (Safarian et al., 2016). Structural alignment supports close similarity between the subunits suggesting that they may have resulted from gene duplication (Safarian et al., 2016). Heterodimer formation between CydA and CydB is supported by hydrophobic interactions between residues on helices 3, 4 and 9 of both subunits (Safarian et al., 2016). The fold observed is found in very few other proteins with similar redox functions with cytochrome *b₅₆₁* being one example (Safarian et al., 2016).

The Q loop consists of a short helix, with a potential pocket for the quinol binding site close to glutamate-257 and lysine-252 residues, along with mostly irregular fold (Safarian et al., 2016). Site directed mutagenesis experiments have also identified this area as the possible quinol binding site (Mogi et al., 2006). Two *b*-type haems (low-spin *b₅₅₈* and high spin *b₅₉₅*) and a chlorin-type haem *d* are contained in a triangular arrangement in CydA (Safarian et al. 2016). Haem *d* is the site of oxygen reduction to water (Safarian et al. 2016).

The third subunit designated CydS in *G. thermodenitrificans* contains a single transmembrane α -helix positioned close to helices α -5-6 of CydA (Safarian et al., 2016). It is proposed to act as a stabiliser of the haem b_{558} in the 4-helix bundle α -5-8 of CydA for possible structural rearrangements during quinol binding to the Q loop and its subsequent oxidation (Safarian et al., 2016). There is no sequence similarity between *E. coli* CydX and



G. thermodenitrificans CydS but the structures of *E. coli* cytochrome *bd* show that CydX is in a very similar position to CydS and so may have the same function (Safarian et al. 2019).

Figure 1.8 - *G. thermodenitrificans* cytochrome *bd* structure. Adapted from pdb structure 5doq. Haem ligands coloured by element (carbon = green, iron = orange and nitrogen = blue).

The published structure of *E. coli* cytochrome *bd*-I (Safarian et al. 2019) (Fig. 1.9) was solved using cryo-EM, as were all of the other cytochrome *bd* structures apart from the X-ray structure of *G. thermodenitrificans* (Friedrich et al. 2022). The resolution is 2.68 Å allowing the backbone and many side chains to be resolved (Safarian et al. 2019). The overall fold of CydA and CydB is the same as in the *G. thermodenitrificans* structure displaying two-fold rotational symmetry between CydA and CydB (Safarian et al. 2019; Theßeling et al. 2019). The same haem ligands and triangular arrangement is present

compared to the *G. thermodenitrificans* structure (Safarian et al. 2019; Theßeling et al. 2019). According to the structures glutamate 445 is the axial ligand to haem *b*₅₉₅ (Safarian et al. 2019; Theßeling et al. 2019) whilst either histidine 19 (Safarian et al. 2019) or glutamate 99 (Theßeling et al. 2019) is the axial ligand for haem *d*. Both glutamate 445 and 99 have undergone side directed mutagenesis with neither concluded to be strongly ligated to the haems but are both important in proton transfer (Murali and Gennis 2018). Tryptophan 441 acts as the bridge between the low spin haem *b*₅₅₈ where quinols binds and the high spin haem *b*₅₉₅ and haem *d* (Safarian et al. 2019; Theßeling et al. 2019). Interestingly the haem ligand position is different with haems *d* and *b*₅₉₅ in the opposite positions between the two structures (Safarian et al. 2019; Theßeling et al. 2019). Additionally, the extra single alpha-helix subunit CydH is present close to haem *b*₅₉₅ (Safarian et al. 2019; Theßeling et al. 2019). The long Q-loop starts at the helix breaking aspartate 239 of transmembrane helix 6 near haem *b*₅₅₈ (Safarian et al. 2019; Theßeling et al. 2019). The N-terminal section of the Q-loop is predicted to be flexible with sections of it being unresolved in the structure including the loop between aspartate 239 and lysine 252 (Safarian et al. 2019; Theßeling et al. 2019). The more rigid C-terminal section of the Q-loop covers the periplasmic side of CydA in a helix-turn-helix motif (Safarian et al. 2019). A ubiquinone-8 molecule is present in a central cavity in CydB which is not present in the *G. thermodenitrificans* structure (Theßeling et al. 2019).

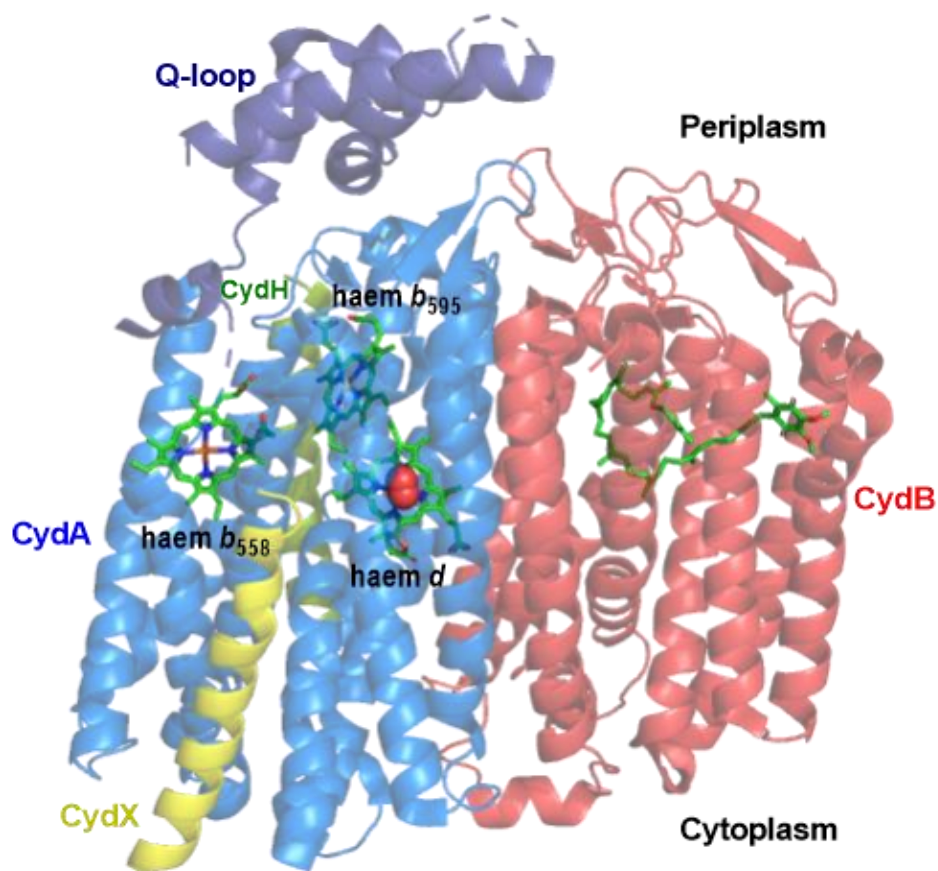


Figure 1.9 - *E. coli* cryo-EM cytochrome *bd*-I structure. Adapted from pdb structure 6RKO. Oxygen next to haem *d* shown by red spheres and haem ligands in CydA/ubiquinone-8 in CydB coloured by element (carbon = green, iron = orange and nitrogen = blue).

1.3.3. Known inhibitors of cytochrome *bd*

Several known inhibitors of cytochrome *bd* exist. These are generally small molecule inhibitors that target either the quinol binding site or haem *d* oxygen reduction site (Fig. 1.10) (Borisov et al. 2011a). Some of the inhibitors also target cytochrome *bo'* or mitochondrial electron transport chain complexes (Meunier et al. 1995). Examples that are proposed or known to target the quinol binding site include antimycin A, aurachin A/C/D, stigmatellin, 2-Thenoyltrifluoroacetone (TTFA) and MOA-stilbene (Meunier et al. 1995). Aurachin C and D are seen bound to the quinol binding site for one of the cryo-EM structures for *E. coli* cytochrome *bd*-II (Grauel et al. 2021). Known examples that target the haem *d* site include nitric oxide (Borisov et al. 2004), carbon monoxide (Hill et al. 1994; Forte et al.

2019), potassium cyanide (Kita et al. 1984) and sodium azide (Kita et al. 1984; Meunier et al. 1995).

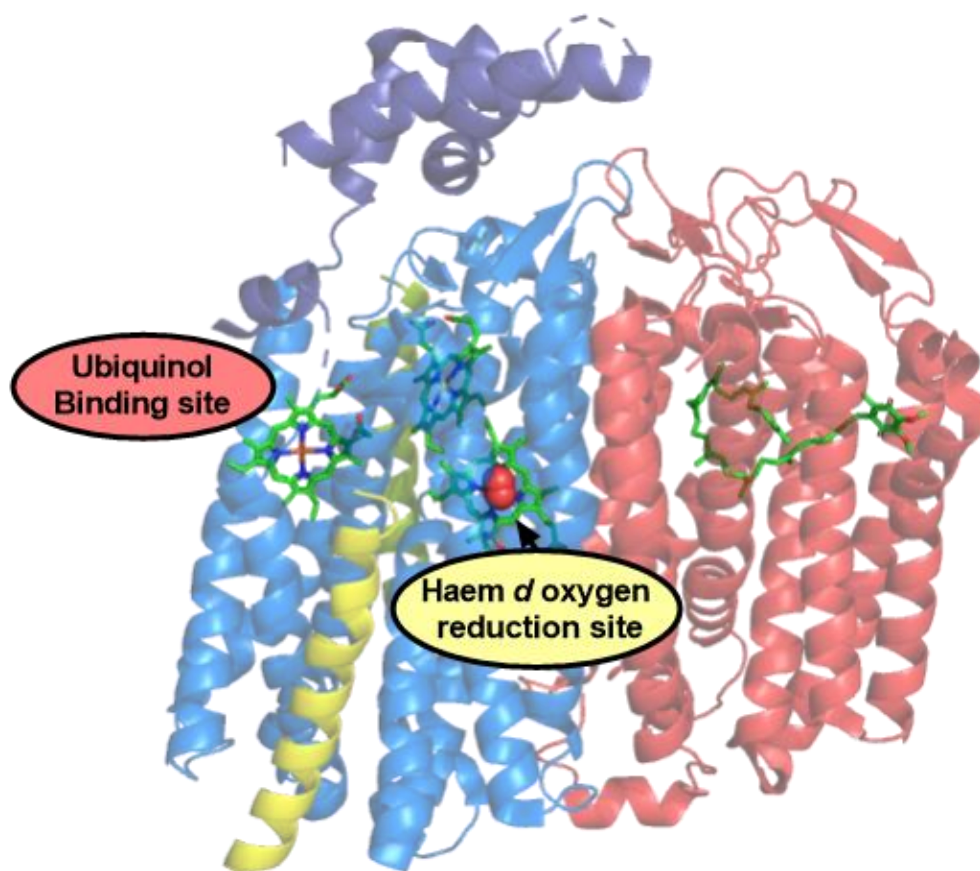


Figure. 1.10 - Main target sites of known inhibitors for cytochrome *bd*-I. *E. coli* cytochrome *bd*-I structure shown.

One example of a quinol binding site inhibitor is antimycin A, which is synthesised by various *Streptomyces* species (Shiomi et al., 2005) in a group of compounds that are known respiratory inhibitors of the mitochondrial electron transport chain (Campo, Kinnally and Tedeschi, 1992). The crystal structure of the cytochrome *bc*₁ complex (i.e. complex III) from bovine heart mitochondria shows that Antimycin A binds to the Q₁ site blocking electron transfer between cytochrome *b* and *c* by preventing ubiquinol oxidation (Xia et al., 1997). In *E. coli*, Antimycin A acts to inhibit the electron flow between ubiquinol and molecular oxygen (Miller and Gennis, 1983). Non-competitive inhibition is observed for

antimycin A when using purified cytochrome *bd*-I from *Azotobacter vinelandii* (Jünemann and Wrigglesworth, 1994). Antimycin A binds to haem *b*₅₅₈ on the low potential side inhibiting electron transfer from ubiquinol to haem *b*₅₅₈ (Jünemann and Wrigglesworth, 1994).

Other small molecule inhibitors include the five prenylphenols labelled LL-Z1272 β , γ , δ , ϵ and ζ identified to be new inhibitors of cytochrome *bd* from *E. coli* (Mogi, Ui, et al., 2009). LL-Z1272 β and ϵ , the dechlorinated derivatives, are most active against cytochrome *bd* whilst the others are more active against cytochrome *bo'*. LL-Z1272 γ demonstrates 84% inhibition of cytochrome *bd*-I at 5 μ g/ml compared to 50% for antimycin A (Mogi, Ui, et al., 2009).

Anti-microbial peptides (AMPs) may also target cytochrome *bd*. AMPs are involved in innate immunity generally with a broad spectrum of activity (Ganz, 2003). They are found in epithelial cells, leukocytes and granules within phagocytes from which they can be released into the phagolysosome, blood or surrounding tissues (Ganz, 2003). The mechanism of action for AMPs is to disrupt the membrane integrity of the target pathogen (Ganz, 2003). Some AMPs have been shown to increase ROS production under aerobic growth conditions and are therefore potential candidates to increase antibiotic lethality (Flannagan, Cosío and Grinstein, 2009). Microcin J25 is a low molecular weight antimicrobial peptide produced by strain AY25 of *E. coli* (Galván et al., 2018). Microcin J25 has been shown to inhibit both cytochromes *bo'* and *bd*-I (Galván et al., 2018). When the membranes of a strain only containing cytochrome *bd*-I are exposed to microcin J25 a large overproduction of ROS is seen in the form of superoxide when reduced quinones are used as the electron source (Galván et al., 2018). No ROS overproduction is observed with membranes of a strain only containing cytochrome *bo'* (Galván et al., 2018). Microcin J25 may act to both induce ROS production by cytochrome *bd*-I and perturb quinol binding possibly by interacting with the Q-loop of cytochrome *bd*-I (Galván et al., 2018). This second effect would lead to the build-up of reduced species upstream and further production of ROS (Galván et al., 2018). However, cytochrome *bd*-II appears to protect *E. coli* from the harmful effects of Microcin J25 possibly by de-toxification of the ROS products via catalase activity (Galván et al., 2018).

Human cathelicidin LL-37 is another antimicrobial peptide that inhibits cytochrome *bd-I* in *E. coli* (Choi, Yang and Weisshaar, 2017).

1.4. Drug Repurposing to develop new antimicrobials

1.4.1. Advantages of drug repurposing

The ever-growing problem of anti-microbial resistance leads to the pressing need for new antimicrobials (Coates et al. 2011). One of the promising strategies for developing new antibiotics is the repurposing of existing drugs for new uses such as to target pathogenic bacteria. Drug repurposing offers several advantages over *de novo* drug design regarding antimicrobials (Farha and Brown 2019). These include a potential reduction in development time due to the accelerated development process (Farha and Brown 2019). FDA-approved drug databases provide a useful starting point for drug repurposing studies (Oprea and Overington 2015). For repurposing FDA-approved drugs some stages of the drug development pipeline can be bypassed or much condensed such as drug discovery, lead optimization, pre-clinical and phase I clinical trials (Fig. 1.11) (Pushpakom et al. 2018; Farha and Brown 2019). This is due to the safety/toxicity, pharmacodynamic and pharmacokinetic data already being known (Farha and Brown 2019). Only an indication of efficacy to the new target is needed at the pre-clinical stage whilst Phase I clinical trials aimed at evaluating drug safety can usually be bypassed as these have already been completed for the original intended use (Issa et al. 2013; Pushpakom et al. 2018; Farha and Brown 2019). *De novo* drug design is estimated to take between 10-17 years which can be reduced to between 3-12 years for repurposed drugs (Fig. 1.11) (Farha and Brown 2019). Reduced risk is present with drug repurposing compared to *de novo* drug design where a large percentage of drugs fail to be approved due to safety problems (Ashburn and Thor 2004). Large cost savings can also be accomplished with drug repurposing due to the reduced development times along with the manufacturing process and administration routes (formulation) likely to already have been set up (Ashburn and Thor 2004).

There are some problems and challenges associated with drug repurposing. These include varying dosage compared to the original use for the repurposed drug (Oprea and Overington 2015). This is particularly the case for repurposed antimicrobials as antibiotics are generally dosed at a higher level than other non-antibiotic drugs (Farha and Brown 2019). Use of higher doses can in turn lead to toxicity problems (Oprea and Overington 2015). Also, low efficacy of repurposed drugs can be a problem due to the drugs not being designed against the antimicrobial target (Sun et al. 2016). Pharmacokinetic properties such as tissue distribution, plasma protein binding and half-life of repurposed drugs may not also be ideal for antimicrobial use (Zeitlinger et al. 2004; Oprea and Overington 2015).

Some successful examples of drug repurposing related to antimicrobials include miltefosine that was originally used for skin metastases and repurposed to target visceral leishmaniasis, a protozoan disease (Soto and Berman 2021). The antiprotozoal pentamidine that has shown activity to sensitize Gram-negative pathogens to colistin by acting as a perturbant to the outer membrane (Stokes et al. 2017). Another example is eflornithine which was approved for treating African trypanosomiasis in 1990, which was originally developed as an anti-cancer agent in the 1970s (Simarro et al. 2012).

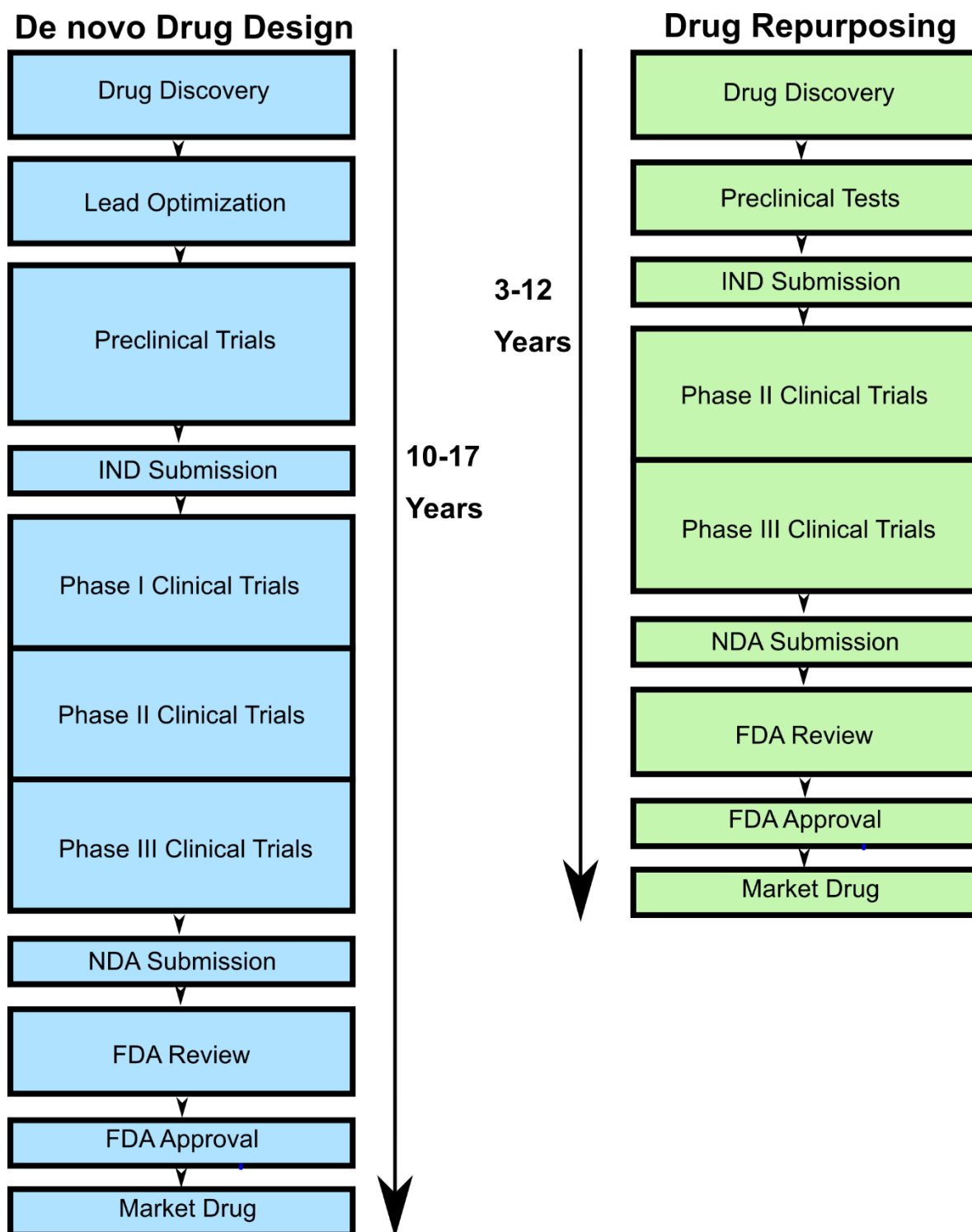


Figure 1. 11 - Timeline for drug repurposing compared to de novo drug design for the USA drug market. IND = Investigational New Drug Application. NDA = New Drug Application. Adapted from (Farha and Brown 2019).

1.4.2. Molecular docking for drug repurposing

Drug repurposing can include many approaches such as phenotypic screening or *in silico* screening (Farha and Brown 2019). *In silico* methods for the repurposing of drugs include literature-based models (Sadeghi and Keyvanpour 2021), drug-drug similarity screening (Pushpakom et al. 2018) and structure-based drug screening (Lionta et al. 2014; Batool et al. 2019). Structure-based drug design involves screening a library of existing compounds against a specific structural target using *in silico*-based methods before selecting the most promising compounds to be screened using laboratory approaches (Batool et al. 2019). For the *in silico* screening, molecular docking represents a simple way to screen many compounds for a potential drug-binding site (Brogi et al. 2020). Analysis of the drug binding site is needed to determine docking box size and position (Feinstein and Brylinski 2015) before compounds are docked into the binding site and a free energy of binding value calculated (Pagadala et al. 2017). This allows for the identification of ligands that are most likely to bind at the target site (Brogi et al. 2020). Further filtering steps are then needed to produce a list of the most promising candidates to screen in the laboratory (Lionta et al. 2014). These filtering steps can include toxicity analysis, pharmacokinetic data and specific interactions in the binding site (Lionta et al. 2014).

Examples of molecular docking software include Glide, GOLD, MOE-Dock, AutoDock4 and AutoDock Vina (Pagadala et al. 2017). AutoDock4 (Morris et al. 2009) and AutoDock Vina (Trott,O., Olson 2010) are examples that are freely available. AutoDock Vina can be automated using Python coding to dock whole ligand libraries outputting 9 possible binding modes for each ligand and the associated free energy of binding values (Trott,O., Olson 2010; Baba and Akaho 2011; Jaghoori et al. 2016). Some molecular docking tools such as AutoDock Vina also allow a degree of flexibility in the ligand and binding site side chains (Trott,O., Olson 2010).

Advantages of structure-based drug design for drug repurposing using molecular docking include much reduced time and cost of screening compared to high-throughput experimental screening methods (Lionta et al. 2014). Due to the lower cost and reduced

time needed, far larger libraries can be screened than with laboratory methods. A large number of different approaches and docking software are also available (Pagadala et al. 2017). Homology modelling to provide the input receptor structure can be used if no known structures are available (Anderson 2003). However, disadvantages include problems due to limited receptor structure flexibility compared to molecular dynamics or experimental screening (Lionta et al. 2014; Forli et al. 2016). Difficulty can also arise in the accuracy of predicting the binding score function between ligand and receptor (Lionta et al. 2014; Pagadala et al. 2017).

Successful examples of structure-based drug design using molecular docking include the anti-cancer drug raltitrexed that was designed to target thymidylate synthase (Anderson 2003). Other examples include STX-0119 that was developed as a new STAT3 inhibitor for lymphoma (Matsuno et al. 2010), and 5,6-Diaryl-1,2,4-triazine-3-ylthioacetate derivatives were screened against cyclooxygenase-2 (COX-2) with several showing inhibition against β -amyloid fibril formation (Dadashpour et al. 2015). Regarding cytochrome *bd*, a previous attempt at structure-based drug design was conducted for the menaquinol binding loop of the *Mycobacterium tuberculosis* cytochrome *bd* oxidase (Harikishore et al. 2020). This work was undertaken before the *Mycobacterium tuberculosis* cytochrome *bd* structure had been published, so a homology model of the oxidase was used for the molecular docking (Harikishore et al. 2020; Friedrich et al. 2022). A novel inhibitor with potency in the micromolar range called MQL-H₂ was identified (Harikishore et al. 2020).

1.5. Project Aims

The susceptibility of Gram-negative bacteria to aminoglycoside antibiotics has been previously linked to both the production of ROS and rate of antibiotic uptake that is mediated by the PMF. The first aim of this project was to quantify the relative contributions between ROS and the PMF to aminoglycoside toxicity, as this balance was poorly understood.

Cytochrome *bd* is a prokaryotic quinol:O₂ oxidoreductase that is important during infection, although several variants exist so it was of interest to conduct a structural and phylogenetic study into cytochrome *bd* oxidases in bacteria and archaea to map out this diversity. This work intended to identify any sequence and structural differences between cytochrome *bd* oxidases from bacteria of different clades as well as archaea. Also, a particular focus was on structural features of cytochrome *bd* that may be important in drug discovery.

Finally, cytochrome *bd* is a promising target for antibiotic drug discovery due to its role in infection in many pathogenic bacteria and that it is unique to prokaryotes being evolutionarily and structurally distant from mitochondrial cytochrome *c* oxidases. Hence, the final aim was to screen known FDA-approved drugs for candidates that could be repurposed (including derivatisation) to target cytochrome *bd*-I in pathogenic *E. coli*. The strategy was to first screen an FDA library using an *in silico* docking pipeline and then to screen potential hits using a combination of molecular genetics and an oxygen consumption assay.

Chapter 2

Materials and Methods

2.1. DNA manipulation and genome editing

2.1.1. Isolation of plasmid DNA

Pure plasmid DNA was isolated from overnight *E. coli* 10 ml LB cultures using a QIAprep Spin Miniprep Kit (QIAGEN) following the manufacturer's instructions.

2.1.2. Agarose gel electrophoresis

Analysis of DNA samples was carried out using agarose gels containing 1% (w/v) agarose, 1x TAE buffer and 0.5 mg/mL ethidium bromide stain. Agarose gels were run at 150V for 40 min in 1X TAE. DNA samples were mixed with 6X loading dye (Promega or NEB) before loading. For size determination of the DNA bands present an aliquot (5 µl for 30 ml gel) of 1kb DNA ladder was loaded (Promega). To estimate the mass of DNA bands required for Gibson Assembly an aliquot of Hyperladder I (Bioline) was loaded. DNA bands were visualised under UV light using a G:BOX gel imager (Syngene).

2.1.3. Preparation of chemically competent *E. coli* cells

20 ml of fresh LB medium was inoculated with 200 µl (1% v/v) overnight starter culture. The culture was incubated at 37°C and 180 rpm until an OD₆₀₀ of 0.4-0.5 was reached. The culture was then centrifuged at 3000 rpm at 4°C for 8 min and the supernatant discarded. The pellet was resuspended in 10 ml ice cold 100 mM CaCl₂ and kept on ice for 10 min. The cells were then pelleted, resuspended and incubated again to the same protocol above. Finally, the cells were pelleted as above and resuspended in 2ml ice cold 100 mM CaCl₂ before dividing into 100µl aliquots for each transformation reaction. Chemically competent *E. coli* cells to be frozen at -80°C had 10% glycerol added to the 100 mM CaCl₂ for the final resuspension step.

2.1.4. Transformation of chemically competent *E. coli* cells

Plasmid DNA (1-2 µl) was gently mixed with 100 µl competent *E. coli* cells and incubated for 30 min on ice. This was followed by a heat shock at 42°C for 45 s and then a 2 min incubation on ice. SOC or LB media recovery media (900 µl) was then added and the cells incubated at 37°C or 28°C for a temperature sensitive plasmid and 180 RPM for 1 h . 100 µl of the cells were then spread onto an LB-agar plate with appropriate selective

antibiotic. The remaining 900 µl was centrifuged at 5000 RPM for 5 min, 800 µl of supernatant removed and the cells resuspended in the final 100 µl of media before also being plated onto a selective LB-agar plate.

2.1.5. Preparation of electrocompetent *E. coli* cells

50 ml LB medium was inoculated with 0.5 ml (1% v/v) overnight starter culture in a 250 ml conical flask. The culture was incubated at 37°C and 180 RPM until an OD₆₀₀ of 0.5-0.6 was reached. The culture was then centrifuged at 3000 xg for 10 min at 4°C, the supernatant was discarded and the pellet was resuspended in 10 ml sterile cold 10% glycerol. This was repeated a further 2 times with the pellet resuspended in 100 µl sterile cold 10% glycerol on the final repeat. Electrocompetent cells were then used immediately for electroporation.

2.1.6. Transformation of electrocompetent *E. coli* cells (electroporation)

9 µl of purified DNA (ligation mix (varying amounts)/plasmid (20 ng)/PCR product (varying amounts up to 1000ng)) was added to a chilled 2 mm electroporation cuvette (cell projects EP-102). Electro-competent cells (40 µl) were then added over the purified DNA and the cuvette tapped to ensure that the mixture falls to the bottom before placing in the chamber of a Bio-Rad electroporator. The electrocompetent *E. coli* was pulsed at 2450 V, 25 µF capacitance and 200 Ω resistance. SOC or LB media recovery media (900 µl) was then added and the cells incubated at 37°C or 28°C for a temperature sensitive plasmid and 180 RPM for 1 h. Cells were then plated as in section 2.1.4 on LB-agar plates supplemented with the appropriate selective antibiotics.

2.1.7. Lambda-red mutagenesis overview

The recombination-based method Lambda-Red mutagenesis (Datsenko and Wanner 2000) was used to generate a series of knockout mutations in the genome of *E. coli* EC958 ST131. The phage λ-Red recombinase was used to replace the *cyoA* gene, the *cydAB* operon and the *appCB* operon with a chloramphenicol resistance cassette. Single *E. coli* EC958 ST131 terminal oxidase deletion mutants ($\Delta cydAB$ or $\Delta cyoA$ or $\Delta appCB$) were used to provide both the starting point for creation of a double terminal oxidase mutant and a source of chloramphenicol resistance cassettes for λ-Red mutagenesis (Fig. 2.1).

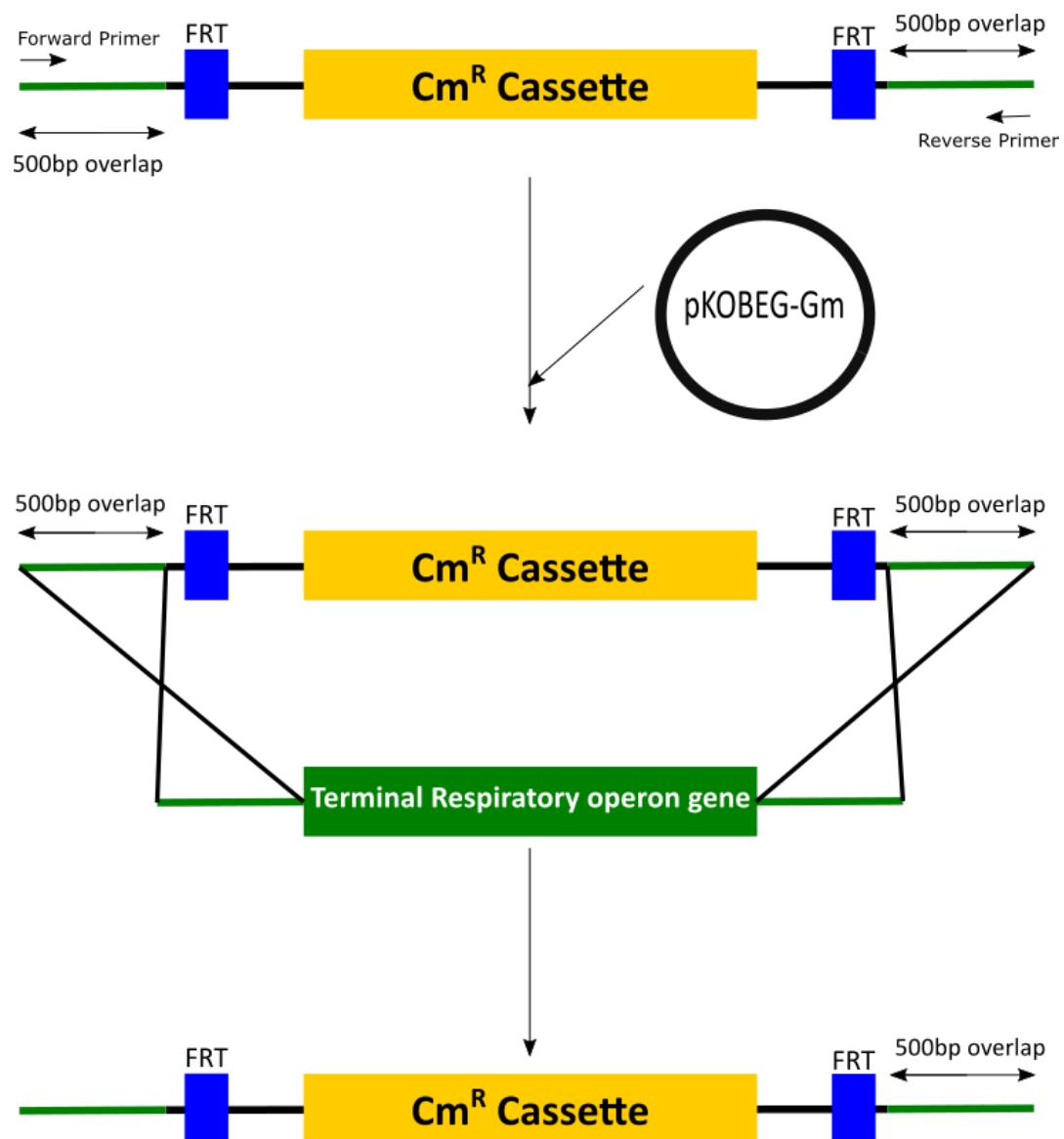


Figure 2.1 - Lambda red mutagenesis overview. Primers labelled as forward and reverse were used to generate Cm^R cassettes with 500bp overhang. Cm^R cassettes were then electroporated into single *E. coli* EC958 ST131 terminal oxidase deletion mutants (Δ *cydAB* or Δ *cyoA* or Δ *appCB*) along with pKOBEG containing the lambda red machinery. λ -Red recombination leads to the target terminal respiratory operon gene being removed and replaced with the Cm^R cassette.

2.1.8. λ -Red mutagenesis preparation - Removal of chloramphenicol cassette

The single respiratory mutants used as a starting point had a chloramphenicol cassette (Cm^R) in the place of the deleted oxidase gene which first had to be removed. The

temperature sensitive FLP recombinase containing plasmid pCP20-Gm (Gm^R) was transformed by electroporation into the single *E. coli* EC958 ST131 terminal oxidase deletion mutants. The chloramphenicol cassettes are flanked by FRT (flippase recognition targets) sites allowing the pCP20-Gm (Gm^R) encoded FLP recombinase to remove the chloramphenicol cassettes (Datsenko and Wanner 2000) (Fig. 2.2).

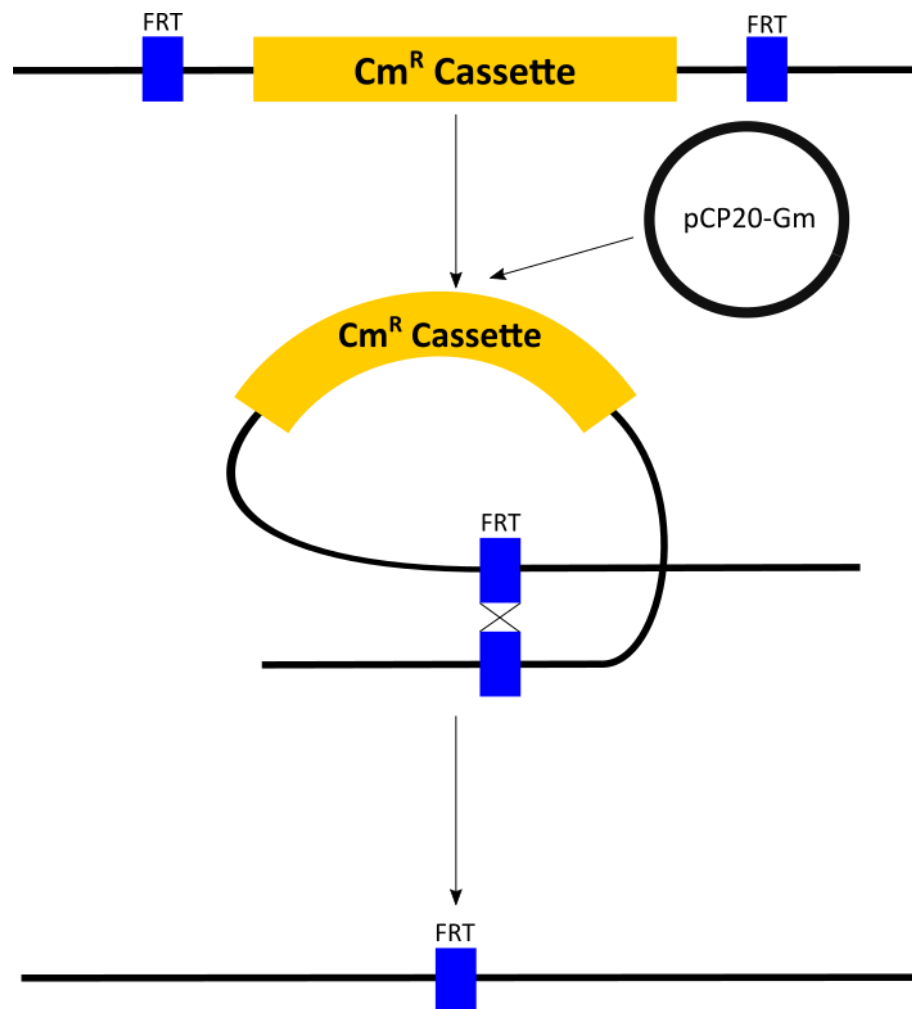


Figure 2.2 - FRT mediated recombination to remove chloramphenicol cassette. FLP recombinase encoded by vector pCP20-Gm recognises the FRT sites resulting in homologous recombination and removal of the Cm^R cassette. A small scar sequence including a single FRT site remains in the genome.

2.1.9. Removal of pCP20-Gm and introduction of λ -Red recombinase plasmid

The phage λ -Red recombinase machinery is encoded by the temperature sensitive plasmid pKOBEG controlled by an arabinose induced promoter. Before pKOBEG could be transformed into each of the *E. coli* single terminal knockout mutants ($\Delta cydAB$, $\Delta cyoA$ and $\Delta appCB$) the pCP20-Gm flippase-encoding plasmid had to be removed due to both plasmids using gentamicin selection. This was completed by passaging at 42°C for 48 h s in 10 ml LB media cultures with a media change every 12 h (500 μ l of culture used to inoculate fresh 10ml LB media). Patching onto LB-ampicillin and LB-gentamicin plates allowed for selection of colonies that had lost pCP20-Gm. The *E. coli* single terminal knockout mutants ($\Delta cydAB$, $\Delta cyoA$ and $\Delta appCB$) were then transformed with the pKOBEG plasmid via chemical (section 2.1.4) or electroporation methods (section 2.1.6).

2.1.10. PCR amplification of chloramphenicol cassettes for λ -Red mutagenesis

PCR was carried out to amplify the Cm^R cassettes from $\Delta cydAB$ or $\Delta cyoA$ and $\Delta appCB$ single knockout mutants with 500bp overlap on each end to allow λ -Red mutagenesis to generate double terminal respiratory mutants. Each PCR reaction was prepared with the following reagents: 25 μ l 2X Q5[®] High-Fidelity DNA Polymerase 2X Master Mix (NEB), 300 nM of the specific forward primer, 300 nM of the specific reverse primer, 0.5 μ l of resuspended *E. coli* single terminal knockout mutant colony (in 50 μ l Milli-Q H₂O) and sterile Milli-Q water to 50 μ l. PCR reactions were carried out in PCR tubes placed into either a T3000 Thermocycler (Biometra[®]) or Veriti Thermal Cycler (Applied Biosystems) machine. PCR conditions were as follows: 94°C for 5 min, 35 cycles of 94°C for 15 s, 50°C for 30 s and 72°C for 5 min before a final step at 72°C for 5 min. PCR reactions were purified using a QIAquick PCR Purification Kit with elution in 30 μ l sterile Milli-Q water.

2.1.11. Expression of λ -Red recombinase and λ -Red mutagenesis

50 ml SOB/LB/M9 medium was inoculated with 0.5 ml (1% v/v) overnight starter culture in a 250 ml conical flask supplemented with 30 μ g/ml gentamicin. The culture was incubated at 28 °C and 180 RPM with 20 mM L-arabinose added at 0, 1 or 2 h s to induce expression of the λ -Red recombinase machinery from the araBAD promoter. The culture was incubated until an OD₆₀₀ of 0.5-0.6 was reached when the cells were made electro-

competent as per section 2.1.5. Electroporation used 9 µl PCR product and the protocol from section 2.1.6. Electroporation using 9 µl sterile Milli-Q water was used as a negative control. LB-agar plates with 25 µg/ml chloramphenicol were used to select for positive deletion mutants as a chloramphenicol resistance cassette replaces the deleted gene.

2.1.12. PCR screening assay

Colony PCR was used to confirm colonies with correct deletion mutants. PCR reagents included: 2x PCRBIO Taq Mix Red (PCRBIO SYSTEMS), 300 nM of the specific forward primer, 300 nM of the specific reverse primer, 0.5 µl of resuspended *E. coli* potential mutant colony and sterile Milli-Q water to 50 µl. PCR conditions were the same as section 2.1.10 apart from annealing temperature and extension time which varied depending on the primer combinations used. Two PCR reactions were used for each deletion mutant utilizing two different sets of primers. The first primer pair bind 200 bp either side of the deleted gene whilst the second primer pair binds internally to the inserted Cm^R gene and 200 bp downstream of the inserted Cm^R gene.

2.1.13. DNA sequencing

PCR was used to amplify the knockout region using the same primers and conditions as section 2.1.10. PCR products (5µM) were sent to GENEWIZ pre-mixed with primers (5µM) that bind 200bp either side of the gene to be knocked out for sanger sequencing. Results were analysed using SnapGene.

2.1.14. Cloning of *E. coli* terminal respiratory oxidases into pSU2718G using Gibson assembly

2.1.14.1. PCR reactions to amplify respiratory oxidases and vector backbone

A colony PCR reaction was used to amplify the pSU2718G vector backbone from *E. coli* TOP10 strain containing pSU2718G-*cydABX* using the primers 2F_pSU2718 and 2R_pSU2718. PCR reaction contained the following components: 25 µl 2X Q5® High-Fidelity DNA Polymerase 2X Master Mix (NEB), 300 nM of the specific forward primer, 300 nM of the specific reverse primer, 0.5 µl of resuspended *E. coli* TOP10 (pSU2718G-*cydABX*) colony (in 50 µl Milli-Q H₂O) and sterile Milli-Q water to 50 µl. PCR reactions were carried out in PCR

tubes placed into either a T3000 Thermocycler (Biometra®) or Veriti Thermal Cycler (Applied Biosystems) machine. PCR conditions were as follows: 94°C for 5 min, 35 cycles of 94°C for 15 s, 50°C for 30 s and 72°C for 5 min before a final step at 72°C for 5 min.

Another PCR reaction was used to amplify the *E. coli* terminal respiratory operons *cydABX* and *cyoABCD* to include a 6*His Tag. The forward primers (*CydABX_F_EC958* and *CyoABCD_F_EC958*) added a 28 bp Gibson assembly overlap region to the pSU2718G vector backbone. The reverse primers (*1RNew_pSU2718* and *CyoABCD_R_EC958*) added a 6*His Tag to the C-terminus of the last gene in the operons (*cydX* and *cyoD* respectively) and a 25 bp overlap to the pSU2718G backbone. PCR reactions contained the same components and used the same conditions as above apart from 0.5 µl of *E. coli* EC958 genomic DNA (8.35 ng) used instead of a resuspended colony. *E. coli* EC958 genomic DNA was extracted using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) following the manufactures' instructions.

2.1.14.2. Gibson Assembly

A Gibson Assembly kit (NEB) was used to join the amplified *E. coli* terminal respiratory operons *cydABX* and *cyoABCD* with His Tag to the linearized pSU2718G vector backbone using the overlapping regions added during the PCR reactions (section 2.1.14.1). The reaction mixture contained: 0.02 to 0.5 pmols of the two DNA fragments, 10 µl Gibson Assembly Master Mix and Milli-Q H₂O to a total of 20 µl. The reactions were incubated at 50°C for 60 min in a Veriti Thermal Cycler (Applied Biosystems). The assembly product was then chilled on ice before adding 10 µl to 50 µl 5-alpha competent *E. coli* (Subcloning Efficiency NEB C2988J) and incubating for 30 min on ice. This was followed by a heat shock at 42°C for 30 s and then a 2 min incubation on ice. SOC recovery media (950 µl) was then added and the cells incubated at 37°C and 180 rpm for 1 h . 100 µl of the cells were then spread onto an LB-agar gentamicin (30 µg/mL) plate for selection. The remaining 900 µl was centrifuged at 5000 RPM for 5 min, 800 µl of supernatant removed and the cells resuspended in the final 100 µl of media before also being plated onto a selective LB-agar gentamicin plate.

2.1.14.3. PCR and Sequencing Confirmation

Colony PCR was used to identify colonies with the correct cloned plasmid using the same components and conditions as section 2.1. Primer pairs were selected so 1 primer binds to the vector backbone and 1 primer binds to the insert (see Table 2.3). To confirm that the successful fragment had been cloned into pSU2718G the plasmids were sent for DNA sanger sequencing (GENEWIZ) using primers that allow the ligation region to be sequenced (see Table. 2.3). The sequencing data was analysed using SnapGene.

2.2. Bacterial growth and physiology

2.2.1. Bacterial Strains

E. coli strains used are listed in Table 2.1.

Table 2.1 - *E. coli* strains

Strain number	Characteristics	Source or reference
MS10	EC958; Uropathogenic (UPEC) wild type strain belonging to clonal group ST131	(Totsika et al. 2011)
MS11	EC958 containing pKOBEG	Prof. Mark Schembri (Sarkar et al. 2016)
MS16	EC958 <i>cydAB</i> ::Cm	(Shepherd 2016)
MS107	EC958 with <i>gfp</i> inserted into the <i>lac</i> operon also containing pCP20-Gent	(Shepherd 2016)
MS402	TOP10 containing pSU2718G- <i>cydABX</i>	(Shepherd 2016)
MS506	EC958 <i>cyoA</i> ::Cm containing pKOBEG	Dr Cláudia A. Ribeiro (Shepherd laboratory)
MS605	EC958 <i>appCB</i> ::Cm containing pKOBEG	This work
MS612	EC958 <i>cyoA</i> ::Cm	This work
MS613	EC958 <i>appCB</i> ::Cm	This work
MS614	EC958 $\Delta cydAB$ (MS16 with Cm removed)	This work

MS616	EC958 $\Delta cyoA$ (MS506 with Cm removed)	This work
MS623	EC958 $\Delta cyoA$ containing pKOBEG	This work
MS624	TOP10 containing pKOBEG	This work
MS625	EC958 $\Delta cydAB$ containing pKOBEG	This work
MS626	EC958 $\Delta cyoA$ <i>appCB::Cm</i> containing pKOBEG	This work
MS627	Failed attempt to generate EC958 $\Delta cydAB$ <i>cyoA::Cm</i> containing pKOBEG	This work
MS628	EC958 $\Delta cyoA$ <i>appCB::Cm</i>	This work
MS629	EC958 $\Delta cydAB$ <i>appCB::Cm</i>	This work
MS630	Failed attempt to generate EC958 $\Delta cydAB$ <i>cyoA::Cm</i>	This work
MS631	DH5 α containing pSU2718-G- <i>cydABX</i> -6*HIS	This work
MS632	DH5 α containing pSU2718-G- <i>cyoABCD</i> -7*HIS	This work

2.2.2. Bacterial Plasmids

Table. 2.2 - List of plasmids

Plasmid	Characteristics	Antibiotic Resistance	Source
pKOBEG	λ -red recombinase, temperature sensitive (Ts) replicon	Gent ^R	(Chaverroche et al. 2000)
pCP20-Gent	<i>S. cerevisiae</i> flp recombinase, temperature sensitive (Ts) replicon	Cm ^R , Gent ^R	(Datsenko and Wanner 2000)
pSU2718-G-<i>cydABX</i>	Complementation plasmid for $\Delta cydAB$	Gent ^R	(Shepherd 2016)
pSU2718-G-<i>cydABX</i>-6HIS	Expression plasmid for EC958 <i>cydABX</i> -6HIS	Gent ^R	This work

pSU2718-G- <i>cyoABCD-7HIS</i>	Expression plasmid for EC958 <i>cyoABCD-7HIS</i>	Gent ^R	This work
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2.2.3. Oligonucleotides

Oligonucleotides used (Table 2.3) were designed using SnapGene ideally with no more than a 5°C difference in melting temperature (T_m) and a GC content between 40-60%. ThermoFisher Multiple Primer Analyzer was used to test for primer dimers before the finalized oligonucleotides were ordered from Integrated DNA Technologies (IDT) with standard desalting purification.

Table 2.3 - List of oligonucleotides used

Primer Name	Sequence (5'-3')	Purpose	Shepherd Lab Primer Number	Direction
pSU2718seq_200_F	GTCGGGTGATGCTGC CAACT	Sequencing pSU2718-G- <i>cydABX</i> -6HIS and pSU2718-G- <i>cyoABCD</i> -7HIS	275	Forward
pSU2718seq_200_R	GCAGCTGGCAGACA GGTTT		276	Reverse
AppCB_500_F	TCGTCAC TTTATCAAT ACGCTTCT	Amplification of Cm ^R cassette with 500 bp overhangs from MS605 EC958 <i>appCB::Cm</i>	392	Forward
AppCB_500_R	AGTTTGATTGCGGGA AATTAAGCA		393	Reverse
cyoA_F_500_New	CGAGAGAAACGTAA CCAGAATCA	Amplification of Cm ^R cassette with 500 bp overhangs from MS506 EC958 <i>cyoA::Cm</i>	397	Forward
cyoA_R_500_New	GTTTGCTGGATTATCT GGCGCTAC		398	Reverse
cydAB_F_600bp	GTGACGGATTACAG TACGTTGCT	Amplification of Cm ^R cassette with 500-600 bp overhangs from MS16 EC958 <i>cydAB::Cm</i>	394	Forward
CydAB_R_500bpnew	CTGCTACTATACGCG CGCGATTGA		417	Reverse
1RNew_pSU2718	ATGCCTGCAGGTCTGA CTCTAGAAATTCAAT GGTGATGGTGATGGT	Amplification of <i>cydABX</i> from EC958 with 6*His-	406	Forward

	GGATGTCTTCTTGAC CGGCTTTGCCTGATT C	tag and Gibson assembly overlap region to pSU2718-G		
<i>CydABX_F_EC958</i>	CGGGGATCCTTAAAG AGGAG AAAGGTACATGATGT TAGATATAGTCGAAC TGTCGCG		407	Reverse
<i>CyoABCD_F_EC958</i>	CGGGGATCCTTAAAG AGGAGAAAGGTACA TGAGACTCAGGAAAT ACAATAAAAGTTTG	Amplification of <i>cyoABCD</i> from EC958 with 7*His- tag and Gibson assembly overlap region to pSU2718-G	408	Forward
<i>CyoABCD_R_EC958</i>	ATGCCTGCAGGTCTGA CTCTAGAAATTTAAT GGTGATGGTGATGGT GGTGCATCATCATGT TGTAATTGAGGTTCC A		409	Reverse
<i>RvSc1_cydABX_His</i>	AGGTCGACTCTAGAA ATTCAATGGTGATGG	Screening of <i>cydABX</i> -6His into pSU2718G	416	Reverse Forward primer = pSU2718se q_200_R
pKOBEG_Sc_F	TGGGCTAGCGAAAA GATGTTTCGT	To detect pKOBEG	419	Forward
pKOBEG_Sc_R	ATCATCGCCATTGCTC CCCAAATAC		418	Reverse
AppCB_200_F	GGCATTACGCTGTA CGGAC	Screening and sequencing Cm ^R cassette insertion into <i>appCB</i> locus in EC958	420	Forward
AppCB_200_R	TGGCGTTGGTAATGT CCGAG		421	Reverse
CmR_Sc1`	CATTCATCAGGCGGG CAAGAATGTG	Screening Cm ^R cassette insertion into <i>appCB</i> locus – binds inside Cm ^R cassette	422	Forward Reverse primer = AppCB_200 _R
CyoA_Internal	CGATCAGATGCAGGC GAGTC	Binds internally to EC958 <i>cyoA</i> for respiratory mutant screening	423	Forward Reverse Primer = cyoA_R_50 0_New
AppC_Internal	AACGGTGCAGGTGCA GGTTC	Binding internally to EC958 <i>appC</i> for respiratory mutant screening	424	Reverse Forward Primer = AppCB_200 _F
<i>cyoA_Sc_fwN</i>	TTATGGTCAACGGAG GTCAG	Screening and sequencing Cm ^R	425	Forward

cyoA_Sc_revN	CCTTCTGCACTTCCT TTCG	cassette insertion into <i>cyoA</i> locus in EC958	426	Reverse
2F_New_pSU2718	ATTCTAGAGTCGAC CTGCAGGCATGCAAG	Amplification of pSU2718-G backbone Amplification of pSU2718-G backbone	431	Forward
2R_New_pSU2718	GTACCTTTCTCCTCTT TAAGGATCCCCGGG		432	Reverse

2.2.4. Chemicals and water

Milli-Q water was used for high purity applications such as PCR with distilled-deionized water used for all other experimental work. Chemicals were purchased from Sigma-Aldrich, ThermoFisher or other as stated. The media components nutrient agar, yeast extract and tryptone were from Oxoid.

Sterile solutions were prepared using either autoclaving or filter sterilization. Autoclaving was completed at 121°C for 15 min at 15 psi. Solutions below 50 mL were filter sterilized using 0.22 µm pore size Millipore filters. Solutions over 50 mL that could not be autoclaved were filter sterilized using a Nalgene Rapid-Flow 150 mL bottle top 0.2 µm SCFA membrane filter with vacuum provided by a Innovac 40 vacuum pump.

2.2.5. Buffer solutions

2.2.5.1. Phosphate-Buffered Saline (PBS)

PBS contained 8 g NaCl, 0.2 g KCl, 1.54 g Na₂HPO₄·2H₂O and 0.24 g KH₂PO₄ per litre with the pH adjusted to 7.4.

2.2.5.2. HEPES Buffers

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was added to the required concentration and the pH adjusted to the desired value using 1M NaOH before any additions. For the membrane potential assay 5 mM HEPES buffer, pH 7.2, containing 5 mM glucose, 100mM KCl, 1% DMSO and 2µM DiSC3(5) ((3,3'-Dipropylthiadicarbocyanine Iodide) was used.

2.2.5.3. Tris Buffers

Tris buffers were made using Trizma base and adjusted to the required pH using 1M HCl.

2.2.5.4. Sonication Buffer

Sonication buffer contained 20 mM Tris HCl at pH 7.4, 2 mM MgCl₂ and 1 mM EGTA.

2.2.5.5. Potassium Phosphate Buffer (50 mM)

Potassium phosphate buffer (50 mM) contained 30.93 mM KH₂PO₄ and 19.07 mM K₂HPO₄ with 1M KOH used to adjust the buffer to pH7.

2.2.6. Media and supplements

2.2.6.1. Lysogeny Broth (LB) medium

LB medium contained 10 g NaCl, 10 g tryptone and 5 g yeast extract per litre.

2.2.6.2. LB agar plates

LB agar for plates contained 15 g of nutrient agar per litre of LB medium. Agar plates were dried inverted at 42°C for 40 min after setting before use.

2.2.6.3. SOB (Sugar Optimal Broth) medium

SOB medium contained 20 g tryptone, 5 g yeast extract, 2.4 g MgSO₄, 0.5 g NaCl and 0.186 g KCl per litre.

2.2.6.4. SOC medium

SOC medium (1 L) was prepared by adding 10 mL of a 2 M Mg²⁺ solution (20.33 g MgCl₂ and 24.65 g MgSO₄ per litre) and 20 mL of a 1 M glucose solution to 970 mL of SOB medium. SOB medium was autoclaved whilst the 2 M Mg²⁺ and 1 M glucose solution were filter sterilized before addition.

2.2.6.5. M9 minimal medium with casamino acids

A 5x M9 salts stock was prepared with 80.24g Na₂HPO₄·2H₂O, 15g KH₂PO₄, 2.5g NaCl and 5.0g NH₄Cl per litre. 5x M9 salts were autoclaved and stored at room temperature. To

prepare 1 litre of 1x M9 minimal medium the following filter sterilized components: 100 μ l of a 1 M CaCl_2 solution, 2 mL of a 1 M MgSO_4 and 20 mL of glucose (20% w/v) were added to 200 mL of 5x M9 salts and the volume adjusted to 1 litre using autoclaved ddH₂O. For M9 minimal media with 0.1% casamino acids 50 mL of autoclaved casein hydrolysate (2% w/v) was added per litre. Where additions of 0.1% casamino acids, 0.1 mM hydrogen peroxide, 1mM DNP or 5% ethanol were made to the M9 minimal media the ddH₂O volume was adjusted to preserve the concentration of the other components.

2.2.7. Antibiotics

The antibiotics used are shown in Table 2.4 at final working concentrations. These were added to *E. coli* cultures to maintain plasmids or to select for transformants and mutants. Gentamicin was also used at variable concentrations in the antibiotic susceptibility assays.

Table 2.4 - List of antibiotics used

Antibiotic	Solvent	Stock Concentration (mg/mL)	Working Concentration (μ g/mL)
Ampicillin	Milli-Q water	125	125
Chloramphenicol	Ethanol	34	25
Gentamicin	Milli-Q water	30	30 and variable

2.2.8. Glycerol stocks

E. coli glycerol stocks were prepared by mixing 500 μ l of sterile 50% (v/v) glycerol with 500 μ l of a specific overnight culture in a cryotube and stored at -80°C.

2.2.9. Nitric oxide delivery

2.2.9.1. GSNO preparation

The NO exogenous source *S*-Nitrosoglutathione (GSNO) was prepared as previously described (Hart 1985). A solution containing 3.08 g of reduced glutathione, 0.69 g of sodium

nitrite (NaNO_2), 0.83 mL of 12.1 M HCl and 18 mL of distilled water was prepared in a 100 mL conical flask kept on ice. The solution was stirred for 40 min on ice before 20 mL of acetone was added. The solution was stirred for a further 10 min leading to a red precipitate forming. The precipitate was extracted by vacuum filtration and then washed 5 times with 2 mL cold distilled water, 3 times with 10 mL acetone and then finally 3 times with diethyl ether. The precipitate was dried overnight in a foil wrapped vacuum desiccator and then stored at -80°C for a maximum of one month.

Before use, the GSNO was dissolved in distilled water and filter sterilized with a $0.22\ \mu\text{m}$ syringe filter. GSNO was quantified at 1:20 dilution via the absorbance measured at 545nm (Agilent Cary 60 UV-vis spectrophotometer) and the extinction coefficient at 545 nm ($15.9\ \text{M}^{-1}\text{cm}^{-1}$)(Mohr et al. 1999).

2.2.9.2. NO gas preparation

To prepare one 60 mL syringe of NO gas 0.25 g of solid NaNO_2 and 3 mL of acidic ferrous sulphate solution containing 1.8 M H_2SO_4 and 1.2 M $\text{Fe}^{2+}(\text{aq})$ were used with preparation taking place in a fume cupboard. To prepare enough acidic ferrous sulphate solution for 10 syringes (60 mL) of NO gas 13.5 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ was added to 28 mL of distilled water. Following this 12 mL of 6 M H_2SO_4 was added slowly with stirring until all the solid dissolved.

0.25 g of solid NaNO_2 was added to a 50 mL syringe against the plunger and then 3 mL acidic ferrous sulphate solution was taken up into the syringe via a needle that was capped immediately afterwards. Vigorous shaking resulted in NO gas production to a volume of 60 mL. To remove contaminating chemicals 5 mL of distilled water was drawn into the syringe and used to dissolve the contaminants before discharging without release of NO gas. NO gas could be disposed of by bubbling slowly through 1 M NaOH. For experimental use NO gas was added to media in serum bottles by inserting a syringe with needle into the media and slowly bubbling the NO gas through the media.

2.2.10. Aerobic Growth Conditions

E. coli EC958 ST131 wild-type was grown in 10 mL LB in a 50 mL falcon tube at 37 °C and 180 rpm overnight to stationary phase. The stationary phase cultures were used to inoculate (at 1% v/v) M9 minimal medium supplemented with 0.1% casamino acids (w/v) (see section 2.2.6.5). Cultures were incubated at 37 °C and 180 rpm until mid-exponential phase before use in the following PMF related experiments (sections 2.2.12-2.2.17).

2.2.11. Anaerobic growth curves

E. coli EC958 was grown in 10 mL M9 minimal media supplemented with 0.1% casamino acids (w/v), 0.2% glycerol (v/v) and +/- 100 mM sodium nitrate (to provide the exogenous electron acceptor) in a 50 mL falcon tube at 37 °C and 180 rpm aerobically overnight to stationary phase. The stationary phase cultures were used to inoculate (0.2% v/v) 30 mL of M9 minimal media supplemented with 0.1% casamino acids (w/v), 0.2% glycerol (v/v) and +/- 100 mM sodium nitrate in sealed serum bottles. Cultures were then sparged with nitrogen gas for 3 min and incubated at 37 °C in a static incubator. Samples (1 mL) from 3 biological repeats were taken every h for 8 h and then again after 24 h and OD₆₀₀ was measured on a Cary 60 UV-Vis spectrophotometer (Agilent Technologies).

2.2.12. Catalase Assay

E. coli EC958 ST131 wild-type cultures were grown using the aerobic growth conditions (section 2.2.10) splitting at mid-exponential phase (approx 1.6×10^8 cells/mL) into 60 mL suspensions in 250 mL conical flasks. These suspensions were exposed to DNP (0 or 1mM), hydrogen peroxide (0.1 mM) or varying concentrations of gentamicin for 30 min at 37 °C. Cell suspensions were harvested (5000 xg for 5 min at 4°C) and washed in 50 mM Potassium Phosphate buffer a total of 3 times before sonication at 18000 Hz for 5 x 30 s intervals with 30 s incubation on ice in between. Cell debris was removed via centrifugation at 15000 rpm for 20 min at 4°C. For the catalase assay, 50 µL of cell-free supernatant was added to 950 µL of 0.036 % (w/w) H₂O₂ in a quartz cuvette and the linear decrease in absorbance at 240 nm was measured that corresponded to consumption of

H₂O₂. Conversion of $\Delta A_{240} \text{ min}^{-1}$ to $(\mu\text{moles H}_2\text{O}_2)\text{min}^{-1}$ was achieved using the extinction coefficient $\epsilon_{240} = 36 \text{ M}^{-1}\text{cm}^{-1}$ (Anderson et al. 1995). A Markwell assay (Markwell et al. 1978) was used to determine protein concentrations to convert units to $\mu\text{moles H}_2\text{O}_2 \text{ min}^{-1}(\text{mg protein})^{-1}$. Three biological repeats were carried out for each condition with three technical repeats completed for each biological repeat.

2.2.13. Native-PAGE KatE and KatG Catalase Assay

The same *E. coli* EC958 ST131 wild-type cell extract samples used for the catalase assay exposed to 0 $\mu\text{g/mL}$ gentamicin, 100 $\mu\text{g/mL}$ gentamicin or 0.1 mM H₂O₂ were also used for the Native-PAGE (polyacrylamide gel electrophoresis) *KatE* and *KatG* assay. Native-PAGE gels used comprised of 5% stacking and 6% resolving acrylamide gels. The exact gel components, loading sample buffer and running buffer used are as described in (Pezzoni et al. 2018). A Markwell assay (Markwell et al. 1978) was used to determine protein concentrations with 10 μg of cell extract loaded into each lane. For the positive control 10 units (0.26 ng) of bovine catalase were used (one unit of catalase will decompose 1 μmole of H₂O₂ per minute at pH 7.0 at 25 °C). A Bio-RAD Mini-PROTEAN Tetra Cell was used to run two identical gels at 15 mA for 2 hr 45 min at 4 °C. One gel was stained with Coomassie stain (1 g/L Coomassie Brilliant blue R, 40% methanol, 10% acetic acid and 50% ddH₂O) overnight before applying de-stain (40% methanol, 10% acetic acid and 50% ddH₂O) and then imaging use a G-box gel doc system. The second identical gel was stained for catalase activity. This gel was soaked in distilled water for 5 min before incubating with 100 mL 4 mM H₂O₂ for 10 min at room temperature. After H₂O₂ incubation the gel was washed with distilled water before soaking in a 100 mL solution containing 1% (w/v) ferric chloride and 1% (w/v) potassium ferricyanide (III) at room temperature. As soon as the gel turned dark green (~ 5 min) the ferric chloride and potassium ferricyanide solution was removed and the gel rinsed with distilled water before imaging. ImageJ v1.53 was used to analyze the band intensities of the *KatE/G* bands. The native-PAGE gel image was converted to a TIF(8-bit)(greyscale) with each lane separated into an equal strip and a profile plot generated for each. The area of the *KatE/G* peaks were determined from the profile plots and reported as band intensity.

2.2.14. ROS flow cytometer assay

E. coli EC958 ST131 wild-type cultures were grown using the aerobic growth conditions (section 2.2.10) to mid-exponential phase when suspensions of 10^8 cells/mL were prepared in fresh M9 minimal medium supplemented with 0.1% casamino acids (w/v). These suspensions were incubated at 37 °C for 30 min and then mixed with 1X ROS dye (Thermo Fisher Total Reactive Oxygen Species Assay Kit 520 nm – catalog number = 88-5930-74) a DCFDA analogue based dye that is well-suited for detection of free radical containing ROS such as superoxide. Aliquots (200 µL) of the ROS dyed suspension were added to a foil covered Greiner Bio-One 96-well, F-bottom (chimney well) microplate. Microplates were incubated at 37 °C for 30 min before exposing to gentamicin (100 µg/mL), Milli-Q water (for no treatment negative control sample) or 1 mM H₂O₂ (positive control). A 20 µL sample was taken at 30 and 90 min after exposure. Samples were diluted 1:10 in Phosphate Buffered Saline (PBS) and fixed with DAPI (5 µg/mL) in 4% PFA (paraformaldehyde)-PBS solution. A BD FACSJAZZ™ flow cytometer was used to analyse the fixed samples capturing 100,000 events for each sample. DAPI was excited by a 405 nm laser and detected with a 450/50 nm band-pass emission filter. ROS dye was excited by a 488 nm laser and detected by a 513/17 nm band-pass emission filter. A two-gate system partially adapted from (McBee et al. 2017) was used to analyse the samples (Figure 2.3) using NovoExpress 1.3.0 software. The first gate used FSC (Forward Scatter) against FSC-Width to determine single cells, removing doublets and debris. All events that passed the first gate were then screened by the second DAPI gate to identify intact cells (DAPI binds to DNA). Fluorescent intensity of the ROS dye was then measured in all cells that passed the DAPI gate (minimum of 60,000) and mean values reported.

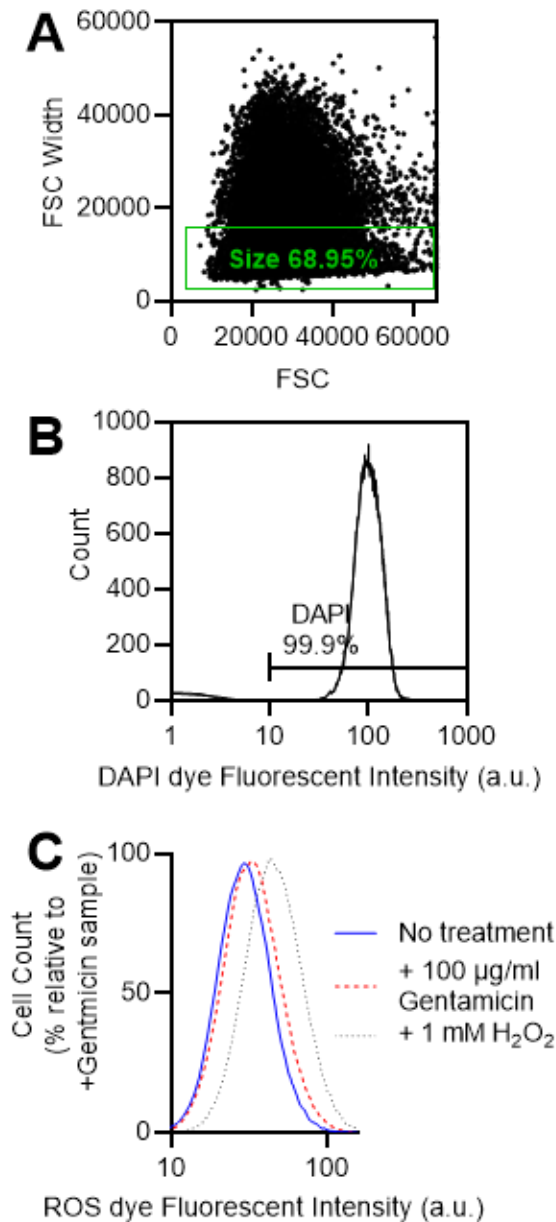


Figure 2.3 - Overview of ROS flow cytometry gating method showing example data. (A) Stage 1 (Forward scatter (FSC) against FSC width) selects for bacterium-sized particles removing doublets, multiples and large debris (Size Gate). Events in the size gate are passed to (B) Stage 2 (DAPI gate) which selects for intact cells that contain DNA. (C) Stage 3 measures the ROS using the fluorescent intensity of the ROS dye. Data in (C) is shown for the 90 min exposure sample to no treatment (+ milliQ water), + 100 µg/mL gentamicin or positive control (+ 1 mM H₂O₂). Method was partially adapted from (McBee et al. 2017) (Stages 1 + 2).

2.2.15. DNP gentamicin susceptibility assays

For survivability assays under aerobic conditions, *E. coli* EC958 ST131 wild-type cultures were grown using the aerobic growth conditions (section 2.2.10) to mid-exponential phase when suspensions of 10^8 cells/mL were prepared in fresh M9 minimal medium supplemented with casamino acids (w/v). These suspensions were exposed to DNP (0 or 1mM) for 30 min at 37 °C before incubation with various concentrations of gentamicin for 90 min. After gentamicin exposure, serial dilutions were carried out in 1X PBS and cells were spotted on LB-agar in triplicate for overnight incubation at 37 °C for colony counting the next day. Six repeats including two biological repeats were performed for each condition.

For survivability assays under anaerobic conditions *E. coli* EC958 was grown in 10 mL M9 minimal media supplemented with 0.1% casamino acids (w/v), 100 mM sodium nitrate and 0.2% glycerol (v/v) in a 50 mL falcon tube at 37 °C and 180 rpm aerobically overnight to stationary phase. The stationary phase cultures were used to inoculate (1% v/v) 30 mL of M9 minimal media supplemented with 0.1% casamino acids (w/v), 100 mM sodium nitrate and 0.2% glycerol (v/v) in sealed serum bottles. Cultures were then sparged with nitrogen gas for 3 min each before incubation in a static incubator at 37°C to until mid-exponential phase. Cultures were then diluted to 10^8 cells/mL in serum bottles and pre-exposed to +/- 1mM DNP for 30 min before incubation with varying concentrations of gentamicin for 90 min at 37°C in a static incubator. Cultures were re-sparged with nitrogen gas for 1.5 min both after DNP and gentamicin addition to maintain anaerobic conditions. After gentamicin exposure, serial dilutions were carried out in 1X PBS and cells were spotted on LB-agar in triplicate for overnight incubation at 37 °C for colony counting the next day. Six repeats including two biological repeats were performed for each condition.

2.2.16. Assay of the membrane electrical potential

E. coli EC958 ST131 wild-type cultures were grown using the aerobic growth conditions (section 2.2.10) to mid-exponential phase when suspensions of 10^8 cells/mL were prepared in fresh M9 minimal medium supplemented with 0.1% casamino acids (w/v). These suspensions were exposed to DNP (0 or 1mM) or GSNO (0 or 15mM) for 30 min at

37°C. Cells were harvested (4500 xg for 3 min) and washed in 5 mM HEPES buffer pH 7.2 containing 5 mM glucose a total of 3 times. The reaction buffer was added to a Greiner Bio-One 96-well, F-bottom (chimney well), black, fluotrac, medium binding microplate so that the final concentration of the reaction buffer components after addition of cells is 5 mM HEPES buffer, pH 7.2 with 5mM glucose, 100mM KCl and 2 μ M of DISC3. The fluorescence was monitored on a FLUOstar Omega plate reader for 60 s at 30 s intervals using excitation and emission wavelengths of 584 nm and 655 nm, respectively to gather a baseline. Cells were then added at a final concentration to 4×10^7 cells/mL in volume 200 μ l. Fluorescence was then monitored for a total of 45 min at 30 s intervals. 200 μ l of 5mM HEPES buffer with 5mM glucose was used for the negative controls.

2.2.17. Oxygen consumption assay for DNP and FCCP

For the DNP oxygen consumption assay, *E. coli* EC958 ST131 wild-type cultures were grown using the aerobic growth conditions (section 2.2.10) to mid-exponential phase when cells were harvested (5000 xg for 5 min) and resuspended in fresh M9 minimal media supplemented with 0.1 % casamino acids (w/v). A Rank Brothers oxygen electrode (Fig. 2.4) was baselined using 4 mL of distilled water and calibrated with sodium dithionite essentially as previously described (Gilberthorpe and Poole 2008) using the following parameters: polarizing volts = 0.6, stirring to setting 6, 37 °C. Voltage data was then recorded until a reading of 0 V was reached. The concentration of oxygen for the air-saturated growth media was 200 μ M as previously used (Gilberthorpe and Poole 2008; Shepherd et al. 2010). 3.6 mL of M9 minimal medium supplemented with 0.1% casamino acids (w/v) was added to the chamber and the baseline voltage was recorded for 5 min before cells were added to a final concentration of 8×10^7 cells/mL. Voltage data was gathered for 8 min before the plunger was inserted to eliminate external oxygen for 10 min prior to addition of 1 mM DNP or ethanol (5 % v/v) for the negative control. A separate oxygen consumption assay was carried out using using 10 μ M carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). The assay was the same as for DNP apart from a 2 mL final volume being used instead of 4 mL in the electrode due to limited FCCP being available.



Figure 2.4 - Oxygen electrode arrangement. Oxygen electrode in the bottom right containing 2 chambers connected to thermostatically controlled water bath and pump. Voltage data recorded by computer on the left.

2.2.18. *E. coli* membrane preparation

E. coli EC958 WT, $\Delta cyoA$ *appCB::Cm* (cytochrome *bd-I* only) and $\Delta cydAB$ *appCB::Cm* (cytochrome *bo'* only) mutants membranes were prepared by growing one colony of each strain in 10 mL LB in a 50 mL falcon tube at 37 °C and 180 rpm for 8 h s to stationary phase. The stationary phase cultures were used to inoculate (at 0.1% v/v) 1 L of LB in a 2 L conical flask. Ampicillin was added to the WT culture whilst chloramphenicol was added to the respiratory mutants at working concentration (Table 2.4). The 1 L *E. coli* cultures were grown overnight at 37 °C and 150 rpm to stationary phase. Cell suspensions were harvested (5000 xg for 20 min at 4°C) and resuspended in sonication buffer before sonication at 15000 Hz for 6 x 30 s intervals with 30 s incubation on ice in between. Cell debris was removed via centrifugation at 12000 rpm for 20 min at 4°C followed by centrifugation at 44,000 rpm for 1 h at 4°C in an ultracentrifuge (Beckman Ti45 with rotor 70Ti). Following ultracentrifugation, the supernatant was disposed and the membrane pellet weighted. *E. coli* membrane pellets were kept frozen at -20°C. Before use the membrane pellets were resuspended in 20 mM Tris pH 7.4 using a homogeniser to 100 mg/mL and aliquoted.

2.2.19. Reduced *minus* oxidised difference spectra

To prepare samples for whole cell difference spectra, *E. coli* EC958 was grown overnight in 90 mL LB and then harvested (5000 xg for 5 min at 4°C). Cell pellets were

resuspended in 4 mL Tris pH 7.5 and then diluted until the photomultiplier (PMT) voltage was close to 800 V, which is indicative of a cell suspension of the appropriate turbidity (i.e. $OD_{600} = 40-60$). For the reduced sample, a few grains of sodium dithionite were added whilst for the oxidised sample a few grains of ammonium persulphate were added. Spectra were then recorded between 400-700 nm in 0.5 nm increments (1 cm path length) using a SDB-4 dual-beam spectrophotometer (Current Designs, Inc) largely following the method of (Poole et al. 1989). The oxidised spectrum was subtracted from the reduced spectrum to give the final difference spectrum which was smoothed using 2nd order exponential smoothing between 4-12 neighbours averaged on GraphPad Prism version 9.3.1. A similar approach was also used for analysing membrane samples (resuspended in 20 mM Tris pH 7.4 and diluted to 50 mg/mL).

2.2.20. CO difference spectra

E. coli whole cells and membranes were prepared as detailed in section 2.2.18 and reduced samples were subsequently prepared with a few grains of sodium dithionite. A 'reduced' spectrum was then recorded between 400-700 nm in 0.5 nm increments (1 cm path length) using a SDB-4 dual-beam spectrophotometer (Current Designs, Inc) largely following the method of (Poole et al. 1989). To generate the 'CO reduced' sample, the reduced sample was bubbled with CO gas for 5 min at an approximate rate of 1 bubble per second. The CO reduced spectrum was then recorded and subtracted from the reduced spectrum to give the final CO difference spectrum which was then smoothed as described in section 2.2.19.

2.2.21. Oxygen consumption assay for potential inhibitors with *bd-I* only membranes

The oxygen consumption assay used to test potential cytochrome *bd-I* inhibitors was adapted from (Beard et al. 1995). Oxygen consumption of cytochrome *bd-I* only containing membranes isolated from the *E. coli* EC958 $\Delta cyoA/\Delta appCB$ mutant strain was measured in a Clarke type oxygen electrode. A Rank Brothers oxygen electrode was baselined using 2 mL of 50 mM HEPES pH 7.5 and calibrated as in section 2.2.17. A 2 mL reaction volume was used for the assay with cytochrome *bd-I* only containing membranes added to 50 mM HEPES pH 7.5 to give a final membrane concentration of 0.5 mg/mL in the electrode chamber.

Potential inhibitors solubilised in DMSO were then added at varying concentrations and the plunger inserted to exclude external oxygen. The baseline voltage was recorded for 2 min. Succinate (8 mM) was then added as the substrate for the aerobic electron transport chain from a 160 mM stock prepared using succinic acid (disodium salt) with the pH adjusted using 1 M HCl to pH 7.5. Addition of succinate was via a Hamilton syringe and the voltage data was gathered for a further 30 min. The oxygen consumption rate was measured between 400-700 s after succinate addition and compared to a 5% DMSO negative control to determine % activity.

2.3. *In silico* approaches

2.3.1. hhsearch pipeline for cytochrome *bd* oxidases in bacteria

The terminal respiratory oxidase sequences from *E. coli* EC958 ST131 were downloaded from the UniProt database (Apweiler et al. 2014). These consist of the operons *cydABX*, *cyoABCD* and *appCBX* for cytochromes *bd*-I, *bo'* and *bd*-II, respectively. A BLASTP search was run against the UniRef90 database (Apweiler et al. 2014) for each of the genes above and the top 250 results for each used as the sequence input for the hhsearch protocol. The proteomes to be investigated, which were mostly highly annotated reference proteomes, were downloaded from UniProt (Apweiler et al. 2014).

The hhsearch pipeline, derived from earlier work by (McGreig 2017), is combined into a single Python script. The 250 input sequences for each terminal oxidase gene were aligned using MAFFT with aligned method L-INS-I (Yamada et al. 2016). The multiple sequence alignments (MSAs) were then used to generate hidden Markov models (Söding 2005) forming the database which each proteome to be investigated was tested against. The individual proteins were first then extracted for each proteome and entered into the hhsearch.

The results were filtered by >80% probability score with the full length proteins for each match outputted to a separate results folder along with a text document detailing the probability scores of all hits. The results were run through Interproscan version 5.16-55

(Jones et al. 2014) locally using another Python script to search for protein family and domains to assist in identification. Finally, NCBI genome viewer (Rangwala et al. 2021) was used to identify if the hits were part of *cydABX*, *appCBX* or *cyoABCD* operons.

2.3.2. BLAST search for cytochrome *bd* oxidases in archaea

A BLASTP search was run using *E. coli* EC958 ST131 CydA against the UNIPROTKB_ARCHAEA database (Apweiler et al. 2014) with the default settings (E-Threshold = 10). All 459 results were downloaded. Strains were manually selected from the 459 results with the aim of generating a diverse range of archaea species that contain *cydA*. 23 CydA proteins were selected for phylogenetic analysis. The NCBI genome browser was used to check if the identified gene was part of a *cydAB* operon.

2.3.3. Phylogenetic analysis

The identified *cydA* sequences from sections 2.3.1 and 2.3.2 were used for the phylogenetic analysis. MSAs were produced using MAFFT (Yamada et al. 2016) with maximum iterations set to 100. Jalview version 2.11.0 (Waterhouse et al. 2009) was used to view and edit the alignments. Gap regions and poorly aligned sections were cut out. BioEdit (Mansour Alzohairy 2011) was used to convert the MSAs into PHYLIP4 file format before the edited alignments were submitted to PhyML 3.0 (Guindon et al. 2010) to construct phylogenetic trees. The LG substitution model was used with 100 bootstrap replicates. FigTree software (<http://tree.bio.ed.ac.uk/software/Figtree/>) was used to view the trees with a Python script used to add branch tip labels. Final annotation of the phylogenetic trees was completed in the vector graphics software Inkscape.

2.3.4. Modelling oxygen entry tunnels

Potential oxygen entry tunnels were modelled for the *E. coli* cytochrome *bd*-I cryo-EM structure 6RKO and *G. thermodenitrificans* cytochrome *bd*-I cryo-EM structure 5DOQ using MOLE 2.5 (Sehna et al. 2013). The predicted channels were set to start from haem *d* with no end point being selected. The origin radius was set to 5Å, surface cover radius to 8Å and the weight function was set to the Voronoi scale. The bottleneck radius was set to 1.4Å, bottleneck tolerance (length) to 3Å and the minimum tunnel length to 0Å. The results were analysed using Pymol and the channel profiles generated by MOLE 2.5. BioEdit

sequence alignment editor was used to produce the related MSA figures (Mansour Alzohairy 2011).

2.3.5. Proton channel modelling

Potential proton pathways were modelled for the *E. coli* cytochrome *bd-I* cryo-EM structure 6RKO using the water molecules already in the cryo-EM structure as a guide. MOLE 2.5 was also used to predict any cavities with the probe radius set to 5Å, interior threshold to 1.1Å and minimum depth to 5Å. Previous analysis conducted by (Safarian et al. 2016) was also used to help identify the key proton channel residues. BioEdit sequence alignment editor was used to produce the related MSA figures (Mansour Alzohairy 2011).

2.3.6. CydB ubiquinone-8 site analysis

3DLigandSite (Wass et al. 2010) was used to predict potential ligand clusters that may bind to the CydB ubiquinone-8 cleft of cytochrome *bd-I*. The ubiquinone-8 ligand in the *E. coli* cytochrome *bd-I* PDB file 6RKO was removed before submitting the CydB structure to 3DLigandSite. Molecular docking (see section 2.3.7) was also used to dock ubiquinone-8 to the empty CydB cleft of cytochrome *bd-I*.

2.3.7. Structural Modelling and analysis

Structural models of *E. coli* cytochrome *bd-I* were constructed using Phyre2 for homology modelling (Kelly et al. 2015) to the existing protein data bank (PDB) structures 6RKO (Safarian et al. 2019) and 6RX4 (Theßeling et al. 2019). Both normal and intensive modes of Phyre2 were used to construct models (Kelly et al. 2015). AlphaFold2 protein structure database (Varadi et al. 2021) was used for retrieval of AlphaFold2 modelled *E. coli* K12 CydA. PyMOL v2.4 was used for structural alignments to create a composite model using AlphaFold2 modelled CydA and the other subunits from PDB structure 6RKO. PyMOL was also used for structural analysis and alignments to *E. coli* cytochrome *bd-II* structures (Grauel et al. 2021; Grund et al. 2021).

2.3.8. Molecular docking and Drug Repurposing pathway

All molecular docking work was undertaken using AutoDock Vina (Trott,O., Olson 2010) with preparation work done in AutoDockTools (Morris et al. 2009) and PyMOL

(Seeliger and de Groot 2010). AutoDockTools (Morris et al. 2009) was used to define the docking box used to dock ligands into different sites on cytochrome *bd-I*.

For the drug-repurposing pipeline FDA-approved drugs from the e-Drug3D online data base (all FDA approved with molecular weight ≤ 2000 g/mol) (Pihan et al. 2012) were downloaded in 3D .sdf format. These were converted into .pdb format using PyMOL and then onto a flexible (.pdbqt) format using python script (prepare_ligand4.py) from MGLTools (Morris et al. 2009). Cytochrome *bd-I* Input structures for molecular docking had the polar hydrogens added using PyMOL. Ligands below 10KB file size were docked to the quinol binding site of *E. coli* cytochrome *bd-I* Phyre2 and AlphaFold2 models using Autodock Vina. The docking box size was $27 \text{ \AA} * 30 \text{ \AA} * 30 \text{ \AA} = 24300 \text{ \AA}^3$ for the Phyre2 produced model of the *E. coli* cytochrome *bd-I* structure and $28 \text{ \AA} * 28 \text{ \AA} * 30 \text{ \AA} = 23520 \text{ \AA}^3$ for the AlphaFold 2 model with the different sizes due to the slight differences in quinol binding cleft seen in each model. Known inhibitor and substrate docking was used to help determine the correct docking box position and size. The Bash script (Vina_auto.sh) was used for automation of the ligand docking.

2.3.9. Molecular docking results analysis

AutoDock Vina docking results were first analysed by predicted binding affinity (kcal/mol). The python script (parse_results_eDrug3D.py) extracted the binding affinity for the top docking pose for each ligand and ranked them in order of binding affinity. For the known inhibitors the equation $K_d = \exp(\Delta G/(R*T))$ with ΔG = binding energy (kcal/mol), R = gas constant = (1.986 cal/mol*K) and T = temperature (298K) was used to estimate the binding K_d . The top 70 ligands by binding affinity were manually analysed in PyMOL looking for specific interactions to the quinol binding site. LIGPLOT v 4.5.3 was also used to analyse the binding site interactions (Wallace et al. 1995). Hydrophobicity taken from the MolLogP values, known drug function and pharmacokinetic data available from e-Drug3D (Pihan et al. 2012) and PubChem (Kim 2016) databases was taken into account before decided on which compounds to purchase for experimental screening.

Chapter 3

**Role of PMF and ROS on
aminoglycoside susceptibility of
E. coli EC958 ST131**

3.1. Summary

It is well-known that loss of aerobic respiration in Gram-negative bacteria can diminish the efficacy of a variety of bactericidal antibiotics, which has led to subsequent demonstrations that the formation of reactive oxygen species (ROS) and the proton motive force (PMF) can both play a role in antibiotic toxicity. The susceptibility of Gram-negative bacteria to aminoglycoside antibiotics, particularly gentamicin, has previously been linked to both the production of ROS and the rate of antibiotic uptake that is mediated by the PMF, although the relative contributions of ROS and PMF to aminoglycoside toxicity has remained poorly understood. Herein, gentamicin was shown to elicit a very modest increase in ROS levels in an aerobically-grown *Escherichia coli* clinical isolate. The well-characterised uncoupler 2,4-dinitrophenol (DNP) was used to disrupt the PMF, which resulted in a significant decrease in gentamicin lethality towards *E. coli*. DNP did not significantly alter respiratory oxygen consumption, supporting the hypothesis that this uncoupler does not increase ROS production via elevated respiratory oxidase activity. These observations support the hypothesis that maintenance of PMF rather than induction of ROS production underpins the mechanism for how the respiratory chain potentiates the toxicity of aminoglycosides. This was further supported by the demonstration that the uncoupler DNP elicits a dramatic decrease in gentamicin lethality under anaerobic conditions. Together, these data strongly suggest that maintenance of the PMF is the dominant mechanism for the respiratory chain in potentiating the toxic effects of aminoglycosides.

3.2. Introduction

It is well-known that the abolition of aerobic respiration dramatically reduces the toxic effects of bactericidal antibiotics ((Lobritz et al. 2015); reviewed in (Stokes et al. 2019)). The lethality of bactericidal antibiotics has been shown to be linked to the activation of pathways that result in oxidative damage via elevation of aerobic respiratory rates (Dwyer et al. 2007, 2014; Foti et al. 2012), with superoxide causing destabilisation of iron-sulphur complexes (Imlay and Fridovich 1991; Keyer and Imlay 1996) leading to iron dysregulation and elevated Fenton chemistry. While different classes of bactericidal antibiotics have

different primary cellular targets, most are able to induce the formation of hydroxyl radicals which is thought to be linked to the aerobic respiratory chain (Kohanski et al. 2007, 2010). The bactericidal aminoglycoside gentamicin directly targets the 30S subunit of the ribosome leading to protein mistranslation with the toxicity believed to be due to either irreversible uptake leading to complete ribosome inhibition (Davis 1987) or insertion of mis-translated proteins into the inner membrane (Davis et al. 1986). Evidence has suggested that mistranslated proteins brought to the membrane activate the two-component stress response sensor Cpx which then may also activate ArcA a redox-responsive two-component transcription factor leading to respiratory and metabolic changes (Kohanski et al. 2008). These changes are proposed to lead to oxidative stress, production of hydroxyl radicals and cell death (Kohanski et al. 2008). However, for aminoglycosides such as gentamicin there is strong evidence for a link between antibiotic uptake provided by the PMF (Taber et al. 1987) and toxicity that is independent of ROS formation. It is suggested that $\Delta\psi$, the electrical component of the PMF, provides the main driving force for the uptake of positively-charged aminoglycosides (Taber et al. 1987; Farha et al. 2013). Indeed, aminoglycoside uptake has been shown to be related to $\Delta\psi$ in *E. coli*, *Staphylococcus aureus* and *Bacillus subtilis* (Taber et al. 1987). Furthermore, the production of ROS by antibiotics has been disputed with one study showing that antibiotic treatment did not result in increased hydrogen peroxide formation or increased intracellular free iron in *E. coli* (Liu and Imlay 2013).

There are a number of well-characterised uncouplers of the bacterial PMF such as 2,4-dinitrophenol (DNP) and carbonylcyanide m-chloromethoxy phenylhydrazone (CCCP) (Lewis et al. 1994). DNP acts as a proton ionophore providing a path to transport protons across the membrane uncoupling the PMF (Gage and Neidhardt 1993). Dissipation of the electrical component of the PMF in *E. coli* K-12 using DNP has been shown to inhibit dihydrostreptomycin uptake and susceptibility (Campbell and Kadner 1980). The respiratory inhibitor nitric oxide (NO) provides resistance to gentamicin in pathogenic *E. coli* (Ribeiro et al. 2021) and *Salmonella enterica* (McCollister et al. 2011), and the latter study attributed an observed decrease in gentamicin uptake resulted from NO-mediated inhibition of the terminal oxidase complexes of the respiratory chain (McCollister et al. 2011). While there is considerable evidence in separate studies to implicate both the

maintenance of PMF and ROS production by the respiratory chain as potentiators of aminoglycoside lethality, there is a notable lack of comparative studies where the two mechanisms are assessed side-by-side. Hence, this chapter sought to assess how the interplay between ROS, PMF and respiratory activity affects the lethality of gentamicin.

3.3. Results

3.3.1. Gentamicin elicits a small increase in ROS in aerobically-grown *E. coli*

Previous studies have reported that gentamicin leads to increased ROS levels in nonpathogenic *E. coli* with H₂O₂ being the most notable increase, although these statistically significant increases were marginal and drastically lower than for other antibiotics (Dwyer et al. 2014). Hence, it was of interest to quantify gentamicin-mediated increases in ROS in the multidrug-resistant *E. coli* clinical isolate, with a particular focus on H₂O₂. The first approach was an indirect measure using a catalase assay to measure the catalase activity in the cytosol (*KatE* and *KatG*) by the decomposition of H₂O₂ (Fig. 3.1A): if elevated peroxide is present then these cells will express higher levels of catalase. Catalase-peroxidase hydroperoxidases I (*KatG*) and II (*KatE*) are both known to be located in the cytoplasm (Hillar et al. 1999; Kumar and Imlay 2013). NADH peroxidase (*ahpCF*) can also scavenge H₂O₂ and is located in the cytoplasm (Kumar and Imlay 2013). To account for the known effects of gentamicin on protein translation a markwell assay was first conducted to allow the catalase assay results to be adjusted to the total protein present in the cell extract. The total protein level in the cell extract was lower for the 100 µg/mL gentamicin exposed sample (58% of the the 0 µg/mL gentamicin exposed sample). One-way ANOVA results showed there was a significant difference between the three treatment groups of the the catalase assay ($F(2,24) = 88.34$, $P < 0.0001$) (Fig. 3.1A). As a positive control, exposure to H₂O₂ (0.1 mM) was shown to result in a 4-fold increase in catalase activity compared to the 0 µg/mL gentamicin sample (Tukey post-hoc test $P < 0.0001$). However, on addition of 100 µg/mL gentamicin only a small increase in catalase activity was observed that is not statistically significant (Tukey post-hoc test $P = 0.3117$). During initial trials other gentamicin concentrations were also tested with little impact seen on the catalase activity (Appendix A.1). As an alternative approach to measure gentamicin-mediated ROS generation, a 2',7'-

dichlorodihydrofluorescein diacetate based fluorescent dye was used to directly measure the ROS present in a flow cytometer (Figure 2.3). A small increase in ROS production can be seen at both 30 min and 90 min after the addition of 100 µg/mL gentamicin (Fig. 3.1B) compared to the negative control sample (+ Milli-Q H₂O). A native-PAGE gel catalase assay using the cell extract from (Fig. 3.1A) was then carried out as a more direct method of measuring the KatE and KatG expression levels (Fig. 3.1C+D). Cell extract from *E. coli* exposed as in Figure 3.1A to 0 µg/mL gentamicin, 0.1 mM H₂O₂ or 100 µg/mL gentamicin was run on a native-PAGE gel before incubating the gel with H₂O₂ and then stained by a ferric chloride-potassium ferricyanide (III) solution. Potassium ferricyanide (III) reacts with H₂O₂ being reduced to potassium ferricyanide (II). Ferric chloride then reacts with potassium ferricyanide (II) to form a blue/green insoluble precipitate. This reaction does not happen where KatE and KatG have decomposed the H₂O₂ leaving a white band proportional in size to KatE and KatG activity. For a loading control an additional identical native-PAGE gel was run and stained with Coomassie blue which displayed identical loading of each cell extract sample (Appendix A.2). The bovine catalase positive control produced a bright band on the catalase activity gel (Fig. 3.1C) but did not show on the Coomassie stained gel (Appendix A.2) likely due to the low amount loaded (0.26 ng) being below the detection limit. *E. coli* KatG is 320 kDa whilst KatE is 336 kDa with the theoretical pI calculated from uniprot being 5.14 for KatG and 5.54 for KatE. As these values are close KatE and KatG are likely to migrate to a similar point on the native-PAGE gel. *E. coli* KatG has previously been shown to migrate further into a native-PAGE gel than KatE (Dong et al. 2009) which would be expected due to the lower molecular weight and lower pI. The brighter band is likely therefore to be KatE whilst the duller band below is likely to be KatG. A clear increase in KatE/KatG activity is seen when exposed to exposure to H₂O₂ (0.1 mM). A small increase in catalase activity is also seen in the 100 µg/mL gentamicin exposed samples compared to the 0 µg/mL gentamicin exposed samples. However, analysis on the band intensities (Fig. 3.1D) again showed this to be not statistically significant (One-way ANOVA ($F(2,6) = 376.8$, $P < 0.0001$, Tukey post-hoc test $P = 0.7776$).

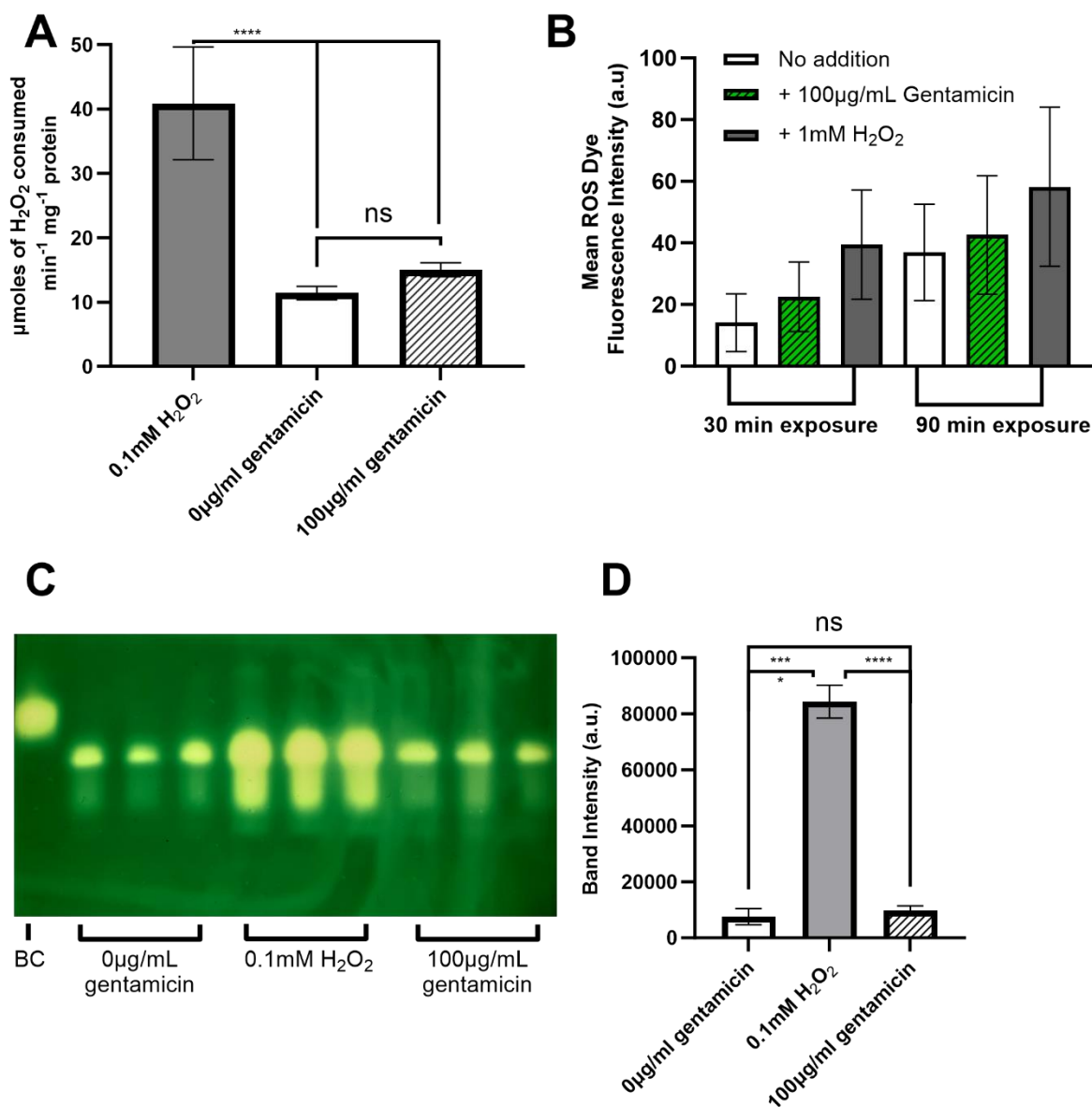


Figure 3.1 - Gentamicin elicits a minimal increase in reactive oxygen species in aerobically grown *E. coli*. (A) Suspensions of 1.6×10^8 cells/ml of *E. coli* EC958 grown in M9 minimal medium supplemented with 0.1% casamino acids were exposed to a sub-lethal level of hydrogen peroxide (0.1 mM), 0 μg/mL gentamicin or 100 μg/ml gentamicin for 30 min with three biological repeats each with three technical repeats completed for each. Cells were then washed, sonicated, cell debris was removed and catalase activity was measured in the cell-free extract. (One-way ANOVA with Tukey post-hoc tests: ****: P -value <0.0001 , n.s (not significant): P -value >0.05). (B) A fluorescent sensitive dye was used to detect ROS in aerobically-growing *E. coli* EC958 using a BD FACSJAZZ™ flow cytometer. Whole cells were selected using DAPI staining and gentamicin-mediated changes in fluorescence were analysed using fluorescent intensity of the ROS dye. At least 60000 cells were sampled for each condition with mean values displayed. (C) Native-PAGE catalase activity gel. *E. coli* EC958 cells exposed and extract prepared as in (A). 10 μg of each cell extract was loaded

from cells exposed to hydrogen peroxide (0.1 mM), 0 µg/mL gentamicin or 100 µg/mL gentamicin. 10 units of bovine catalase (BC) loaded as a positive control. Gel exposed to 4 mM H₂O₂ before staining with ferric chloride-potassium ferricyanide solution. (D) ImageJ analysis of band intensities conducted on the KatE/KatG activity bands (One-way ANOVA with Tukey post-hoc tests: ****: *P*-value <0.0001, n.s (not significant): *P*-value >0.05).

3.3.2. Dissipation of the proton motive force diminishes gentamicin lethality in aerobically-grown cells

The effect of the PMF uncoupler DNP on gentamicin susceptibility was tested on aerobically-grown *E. coli* EC958 cells. Exponentially-growing cells were pre-exposed to 1mM DNP for 30 min before addition of varying gentamicin concentrations. The presence of DNP resulted in a significant decrease in gentamicin lethality (Fig. 3.2A). The IC₅₀ value without DNP was 5 µg/mL which is consistent with the EUCAST MIC values for gentamicin and *E. coli* at >4 mg/L (Jakobsen et al. 2007). The IC₅₀ value with DNP was 45 µg/mL resulting in a 600-fold (3.15 log₁₀) increase in % survival at 50 µg/mL gentamicin.

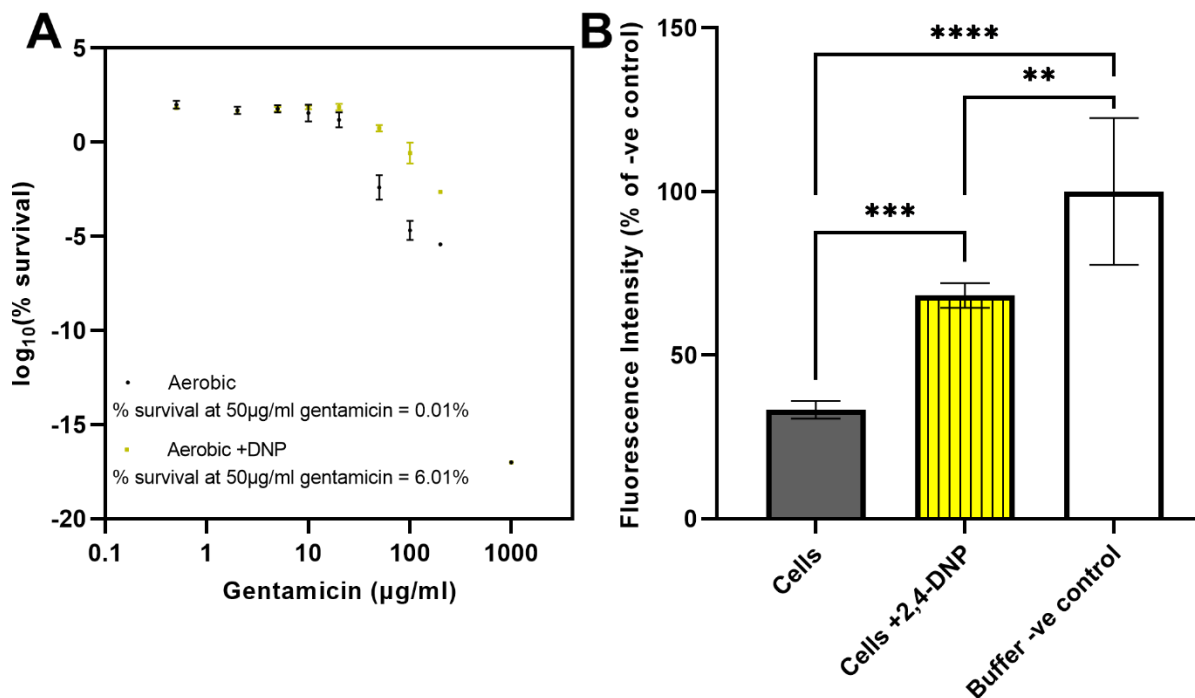
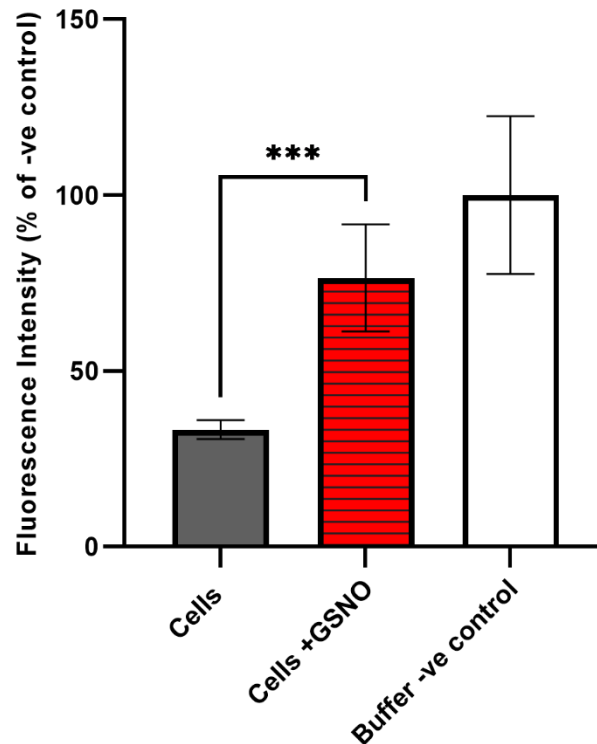


Figure 3.2 - Dissipation of the proton motive force leads to a significant decrease in gentamicin toxicity (A) *E. coli* EC958 cells grown aerobically in M9 minimal media supplemented with 0.1% casamino acids were exposed to +/-1 mM DNP for 30 min followed by incubation with varying concentrations of gentamicin for 90 min. This was followed by serial dilutions, spotting on LB-agar and overnight incubation at 37 °C to enumerate CFU/mL. Mean % survival values are shown +/- standard deviation from 2 biological repeats each with 3 technical repeats. (B) *E. coli* EC958 cells grown aerobically in M9 minimal media supplemented with 0.1% casamino acids were exposed to +/-1 mM DNP for 30 min. Cells were harvested and the electrical potential of the PMF was measured with a plate reader using the fluorescent dye DiSC₃(5) (2 µM). Control experiments were performed without DNP and without cells (i.e. buffer only). Data in the figure are at time point 2452s and are averages of six repeats including two biological repeats for each condition. Data expressed as % relative to the negative control. (One-way ANOVA with Tukey post-hoc tests: ****: *P*-value <0.0001, ***: *P*-value <0.001, **: *P*-value <0.01).

To confirm that DNP is indeed dissipating the PMF, a fluorescent voltage sensitive dye DiSC₃(5) was used to measure the electrical component of the PMF: DiSC₃(5) associates with the negatively charged inner membrane when the PMF is high and is released upon membrane depolarization resulting in an increase in fluorescence (te Winkel et al. 2016). *E. coli* EC958 ST131 was again pre-exposed to 1 mM DNP for 30 min before PMF quantitation with DiSC₃(5), and a positive control was performed for 'cells without DNP' (Fig. 3.2B). A negative control was performed without cells or DNP (i.e. buffer only) to measure the maximum fluorescence of the free dye. A clear increase in fluorescence is observed of approximately 2-fold between cells exposed to DNP and those not exposed to DNP. Since the NO-donor GSNO, a respiratory inhibitor, has previously been shown to provide resistance to gentamicin in the pathogenic *E. coli* EC958 ST131 strain (Ribeiro et al. 2021),

it was also of interest to confirm that GSNO can dissipate the PMF, which indeed it can (Fig. 3.3).

Figure 3.3 - GSNO dissipates the proton motive force. Suspensions of 10^8 cells/mL of *E. coli* EC958 ST131 grown in M9 minimal media supplemented with 0.1 % casamino acids were exposed to +/- 15mM GSNO for 30 min. Cells were re-suspended in 5 mM HEPES buffer, pH 7.2, containing 5 mM glucose to 1.6×10^8 CFU/mL. 5mM HEPES buffer, pH 7.2, containing 5mM glucose, 100mM KCl, 1% DMSO and $2\mu\text{M}$ DiSC₃(5) was added to wells of a 96-well plate and baseline fluorescence was monitored for 90 s in a plate reader using excitation and emission wavelengths of 584 nm and 655 nm, respectively. Cells were then added to a



final density of 4×10^7 CFU/mL and fluorescence was recorded every 30 s for a further 2610s. Data are averages of six repeats including two biological repeats for each condition. Negative control was included of 5 mM HEPES buffer, pH 7.2, containing 5 mM glucose, 100mM KCl, 1% DMSO and $2\mu\text{M}$ DiSC₃(5). Data taken at time point 2452s. Data expressed as % relative to the negative control. (One-way ANOVA with Tukey post-hoc tests: ***: P -value <0.001).

3.3.3. The uncoupler DNP does not alter respiratory oxygen consumption but results in an increase in ROS production

The influence of 1 mM DNP upon the respiration rate of *E. coli* EC958 was measured via monitoring oxygen consumption in an oxygen electrode (Fig. 3.4A, Appendix A.3). A

minor decrease in oxygen consumption of aerobically-grown cells was observed in response to 1 mM DNP that is not statistically significant (One-way ANOVA ($F(3, 8) = 1.76$, $P=0.2322$)). An ethanol control was also performed as DNP was solubilised in this solvent, which elicited a small rise in oxygen consumption that was not statistically significant (One-way ANOVA ($F(3, 8) = 1.76$, $P=0.2322$)).

The influence of the alternative more potent uncoupler FCCP upon the respiration rate of *E. coli* EC958 was also studied (Fig. 3.5). At 10 μ M only a minor increase in the respiration rate was seen that is not statistically significant (unpaired t-test $P=0.7871$).

Interestingly, DNP treatment produced a noticeable increase in catalase activity (Fig. 3.4B) indicating that DNP is promoting the production of ROS species (One-way ANOVA ($F(3, 7) = 734.9$, $P<0.0001$, Tukey post-hoc test $P<0.0001$)). The ethanol control also resulted in an increase in catalase activity, although DNP exposure did result in a statistically significant increase in catalase activity compared to this control ((One-way ANOVA ($F(3, 7) = 734.9$, $P<0.0001$, Tukey post-hoc test $P=0.0013$)).

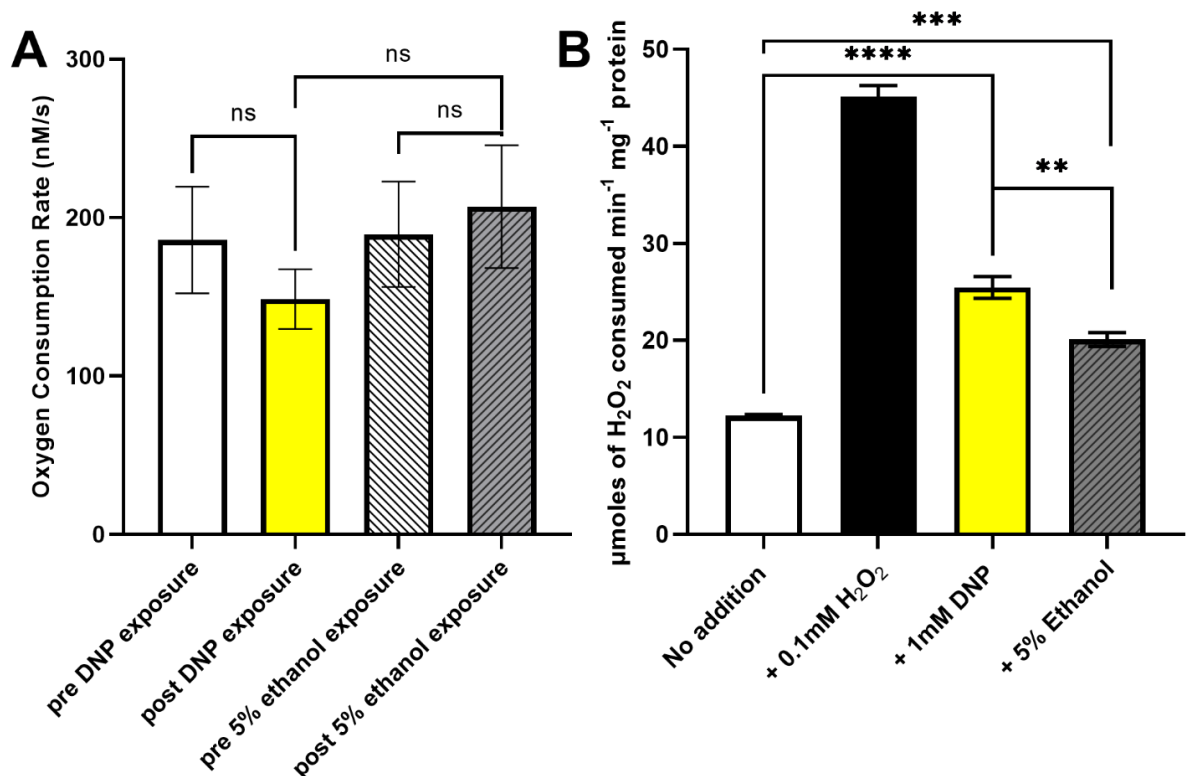


Figure 3.4 - Dissipation of the proton motive force does not alter respiratory oxygen consumption but increases ROS. (A) Suspensions of 4.8×10^8 CFU/mL of *E. coli* EC958 grown aerobically were diluted into fresh M9 minimal medium supplemented with 0.1 % casamino acids to 8.0×10^7 CFU/mL in the Rank Brothers Clark oxygen electrode chamber after a baseline of 5 min was recorded. Voltage data was collected for 8 min followed by the addition of the plunger to exclude external oxygen and data was recorded for another 10 min. DNP (1 mM final) was injected and data recorded until all oxygen was consumed. Since, DNP was dissolved in ethanol, control experiments were performed with this solvent. (One-way ANOVA with Tukey post-hoc tests: n.s (not significant): P -value >0.05) (B) Suspensions of 1.6×10^8 cells/ml of *E. coli* EC958 grown in M9 minimal medium supplemented with 0.1% casamino acids were exposed to a sub-lethal level of hydrogen peroxide (0.1 mM), +/- 1mM DNP or 5% ethanol for 30 min. Cells were then washed, cell debris was removed and catalase activity was measured in the cell-free extract. (One-way ANOVA with Tukey post-hoc tests: ****: P -value <0.0001 , ***: P -value <0.001 , **: P -value <0.01).

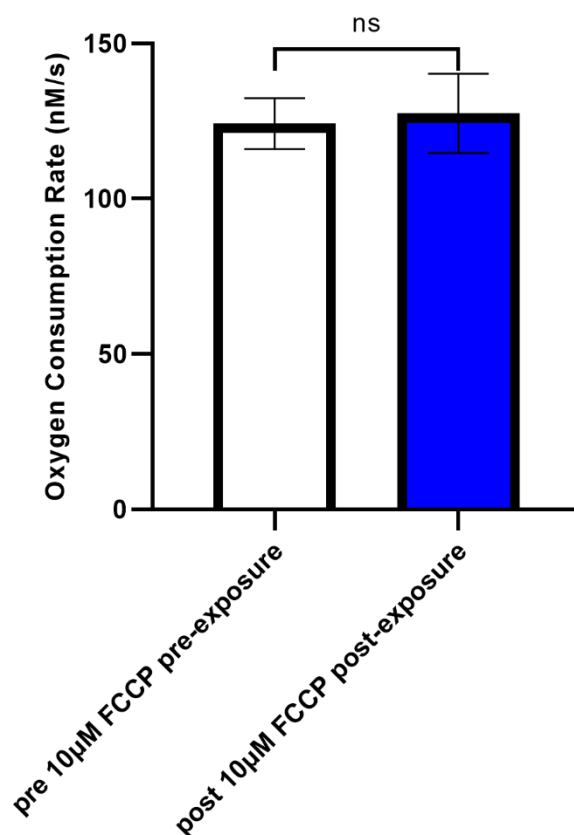


Figure 3.5 - Measurement of respiratory oxygen consumption in response to FCCP. Suspensions of 4.8×10^8 CFU/mL of *E. coli* EC958 grown aerobically were diluted into fresh M9 minimal medium supplemented with 0.1 % casamino acids to 8.0×10^7 CFU/mL in the Rank Brothers Clark oxygen electrode chamber after a baseline of 5 min was recorded. Voltage data was collected for 8 min followed by the addition of the plunger to exclude external oxygen and data was recorded for another 10 min. FCCP (10 µM final) was injected and data recorded until all oxygen was consumed. Since, FCCP was dissolved in ethanol, control experiments were performed with this solvent at 5%. (Student's two-tailed unpaired *t*-test n.s (not significant): *P*-value >0.05)

3.3.4. Dissipation of the proton motive force diminishes gentamicin lethality in anaerobially-grown cells

As demonstrated above, dissipation of the PMF by DNP under aerobic conditions diminishes gentamicin lethality (Fig. 3.2A), and this effect was not due to alleviation of ROS production by DNP (Fig. 3.4B): indeed, quite the opposite. To analyse the effects of DNP-mediated PMF disruption upon gentamicin lethality in isolation from ROS, viability assays

were performed under anaerobic conditions (i.e. where ROS production by the respiratory chain is excluded). To ensure that cells were performing anaerobic respiration, control experiments were performed in the absence of exogenous electron acceptors which abolished growth (Fig. 3.6). Pre-exposure of *E. coli* to 1mM DNP for 30 min at OD₆₀₀ = 0.1 prior to addition of varying gentamicin concentrations resulted in a large decrease in gentamicin lethality (Fig. 3.7). The change in IC₅₀ value resulting from DNP exposure could not be quantified due to the complete abolition of gentamicin toxicity by DNP, although it was noted that in the absence of DNP gentamicin toxicity was diminished (IC₅₀ = 91.19 µg/mL) compared to aerobic growth (IC₅₀ = 5 µg/mL, Fig. 3.2A). This might suggest a role for anaerobic respiratory complexes in gentamicin lethality, although this is speculative at this stage and will require further work to investigate this hypothesis.

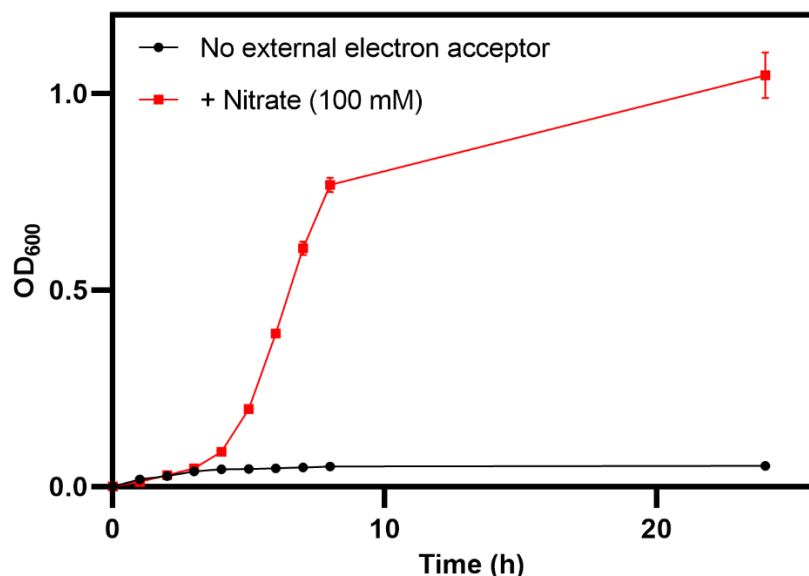


Figure 3.6 - Lack of exogenous electron acceptor prevents growth in anaerobic conditions. *E. coli* EC958 was grown under anaerobic conditions in M9 minimal medium supplemented with 0.1 % (w/v) casamino acids (no external electron acceptor) or 0.1 % casamino acids and sodium nitrate (100 mM) to provide the exogenous electron acceptor. Mean OD₆₀₀ values are shown +/- standard deviation from 3 biological repeats.

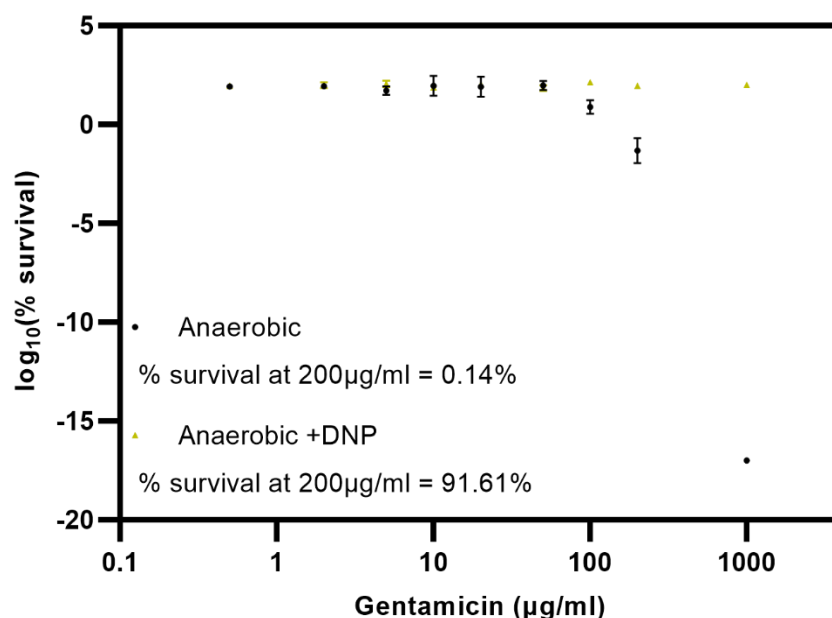


Figure 3.7 - The uncoupler DNP elicits a decrease in gentamicin toxicity under anaerobic conditions. *E. coli* EC958 cells grown anaerobically in M9 minimal media supplemented with 0.1% casamino acids (w/v), 100 mM sodium nitrate and 0.2% glycerol (v/v) were exposed to +/-1 mM DNP for 30 min followed by incubation with varying concentrations of gentamicin for 90 min. This was followed by serial dilutions, spotting on LB-agar and overnight incubation at 37 °C to enumerate CFU/mL. Mean % survival values are shown +/- standard deviation from 2 biological repeats each with 3 technical repeats.

3.4. Discussion

It is well-known that respiratory activity can promote the lethality of many antibiotics, and there is strong evidence to support the role of ROS generation by the aerobic respiratory chain in the lethality of a number of different antibiotic classes (reviewed in (Stokes et al. 2019)). However, the interplay between ROS, respiratory activity and aminoglycoside lethality has remained less clear, and the maintenance of PMF provides an alternative mechanism for the respiratory chain to potentiate the lethality of aminoglycosides (i.e. via promoting the uptake of positively charged aminoglycosides). Hence, this chapter sought to investigate the competing ROS vs. PMF hypotheses under comparable conditions to assess the underpinning mechanism for gentamicin lethality for a clinically-relevant *E. coli* strain. The first question to answer was: does exposure of *E. coli* to gentamicin result in the production of ROS? To answer this simple question, the three approaches of a catalase assay, direct ROS measurement and a native-PAGE KatE/KatG

catalase assay were undertaken and all approaches demonstrated that gentamicin exposure does not result in a significant increase in ROS under the conditions tested (Fig. 3.1). It has previously been shown that the 30 min exposure to 0.1 mM H₂O₂ used in this investigation can upregulate *E. coli* KatG and increase survivability against oxidative stress (Rodríguez-Rojas et al. 2020). The experiments were performed over a concentration range that is known to be toxic to this strain under the conditions tested and provides very good evidence to support the hypothesis that lethal doses of gentamicin do not elicit significant increases in ROS. The small increase in ROS production seen in the direct ROS measurements (Fig. 3.1B) could be due to the detection of superoxide that the 2',7'-dichlorodihydrofluorescein diacetate based fluorescent dye can detect (Freemerman et al. 2014) as opposed to the catalase assays being specific for H₂O₂ detection. Another aminoglycoside kanamycin has also been shown not to increase hydrogen peroxide levels in *E. coli* (Lio and Imlay 2013).

The respiratory uncoupler DNP has been previously used to demonstrate that dissipation of the PMF significantly diminishes the lethality of the aminoglycoside dihydrostreptomycin (Campbell et al. 1980). To verify that this is the case under the same conditions that ROS species were measured, aerobically-grown *E. coli* EC958 was exposed to 1 mM DNP over a range of gentamicin concentrations and viable counts were measured. These data confirm that DNP elicits a significant decrease in gentamicin lethality (Fig. 3.2A), consistent with the hypothesis that maintenance of the PMF by respiratory activity potentiates the bactericidal effects of gentamicin. To further investigate this, 1 mM DNP was confirmed to dissipate the electrical potential of the inner membrane as expected (Fig. 3.2B). Since the respiratory inhibitor and NO-donor GSNO has previously been shown to diminish gentamicin lethality (Ribeiro et al. 2021), it was of interest to investigate the hypothesis that exposure to GSNO could dissipate the PMF. This was tested under the conditions used in this previous study, and a significant dissipation of the membrane electrical potential was observed in response to GSNO (Fig. 3.3), a magnitude change comparable to that of 1 mM DNP. Significant effort was made to investigate whether GSNO elevated ROS production using the fluorescent dye approach described herein, but the background fluorescence generated by GSNO was far too high to generate reliable and

reproducible results. Furthermore, the catalase assay could not be used as this enzyme utilises a haem cofactor and is well-known to be inhibited by NO (Titov and Osipov 2017). Nevertheless, transcriptomics studies have been undertaken where *E. coli* was exposed to GSNO and no perturbations in ROS stress responses were detected (Flatley et al. 2005). Taken together with the data described herein, this supports the hypothesis that inhibition of aerobic respiration by GSNO diminishes the lethality of gentamicin through dissipation of the PMF and not alleviation of ROS generation by the respiratory chain. Indeed, our observations are consistent with previous work on *Salmonella* where NO-mediated respiratory inhibition diminished the toxicity of aminoglycosides through a mechanism involving diminished gentamicin uptake (McCollister et al. 2011).

It has previously been reported that protonophores such as FCCP and DNP can block translocation of periplasmic and membrane proteins in *E. coli* leading to aggregation and induced heat shock response with an increased level of the heat shock regulator protein sigma-32 (Jana et al. 2009). The heat shock protease ClpP is implicated in this process (Jana et al. 2009) and upregulation of the heat shock proteases has been linked to increased resistance to antimicrobial stress in *Acinetobacter baumannii* (Lazaretti et al. 2020)(Cardoso et al. 2010) and *E. coli* (Yamaguchi et al. 2003)((Kohanski et al. 2008). DNP has previously been shown to upregulate several heat shock responses in *E. coli* (Gage and Neidhardt 1993), so it is important to consider that DNP-induced stress response may contribute to the DNP-mediated increases in resistance to gentamicin observed herein (Fig.3.2A and Fig 3.7). However, the well-characterised uncoupling action (and resulting diminished aminoglycoside uptake) seems a more probable explanation for the DNP-mediated decrease in gentamicin lethality.

To further investigate potential influences in oxygen utilisation by DNP, oxygen electrode experiments were performed. These data demonstrate that DNP did not elicit any significant change in respiratory oxygen consumption at 1 mM (Fig. 3.4A), which is consistent with previous studies using 1 mM DNP (Goto and Anraku 1974) as DNP is a protonophore that allows protons to leak across the normally proton-impermeable cytoplasmic membrane inhibiting PMF-dependent transport systems such as gentamicin uptake with little effect on the cellular respiration (Taber et al. 1987). As a control the more

potent uncoupler FCCP also did not show any significant change in oxygen consumption at 10 μ M (Fig. 3.5). It is acknowledged that for these experiments cells were exposed to uncouplers for slightly less time than the 30 min pre-exposures used for some other figures, although it is not anticipated that a marginal increase in exposure time would elicit an increase in oxygen consumption. It was interesting to observe an increase in catalase activity in cells that were exposed to DNP, especially as DNP protects the cells against gentamicin lethality (Fig. 3.2A). Two explanations therefore present themselves: i) The effects of DNP-mediated increase in ROS upon cell survival are insignificant compared to the DNP-mediated decrease in PMF that protect *E. coli* from gentamicin uptake ROS are not; ii) pre-exposure to DNP activates the ROS stress response systems and renders the cells more tolerant to gentamicin-mediated ROS toxicity possibly through upregulation of KatE and KatG. However, given that gentamicin does not elicit the formation of significant levels of ROS in this study (Fig. 3.1), it is more likely that the protective effects of DNP-mediated membrane depolarisation (Fig. 3.2B) outweigh any toxicity issues resulting from elevated ROS due to DNP.

Finally, as aminoglycoside antibiotics work on *E. coli* grown under anaerobic conditions, it is logical to hypothesise that DNP will also diminish the lethality of gentamicin under these conditions. To test this hypothesis, *E. coli* EC958 was grown under anaerobic conditions and viability assays were performed following pre-exposure to 1 mM DNP followed by addition of various concentrations of gentamicin (Fig. 3.7). These data support the hypothesis that dissipation of the PMF by DNP can abolish the lethality of gentamicin under conditions that exclude the possibility of ROS generation by the respiratory chain. This further supports the hypothesis that DNP diminishes the lethality of gentamicin through dissipation of the PMF and not alleviation of ROS generation by the respiratory chain.

Chapter 4

**Phylogenetic and structural analysis
of cytochrome *bd***

4.1. Summary

Cytochrome *bd* is a terminal respiratory oxidase that is present in many pathogenic bacteria, with several structural variants having been characterised. Cytochrome *bd* is also present in some archaeal aerobic respiratory chains. Cytochrome *bd* is an important target for the development of new antimicrobial drugs due to its implications in bacterial virulence, resistance to external stress and that it is only present in prokaryotes. This chapter seeks to understand the diversity of cytochrome *bd* variants across bacterial and archaeal phyla, and to provide a structural focus that could in future be applied to antimicrobial drug discovery.

Firstly a pipeline for identifying remote homologues using hhsearch was performed on a variety of bacterial proteomes using *E. coli* cytochrome *bd*-I and *bd*-II as the search templates. A BLAST search was also conducted to detect archaeal cytochrome *bd* homologues. The phylogenetic results confirmed that cytochrome *bd* oxidases can be grouped into short and long Q-loop oxidases, as previously reported. Further analysis revealed that the short loop oxidases are shown to be split into 2 phylogenetically related groups. An attempt was made to characterise the cyanide insensitive oxidases (CIOs), and a small selection of bacteria were found to encode cytochrome *bd* complexes that are similar to the CIO complex of *Pseudomonas aeruginosa*. However, no sequence motifs related to the CIOs could be determined with both short and long Q-loop CIO being present.

E. coli cytochrome *bd*-I and *G. thermodenitrificans* cytochrome *bd* structures have the haem *b*₅₉₅ and haem *d* cofactors in the opposite arrangement. Phylogenetic evidence was generated that supports the hypothesis that the presence or absence of a residue at position 101 (*E. coli* *bd*-I numbering) provides an indication of which haem arrangement is present in the structure. Furthermore, the oxygen entry tunnels were modelled for both *E. coli* cytochrome *bd*-I and *G. thermodenitrificans* cytochrome *bd* structures. Conservation of residues in the oxygen entry channel regions was used to provide further clues to haem arrangement. The *E. coli* oxygen entry channel has a small hydrophobic exit pore potentially suitable to target with small hydrophobic inhibitors.

The quinol binding site in CydB of *E. coli* cytochrome *bd*-I was investigated as to its potential role. Prediction of the proton pathways was used along with 3DLigandSite to predict that this second ubiquinone-8 probably has a predominantly structural role. Molecular docking analysis suggests that ubiquinone-8 binds very tightly to the CydB site and consequently would be a difficult target for competitive inhibitors.

4.2. Introduction

Cytochrome *bd* is a prokaryotic terminal respiratory oxidase involved in virulence in several drug resistant bacterial pathogens including strains of including *E. coli* (Shepherd et al. 2016), *Mycobacterium* (Cai et al. 2021) and *Salmonella* (Jones-Carson et al. 2016).

Previous phylogenetic analysis has shown that cytochrome *bd* is present in both bacteria and archaea (Borisov et al. 2011a). Cytochrome *bd* oxidases can be sub-divided based on the Q-loop size (the Q-loop is located between the transmembrane helices 6 and 7 of CydA forming part of the quinol binding site (Safarian et al. 2016)). Long Q-loop oxidases having an extended C-terminal part of the Q-loop whilst short Q-loop oxidases are missing this section (Borisov et al. 2011a). Multiple duplications of cytochrome *bd* genes have been observed leading to some organisms having multiple different cytochrome *bd* operons, including *E. coli* which encodes both a *bd*-I (CydABXH) and *bd*-II (AppCBX) (Esposti et al. 2015; Murali Ranjani et al. 2021). There is also CydAA' type cytochrome *bd* oxidases only found in archaea resulting from *cydA* duplication (Murali Ranjani et al. 2021). Evidence for horizontal gene transfer of whole operons (Esposti et al. 2015) and *cydX* (Allen et al. 2014) has also been proposed, which complicates the evolutionary picture. Furthermore, asymmetric evolution has been reported, with *cydB* reported to have evolved 1.2 times faster than *cydA* (Hao and Golding 2006).

Cytochrome *bd* oxidases that show no spectroscopic signature of haem *d* (Borisov et al. 2011a) and usually display resistance to cyanide are classed as cyanide insensitive oxidases (CIO). The CIO from *Gluconobacter oxydans* is currently the only reported CIO to produce the spectral peaks for haem *d* (Miura et al. 2013). Currently no sequence motifs have been reported that can be used to identify cytochrome *bd* complexes as being CIO,

and no structures exist (Murali et al. 2021). CIO have been identified in *B. subtilis* (Azarkina et al. 1999), *P. aeruginosa* (Cunningham et al. 1997) and *C. jejuni* (Jackson et al. 2007). CIO in Proteobacteria were categorised into 3 groups by (Esposti et al. 2015) using gene clusters.

Recently solved cryo-EM or X-ray crystallography cytochrome *bd* structures have become available for *G. thermodenitrificans* (Safarian et al. 2016) and *E. coli* (Safarian et al. 2019; Theßeling et al. 2019; Grauel et al. 2021; Grund et al. 2021). This has allowed a more structure-based approach, which complements the phylogenetic analyses conducted in this chapter.

E. coli cytochrome *bd*-I and *bd*-II both have a long Q-loop whereas the *G. thermodenitrificans* cytochrome *bd* has a short Q-loop. Interestingly, the arrangement of the haem ligands *b*₅₉₅ and haem *d* is reversed between the *G. thermodenitrificans* cytochrome *bd* structure (Safarian et al. 2016) and that of *E. coli* cytochrome *bd*-I (Safarian et al. 2019; Theßeling et al. 2019) and cytochrome *bd*-II (Grauel et al. 2021; Grund et al. 2021). *E. coli* cytochrome *bd*-I also has an extra single alpha helix subunit called CydH encoded by the gene *ynhF* that is not part of the *cydABX* operon (Safarian et al. 2019). The observed differences in the structures and subunit composition have led to the suggestion that different proton and oxygen channels are present in cytochrome *bd* complexes from these two organisms (Safarian et al. 2019). *E. coli* cytochrome *bd*-I has a second quinol binding site located in CydB with ubiquinone-8 bound in the cleft (Theßeling et al. 2019). The function of this site has not been determined but is suggested as possibly structural (Theßeling et al. 2019). As a supplement to the main quinol binding site in CydA the oxygen and proton channels offer further potential drug targets along with the CydB quinol cleft (Friedrich et al. 2022).

4.3. Results

4.3.1. Development of a pipeline for identifying remote homologues using a cytochrome *bd* template

In order to map cytochrome *bd* variants across different bacterial phyla, a series of 55 bacterial strains were analysed (Table 4.1) using a pipeline for identifying remote

homologues (Fig. 4.1). Representative strains were chosen from a wide variety of bacteria phyla, including a strain from each of the 30 recognised bacteria phyla at the time of the analysis from the LIST (List of Prokaryotic names with Standing in Nomenclature) database (Parte 2014). In addition to this, all of the ESKAPE pathogens were added as cytochrome *bd* is important in pathogenesis of many strains (Borisov et al. 2011a). Also added were strains that were known to contain cytochrome *bd* and were of biochemical interest for further work such as *Pseudomonas aeruginosa* PA14 and *Staphylococcus aureus* USA300 in order to include them in the phylogenetic analysis. Finally, as Eng et al (Eng et al. 2018) reported that cytochrome *bd*-I (CydABXH) and the ABC transporter CydDC do not always co-occur: out of 1965 bacterial genomes searched using the MicrobesOnline tools (Alm et al. 2005), 407 species were found to contain *cydC* whilst only 53 contained a *cydB* homologue (Eng et al. 2018). CydDC has been suggested as being important for biosynthesis of haem *d* (Poole et al. 2019) as a haem transporter (Wu et al. 2022) required for assembly of cytochrome *bd* so the result by Eng and coworkers was surprising, even considering that some cytochrome *bd* do not contain haem *d* being called cyanide-insensitive oxidases (CIO) (Borisov et al. 2011a). Hence, this was investigated further in the current study. Several strains are included that (Eng et al. 2018) report as being *cydDC* positive but *cydB* negative including *Corynebacterium diphtheriae*, *Deinococcus geothermalis*, *Yersinia pestis*, *Salmonella typhimurium*, *Shewanella oneidensis*, *Haemophilus influenzae* and *Bordetella pertussis*. Additional strains were also included that (Eng et al. 2018) reported as negative for both *cydDC* and *cydB* including *G. thermodenitrificans*, *Bacillus subtilis*, *Rickettsia rickettsia*, *Rhodobacter sphaeroides*, *Neisseria meningitidis* and *Helicobacter pylori*. *G. thermodenitrificans* has a known cytochrome *bd* structure (Safarian et al. 2016) and was included to determine if the pipeline for identifying remote homologues protocol would detect it.

The pipeline for identifying remote homologues is shown below in Figure 4.1. The pipeline allows multiple proteomes to be mined for homologues to all of the *E. coli* terminal respiratory complexes using the same python script. hhsearch allows remote homologs to be identified.

The results were first analysed using Interproscan which searches for protein domains and motifs in order to categorise the hhsearch gene results. Furthermore, the NCBI genome browser was used to determine if the identified gene was part of an operon (*cydABX*, *cioAB* or *appCBX*). Using this information, phylogenetic analysis was conducted on the CydA results. MSA analysis was conducted for CydA and CydB.

As some archaea are known to encode cytochrome *bd* (Borisov et al. 2011a) a BLASTP search was conducted using *E. coli* CydA against the UniProtKB Archaea database. Strains were manually selected from the 459 results with the aim of generating a diverse range of archaeal species that contain CydA. The NCBI genome browser was used to check if the identified gene was part of a *cydAB* operon. The pipeline for identifying remote homologues was not utilised for archaea as the main focus of this investigation is targeting cytochrome *bd* in pathogenic *eubacteria*. The BLASTP search allowed a quick sample of archaea cytochrome *bd* to be added to the phylogenetic trees.

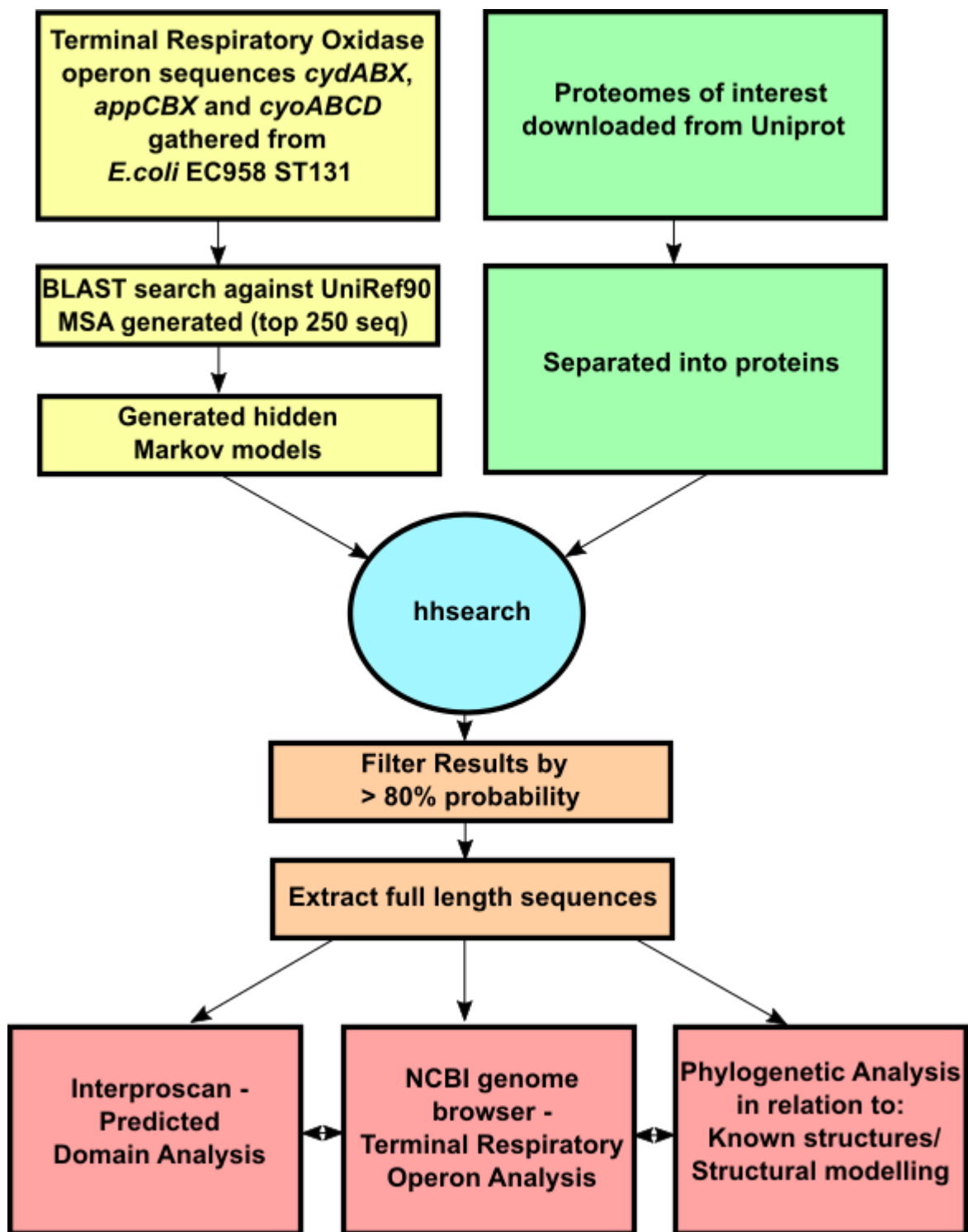


Figure 4.1 - Pipeline for identifying remote homologues for terminal oxidases.

A total of 52 CydA and 51 CydB proteins were identified from the bacteria for further analysis. In addition to this, 23 CydA proteins from archaea were detected via the BLASTP search using *E. coli* EC958 ST131 cytochrome *bd-I* as the input along with 20 CydB proteins.

Phylogenetic and structural analysis of the identified *cydAB* operons was then carried out in the following sections.

Table. 4.1 - List of bacterial strains used for the pipeline for identifying remote homologues. The Cytochrome *bd*? column displays the bacterial strains containing cytochrome *bd* as identified by the hhsearch analysis.

Bacterium	Phyla	Cytochrome <i>bd</i>?
<i>Aminobacterium colombiense</i>	Synergistetes	No
<i>Bacillus Anthracis</i>	Firmicutes	Yes
<i>Bacillus subtilis</i>	Firmicutes	Yes
<i>Bordetella Pertussis</i>	Betaproteobacteria	Yes
<i>Borrelia burgdorferi</i>	Spirochaetes	No
<i>Caldilinea aerophila</i>	Chloroflexi	No
<i>Campylobacter jejuni</i>	Epsilonproteobacteria	Yes
<i>Chlamydia trachomatis</i>	Chlamydiae	Yes
<i>Chlorobium chlorochromatii</i>	Green sulfur bacteria (Chlorobi)	No
<i>Chlorobium limicola</i>	Green sulfur bacteria (Chlorobi)	Yes
<i>Corynebacterium diphtheriae</i>	Actinobacteria	Yes
<i>Deinococcus geothermalis</i>	Deinococcus–Thermus	Yes
<i>Denitrovibrio acetiphilus</i>	Deferribacteres	Yes
<i>Desulfurispirillum indicum</i>	Chrysiogenetes	Yes
<i>Dictyoglomus thermophilum</i>	Dictyoglomi	No
<i>E. coli</i> O25bH4 ST131 EC958	Gammaproteobacteria	Yes
<i>Elusimicrobium minutum</i>	Elusimicrobia	No
<i>Fibrobacter succinogenes</i>	Fibrobacteres	No
<i>Fusobacterium nucleatum</i>	Fusobacteria	No
<i>Gemmatimonas aurantiaca</i>	Gemmatimonadetes	No
<i>Geobacillus thermodenitrificans</i>	Firmicutes	Yes
<i>Gloeobacter violaceus</i>	Cyanobacteria	Yes
<i>Haemophilus influenzae</i>	Gammaproteobacteria	Yes
<i>Helicobacter pylori</i>	Epsilonproteobacteria	No
<i>Lentisphaera araneosa</i>	Lentisphaerae	No
<i>Mycobacterium tuberculosis</i>	Actinobacteria	Yes
<i>Mycoplasma genitalium</i>	Tenericutes	No
<i>Neisseria meningitidis</i>	Betaproteobacteria	No
<i>Nitrospira moscoviensis</i>	Nitrospirae	Yes
<i>P. aeruginosa</i> PAO1	Gammaproteobacteria	Yes
<i>Porphyromonas catoniae</i>	Bacteroidetes	Yes
<i>Rhodobacter sphaeroides</i>	Alphaproteobacteria	Yes
<i>Rhodopirellula baltica</i>	Planctomycetes	Yes
<i>Rickettsia rickettsii</i>	Alphaproteobacteria	Yes
<i>S. aureus</i> NCTC 8325	Firmicutes	Yes
<i>Salmonella typhimurium</i>	Gammaproteobacteria	Yes
<i>Shewanella oneidensis</i>	Gammaproteobacteria	Yes

<i>Sphaerobacter thermophilus</i>	Thermomicrobia	No
<i>Streptococcus pyogenes</i> serotype M1	Firmicutes	No
<i>Streptomyces pratensis</i>	Actinobacteria	Yes
<i>Synechocystis</i>	Cyanobacteria	Yes
<i>Thermocrinis ruber</i>	Aquificae	No
<i>Thermodesulfobacterium geofontis</i>	Thermodesulfobacteria	Yes
<i>Thermotoga maritima</i>	Thermotogae	No
<i>Verrucomicrobium</i>	Verrucomicrobia	No
<i>Vibrio cholerae</i> serotype O1	Gammaproteobacteria	Yes
<i>Vitreoscilla</i>	Betaproteobacteria	Yes
<i>Yersinia pestis</i>	Gammaproteobacteria	Yes
<i>Acinetobacter baumannii</i>	Gammaproteobacteria	Yes
<i>Azotobacter vinelandii</i>	Gammaproteobacteria	Yes
<i>Enterobacter cloacae</i> S611	Gammaproteobacteria	Yes
<i>Enterococcus faecium</i>	Firmicutes	No
<i>Klebsiella pneumoniae</i>	Gammaproteobacteria	Yes
<i>Pseudomonas aeruginosa</i> PA14	Gammaproteobacteria	Yes
<i>Staphylococcus aureus</i> USA300	Firmicutes	Yes

4.3.2. Diversity of cytochrome *bd* terminal oxidases

The bacterial sequences from the hhsearch (Table 4.1) and archaeal sequences from the BLAST search (Appendix A.4) were combined and the phylogenetic relationships were modelled (see methods section 2.3.3 for details). The phylogenetic tree of CydA sequences (Fig. 4.2) highlights the presence of a short or a long Q-loop. Cytochrome *bd* has a quinol binding Q-loop split into an N-terminal and a C-terminal section. Cytochrome *bd* oxidases that are missing the C-terminal section are classified as short Q-loop oxidases and the others long Q-loop oxidases (Hao and Golding 2006). A single group of long Q-loop oxidases is present (Fig. 4.2) including both *E. coli* cytochrome *bd* subunit I's (*bd*-I CydA and *bd*-II appC). *E. coli* CydA and AppC have a % sequence identity of 61% and are phylogenetically very closely related (Fig 4.2). Most of the bacteria containing the long Q-loop oxidase identified are gammaproteobacteria, which has previously been reported (Hao and Golding 2006). Thermophilic Archaea of the *Thermococcus* genus also contain a long Q-loop cytochrome *bd* oxidase (Fig. 4.2). The *Thermococcus* species all have Q-loops around 20 residues shorter than *E. coli* *bd*-I and are accordingly placed in their own group. *Thermococcus* species are strict anaerobes (Jolivet et al. 2003) and are thus likely to possess cytochrome *bd* to detoxify oxygen. All of the Gram-positive bacteria and most of the archaea searched contain a short Q-loop cytochrome *bd* oxidase. According to (Fig. 4.2) these short Q-loop oxidases are split

into two phylogenetically related groups. One group contains firmicutes and archaea, whilst the other contains a large range of gram positive and negative bacteria species plus some archaea (Fig. 4.2).

Cyanide insensitive oxidases (CIO) were also investigated to see if they could be classified with a number of examples reported in (Azarkina et al. 1999; Jackson et al. 2007; Esposti et al. 2015) highlighted in red (Fig. 4.2). CIO complexes contain only *b*-type haems and confer increased resistance to cyanide (Borisov et al. 2011a). A small group of CIOs was identified that are similar to *Pseudomonas aeruginosa* with a short Q-loop (Fig. 4.2). These include CIO's from the Gammaproteobacteria *Azotobacter vinelandii* and the Alphaproteobacteria *Rickettsia rickettsia* (Fig. 4.2). However, *Campylobacter jejuni* CioA is unrelated to *Pseudomonas aeruginosa* CioA containing a long Q-loop. *Bacillus subtilis* CIO (YthA) is also distant to *Pseudomonas aeruginosa* CIO. Additionally, no sequence features could be found that would determine a CIO. Furthermore, the number of these cyanide insensitive oxidases identified from the literature is small making it difficult to find sequence similarities.

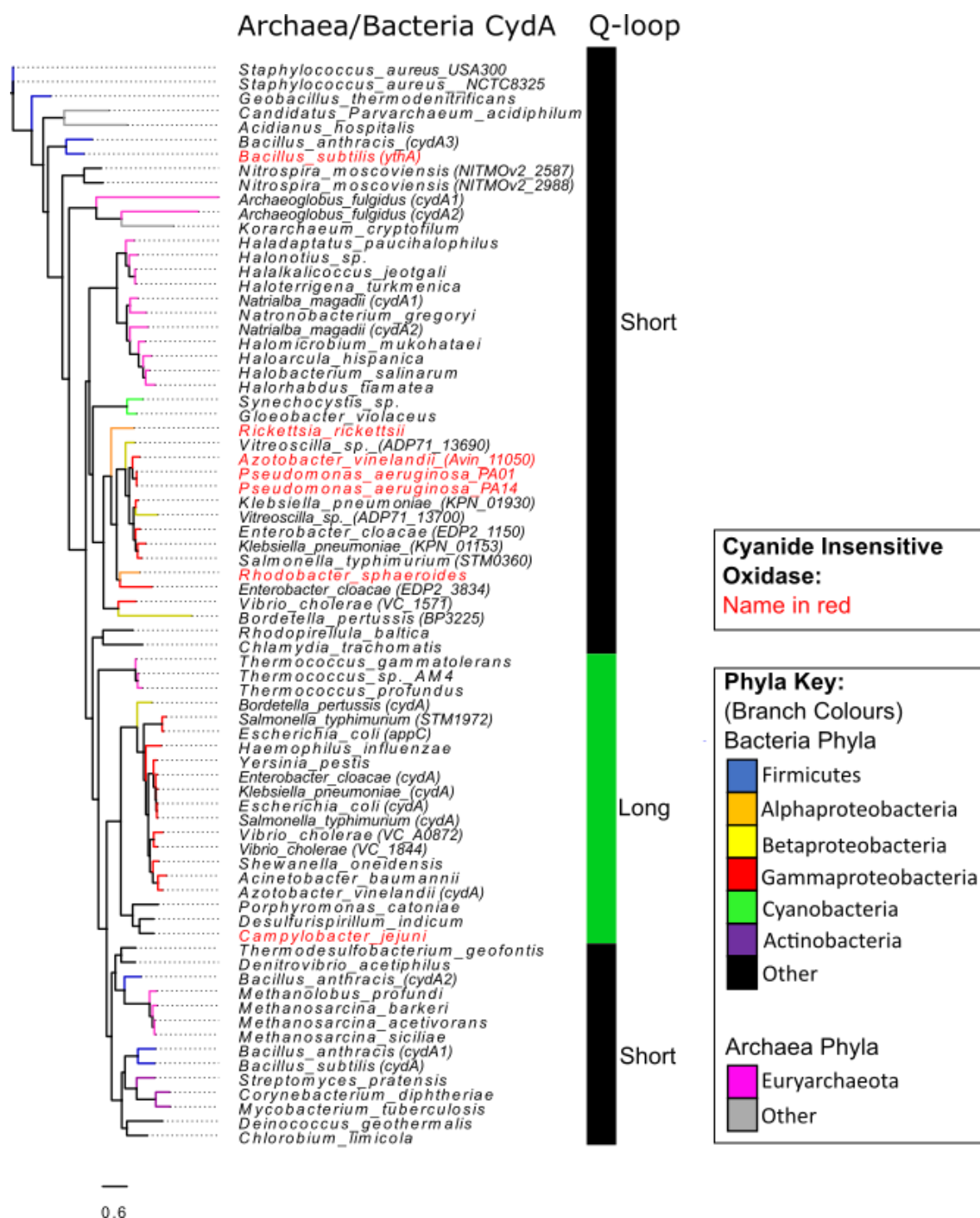


Figure 4.2 - Phylogenetic relationship of the cytochrome *bd*-oxidases CydA subunit from archaea and bacteria. Branches coloured according to phyla. Presence of a long or short Q-loop is indicated. CydA reported as cyanide insensitive are highlighted in red (Azarkina et al. 1999; Jackson et al. 2007; Esposti et al. 2015).

The hhsearch results were compared to previous results by (Eng et al. 2018) which reported that cytochrome *bd*-I (CydABXH) and the ABC transporter CydDC do not always co-occur (see section 4.3.1). Out of the strains that (Eng et al. 2018) reported as being *cydDC* positive but *cydB* negative, all were shown to have a CydA and CydB homologue from the hhsearch (Table 4.2). Searches using the NCBI genome browser showed that all of these strains had the *cydA* and *cydB* genes in a *cydAB* operon. Out of the strains that (Eng et al. 2018) reported as being negative for both *cydDC* and *cydB*, 50% had a CydAB homologue detected by the hhsearch (Table 4.2). This result suggests that the pipeline for identifying remote homologues is more effective at detecting homologues of cytochrome *bd* than high-throughput BLAST based methods such as that provided by MicrobesOnline for comparative genomics.

Table. 4.2 - Results from bacterial strains cytochrome *bd* pipeline for identifying remote homologues using hhsearch compared to results from (Eng et al. 2018). Bacterial strains that (Eng et al. 2018) reported as being negative for both *cydDC* and *cydB* but in which the hhsearch detected a CydAB are highlighted in yellow. Bacterial strains that (Eng et al. 2018) reported as being *cydDC* positive but *cydB* negative but in which the hhsearch detected a CydAB are highlighted in green.

Bacterium	Phyla	<i>cydB</i> homologue to <i>E. coli cydB</i> according to (Eng et al. 2018)	<i>cydDC</i> homologue to <i>E. coli cydDC</i> according to (Eng et al. 2018)	CydAB detected from hhsearch analysis
<i>Aminobacterium colombiense</i>	Synergistetes	No	No	No
<i>Bacillus anthracis</i>	Firmicutes	No	No	Yes
<i>Bacillus subtilis</i>	Firmicutes	No	No	Yes
<i>Bordetella Pertussis</i>	Betaproteobacteria	No	Yes	Yes
<i>Borrelia burgdorferi</i>	Spirochaetes	No	No	No
<i>Caldilinea aerophila</i>	Chloroflexi	N/A	N/A	No
<i>Campylobacter jejuni</i>	Epsilonproteobacteria	No	No	Yes
<i>Chlamydia trachomatis</i>	Chlamydiae	No	No	Yes
<i>Chlorobium chlorochromatii</i>	Green sulfur bacteria (Chlorobi)	No	No	No

<i>Chlorobium limicola</i>	Green sulfur bacteria (Chlorobi)	No	No	Yes
<i>Corynebacterium diphtheriae</i>	Actinobacteria	No	Yes	Yes
<i>Deinococcus geothermalis</i>	Deinococcus–Thermus	No	Yes	Yes
<i>Denitrovibrio acetiphilus</i>	Deferribacteres	No	No	Yes
<i>Desulfurispirillum indicum</i>	Chrysiogenetes	N/A	N/A	Yes
<i>Dictyoglomus thermophilum</i>	Dictyoglomi	No	No	No
<i>E. coli</i> O25bH4 ST131 EC958	Gammaproteobacteria	Yes	Yes	Yes
<i>Elusimicrobium minutum</i>	Elusimicrobia	No	No	No
<i>Fibrobacter succinogenes</i>	Fibrobacteres	No	No	No
<i>Fusobacterium nucleatum</i>	Fusobacteria	No	No	No
<i>Gemmatimonas aurantiaca</i>	Gemmatimonadetes	No	No	No
<i>Geobacillus thermodenitrificans</i>	Firmicutes	No	No	Yes
<i>Gloeobacter violaceus</i>	Cyanobacteria	No	No	Yes
<i>Haemophilus influenzae</i>	Gammaproteobacteria	No	Yes	Yes
<i>Helicobacter pylori</i>	Epsilonproteobacteria	No	No	No
<i>Lentisphaera araneosa</i>	Lentisphaerae	No	No	No
<i>Mycobacterium tuberculosis</i>	Actinobacteria	No	Yes	Yes
<i>Mycoplasma genitalium</i>	Tenericutes	No	No	No
<i>Neisseria meningitidis</i>	Betaproteobacteria	No	No	No
<i>Nitrospira moscoviensis</i>	Nitrospirae	N/A	N/A	Yes
<i>P. aeruginosa</i> PAO1	Gammaproteobacteria	No	No	Yes
<i>Porphyromonas catoniae</i>	Bacteroidetes	N/A	N/A	Yes
<i>Rhodobacter sphaeroides</i>	Alphaproteobacteria	No	No	Yes
<i>Rhodopirellula baltica</i>	Planctomycetes	N/A	N/A	Yes
<i>Rickettsia rickettsii</i>	Alphaproteobacteria	No	No	Yes
<i>S. aureus</i> NCTC 8325	Firmicutes	No	No	Yes
<i>Salmonella typhimurium</i>	Gammaproteobacteria	No	Yes	Yes
<i>Shewanella oneidensis</i>	Gammaproteobacteria	No	Yes	Yes
<i>Sphaerobacter thermophilus</i>	Thermomicrobia	No	No	No
<i>Streptococcus pyogenes</i> serotype M1	Firmicutes	No	No	No
<i>Streptomyces pratensis</i>	Actinobacteria	N/A	N/A	Yes
<i>Synechocystis</i>	Cyanobacteria	No	No	Yes
<i>Thermocrinis ruber</i>	Aquificae	N/A	N/A	No
<i>Thermodesulfobacterium geofontis</i>	Thermodesulfobacteria	N/A	N/A	Yes
<i>Thermotoga maritima</i>	Thermotogae	No	No	No

<i>Verrucomicrobium</i>	Verrucomicrobia	No	No	No
<i>Vibrio cholerae</i> serotype O1	Gammaproteobacteria	No	Yes	Yes
<i>Vitreoscilla</i>	Betaproteobacteria	N/A	N/A	Yes
<i>Yersinia pestis</i>	Gammaproteobacteria	No	Yes	Yes
<i>Acinetobacter baumannii</i>	Gammaproteobacteria	No	No	Yes
<i>Azotobacter vinelandii</i>	Gammaproteobacteria	No	No	Yes
<i>Enterobacter cloacae</i> S611	Gammaproteobacteria	No	Yes	Yes
<i>Enterococcus faecium</i>	Firmicutes	No	No	No
<i>Klebsiella pneumoniae</i>	Gammaproteobacteria	No	Yes	Yes
<i>Pseudomonas aeruginosa</i> PA14	Gammaproteobacteria	No	No	Yes
<i>Staphylococcus aureus</i> USA300	Firmicutes	No	No	Yes

4.3.3. Predicting haem *d* locations for cytochrome *bd* oxidase structures

Structural analysis using PyMOL was conducted on the haem *d* active site region for *E. coli* cytochrome *bd*-I (6RKO) and the same region in the *G. thermodenitrificans* cytochrome *bd* oxidase (Fig. 4.3). Alignment of the 2 structures in PyMOL allowed a comparison of this region (Fig. 4.3). A key difference determined was the flipped positions of haem *b*₅₉₅ and haem *d* in the *E. coli* and *G. thermodenitrificans* structures as reported by (Safarian et al. 2019). The hydrophobic pocket formed that binds oxygen next to haem *d* in *E. coli* cytochrome *bd*-I is absent in *G. thermodenitrificans* cytochrome *bd* (Fig. 4.3). This pocket is formed in *E. coli* by the hydrophobic residues phenylalanine 104, isoleucine 144 and alanine 141 around glutamate 99 in CydA (Phe104^A/Ile144^A/Ala141^A/Glu99^A) (Fig. 4.3). In *G. thermodenitrificans* cytochrome *bd*, these residues correspond to phenylalanine 103, isoleucine 146, alanine 143 and glutamate 101 in CydA (Phe103^A/Ile146^A/Ala143^A/Glu101^A) (Fig. 4.3). The presence of leucine 101 in *E. coli* CydA (Leu101^A) causes a kink being in transmembrane helix 3 that results in Glu99^A being moved away from haem *d* allowing oxygen to bind (Fig. 4.3). Crucially Leu101^A in *E. coli* is absent in *G. thermodenitrificans* cytochrome *bd* with a single residue gap being present that results in no kink being present in transmembrane helix 3 (Fig. 4.3). This, combined with Phe104^A in *E. coli* facing the opposite direction to the corresponding Phe103^A in *G. thermodenitrificans*, prevents the pocket being formed for oxygen access (Fig. 4.3). Ile144^A (*E. coli*)/ Ile146^A (*G. thermodenitrificans*) blocks oxygen transfer between haems *d* and *b*₅₉₅ in both cytochrome

bd oxidase structures. Due to this it can be assumed that both structures have a different oxygen entry channel (see section 4.3.4). *E. coli* cytochrome *bd*-II also contains leucine 101 and has the same kink in transmembrane helix 3 (Grauel et al. 2021). Using the presence of leucine 101 as a potential marker for assigning the structural positions of *b*₅₉₅ or haem *d* was investigated using the phylogenetic tree of CydA (Fig. 4.4). A group of phylogenetically-related CydA proteins, including *G. thermodenitrificans* CydA, have a one-residue deletion present at the *E. coli* leucine 101 position. This could be an indication of haem *d* position with the oxidases containing the residue 101 deletion having the *G. thermodenitrificans* haem arrangement. This work is followed up with structural analyses in sections 4.3.4 and 4.3.5.

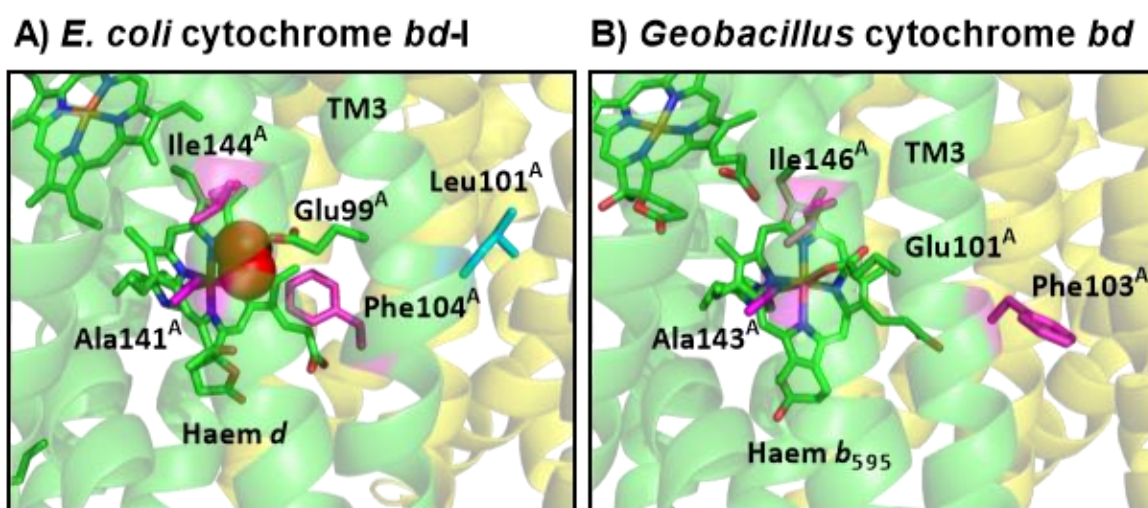


Figure 4.3 - A) *E. coli* cytochrome *bd*-I haem *d* region comparison with the same region in B) *G. thermodenitrificans* cytochrome *bd* (haem *b*₅₉₅ region). Oxygen (red spheres) shown bound to the haem *d* active in the *E. coli* cytochrome *bd*-I. Leucine 101 is shown in cyan whilst labelled hydrophobic residues that make up the oxygen binding pocket in *E. coli* and the equivalent residues in *G. thermodenitrificans* are highlighted in magenta. Transmembrane helix 3 of CydA labelled as TM3.

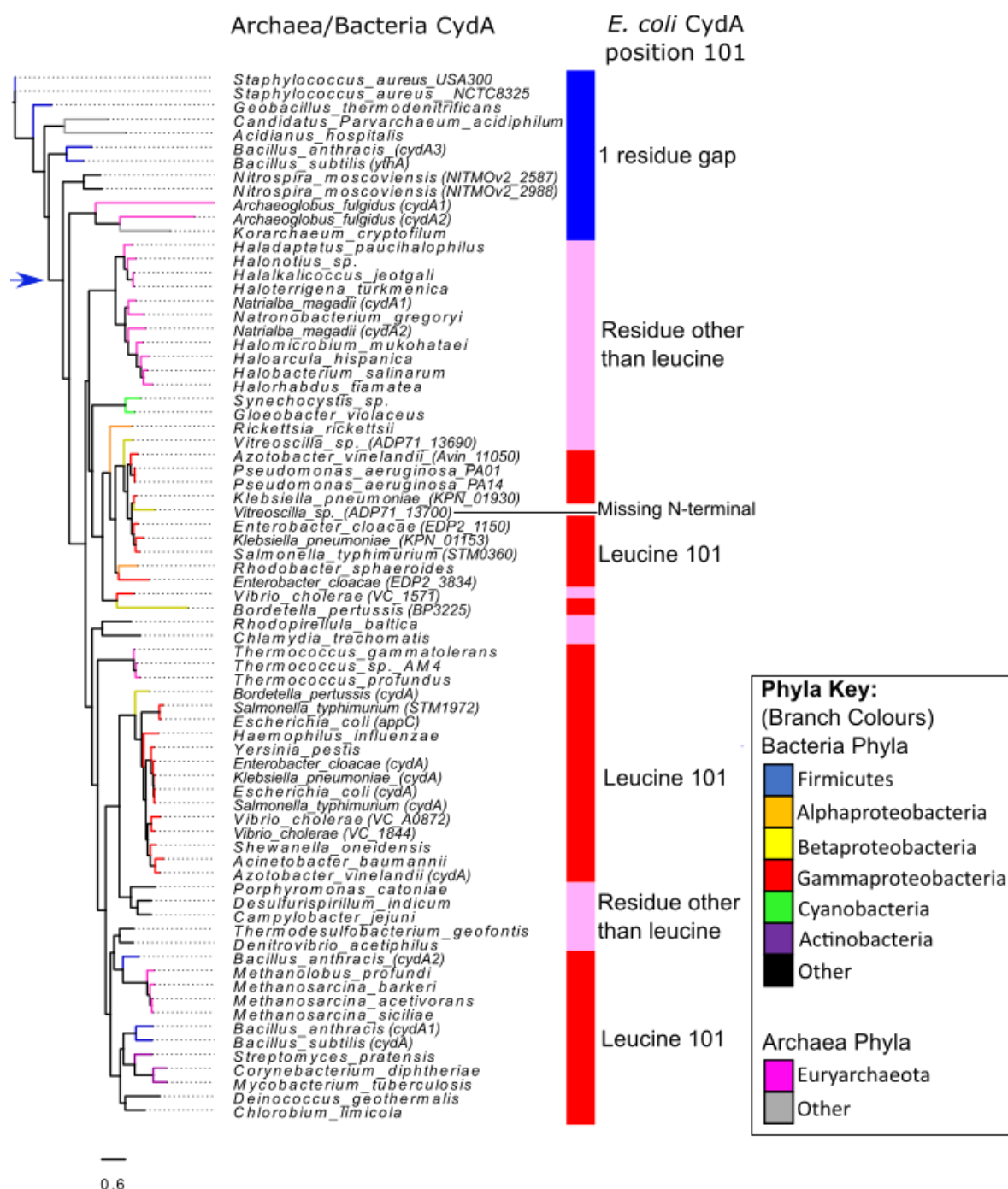


Figure 4.4 - Phylogenetic relationship of the cytochrome *bd* CydA subunit from archaea and bacteria. Branches coloured according to phyla. Presence of leucine 101, alternative residue or gap is indicated. Blue arrow shows divergence of leucine 101 position.

4.3.4. Cytochrome *bd* oxygen entry tunnels as predictors of haem arrangement and drug targets

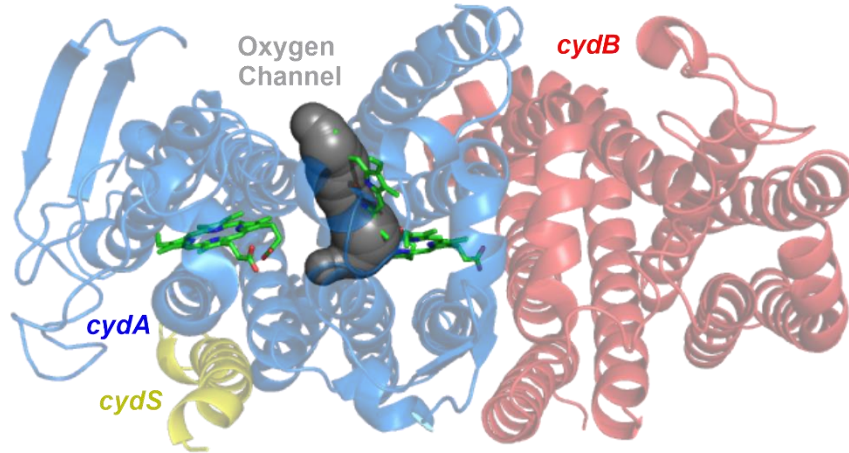
The potential oxygen and proton channels of the *E. coli* and *G. thermodenitrificans* cytochrome *bd* structures were investigated with 2 main aims. The first was to provide further insights into predicting the haem *d* position of cytochrome *bd* oxidases. The second aim was to identify any potential sites that would make good targets for drug screening studies.

Firstly, the online server MOLE 2.5 was used to search for oxygen entry tunnels in both *E. coli* cytochrome *bd*-I and *G. thermodenitrificans* cytochrome *bd* (Fig. 4.5). The different haem *d* positions for the *E. coli* and *G. thermodenitrificans* cytochrome *bd* structures were used as the starting point for the tunnel modelling. The oxygen entry route for *G. thermodenitrificans* CydA (Fig. 4.5A) is blocked in *E. coli* CydA by the presence of the small subunit CydH. An alternative channel is predicted by MOLE 2.5 for *E. coli* cytochrome *bd*-I that runs from the haem *d* oxygen binding site in CydA through CydB (Fig. 4.5B) to exit in a hydrophobic pocket within the transmembrane region (Fig. 4.7). This predicted channel reaches a minimum constriction of 2.4 Å at threonine 66 on CydB (Thr66^B) that is still large enough for oxygen to pass through (Fig. 4.5C). This alternative oxygen entry channel could be another marker for haem arrangement as it could be expected that cytochrome *bd* oxidases that contain the *E. coli* haem arrangement would also contain this channel and those with the *G. thermodenitrificans* haem arrangement would not. To investigate this further MOLE 2.5 was used to see if a similar alternative channel was present in *G. thermodenitrificans* cytochrome *bd*. According to the MOLE 2.5 analysis the alternative oxygen entry channel for *E. coli* cytochrome *bd*-I (Fig 4.6A) is not present in *G. thermodenitrificans* cytochrome *bd* (Fig. 4.6B). The closest predicted channel to the *E. coli* alternative channel in *G. thermodenitrificans* cytochrome *bd* (Fig. 4.6B, green channel) exits into the cytoplasmic side of CydB, is much more constricted (bottleneck radius of 0.9Å) and hydrophilic suggesting that this is more likely to be a proton channel.

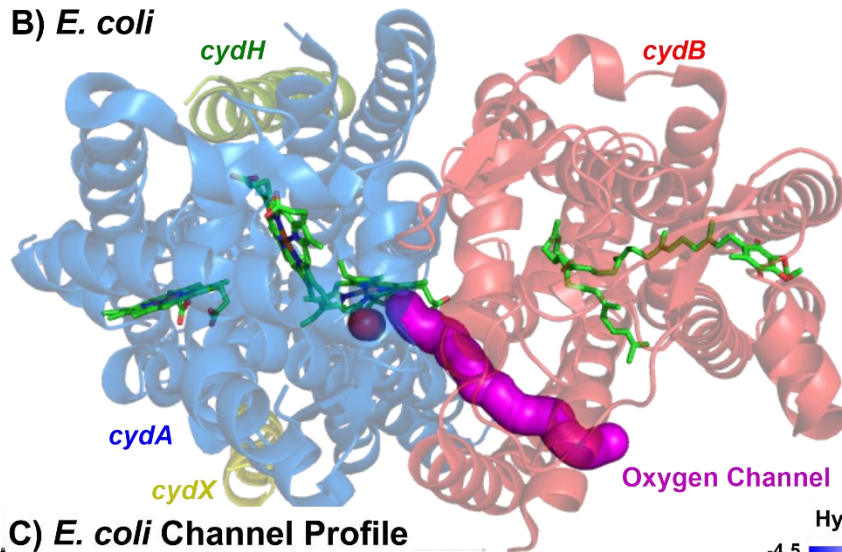
Furthermore, the residues surrounding the *E. coli* cytochrome *bd*-I alternative oxygen entry channel were investigated and compared to the same region in *G. thermodenitrificans* cytochrome *bd* (Fig. 4.6). The 2 structures were aligned for this analysis

using PyMOL allowing the *E. coli* alternative oxygen entry channel to be superimposed onto the *G. thermodenitrificans* structure. The main aim of this was to determine any bulky residues that take the place of the *E. coli* cytochrome *bd* alternative oxygen entry channel in *G. thermodenitrificans* cytochrome *bd* and then investigate the conservation. Any conserved bulky residues in cytochrome *bd* oxidases closely related to *G. thermodenitrificans* cytochrome *bd* could provide additional evidence for predicting haem arrangement. In the *G. thermodenitrificans* structure several large bulky residues are present that block the course of the alternative oxygen entry channel (Fig. 4.6A + B). The three *G. thermodenitrificans* *bd* residues Phe99^A (*E. coli* *bd*-I Ala98^A), Phe62^B (*E. coli* *bd*-I Thr66^B) and Phe308^B (*E. coli* *bd*-I Ala344^B) appear to block the alternative channel (Fig. 4.6A + B). TMH 9 containing Phe308^B is also straighter in *G. thermodenitrificans* cytochrome *bd* than *E. coli* cytochrome *bd*-I which also blocks the alternative oxygen entry channel. Phe62^B is bulkier than Thr66^B and is present at the narrowest region of the alternative oxygen entry channel. Phe62B and Phe308^B are also conserved to the phylogenetically closest CydB proteins to *G. thermodenitrificans* CydB and so these residues may provide another clue to haem *d* position (Fig. 4.6C).

A) *Geobacillus*



B) *E. coli*



C) *E. coli* Channel Profile

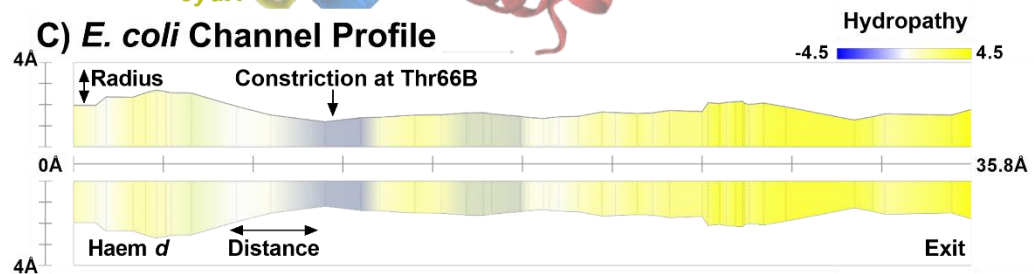
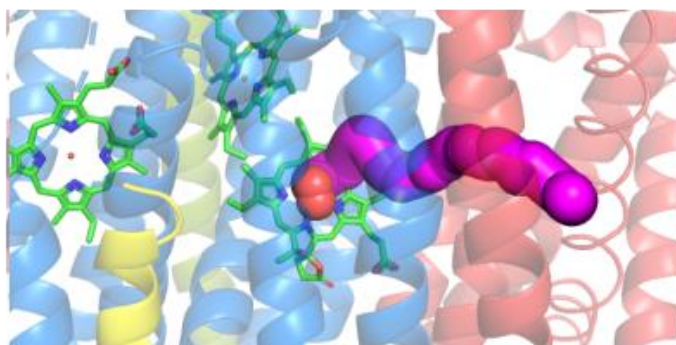
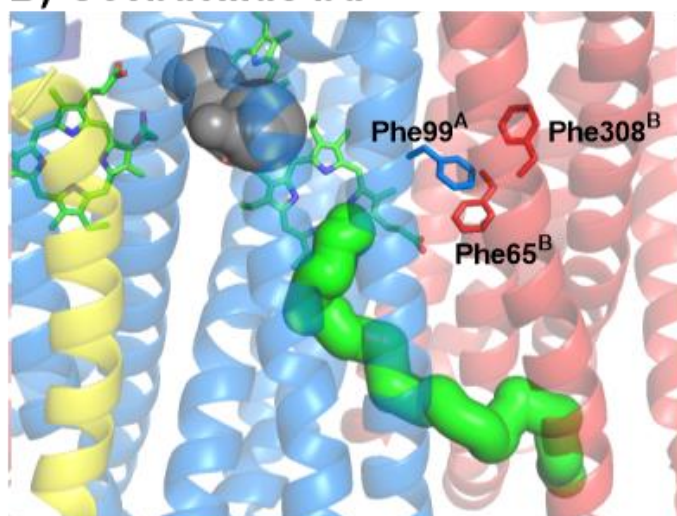


Figure 4.5 - Comparison of oxygen entry tunnels in (A) *G. thermodenitrificans* and (B) *E. coli* cytochrome *bd* oxidases. Direction of view from the periplasm with *E. coli* cytochrome *bd*-I structure 6KRO used. (C) *E. coli* oxygen entry channel dimensions and hydrophobicity profile.

A) *E. coli* *bd-I*



B) *Geobacillus* *bd*



C) *cydB* MSA

	<i>G. therm</i> CydB: Phe65	Phe308
<i>E. coli</i> CydB: Thr66	Ala344	
<i>Geobacillus thermodenitrificans</i> <i>cydB</i>	VW E VTNVFLV F FFVG	ALIVAFIAG
<i>Staphylococcus aureus</i> <i>cydB</i>	VW E VTNVFFV F FFVG	TLVIVFILG
<i>Bacillus anthracis</i> <i>ythB</i>	TW E VTNTFLV F VVVA	ALLIIYAIG
<i>Bacillus subtilis</i> <i>ythB</i>	TW E VTNVFIV A IVVA	ATERALFAT
<i>Escherichia coli</i> <i>cydB</i>	H WDGNQVWLI T AGGA	VMTWVAVVL
<i>Escherichia coli</i> <i>appB</i>	H W E GNQVWLI L AGGA	IMLVIVLIF
<i>Klebsiella pneumoniae</i> KPN 01154	VWDG N ETWLV L GGAG	FMLPGALLI
<i>Haemophilus influenzae</i> <i>cydB</i>	H WDGNQVWLI T AGGA	LMLIHAVVF
<i>Pseudomonas aeruginosa</i> PA14 <i>cioB</i>	VWDG N ETWLV L GGAP	FMLVGALFI
<i>Mycobacterium tuberculosis</i> <i>cydB</i>	VWDG N E V WLI T AGAP	IMTWVTAFF
<i>Azotobacter vinelandii</i> <i>cydB</i>	VWDG N ETWLV L GGAG	FALVGALLI
<i>Haloarcula hispanica</i> <i>cydB</i>	VWD A N E VWLV A FGTM	LVTVMGLPV

Figure 4.6 - Comparison of oxygen entry tunnels key residues in cytochrome *bd* oxidases. (A) *E. coli* cytochrome *bd-I* with predicted oxygen entry channel in magenta. (B) *G. thermodenitrificans* cytochrome *bd* with predicted oxygen entry channel in grey along with the closest predicted channel to the *E. coli* oxygen entry channel in green. Residues highlighted take the position of the *E. coli* oxygen entry channel in *G. thermodenitrificans* cytochrome *bd*. (C) MSA of selected CydB highlighting important residues around the *E. coli* oxygen entry tunnel region.

The *E. coli* cytochrome *bd-I* oxygen entry channel (alternative channel) was further investigated as a potential target for novel inhibitors. The predicted *E. coli* cytochrome *bd-I* oxygen entry channel exits into the transmembrane region. A small hydrophobic pocket is present at the mouth of the oxygen entry channel (Fig. 4.7) close to Trp63^B. This could potentially be targeted by small hydrophobic compounds due to its intermembrane position and the pore being surrounded by hydrophobic residues (Fig. 4.7B).

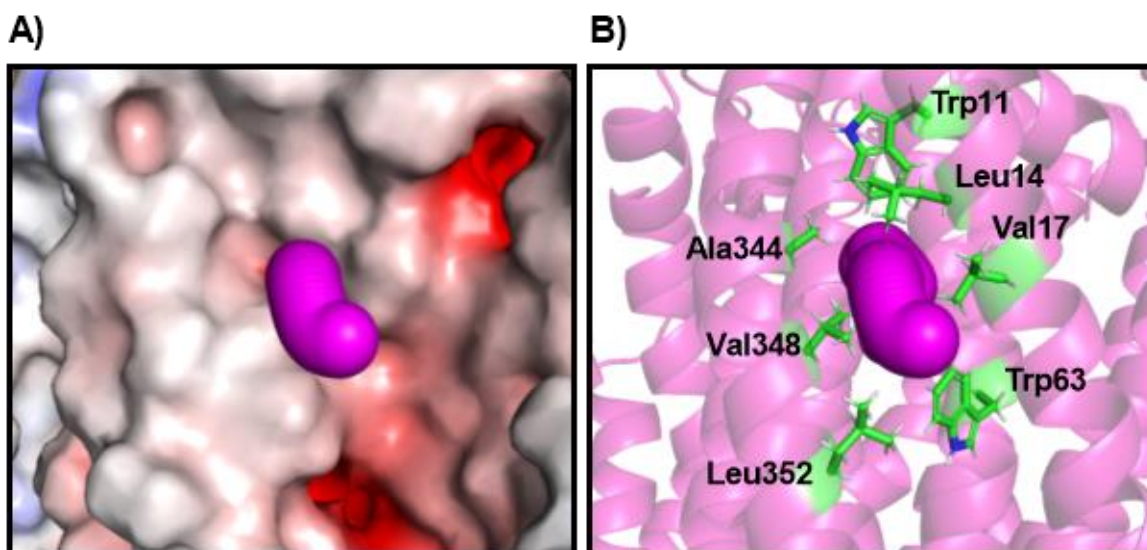


Figure 4.7 - Exit of predicted *E. coli* cytochrome *bd-I* oxygen entry channel (purple spheres) into the transmembrane region. (A) Electrostatic potential molecular surface view with positive charged regions in red, negative in blue and no charge in white. (B) Hydrophobic residues surrounding the oxygen entry site exit.

4.3.5. Predicted Proton Channels

The hydrophilic proton channels for *E. coli* cytochrome *bd-I* were also modelled using the water molecules in the cryo-EM structure 6RKO as a guide (Fig. 4.8A) (Safarian et al. 2019). A CydA channel is present along with a CydB channel. The CydA channel delivers protons directly to haem *d* whilst the CydB channel joins the oxygen channel near Thr66^B (Fig. 4.8A). The oxygen channel is accordingly more hydrophilic from Thr66^B to haem *d* (Fig. 4.5C). The CydB channel includes the conserved WDG^BNQ motif from that starts from Trp57^B (Safarian et al. 2019). Furthermore, a branch connects the 2 channels via aspartate 58 in

CydB (Asp58^B) and the conserved WDG^BNQ motif. Both channels are lined with hydrophilic residues allowing proton transfer from the cytoplasm to haem *d*. This analysis is supported by the two cytochrome *bd*-I structural papers (Safarian et al. 2019; Theßeling et al. 2019) and site directed mutagenesis work (Murali and Gennis 2018).

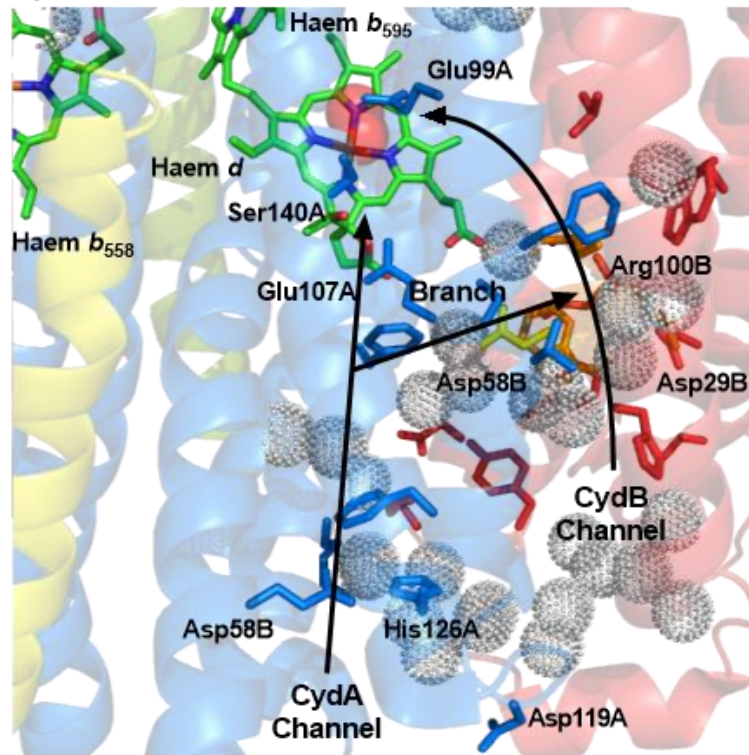
The proton channels for *G. thermodenitrificans* cytochrome *bd* were also predicted (Fig. 4.8B). No water molecules are present in the *G. thermodenitrificans* cryo-EM structure so just the hydrophilic residues were used for proton channel prediction. Previous analysis of the *G. thermodenitrificans* cytochrome *bd* structure identified potential CydA and CydB proton pathways (Safarian et al. 2016). These were also seen in this analysis (Figure 4.8B). Interestingly, a glutamate is present in *G. thermodenitrificans* CydA in the same 3D space that the aspartate 58 occupies in *E. coli* CydB. Also, a similar WEVTN sequence is present at the position of the *E. coli* WDG^BNQ motif. This suggests that the branch between CydA and CydB channels is also present in *G. thermodenitrificans* cytochrome *bd*.

Sequence conservation in the proton channels was analysed (Fig. 4.9) using an MSA of the terminal oxidases from the phylogenetic analysis (Fig. 4.2 and 4.4). Overall, a high degree of conservation exists in the CydA, CydB and branch channels. Key conserved hydrophilic residues in the CydA pathway include *E. coli* *bd*-I residues His126^A (*G. thermodenitrificans* *bd* His128^A), Asp119^A (*G. thermodenitrificans* *bd* Asp120^A), Ser140^A (*G. thermodenitrificans* *bd* Ser142^A) and Glu107^A (*G. thermodenitrificans* *bd* Glu109^A) leading to Glu99^A (*G. thermodenitrificans* *bd* Glu101^A) that is ligated to haem *d*/*b*₅₉₅. Conserved residues in the CydB pathway include Asp29^B (*G. thermodenitrificans* *bd* Asp25^B) and Arg100^B (*G. thermodenitrificans* *bd* Arg99^B). The branch channel residues in CydB are also highly conserved with either Asp or Glu at *E. coli* position 58 forming part of the conserved motif WDG^BNQ in *E. coli* and WEVTN in *G. thermodenitrificans*. Due to the high conservation of the proton channel residues between *E. coli* and *G. thermodenitrificans* cytochrome *bd*, no distinctive features could be identified that could indicate the haem *d* position. It is likely that protons first transfer to the haem *b*₅₉₅ in *G. thermodenitrificans* before transfer onto haem *d*.

Two CydA proteins identified in this analysis were missing the key proton channel residues Asp119^A (*G. thermodenitrificans* *bd* Asp120^A), Ser140^A (*G. thermodenitrificans* *bd*

Ser142^A), Glu107^A (*G. thermodenitrificans* *bd* Glu109^A) and crucially the haem ligating Glu99^A (*G. thermodenitrificans* *bd* Glu101^A). These are *Bordetella pertussis* BP3225 and *Archaeoglobus fulgidus* CydA1. Interestingly, *B. pertussis* BP3225 (the predicted CydB homologue that is in an operon with BP3224) contains the CydB proton channel residues Asp29^B (*G. thermodenitrificans* *bd* Asp25^B), Asp58^B (*G. thermodenitrificans* *bd* Glu57^B) and Arg100^B (*G. thermodenitrificans* *bd* Arg99^B). *Archaeoglobus fulgidus* CydA1 is the second subunit in an operon with CydA2, which does contain the key proton channel residues. This operon is thus likely to be a CydAA' cytochrome *bd*, which are only found in archaea (Murali Ranjani et al. 2021). CydA' (*Archaeoglobus fulgidus* CydA1) is believed to have arisen from gene duplication from CydA and performs the role of CydB (Murali Ranjani et al. 2021).

A) *E. coli* *bd-I*



B) *G. thermodenitrificans* *bd*

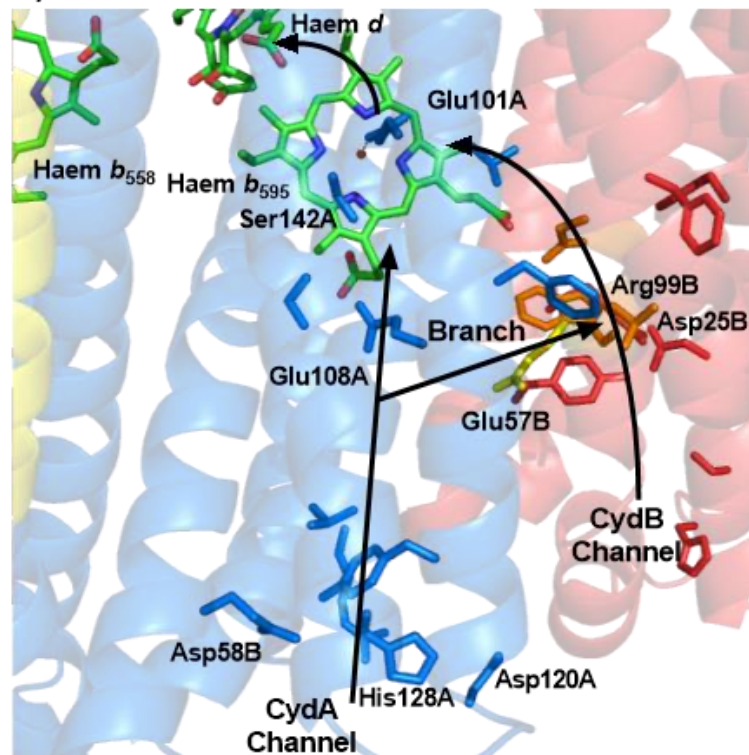


Figure 4.8 - Predicted proton channels for (A) *E. coli* and (B) *G. thermodenitrificans* cytochrome *bd* structures. Proton Channels shown by arrows with key mostly hydrophilic side chains displayed. Colour scheme; CydA (blue), CydB (red), waters (grey spheres), WDGNQ/WEVTN motif (orange) and D58B/E57B (yellow).

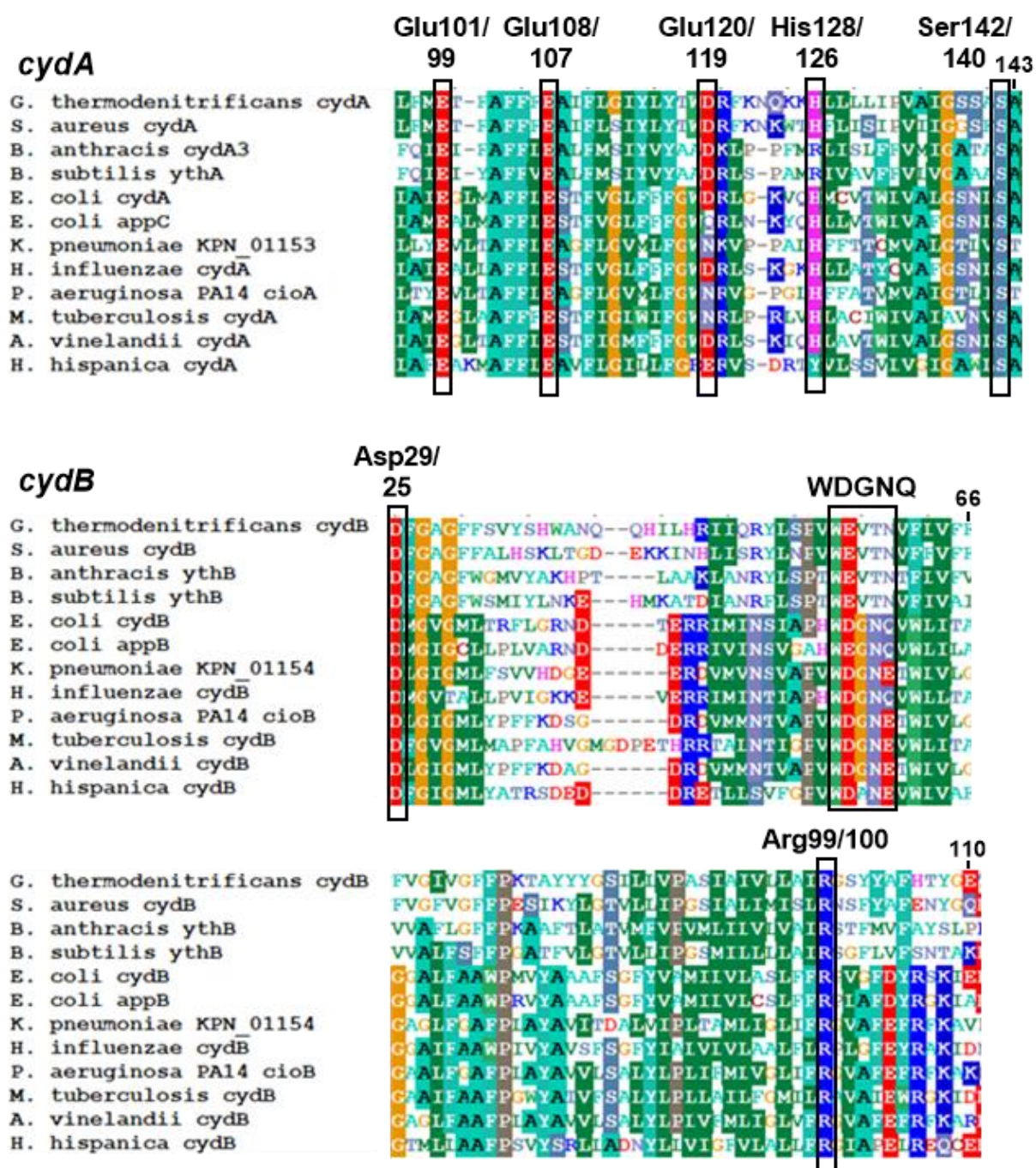


Figure 4.9 - MSA of selected *cydA* and *cydB* sequences highlighting some important conserved residues of the predicted proton channels for cytochrome *bd*. *G. thermodenitrificans* residue numbers displayed first in annotations.

4.3.6. Role of CydB-bound Ubiquinone-8 in cytochrome *bd-I*

The *E. coli* cytochrome *bd-I* cryo-EM structures (Safarian et al. 2019; Theßeling et al. 2019) contain a ubiquinone-8 molecule bound to CydB in a hydrophobic pocket. Ubiquinone-8 in CydB was investigated to determine if it may be involved in the electron transfer for oxygen reduction or proton transfer. Figure 4.10 shows the ubiquinone-8 molecule present in CydB of *E. coli* cytochrome *bd-I*. The cryo-EM structure 6RKO, along with the oxygen and proton channel modelling from sections 4.3.4 and 4.3.5, allows an assessment of the potential functional role of ubiquinone-8 in the CydB site. A large distance (circa 10Å) is observed between the molecule and the proton/oxygen channels. Haem *d* is located even further from ubiquinone-8 than the proton channels (Fig. 4.10A) and the head group for ubiquinone-8 is located pointing away from CydA (Fig. 4.10B). This, along with the hydrophobic residues present between the ubiquinone-8 and the active site, suggests that ubiquinone-8 is more likely to have a structural role, possibly in folding or stabilising CydB. (Theßeling et al. 2019) suggests that as ubiquinone-8 in CydB occupies a similar pocket to the haem ligands in CydA it could be present to provide structural stabilisation to a cavity where haem no longer binds. To investigate this further 3DLigandSite (Wass et al. 2010) was used to predict potential ligands that may bind to the CydB ubiquinone-8 cleft. The ubiquinone-8 ligand in the *E. coli* cytochrome *bd-I* PDB file 6RKO was removed before submitting the CydB structure to 3DLigandSite. Interestingly, two clusters of haem ligands were predicted to be able to bind to the CydB ubiquinone-8 site (Appendix A.7). This provides evidence that ubiquinone-8 may be taking the place of haem groups in CydB providing stabilisation to this vestigial haem cavity in CydB.

Even though the ubiquinone-8 site in CydB is unlikely to be directly involved in catalysis, it could still be a potential drug target to induce complex destabilisation. The ubiquinone-8 site in CydB is fully resolved in the cryo-EM structures allowing easier structure-based drug design than the partially resolved CydA quinol binding site. Autodock Vina was used to dock known quinol binding site inhibitors and ubiquinone-8 to the CydB ubiquinone-8 site. This was intended to determine the affinity of the known inhibitors to the ubiquinone-8 site in comparison with the structural ubiquinone-8. The ubiquinone-8 ligand in the *E. coli* cytochrome *bd-I* PDB file 6RKO was again removed before the docking

to allow the known inhibitors (Table 4.3) to be docked into the CydB quinol cleft. The highest predicted affinity for a known inhibitor was for aurachin A at -11.3 kcal/mol compared to ubiquinone-8 at -11.7 kcal/mol. The docking result suggests that ubiquinone-8 binds very tightly to CydB and may be difficult to remove with a competitive quinol binding site inhibitor.

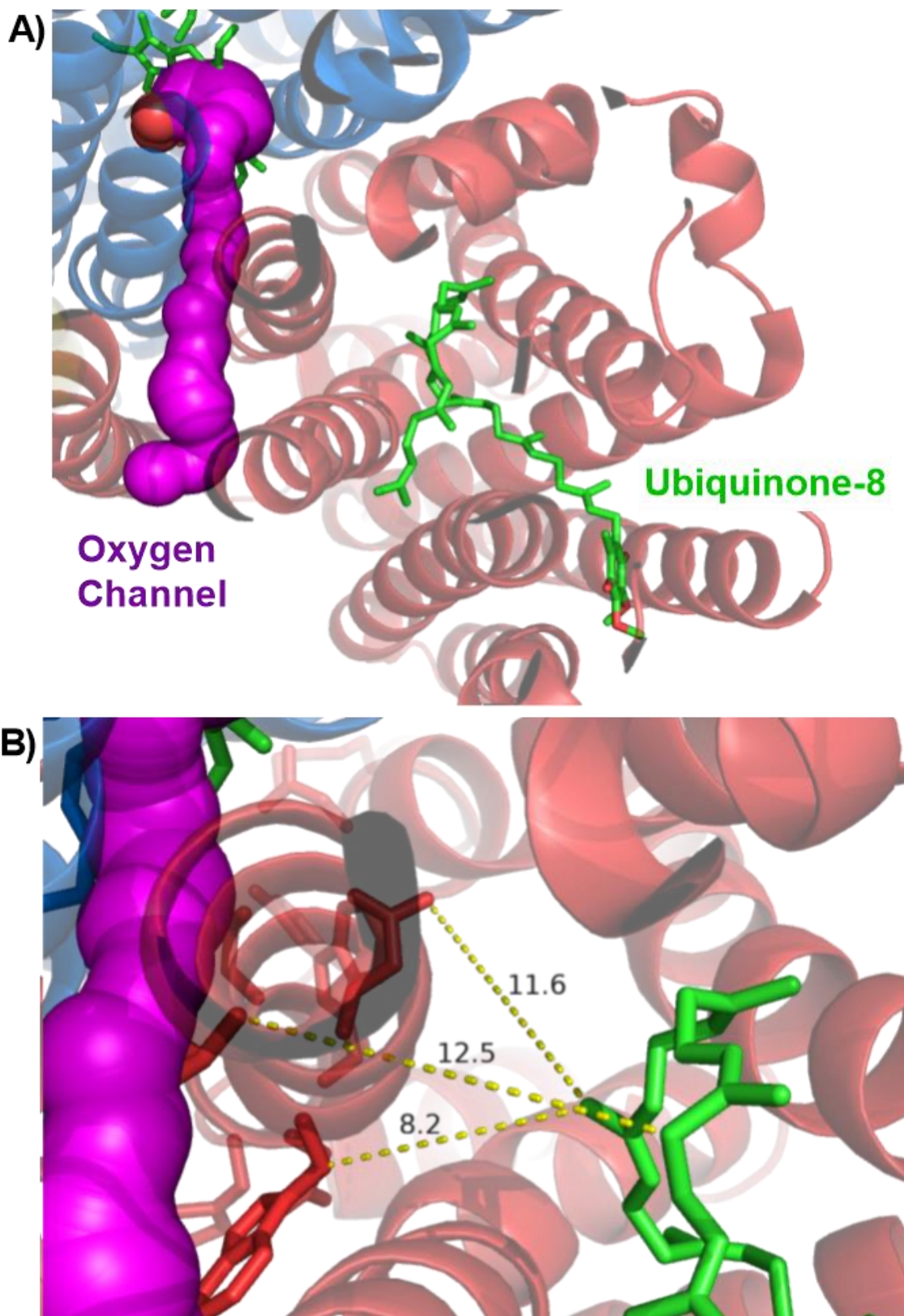


Figure 4.10- Ubiquinone-8 and the oxygen entry channel in *E. coli*. (A) Ubiquinone-8 in relation to the oxygen entry channel (magenta) (B) Distance between ubiquinone-8 and proton channel key residues in Angstroms. CydA shown in blue and CydB in red.

Table 4.3 - Docking of known quinol binding site inhibitors and ubiquinol-8 to the ubiquinol cleft of CydB in *E. coli* cytochrome *bd*-I.

Inhibitor/Ubiquinol-8	Predicted affinity (kcal/mol)	Estimated K_d (<u>nM</u>)
Ubiquinol-8	-11.7	2.60
Aurachin A	-11.3	5.10
Aurachin D	-11.0	8.47
Aurachin C	-10.9	10.0
Stigmatellin	-10.3	27.6
Piericidin A	-9.5	107
Myxothiazol	-9.3	150
Funiculosin	-8.9	294
HOQNO	-8.1	1138
MOA_stilbene	-8.0	1347
UHDBT	-8.0	1347
Antimycin_A	-7.4	3713
Dibromothymoquinone	-7.1	6164
Pentachlorophenol	-6.4	20117
TTFA	-6.4	20117
2,6-Dimethyl-p-benzoquinone	-6.3	23820
p-benzoquinone	-5.1	180935

4.4. Discussion

4.4.1. Development of pipeline for identifying remote homologues for terminal oxidases

A pipeline for identifying remote homologues using hhsearch was adapted for detection of terminal oxidases in bacteria. hhsearch allows more remote homologues to be detected than a BLAST search (Söding 2005). This local pipeline allowed different proteomes to be searched for all of the terminal oxidase proteins at once. The main disadvantage is the much slower processing times compared to BLAST based methods and the manual processing of results, which precludes large numbers of proteomes being searched. To validate the pipeline, the *E. coli* cytochrome *bd*-I was used as the template for the hhsearch to detect the distantly-related *G. thermodenitrificans* cytochrome *bd* which has a known structure (Safarian et al. 2016). The current pipeline for identifying remote homologues identified a greater number of cytochrome *bd* homologues than the MicrobesOnline high-throughput BLAST based method (Alm et al. 2005) used by (Eng et al. 2018).

For future analysis, the pipeline for identifying remote homologues could be expanded to include a diverse range of Archaea. The *cyoABCD* operon could also be investigated to allow a comparison of strains that contain cytochromes *bo'*, *bd*-I, or both. A larger BLASTP search could also be conducted to allow a much larger phylogenetic tree to be constructed perhaps using the results from the hhsearch as the input.

4.4.2. Phylogenetic analysis of cytochrome *bd* terminal oxidases

Phylogenetic analysis of cytochrome *bd* oxidases from the pipeline for identifying remote homologues for bacteria and BLASTP search for archaea results grouped the oxidases into long and short Q-loop types. Archaea of the *Thermococcus* genus form a subgroup of the long Q-loop oxidases with ~20 residue shorter Q-loops.

An attempt was made to phylogenetically distinguish the cyanide insensitive oxidases (CIOs). A small group of CIOs were shown to be phylogenetically related to *Pseudomonas aeruginosa* CIO. However, no specific sequence signature could be found that could be used to identify cytochrome *bd* complexes as being a CIO, with several known CIO complexes (including *Campylobacter jejuni* CioA and *Bacillus subtilis* YthA) being

phylogenetically distanced from the *P. aeruginosa* group. (Esposti et al. 2015) attempted to categorize CIOs in proteobacteria using gene clusters and assigned all potential CIO complexes into three sub-types, with *P. aeruginosa* CIO being in sub-type C. The *B. subtilis* operon *ythABX* was also predicted to be an ancestral operon for the sub-type A group in Proteobacteria (Esposti et al. 2015), which fits with the phylogenetic tree constructed in the current analysis (Fig. 4.2). No CIO has currently been structurally characterised (Murali Ranjani et al. 2021), and other studies have also not been able to identify specific sequence features that are specific to CIOs (Murali Ranjani et al. 2021). In future, structural analysis of a CIO would be helpful in determining differences between CIO and other *bd* type oxidases including what provides the increased resistance to cyanide.

4.4.3. Leucine 101 as a tool for identification of haem *d* position

E. coli cytochrome *bd-I/bd-II* (Safarian et al. 2019; Theßeling et al. 2019; Grauel et al. 2021; Grund et al. 2021) and *G. thermodenitrificans* cytochrome *bd* (Safarian et al. 2016) structures contain the haem *d* and *b*₅₉₅ ligands in the opposite positions. It has previously been hypothesized that an insertion/deletion at *E. coli* CydA leucine 101 leads to the reversal of the haem positions (Fig. 4.3) (Safarian et al. 2019). Phylogenetic analysis was employed to investigate if the presence/absence of position 101 could be used as a predictive tool to assign haem cofactor positions. According to Figure 4.4, a group of phylogenetically related CydA proteins (including *G. thermodenitrificans* CydA) lack the residue that is found at *E. coli* cydA leucine 101. This could be an indication of haem *d* position with the oxidases containing this deletion having the *G. thermodenitrificans* cytochrome *bd* haem arrangement. This observation is also supported by (Murali Ranjani et al. 2021) who conducted a larger CydA phylogenetic investigation conducted after the current work was undertaken. The CydA sequences that contained a quinol binding site were categorized into qOR1 (including *E. coli*), qOR2, qOR3 (including *G. thermodenitrificans*) and qOR4a families (Murali Ranjani et al. 2021). The largest qOR1 subfamily included the extra residue at position 101 whilst the others largely had the single residue deletion (Murali Ranjani et al. 2021). As the qOR1 family was the largest it was suggested that this is the oldest subfamily (Murali Ranjani et al. 2021). However, while this

work was useful to identify homologues of *G. thermodenitrificans* cytochrome *bd*, the fact remained that this was the only example of a cytochrome *bd* that lacked leucine 101 and had been confirmed to have this unique haem arrangement. Hence, further structural analysis was undertaken to develop alternative approaches to assign haem arrangements (discussed in section 4.4.4).

4.4.4. Oxygen Channel Modelling for cytochrome *bd*

Oxygen channel modelling of the current cytochrome *bd* structures of *E. coli* and *G. thermodenitrificans* provided further insights into prediction of haem arrangement. Different oxygen channels were predicted for the *E. coli* and *G. thermodenitrificans* structures which are consistent with previous analyses (Safarian et al. 2019). The *E. coli* cytochrome *bd* CydH subunit blocks oxygen entry using the *G. thermodenitrificans* cytochrome *bd* route and so an alternative channel is predicted that exits through CydB. Further analysis revealed that the three bulky residues in *G. thermodenitrificans* cytochrome *bd*, Phe99A (*E. coli* *bd*-I Ala98A), Phe62B (*E. coli* *bd*-I Thr66B) and Phe308B (*E. coli* *bd*-I Ala344B) occupy the same space as the *E. coli* alternative channel when the structures are superimposed (Fig. 4.6A + B). TMH 9 containing Phe308B is also straighter in *Geobacillus* than *E. coli* cytochrome *bd*-I further occupying the same space as the alternative oxygen entry channel. Phe62B and Phe308B are conserved to the phylogenetically closest CydB proteins to *Geobacillus* (Fig. 4.6C). The above phenylalanine residues as a marker of the *Geobacillus* oxygen entry channel may provide another clue to haem arrangement to be taken into account with the residue 101 insertion/deletion. The recently published cytochrome *bd* structure from *C. glutamicum* also has the *E. coli* cytochrome *bd*-I haem arrangement and contains a very similar oxygen entry channel.

The predicted *E. coli* cytochrome *bd*-I oxygen entry channel was further investigated as a potential drug target. A small highly hydrophobic pocket is located at the mouth of the oxygen entry channel on CydB (Fig. 4.7) that is it in a well-resolved area of the cytochrome *bd*-I structure (Safarian et al. 2019; Theßeling et al. 2019). Due to the oxygen entry channel pockets being located in the intramembrane region and it being surrounded by highly

hydrophobic residues (Fig. 4.7B), future drug screening studies may seek to target this site with small hydrophobic compounds (Fig. 4.7B). Before any drug screening work is undertaken, it would also be interesting to consider mutations around the predicted *E. coli* oxygen entry channel. Thr66B present at the narrowest point in the predicted channel (Fig. 4.5C) could be mutated to a residue with a larger side chain such as a phenylalanine. Oxygen consumption experiments could then be carried out which could confirm if this is indeed the sole route of oxygen entry for *E. coli* cytochrome *bd*-I. In future, *E. coli* cytochrome *bd*-II could also be investigated in a similar way.

4.4.5. Proton Channel Modelling for cytochrome *bd*

Proton channel modelling for cytochrome *bd* identified 2 main proton channels (CydA and CydB pathway) along with a branch between them. This is seen in both *E. coli* and *G. thermodenitrificans* cytochrome *bd* structures and is in agreement with previous work (Safarian et al. 2016, 2019; Theßeling et al. 2019). The full CydB proton pathway in *E. coli* cytochrome *bd*-I identified in this work (Fig. 4.8) was not previously characterised (Safarian et al. 2019). The branch between the CydA and CydB pathways in *G. thermodenitrificans* cytochrome *bd* seen in Figure 4.8 was also not previously identified (Safarian et al. 2016). Overall, a high degree of conservation was observed for most proton channel residues, even in clades that are likely to have different haem cofactor arrangements, so it was concluded that this approach could not be used for prediction of haem ligand arrangement. However, two CydA proteins were identified as missing key proton channel residues. These are *Bordetella Pertussis* BP3225 and *Archaeoglobus fulgidus* CydA1. *Archaeoglobus fulgidus* cytochrome *bd* is likely to be a CydAA' type which are only found in archaea (Murali Ranjani et al. 2021). Gene duplication has been shown to be important in cytochrome *bd* evolution (Esposti et al. 2015) with the CydA' (*Archaeoglobus fulgidus* CydA1) subunit believed to have arisen from gene duplication from CydA (Murali Ranjani et al. 2021) to perform the role of CydB. Future analysis would be needed to identify potential proton and oxygen channels of the CydA' subunit in archaea. The recently published cytochrome *bd* structure from *C. glutamicum* has a slightly different proton channel starting in CydB with the CydA pathway blocked by a C-terminal extension of CydA.

The proton channel is a possible drug target for cytochrome *bd-I*. The conserved nature of the proton channels may enable structure-based design of inhibitors that target multiple pathogenic bacteria species cytochrome *bd* oxidases. However, the redundancy identified due to the CydA and CydB proton channels with a branch between them would be a problem for drug design. Mutational analysis would be useful to determine if both of the predicted proton channels are functional, possibly by mutating some of the key conserved hydrophilic residues to hydrophobic residues.

4.4.6. CydB ubiquinone-8 site as a potential drug target

The quinol binding site in *E. coli* cytochrome *bd-I* present in the CydB subunit (Safarian et al. 2019; Theßeling et al. 2019) was analysed to investigate the functional role, and whether it is likely to be a promising drug target. The proximity of the ubiquinone-8 molecule in CydB relative to the haem *d* cofactor and predicted proton and oxygen channels suggested a non-catalytic role for this quinol. 3DLigandSite (Wass et al. 2010) analysis of the CydB ubiquinol cavity suggested that haem ligands are theoretically able to bind to the CydB cleft, providing evidence for the hypothesis of (Theßeling et al. 2019) that the ubiquinone-8 now performs a structural role in filling a cavity where haem ligands used to bind.

The docking of known quinol site inhibitors to the CydB cavity revealed that ubiquinone-8 is predicted to bind more tightly to the cleft than any of the known inhibitors. This suggests a problem with designing competitive inhibitors against the CydB site due to the likely high affinity of ubiquinone-8 binding. (Theßeling et al. 2019) tried to remove ubiquinone-8 from CydB with excess aurachin C but was unsuccessful, which supports a stable interaction.

Chapter 5

Cytochrome *bd*-I as a target for drug discovery

5.1. Summary

The terminal oxidase cytochrome *bd*, found in a variety of pathogenic bacteria, is an important target for new antimicrobials. The cytochrome *bd* complex is important in virulence and host colonisation in several drug resistant pathogens including strains of *Mycobacterium*, *E. coli*, *Salmonella* and *Brucella*. Cytochrome *bd* represents a particularly attractive target as the structure is unique to prokaryotes and several structures have recently been published.

In this chapter, a drug repurposing approach was used to target the cytochrome *bd*-I oxidase of *E. coli*. An *in silico* drug screening pathway for FDA approved drugs was developed using a molecular docking approach. This produced a list of potential inhibitors, ranked by affinity and hydrophobicity, of which the top candidates were then screened in the laboratory. An oxygen consumption assay was used to measure respiratory activity of isolated membranes from the uropathogenic *E. coli* EC958 strain that contains cytochrome *bd*-I as the sole terminal oxidase (i.e. '*bd*-I only' membranes). Several interesting potential drug candidates were identified including quineestrol, a prodrug that has been used in birth control and to treat breast/prostate cancer. An IC₅₀ in the nM range was observed for '*bd*-I only' membranes. Quineestrol was further shown to be effective against whole cells with an IC₅₀ also in the nM range for '*bd*-I only' cells. This has paved the way for future structural, mutagenesis and drug-derivatisation studies. Furthermore, this approach could be used for targeting cytochrome *bd* complexes from other pathogenic bacteria.

5.2. Introduction

5.2.1. Cytochrome *bd* is a good drug target

Cytochrome *bd* is a respiratory terminal oxidase that is found in many bacteria and archaea (Borisov et al. 2011a). Cytochrome *bd* functions to reduce molecular oxygen to water with the electrons supplied by quinols such as ubiquinol which are consequently oxidised (Safarian et al. 2019). Protons are transferred from the quinols to the periplasm generating an electrochemical proton gradient (Safarian et al. 2019), which is then used to

generate ATP. Cytochrome *bd* promotes growth and survival of a variety of bacterial pathogens during infection, including multidrug-resistant *E. coli* (Shepherd et al. 2016), *Salmonella* (Jones-Carson et al. 2016), *Mycobacterium* (Cai et al. 2021) and *Brucella* (Endley et al. 2001). Cytochrome *bd* has been shown to be important in resistance to various environmental stresses such as sulphide (Korshunov et al. 2016) and cyanide (Borisov et al. 2015a). Furthermore, cytochrome *bd* is involved in bacterial resistance to reactive oxygen and nitrogen species (Borisov et al. 2015a) produced by the host immune system, such as nitric oxide (Mason et al. 2009), hydrogen peroxide (Al-Attar et al. 2016) and peroxyxynitrite (Borisov et al. 2015b). Finally, cytochrome *bd* can promote antibiotic tolerance in biofilms (Beebout et al. 2021).

Cytochrome *bd* is a very promising drug target due to three main reasons. Firstly, as stated above it is important in pathogenesis and virulence of many pathogenic bacteria. Secondly, cytochrome *bd* has a structure unique to prokaryotes (Giuffrè et al. 2014), potentially alleviating problems with drugs also targeting mitochondrial electron transport chain complexes in human cells. Thirdly, this work is very timely as several recently solved cryo-EM or X-ray crystallography cytochrome *bd* structures have become available: *G. thermodenitrificans* (Safarian et al. 2016), *E. coli* *bd*-I (Safarian et al. 2019; Theßeling et al. 2019), *E. coli* *bd*-II (Grauel et al. 2021; Grund et al. 2021), *Mycobacterium smegmatis* (Wang et al. 2021) and *Mycobacterium tuberculosis* (Safarian et al. 2021).

5.2.2. Drug Repurposing for antimicrobial discovery

Antibiotic resistance is becoming an ever-increasing problem. This is coupled with the slowdown in antibiotic discovery in the last few decades leading to the urgent need for new antimicrobials (Coates et al. 2011). The repurposing of existing drugs offers a promising approach to find new antimicrobials (Farha and Brown 2019). Drug repurposing can provide advantages such as a much shorter development time and less risk of failure due to toxicity problems (Pushpakom et al. 2018). Due to the shorter development times possible the cost of repurposing a drug for an alternative use can often be much cheaper than *de novo* drug development (Pushpakom et al. 2018; Sadeghi and Keyvanpour 2021). Examples of successfully repurposed drugs include zidovudine originally researched as a cancer treatment that was re-purposed as an anti-viral to treat HIV (Sperling 1998) and fingolimod

originally used in transplant rejection that was re-purposed to treat multiple sclerosis (Brinkmann et al. 2010). However, this approach is fairly new to the antimicrobial drug discovery field (Farha and Brown 2019). Some promising examples include miltefosine that was originally used for skin metastases repurposed to target the protozoan disease visceral leishmaniasis (Soto and Berman 2021), and the antiprotozoal pentamidine that has shown activity as a perturbant to the outer membrane of Gram-negative pathogens sensitizing them to colistin (Stokes et al. 2017).

5.2.3. Molecular Docking for Drug Discovery

Several computational approaches exist for repurposing drugs for drug discovery including literature-based models (Sadeghi and Keyvanpour 2021), drug-drug similarity screening (Pushpakom et al. 2018) and structure-based drug screening (Lionta et al. 2014). Structure-based drug screening is useful when there is a known structural target as is the case for cytochrome *bd-I*. The focus of this chapter is structure-based drug screening to target the quinol binding cleft of cytochrome *bd-I* in *E. coli*. Structure-based drug screening involves using a known protein structure to screen a library of potential ligands (Lionta et al. 2014; Batool et al. 2019). Firstly, the binding site to be targeted needs to be identified (Seeliger and de Groot 2010; Lionta et al. 2014; Feinstein and Brylinski 2015). A library of ligands then needs to be selected with the FDA approved drug libraries such as that from eDrug3D (Pihan et al. 2012; Douguet 2018) offering a useful source of known approved drugs. Some preparation steps are needed for the structure and ligands for optimal results (Lionta et al. 2014). Molecular docking is then used to dock the ligand library to the binding site with a free energy of binding estimate calculated (Lionta et al. 2014). The results can then be filtered (e.g. for toxicity) (Batool et al. 2019) and analysed (e.g. for specific binding interactions) (Lionta et al. 2014) and then the best candidates selected for experimental screening. AutoDock4 (Morris et al. 2009) and AutoDock Vina (Trott,O., Olson 2010) are examples of molecular docking software that are freely available. Autodock Vina is a popular freely-available molecular docking software that incorporates several improvements on the previous version AutoDock 4 (Morris et al. 2009; Jaghoori et al. 2016). Compared to AutoDock 4, Vina has been shown to achieve a smaller error between predicted and experimental free energies (Trott,O., Olson 2010). Furthermore, the new

optimized algorithm and ability to use multiple cores to run calculations in parallel means that the time taken to analyse each ligand is much lower with AutoDock Vina (Trott,O., Olson 2010) compared to its predecessor. AutoDock Vina can be automated to dock an entire ligand library providing 9 binding modes for each ligand complete with a binding affinity score (Jaghoori et al. 2016). The docking results are then easily viewed and analysed in PyMOL (Seeliger and de Groot 2010). Structure based drug design using molecular docking is possible with *E. coli* cytochrome *bd* due to the published structures (Safarian et al. 2019; Theßeling et al. 2019; Grauel et al. 2021; Grund et al. 2021) and known quinol binding site (Friedrich et al. 2022). A previous attempt was made to dock ligands to the menaquinol binding loop of the *Mycobacterium bd* oxidase which identified the novel inhibitor MQL-H₂ with potency in the micromolar range (Harikishore et al. 2020). This was conducted on a homology model of the *Mycobacterium tuberculosis bd* oxidase (Harikishore et al. 2020) before the structure was known. Now that several structures have been published, more accurate molecular docking is possible for *E. coli* and *Mycobacterium* cytochrome *bd* structures (Friedrich et al. 2022).

5.2.4. Structural Prediction for Molecular Docking

If a protein structure is not available or a region is missing then protein structural prediction can be used to provide the input structure for molecular docking. An example of a region with missing residues is the quinol binding site in *E. coli* cytochrome *bd*-I (Safarian et al. 2019) . Phyre2 (Kelly et al. 2015) and AlphaFold 2 (Jumper et al. 2021) are examples of software that can predict protein structures. Phyre2 uses homology modelling to known protein structures to provide structure predictions (Kelly et al. 2015).

In the latest Critical Assessment of protein Structure Prediction (CASP 14), structures predicted using AlphaFold 2 had a far higher accuracy than the other competing methods, with a median backbone accuracy of 0.96 Å r.m.s.d.₉₅ (C α root-mean-square deviation at 95% residue coverage) compared to the next best method at 2.8 Å r.m.s.d.₉₅ (Jumper et al. 2021).

AlphaFold 2 uses the input protein sequence to search several protein databases and create an MSA (Jumper et al. 2021). AlphaFold 2 also constructs an initial

representation of the structure using proteins that are likely to have a similar structure to the query sequence called the pair representation (Jumper et al. 2021). The MSA and pair representation are then fed through a neural network called the “Evoformer” that refines both the MSA and pair representation (Jumper et al. 2021). This leads into the final stage in which another neural network takes the refined MSA and pair representation combining them into a single structure (Jumper et al. 2021). AlphaFold 2 will refine the predicted structure by putting the finished structure model, refined MSA and pair representation back through both the “Evoformer” and final structure prediction neural networks (Jumper et al. 2021).

AlphaFold 2 has a growing open-access associated database of predicted structures (Varadi et al. 2021). *E. coli* is one of the first 20 model organisms that was selected for structure predicted of the protein sequences between 16-2700 residues in the UniProt reference proteome (*E. coli* K12) (Varadi et al. 2021).

5.3. Results

5.3.1. Generation of *E. coli* EC958 terminal oxidase mutant strains

In order to test potential inhibitors of *E. coli* terminal respiratory oxidases, mutant strains that express only one terminal oxidase (either cytochromes *bd-I*, *bo'* or *bd-II*) are clearly useful. The uropathogenic strain *E. coli* EC958 ST131 was chosen to construct these mutants to allow inhibitors to be tested on a pathogenic strain of *E. coli*. Existing single knockout strains of *E. coli* EC958 that lack either cytochrome *bd-I*, cytochrome *bd-II* or cytochrome *bo'* (Table 2.1) were used as a starting point.

To generate double *E. coli* EC958 ST131 terminal oxidase deletion mutants (i.e. that leaves only one terminal oxidase operon in the genome), λ -red mutagenesis was used. A total of three *E. coli* EC958 mutants were attempted to be constructed (Table 5.1). '*bd-I* only' and '*bo'* only' mutants were successfully constructed. These strains express cytochrome *bd-I* or cytochrome *bo'* respectively as the sole terminal respiratory oxidase. Despite several efforts the '*bd-II* only' strain failed to be constructed.

Table 5.1 - Double *E. coli* EC958 ST131 terminal oxidase deletion mutants

Mutant Name	Terminal Oxidases Deleted	Genes Deleted
'bd-I only'	Cytochrome <i>bd</i> -II	<i>appCB</i>
	Cytochrome <i>bo</i> '	<i>cyoA</i>
'bd-II only'	Cytochrome <i>bd</i> -I	<i>cydAB</i>
	Cytochrome <i>bo</i> '	<i>cyoA</i>
'bo' only'	Cytochrome <i>bd</i> -I	<i>cydAB</i>
	Cytochrome <i>bd</i> -II	<i>appCB</i>

The single respiratory mutants that were used as a starting point had a chloramphenicol (Cm^R) cassette in the place of the deleted oxidase gene. As the same approach would be used to delete other operons in the same strains, the Cm^R cassettes would have to first be removed from the single deletion mutant strains. The temperature sensitive flippase containing plasmid (pCP20-Gm) was used to remove the Cm^R cassette by recombination between flanking FRT sites, as previously described (Datsenko and Wanner 2000). Successful removal of the Cm^R cassettes was confirmed by colony PCR, as a reduction of 930bp is observed when Cm^R is 'flipped out' (Fig. 5.1 & 5.2).

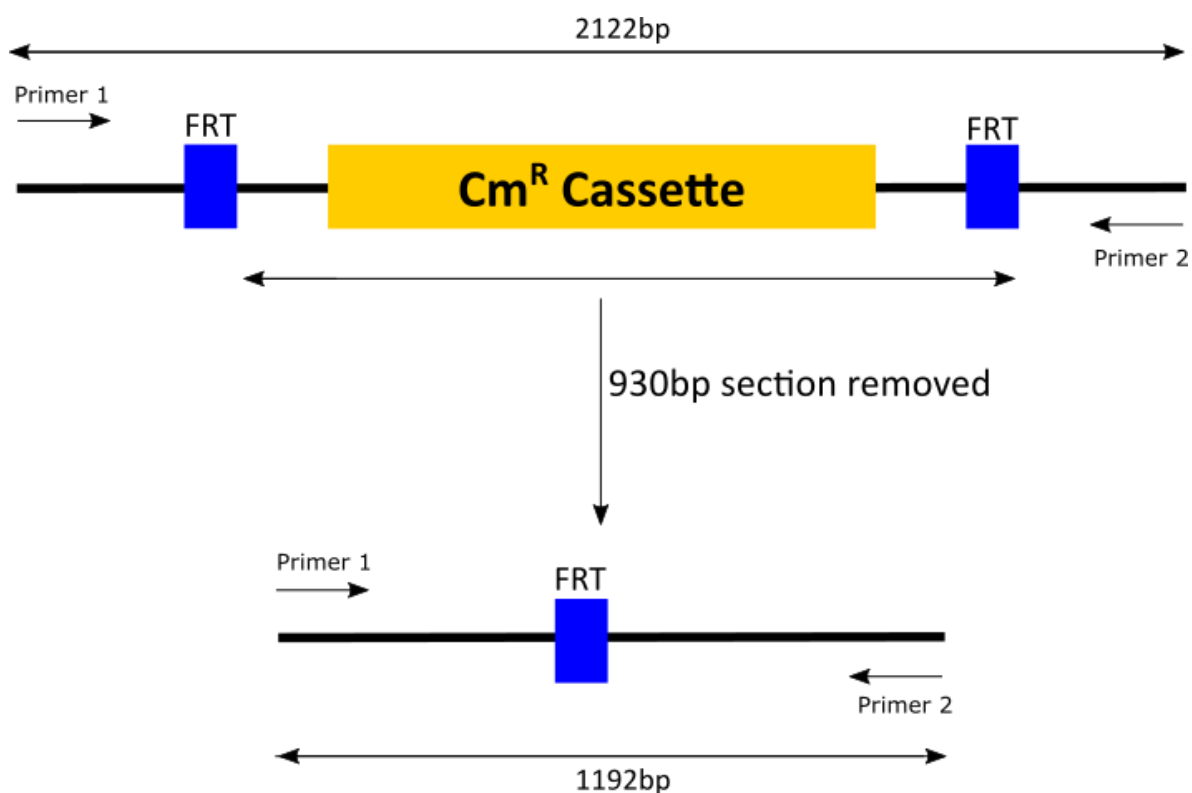


Figure 5.1 - Cm^R cassette removal from a cytochrome *bd-I* knockout strain. PCR primers used for confirmation of Cm^R cassette removal from Δ cytochrome *bd-I* mutant are labelled as Primer 1 (cydAB_F_600bp (394)) and Primer 2 (CydAB_R_500bpnew (417)) with the expected PCR product sizes shown.

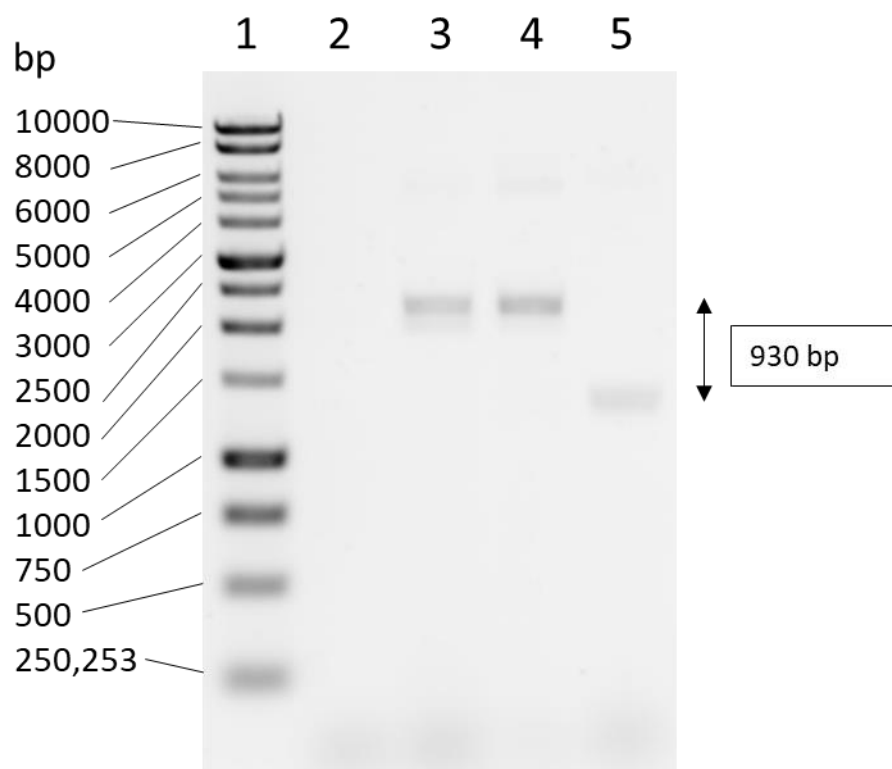


Figure 5.2 - PCR products gel for screening of Cm^R cassette removal from *E. coli* EC958 ST131 Δ cytochrome *bd-I* mutant. Promega 1kbp ladder is in lane 1, blank (lane 2) PCR product before Cm^R cassette removal (lanes 3+4), expected size: 2122 bp and PCR product after Cm^R cassette removal (lane 5), expected size: 1192 bp.

λ -Red mutagenesis allows mutations to be moved from strain to strain. This is achieved via amplification of the Cm^R cassettes from a deletion. An overhang region on each end of the Cm^R cassettes is also included in the amplification allowing λ -Red recombination to then incorporate the mutation into a different strain. Amplification of Cm^R cassettes with 500 bp overhang from each of the single *E. coli* EC958 ST131 terminal oxidase deletion mutants (Δ *cydAB* or Δ *cyoA* and Δ *appCB*) was carried out by PCR to provide the DNA needed for λ -Red recombination to form double terminal respiratory mutants (Fig. 5.3). The

DNA concentration has clearly been increased in the samples where 3 reactions were combined and concentrated (Fig. 5.3 + 5.4) necessary to increase λ -Red recombination efficiency.

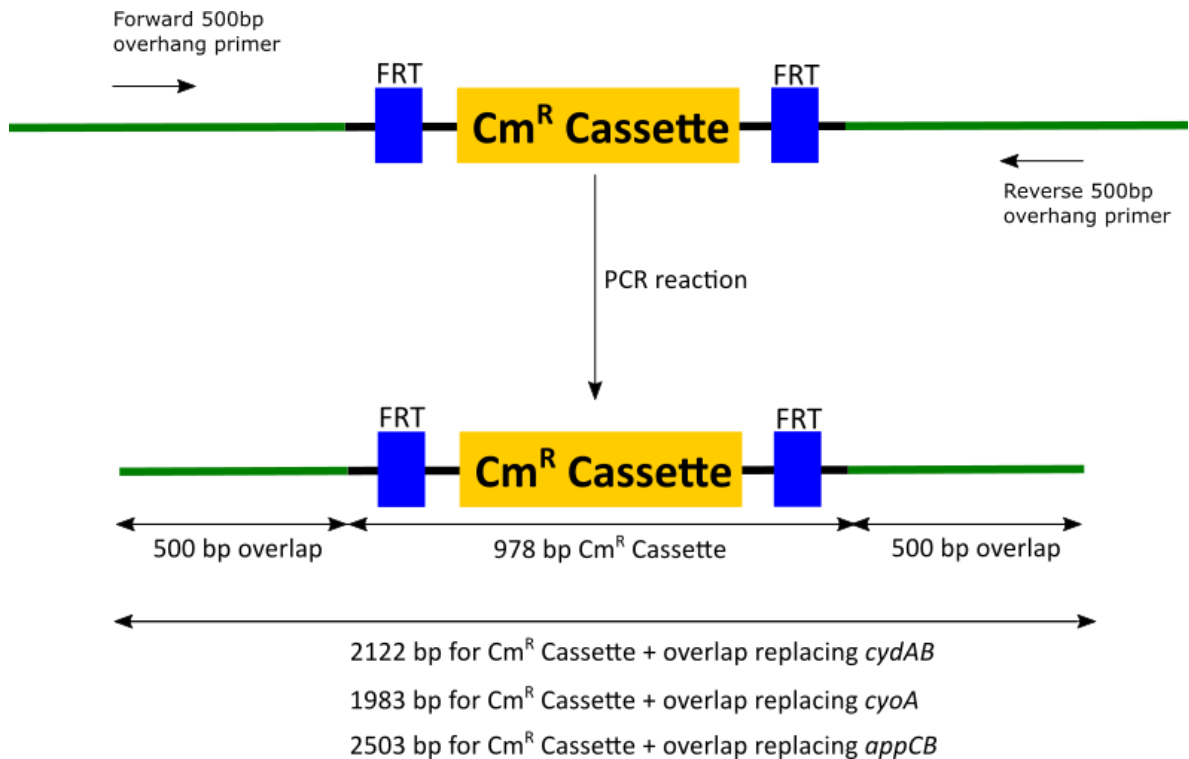


Figure 5.3 - PCR amplification of Cm^R cassettes with 500bp overhangs from single terminal respiratory mutants. Completed Cm^R with 500bp overlap PCR fragments vary slightly in size due to overhang primers not binding exactly 500bp from the Cm^R cassette (sites selected for optimal primer annealing).

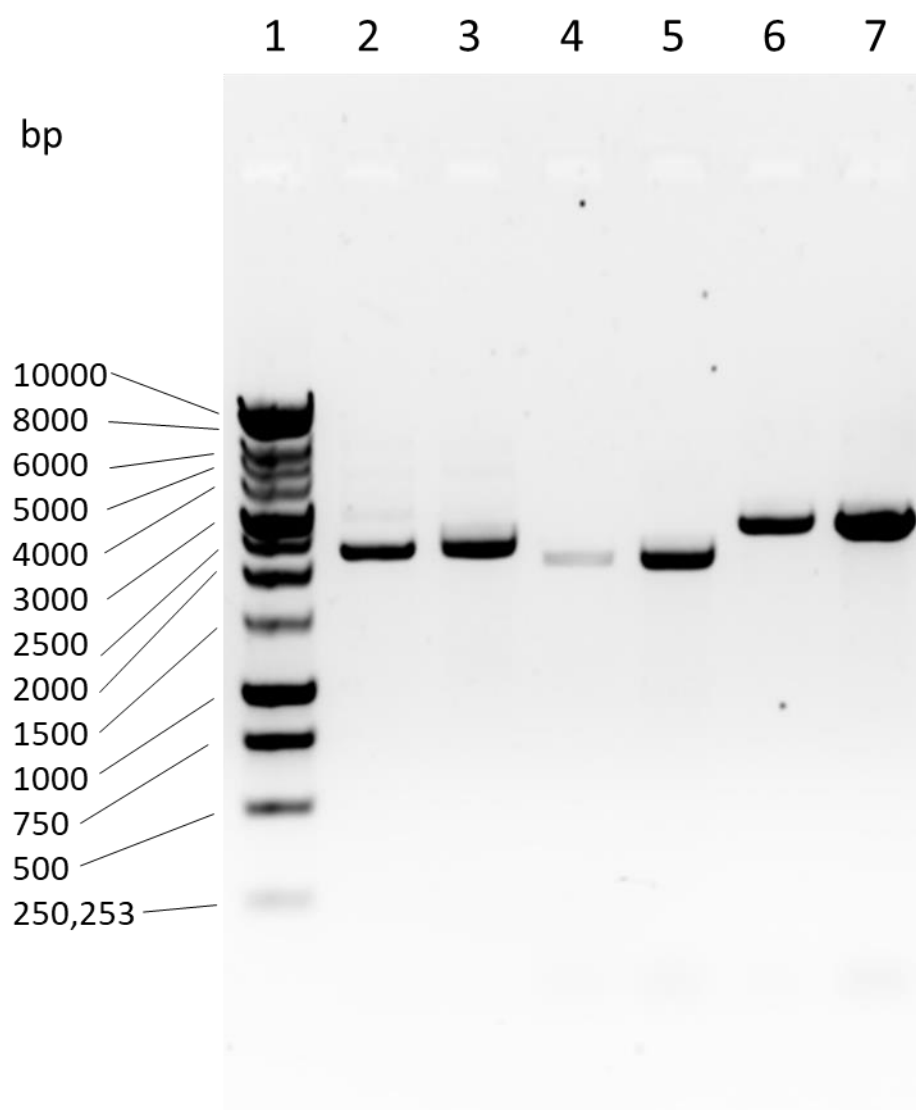


Figure 5.4 - PCR amplification of Cm^R cassettes with 500bp overhangs from ΔcydAB , ΔcyoA and ΔappCB mutants. Promega 1kb ladder is in lane 1, ΔcydAB Cm^R cassette (lane 2), ΔcydAB Cm^R cassette 3 PCR concentrated sample (lane 3), ΔcyoA Cm^R cassette (lane 4), ΔcyoA Cm^R cassette 3 PCR concentrated sample (lane 5), ΔappCB Cm^R cassette (lane 6) and ΔappCB Cm^R cassette 3 PCR concentrated sample (lane 7). Expected sizes in Figure 5.3.

The ΔcydAB , ΔcyoA and ΔappCB mutants (section 2.1.4 and section 2.1.6) were transformed with the temperature sensitive plasmid pKOBEG which contains the phage λ -Red recombinase machinery. The λ -Red recombinase machinery is controlled by an arabinose induced promoter (araBAD). ΔcydAB , ΔcyoA and ΔappCB mutants were grown in M9 medium with 20 mM L-arabinose used to induce expression of the λ -Red recombinase

machinery (section 2.1.11). Electroporation of the Cm^R cassettes into the single terminal oxidase mutants then followed with λ -Red recombination resulting in 'bd-I only', 'bo' only' or 'bd-II only' mutants. After electroporation of the Cm^R cassettes into the single *E. coli* EC958 ST131 terminal oxidase deletion mutants' chloramphenicol resistant colonies were screened using colony PCR via two methods. The first involved screening primers that bind 200bp either side of the inserted Cm^R cassette for the ΔcydAB , ΔcyoA or ΔappCB position in the *E. coli* genome (Fig. 5.5). Figure 5.6 displays colony screening for potential ΔcyoA $\Delta\text{appCB}::\text{Cm}^R$ mutants with 5 positive colonies shown (lanes 2-6). A further PCR screening reaction was completed using one of the positive ΔcyoA $\Delta\text{appCB}::\text{Cm}^R$ colonies using a primer that binds internally to the Cm^R gene (Fig. 5.7). Figure 5.8 shows a positive result in (lanes 4-5) for ΔappCB deletion.

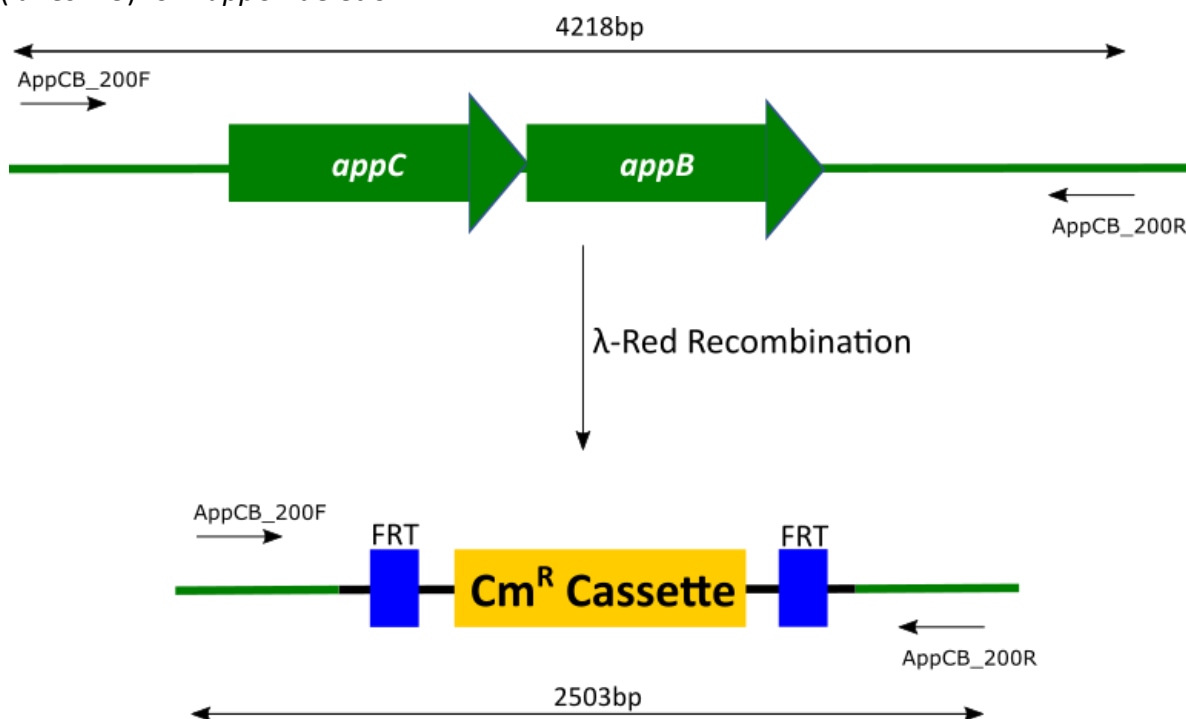


Figure 5.5 PCR confirmation of double terminal respiratory mutant knockouts using primers with 200bp overhang to the ends of the inserted Cm^R gene. This example shows knockout of *appCB* operon with 200bp overhang primers (AppCB_200F and AppCB_200R) used to confirm the deletion and insertion of Cm^R cassette via the size difference in PCR product shown.

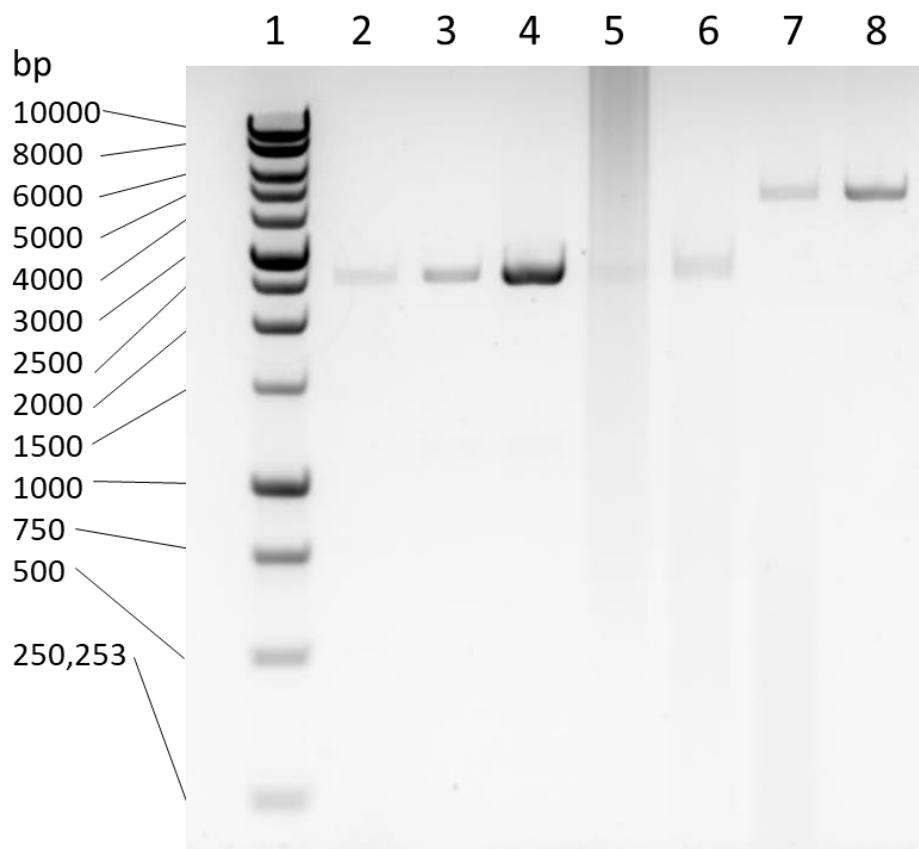


Figure 5.6 - PCR products gel for screening of *E. coli* EC958 ST131 Δ cytochrome *bo'* mutant colonies for Δ *appCB* deletion. Promega 1kbp ladder is in lane 1, PCR product from colonies after λ -Red recombination for deletion of *appCB* and insertion of Cm^R cassette (lanes 2-6), negative control wild type (lane 7) and negative control Δ cytochrome *bo'* (lane 8). *appCB* deletion expected size: 2503 bp, negative controls containing *appCB* expected size: 4218 bp.

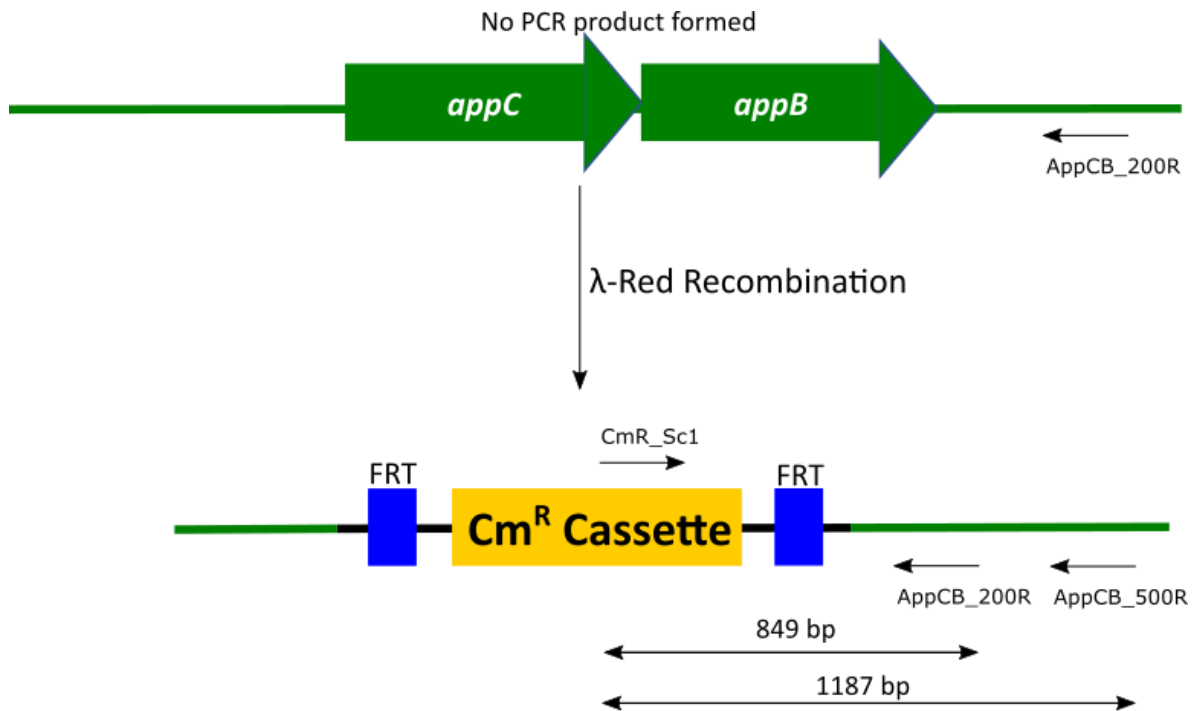


Figure 5.7 - PCR confirmation of *appCB* mutant knockouts using a forward primer that binds internally to the inserted Cm^R gene and a reverse primer that binds with 200bp or 500bp overhang to the end of the Cm^R gene. This example shows knockout of *appCB* operon with 500bp or 200bp reverse overhang primers (AppCB_500R and AppCB_200R) and Cm^R internal binding primer (CmR_Sc1) used to confirm the deletion and insertion of Cm^R cassette via the PCR product shown. Before λ-Red mutagenesis only the reverse primers can bind and so no PCR products are seen in non-mutated colonies.

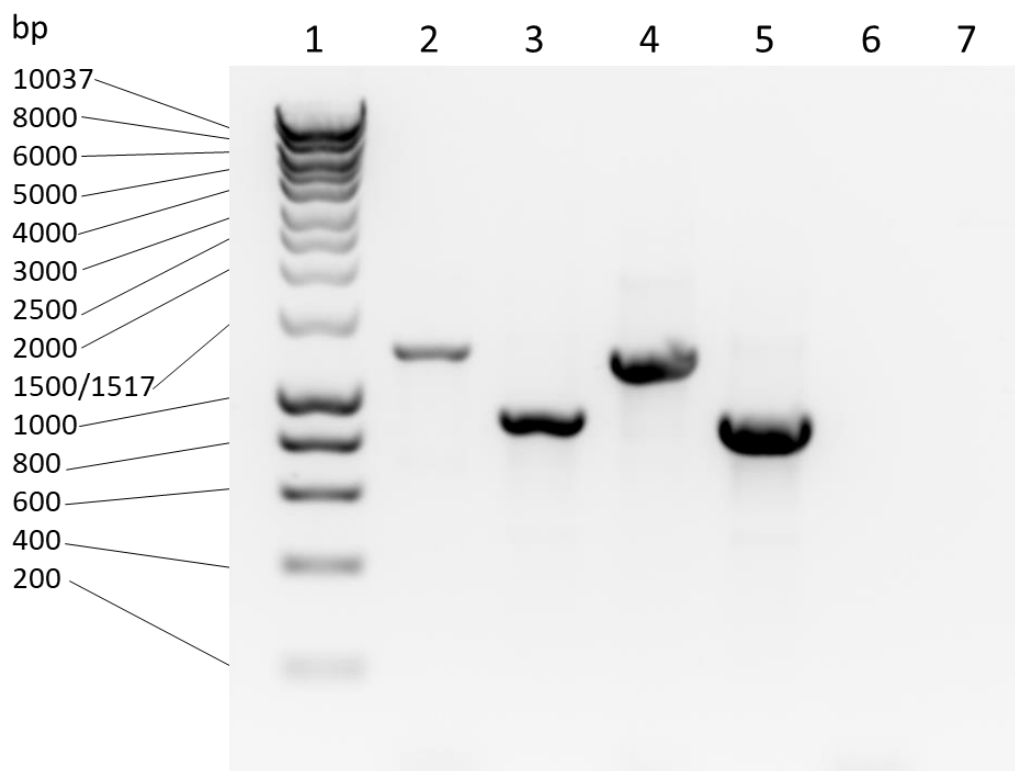


Figure 5.8 - PCR products of screening PCR for $\Delta appCB$ deletion ($\Delta cyoA appCB::Cm$) using internal Cm^R binding primer. Hyperladder 1kb ladder is in lane 1, PCR product from positive control $\Delta appCB::Cm$ colony using 500 bp reverse primer (lane 2), positive control $\Delta appCB::Cm$ colony using 200 bp reverse primer (lane 3), $\Delta cyoA appCB::Cm$ colony using 500 bp reverse primer (lane 4), $\Delta cyoA appCB::Cm$ colony using 200 bp reverse primer (lane 5), negative control wild type colony using 500 bp reverse primer (lane 6) and wild type colony using 200 bp reverse primer (lane 7). *appCB* deletion expected size: 1187 bp for 500 bp reverse primer reaction and 849 bp for 200 bp reverse primer reaction. No bands expected for wild type.

In total, two different *E. coli* EC958 ST131 double terminal respiratory mutants were created by λ -Red mutagenesis: $\Delta cyoA appCB::Cm$ (*bd-I* only) and $\Delta cydAB appCB::Cm$ (*bo'* only). Both were confirmed using colony PCR. To ensure that the knockout mutations were present, all of the double terminal respiratory mutants had the knockout region sequenced by sanger sequencing with the result seen in Figure 5.9 and Appendix A.6. Cm^R successfully replaced the *appCB* operon in the $\Delta cyoA appCB::Cm$ mutant and $\Delta cydAB appCB::Cm$ (Figure 5.9 and Appendix A.6). Despite several attempts, the mutant $\Delta cydAB cyoA::Cm$ (*bd-II* only) could not be constructed.

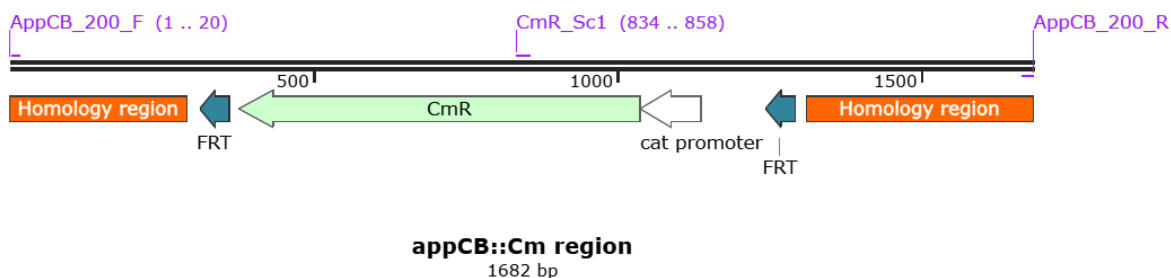


Figure 5.9 - Sanger sequencing result of double terminal respiratory mutants $\Delta cyoA$ *appCB::Cm* (*bd-I* only) and $\Delta cydAB$ *appCB::Cm* (*bo'* only) displaying *appCB* deleted region.

Homology regions are the regions flanking the *appCB* locus used for lambda red mutagenesis. Cm^R cassette consisting of the Cm^R gene, cat promoter and associated FRT sites have been inserted in place of the *appCB* operon. AppCB_200_F and AppCB_200_R are the primers used for sequencing. CmR_Sc1 is the internal primer used for the second colony PCR to confirm *appCB* deletion. Sequence provided in Appendix A.6.

5.3.2. Analysis of spectral characteristics and growth properties for *E. coli* EC958 terminal oxidase deletion mutants

Whole cell reduced *minus* oxidised (Fig. 5.10) and CO difference (Fig. 5.11) spectra were recorded for *E. coli* EC958 WT, $\Delta cyoA$ *appCB::Cm* (*bd-I* only) and $\Delta cydAB$ *appCB::Cm* (*bo'* only) mutants. For the reduced *minus* oxidised spectra (Fig. 5.10), all of the distinctive features of the cytochrome *bd* oxidase are present in the WT and cytochrome *bd-I* only samples. The most distinctive feature for cytochrome *bd-I* is the peak at 630 nm that is characteristic of the ferrous form of haem *d* (Poole et al. 1989). Troughs are present at 650 nm and 680 nm due to the oxygenated (Poole 1983) and peroxy (Poole and Williams 1988) forms of the haem *d* cofactor (Poole et al. 1989). The shoulder of the peak at 558 nm is from cytochrome *b*₅₅₈ which also contributes to the Soret band at 426 nm and the overlapping β bands at 527 nm (Poole et al. 1989). Cytochrome *b*₅₉₅ is responsible for the peak around 590 nm along with most of the peak at 436 nm (Poole et al. 1989). For the reduced *minus* oxidised spectra for the '*bo'* only' strain, the peak for haem *d* at 630 nm and troughs at 650 nm and 680 nm for the oxy- and peroxy- forms of cytochrome *d* are not present as expected. The shoulder on the Soret band at 436 nm is also absent in the '*bo'* only' mutant.

The CO difference spectra (Fig. 5.11) showed a large peak at 640 nm for the WT and 'bd-I only' mutant which corresponds to the ferrous cytochrome *d*-CO complex (Poole et al. 1989). Accordingly, this peak at 640 nm is absent in the spectra for the 'bo' only' mutant. A broad trough is also seen in the WT and 'bd-I only' mutant spectra between around 570 nm and 620 nm with haems *b*₅₉₅ and *d* contributing (Poole et al. 1989). The spectra for the cytochrome *bo*' only mutant is similar to that for purified cytochrome *bo*' with the peak at 414 due to the cytochrome *o*-CO complex (Kita et al. 1984) (Poole et al. 1989).

An aerobic growth assay was also conducted to determine the effect of the double terminal respiratory mutations upon the growth of *E. coli* EC958 (Fig. 5.12). Both the 'bd-I only' and 'bo' only' mutant strains show a small lag in growth before the exponential phase compared to the wild-type. Exponential growth rate is very similar for the mutant strain, with both reaching the same final OD₆₀₀ in stationary phase.

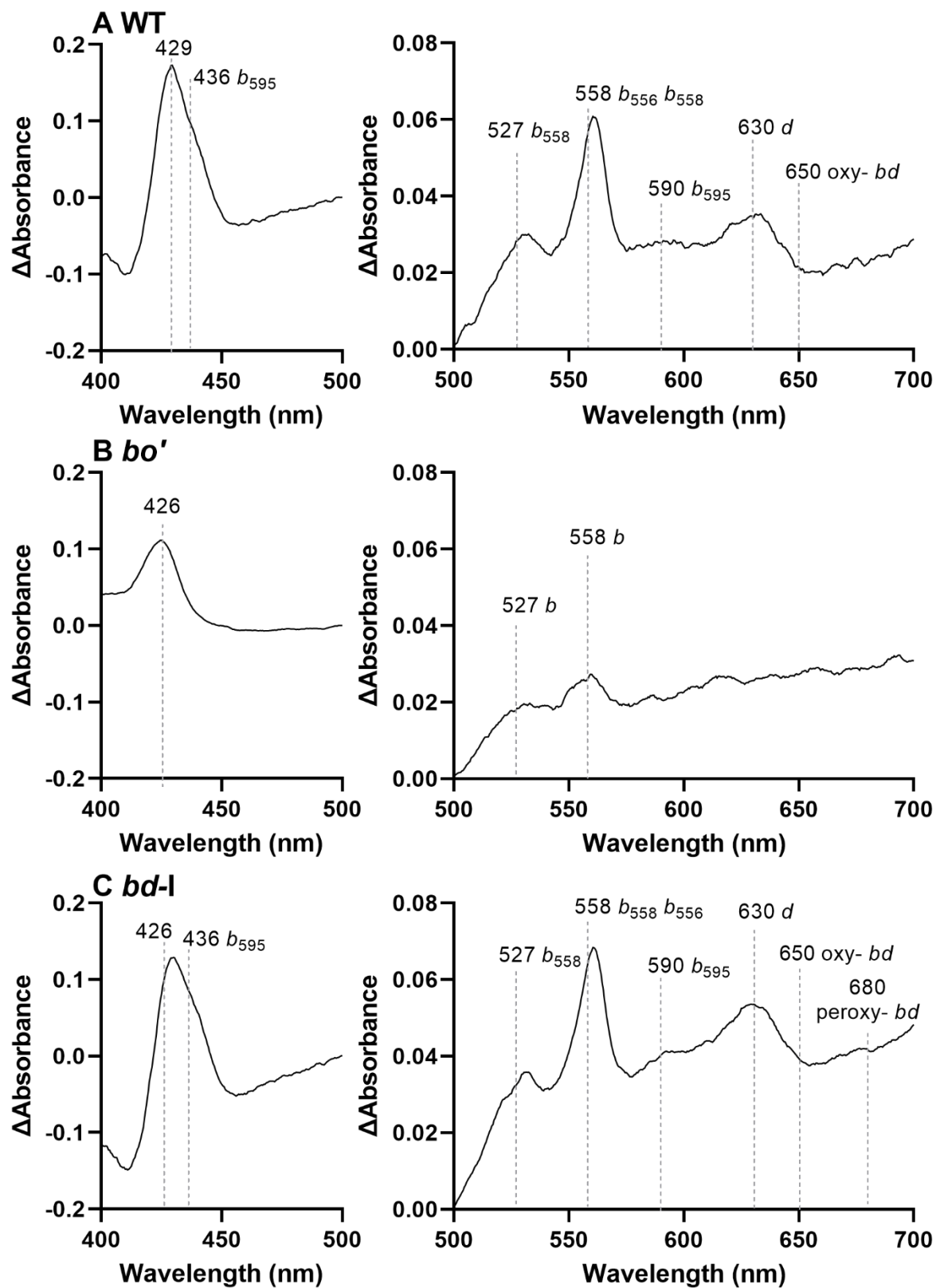


Figure 5.10 - Reduced (dithionite) *minus* oxidised (persulphate) whole cell spectra for *E. coli* EC958 (A) wild-type, (B) '*bo*' only and (C) '*bd-I* only' strains.

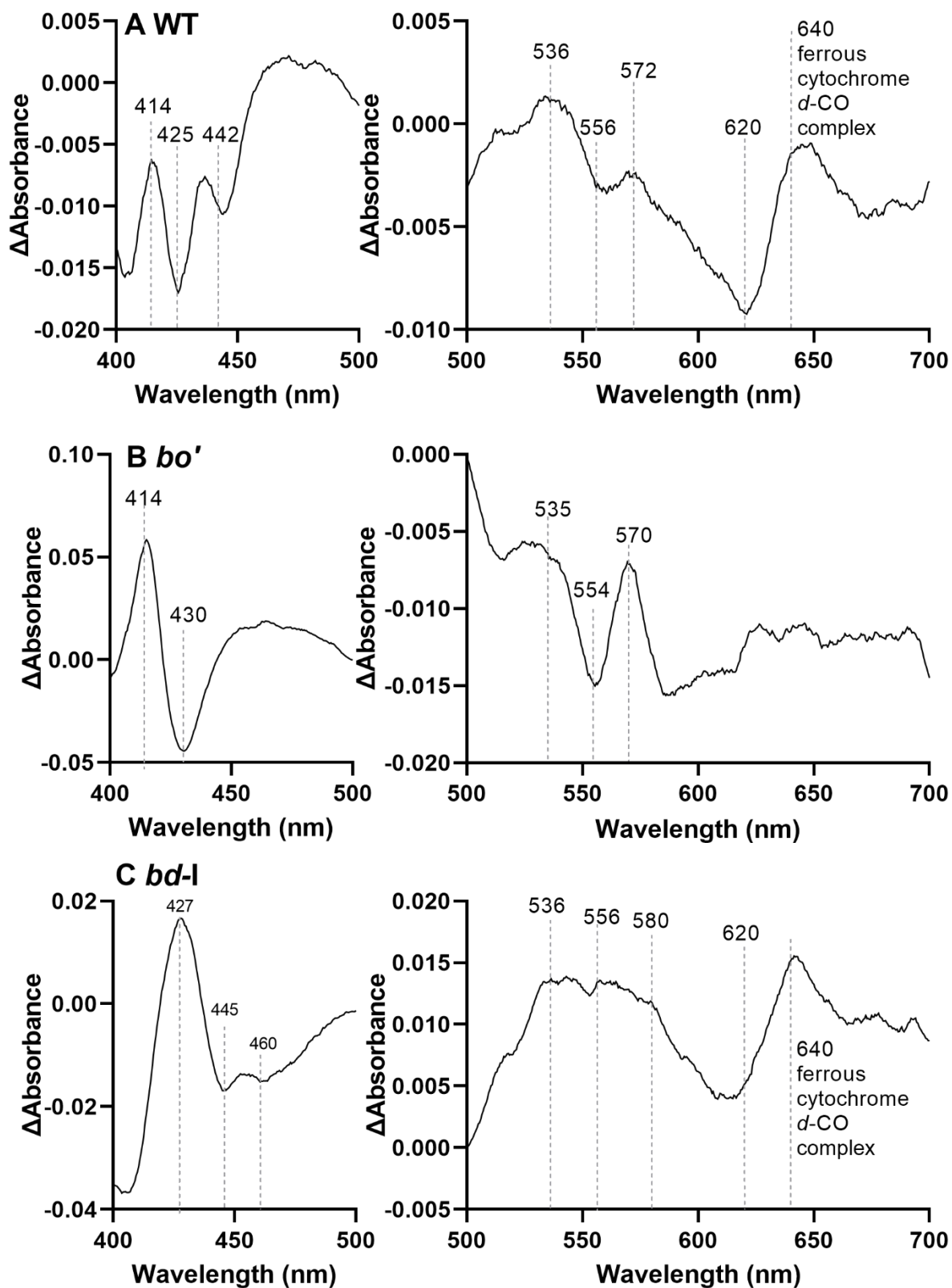


Figure 5.11 - CO difference whole cell spectra for *E. coli* EC958 (A) wild-type, (B) '*bo*' only' and (C) '*bd-I* only' strains.

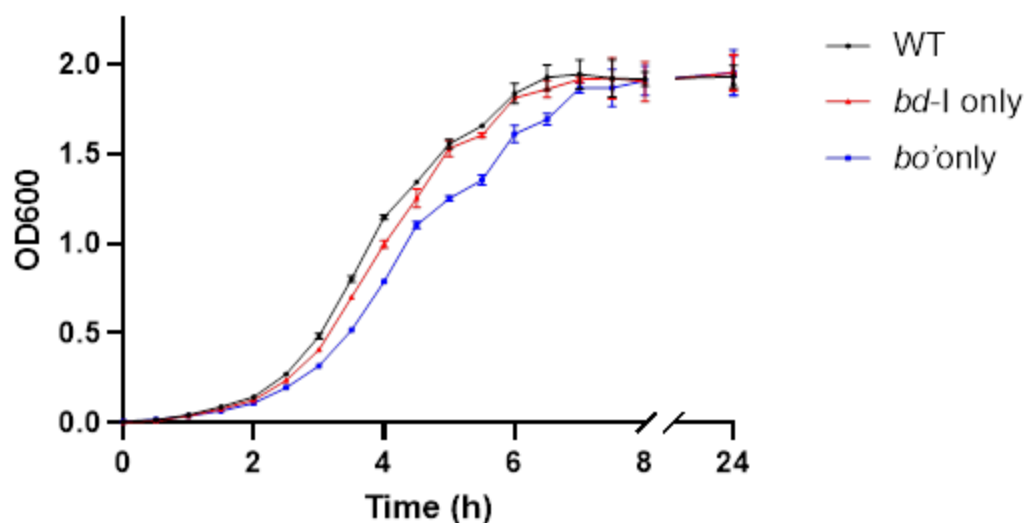


Figure 5.12 - Aerobic growth curves for *E. coli* EC958 ST131 (A) wild-type, (B) '*bo'* only' and (C) '*bd-I* only' strains. *E. coli* was grown under aerobic conditions in M9 minimal medium supplemented with 0.1% casamino acids. Mean OD₆₀₀ values are shown +/- standard deviation from 3 biological repeats.

5.3.3. *E. coli* cytochrome *bd-I* membrane spectra

To screen compounds for inhibitory activity against *E. coli* cytochrome *bd-I*, oxygen consumption assays on purified '*bd-I* only' membranes would have to be set up. The *E. coli* EC958 $\Delta cyoA/\Delta appCB$ mutant (i.e. '*bd-I* only') was grown at low aeration (1 L of LB in a 2 L conical flask at 150 rpm and 37 °C) overnight resulting in membranes containing cytochrome *bd-I* as the sole terminal oxidase. The reduced *minus* oxidised and CO difference spectra were recorded for these membranes (Fig. 5.13). The spectra produced are similar to the whole cell spectra for '*bd-I* only' cells (Fig. 5.10 and 5.11). For the reduced *minus* oxidised spectra (Fig. 5.13A) the peak at 630 nm provided by the ferrous

form of haem *d* (Poole et al. 1989) is present along with the trough at 650 nm due to oxygenated cytochrome *d* (Poole 1983). The shoulder on the Soret band at 436 nm is indicative of the high-spin haem *b*₅₉₅ along with the small peak at 590 nm (Poole et al. 1989). In the CO difference spectra (Fig. 5.13B) the large peak at 640 nm which corresponds to the ferrous cytochrome *d*-CO complex is present along with the trough between around 570 nm and 620 nm with contributions provided from haem *b*₅₉₅ and *d* (Poole et al. 1989).

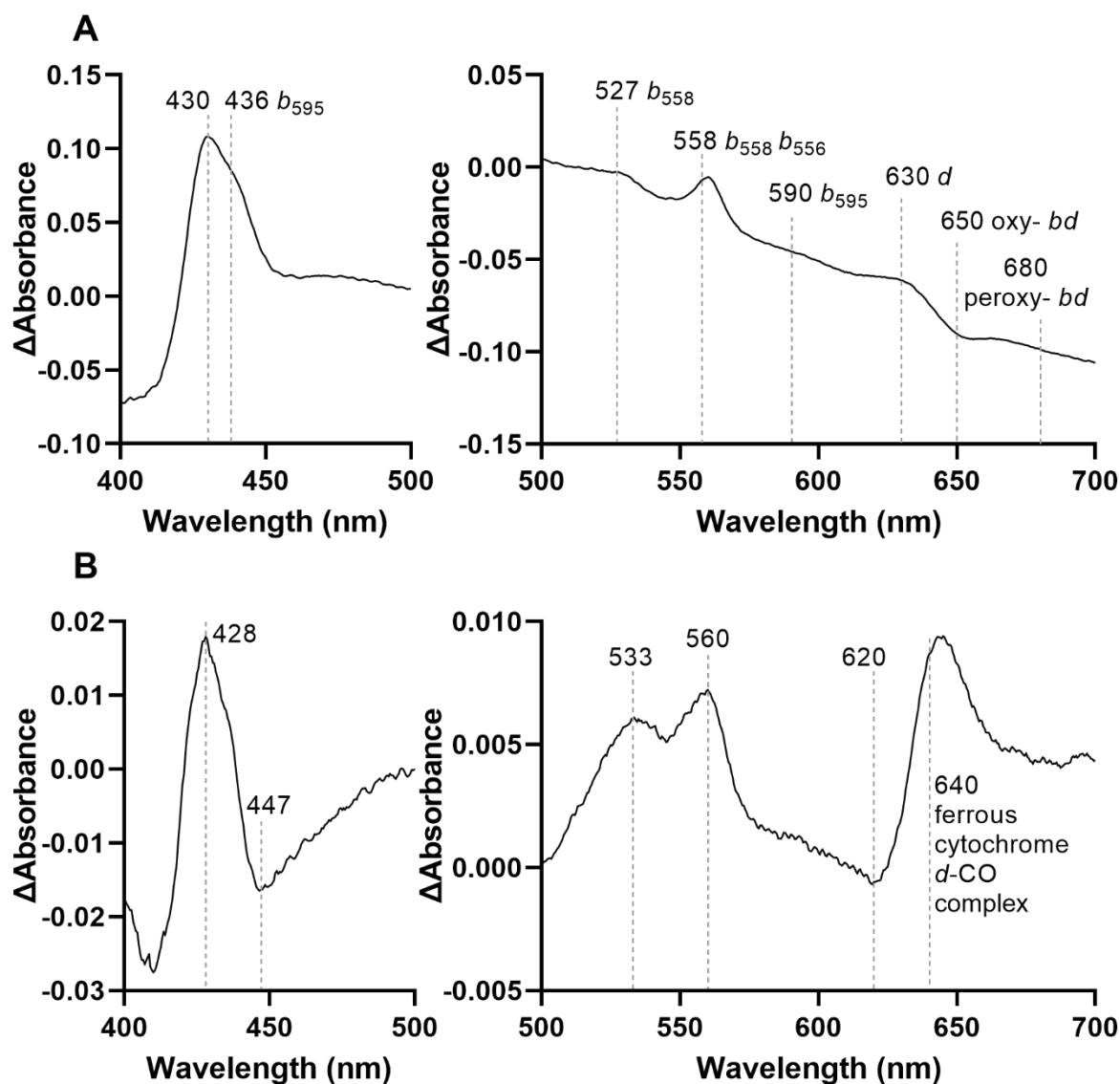


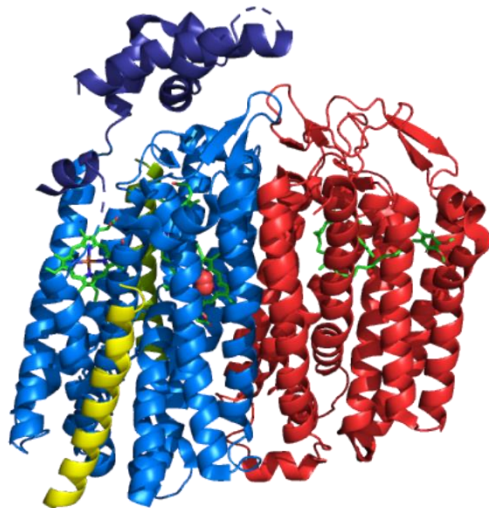
Figure 5.13 - Spectral analysis of membranes isolated from *E. coli* EC958 $\Delta cyoA/\Delta appCB$ (' bd -I only'). (A) Reduced *minus* oxidised difference spectra using 50 mg/mL membranes. Samples were oxidised with ammonium persulphate and reduced with sodium dithionite. Spectra shown are the difference between the reduced and oxidised spectra. Path length was 1 cm with the scan rate set at 300 nm min⁻¹ using 0.5 nm intervals. (B) CO difference spectra using 50 mg/mL membranes suspended in 20 mM Tris pH 7.4. Sample was reduced with dithionite then bubbled with CO for 5 min. Difference spectra shown are the difference between the CO treated reduced sample and the reduced sample before CO treatment. Path length was 1 cm with the scan rate set at 300 nm min⁻¹ using 0.5 nm intervals.

5.3.4. Generating a structural model of *E. coli* cytochrome *bd-I* with an intact Q-loop

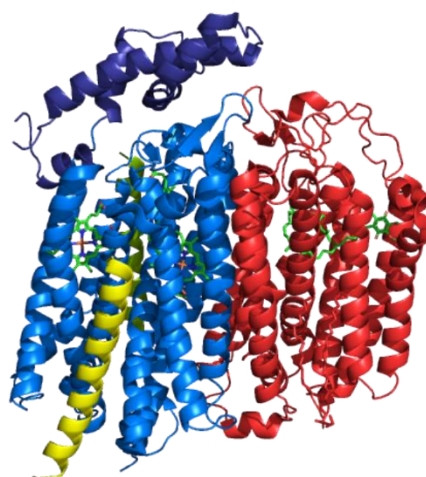
There are currently two cryo-EM structures available for *E. coli* cytochrome *bd-I*. These are the protein data bank structures 6RKO (Safarian et al. 2019) and 6RX4 (Theßeling et al. 2019). However, these structures are both missing sections of the Q-loop of the CydA subunit. Hence, in order to perform in silico drug screening on the quinol binding site these missing sections needed to be resolved. Therefore, a homology model of the CydA subunit was constructed using Phyre2 (Kelly et al. 2015) to ‘fill in the missing parts’. Once CydA was modelled the structure was aligned to the protein data bank structure 6RKO using pymol and the other sub-units CydB, CydX and CydH along with the haem groups, ubiquinol-8 and oxygen molecule in the active site were transferred to make a composite model. Two different models were made, one using Phyre2 normal mode and the other using intensive mode (Fig. 5.14). The normal model uses homology modelling based on the template 6RKO to model CydA along with loop modelling to model any deletions. When the Phyre2 normal mode model is aligned to 6RKO, the RMSD is 0 (Fig. 5.15) due to the template used having nearly exactly the same sequence as the homology model. *E. coli* EC958 *cydA* sequence was used for the modelling which has 3 substitutions compared to *E. coli* K-12 used in the 6RKO structure. These are all in regions away from the haem cofactors and quinol binding site. 6RKO lacks electron density in three regions of the Q-loop: E240-V248, A258-L310 and G334-A339. The normal model used loop modelling to fill the gap between E240-V248, of which only the backbone can be roughly traced in 6RKO. The gap between A258-L310 was too large to be modelled by loop modelling and so this is missing from the normal mode structure. In order to model the entire Q-loop a Phyre2 intensive mode structure was also created (Fig 5.14) with an RMSD to 6RKO of 0.122 Å (Fig. 5.15). Phyre2 intensive mode uses multiple templates that cover different regions of the query sequence to construct a homology model. In this case both of the cryo-EM structures 6RX4 and 6RKO were used which each have different regions of the Q-loop resolved (Fig. 5.15). This still leaves a large gap between Q263-T302 not covered by either 6RX4 or 6RKO. Phyre2 in intensive mode uses *Ab initio* modelling using POING to fill in any large gaps (Kelly et al. 2015). The modelled region between Q263-T302 must therefore be treated with caution as *Ab initio* modelling

is unreliable (Kelly et al. 2015). Due to the more complete Q-loop the Phyre2 intensive model was selected for the first docking screen.

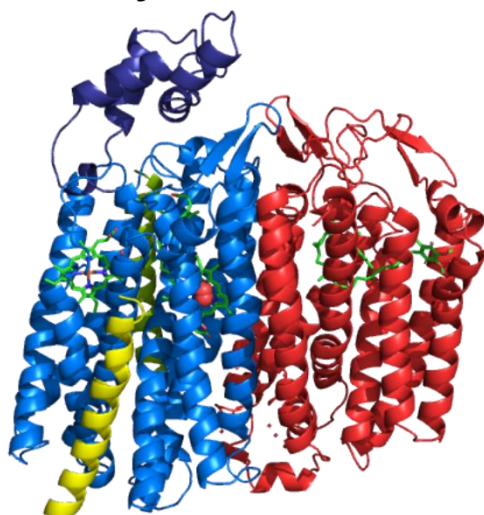
A) 6rko



B) 6rx4



C) *bd-I* Phyre 2 normal



D) *bd-I* Phyre 2 intensive

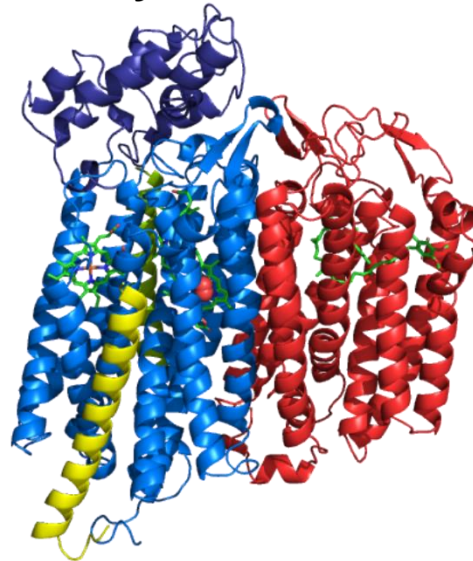
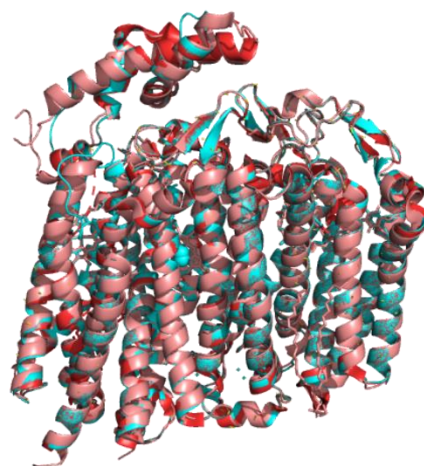
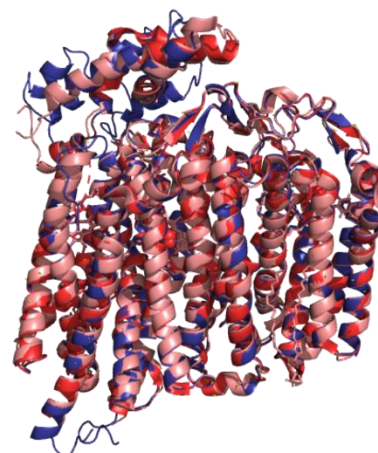


Figure 5.14 - *E. coli* cytochrome *bd-I* structures and models. *E. coli* cytochrome *bd-I* cryo-EM structures from the protein data bank (A) 6RKO and (B) 6RX4. Phyre2 produced *E. coli* cytochrome *bd-I* models in (C) normal mode and (D) intensive mode.

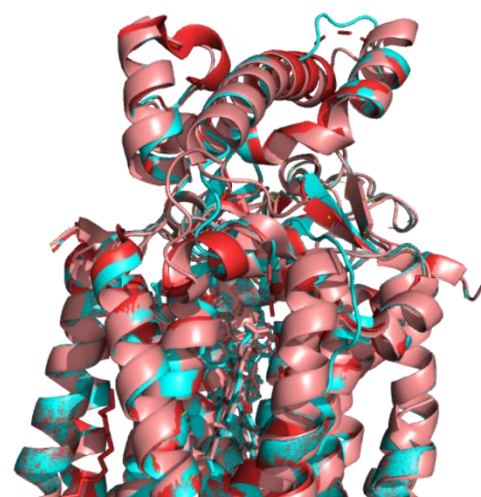
A) *bd-I* Phyre 2 normal
RMSD = 0Å



B) *bd-I* Phyre 2 intensive
RMSD = 0.122Å



C) *bd-I* Phyre 2 normal
Q-loop



D) *bd-I* Phyre 2 intensive
Q-loop

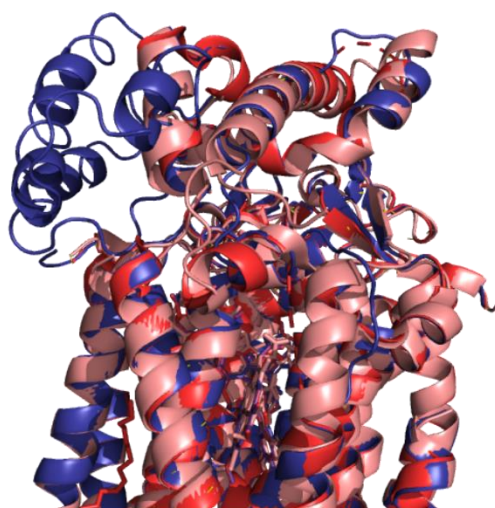


Figure 5.15 - Structural alignment of *E. coli* EC958 cytochrome *bd-I* Phyre2 models to cryo-EM structures. *E. coli* cytochrome *bd-I* cryo-EM structures 6RX4 (pink) aligned to 6RKO (red) for all sub-parts. (A) Overall structure of Phyre2 produced model in normal mode (cyan) aligned to 6RKO (red). (B) Overall structure of Phyre2 produced model in intensive mode (dark blue) aligned to 6RKO (red). Q-loop regions of the Phyre2 normal mode structure (C) and intensive structure (D) aligned to 6RKO (red).

5.3.5. Modelling the *E. coli* cytochrome *bd-I* Q-loop

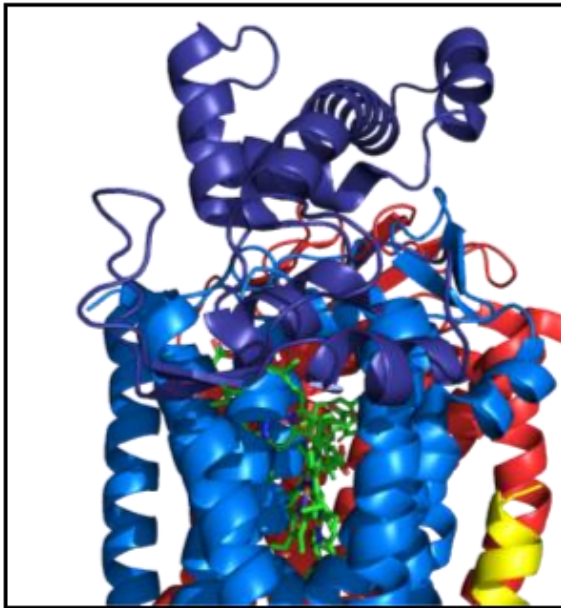
The structure of *E. coli* cytochrome *bd-II* (*appCBX*) was solved at 3Å resolution by cryo-EM in November 2021 (Grauel et al. 2021). Interestingly, this structure was solved with

bound inhibitor aurachin D has a fully resolved Q-loop. The overall structure of *E. coli* cytochrome *bd-II* is very similar to *bd-I* with a high sequence similarity of ~60% including the Q-loop. A further structure of *E. coli* cytochrome *bd-II* with the Q-loop not fully resolved has also been published, which suggests flexibility in the Q-loop (Grund et al. 2021). These two studies suggest that a bound inhibitor (i.e. aurachin D) is required to immobilise the Q-loop of cytochrome *bd-II* to enable this region to be resolved at high resolution via cryo-EM (Grauel et al. 2021; Grund et al. 2021).

A significant difference in the Q-loop fold is seen between the *E. coli* cytochrome *bd-II* structure with the inhibitor aurachin D bound and the Phyre2 intensive model *bd-I* structure (Fig. 5.16). An alpha helix is located in front of the haem *b*₅₅₈ quinol binding site in the cytochrome *bd-II* structure (Ala90 – His98) that forms part of the aurachin D (and quinol) binding site (Fig. 5.16), which is not present in the Phyre2 intensive model *bd-I* structure (Fig. 5.16). Hence, an improved cytochrome *bd-I* model was created using the cytochrome *bd-II* Q-loop as a modelling template. For this purpose, the new AlphaFold protein structure prediction software version 2 (Jumper et al. 2021) was selected. The *E. coli* cytochrome *bd-I* subunit I (CydA) prediction was downloaded in .pdb format and is shown with the *E. coli* cytochrome *bd-II* structure (Fig. 5.17). Nearly all the residues in the prediction have a pLDDT confidence value of >70 (confident), including all residues in the Q-loop. The full AlphaFold 2 *E. coli* cytochrome *bd-I* model was created by aligning the AlphaFold2 CydA structure to the cryo-EM *bd-I* structure 6RKO using Pymol and the other sub-units CydB, CydX and CydH along with the haem groups, ubiquinol-8 and oxygen molecule in the active site were superposed to make a composite model.

The *bd-I* AlphaFold 2 model aligns very closely to the cytochrome *bd-II* with bound aurachin D structure (Fig. 5.18 A+C) with RMSD = 0.695 Å. When the *bd-I* AlphaFold 2 model is aligned to the cytochrome *bd-I* cryo-EM structure, 6RKO and the Phyre2 intensive model (Fig. 5.18 B+D), a clear difference in Q-loop structures can be seen. The Phyre2 intensive model (dark blue) has a large alpha helix in the Q-loop region towards the periplasmic space. However, in the AlphaFold 2 model this alpha helix is not present, with the Q-loop region closely following the Q-loop fold of the cytochrome *bd-II* structure (Fig 5.18 C+D).

A) 7ose - *bd-II*



B) *bd-I* Phyre2 Intensive Model

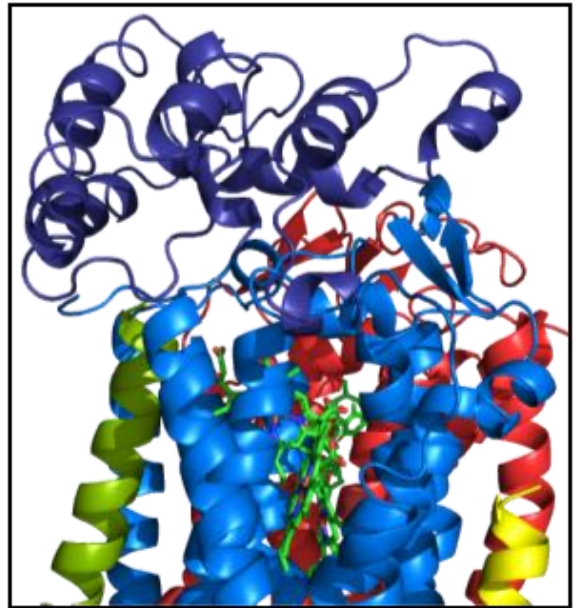
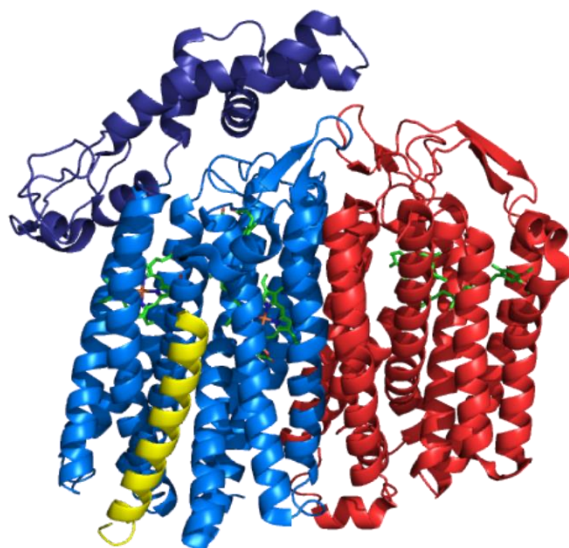
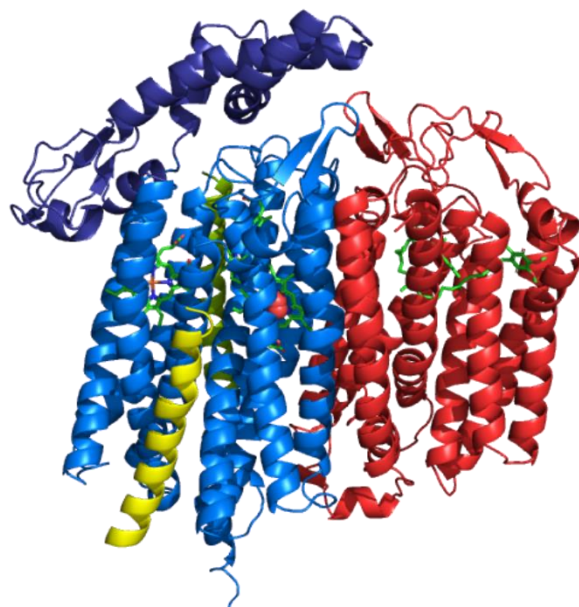


Figure 5.16 - Q-loop differences between *E. coli* cytochrome *bd-I* Phyre2 intensive mode structure and *E. coli* cytochrome *bd-II* cryo-EM structure. CydA Q-loop of both structures highlighted in dark blue against the rest of CydA in blue.

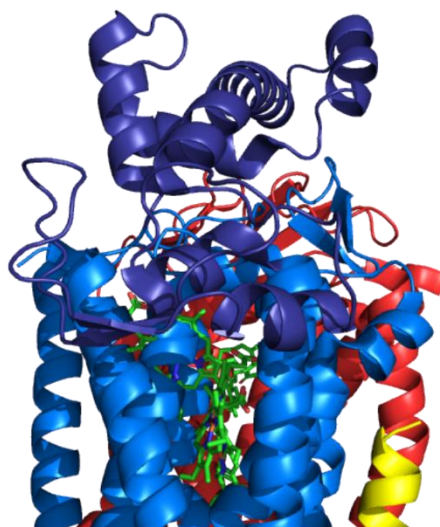
A) 7ose - *bd-II*



B) *bd-I* AlphaFold2



C) 7ose - *bd-II* Q-loop



D) *bd-I* AlphaFold2 Q-loop

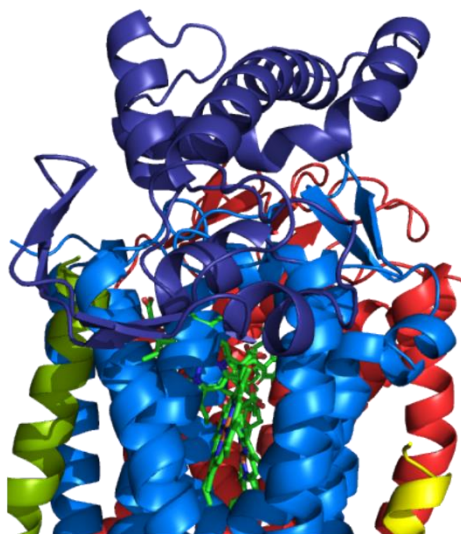
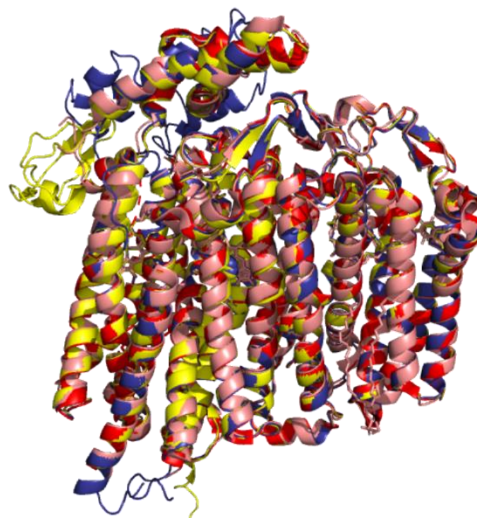


Figure 5.17 - *E. coli* cytochrome *bd-II* structure and improved *bd-I* model. (A) and (C) *E. coli* cytochrome *bd-II* cryo-EM structure 7ose from the protein data bank (Grauel et al. 2021). (B) and (D) the structural model of *E. coli* cytochrome *bd-I* that was produced using AlphaFold2.

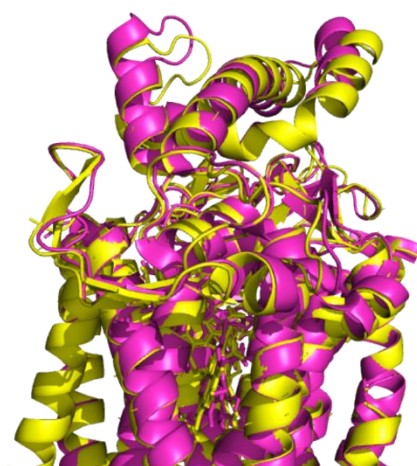
A) *bd-I* AlphaFold2 model with
7ose (*bd-II*) alignment
RMSD = 0.695Å



B) *bd-I* AlphaFold2/Phyre2 intensive
model with 6rko (*bd-I*) alignment
bd-I AlphaFold2 RMSD = 0.184Å



C) *bd-I* AlphaFold2 model
with 7ose (*bd-II*) alignment
Q-loop region



D) *bd-I* AlphaFold2/Phyre2 intensive
model with 6rko (*bd-I*) alignment
Q-loop region

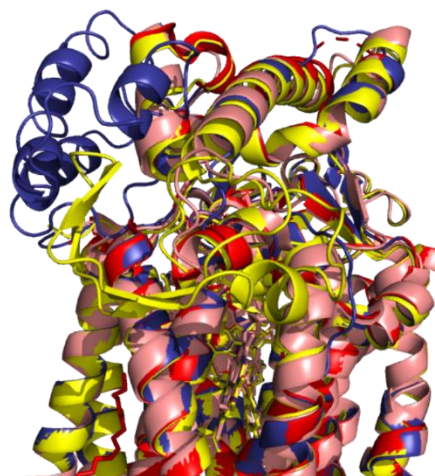


Figure 5.18 - Structural alignment of *E. coli* EC958 cytochrome *bd-I* Alphafold2 structure and cryo-EM structures for *E. coli* cytochrome *bd-I* and *bd-II*. *E. coli* cytochrome *bd-I* cryo-EM structures 6RX4 (pink) aligned to 6RKO (red) for all. (A) Overall structure of Alphafold 2 produced cytochrome *bd-I* model (yellow) aligned to cytochrome *bd-II* structure cryo-EM structure 7ose (magenta). (B) Overall structure of Alphafold2 produced cytochrome *bd-I* model (yellow), Phyre2 intensive mode produced structure (dark blue) and *E. coli* cryo-EM structure 6RX4 (pink) aligned to *E. coli* cryo-EM structure 6RKO (red). (C) + (D) Q-loop regions of (A) and (B) respectively.

5.3.6. Development of a drug docking pipeline using FDA-approved drugs

E. coli cytochrome *bd*-I respiratory terminal oxidase is a promising target for antibacterial agents for several reasons. Firstly, it is only present in prokaryotes and has a unique structure unrelated to the haem-copper oxidases such as mitochondrial cytochrome *c* oxidase (Borisov et al. 2021). Furthermore, cytochrome *bd* is known to be important in infection by providing resistance to various stress conditions including ROS and RNS (Giuffrè et al. 2014). Furthermore, the structure of *E. coli* cytochrome *bd*-I has been solved by cryo-EM allowing its novel structure to be screened for new antimicrobials (Safarian et al. 2019; Theßeling et al. 2019). Indeed, this data has been used herein to produce a structural model for this purpose (section 5.3.5).

In order to screen for new cytochrome *bd*-I inhibitor a library of compounds must first be selected. The aim was to repurpose existing drugs to target *E. coli* cytochrome *bd*-I so a library of FDA-approved drugs was selected. The use of FDA-approved compounds minimizes the risk of toxic side effects and can increase the speed whilst reducing the cost of drug development (Pihan et al. 2012). Some current inhibitors of cytochrome *bd*-I such as the non-FDA approved TTFA (Fig 5.21, 5.22 + 5.23) also target the mitochondrial respiratory complexes (Zhang et al. 2001; Nadanaciva et al. 2007) and are therefore poor drug choices for use in humans. Hence, the use of FDA-approved drugs minimises this problem from the outset. Furthermore, cytochrome *bd* is only found in prokaryotes (Borisov et al. 2021), which reduces the chances of encountering off target effects in mammalian cells.

The e-Drug3D online database, which contains all currently registered FDA-approved drugs with a molecular weight ≤ 2000 g/mol (Pihan et al. 2012; Douguet 2018), was chosen for the in silico drug screening study. The e-Drug3D database contains both 3D structural and pharmacokinetic data for the FDA-approved drugs (Pihan et al. 2012; Douguet 2018).

An *in silico* molecular docking pathway (Fig. 5.19) was set up 'in house' using the molecular docking software AutoDock Vina (Trott,O., Olson 2010) to screen the FDA-approved database for possible inhibitors of cytochrome *bd*. The entire FDA-approved

library was downloaded from the e-Drug3D online database in 3D .sdf format for a total of 1993 compounds. In the e-Drug3D database, the drug structures had all been manually checked to ensure correct stereochemistry with enantiomers present as different structures (Pihan et al. 2012), which avoids errors in converting drug structures from 2D to 3D format. Ligands were converted into 3D .pdb format using PyMOL, polar hydrogens added and then converted to .pdbqt format using a python script (prepare_ligand4.py) from MGLTools (Morris et al. 2009). AutoDock Vina requires the .pdbqt file format for ligands which adds 4 additional features to the standard .pdb format (Morris et al. 2009). Firstly, partial charges are added to each atom and the AutoDock atom types are added to the ATOM record (Morris et al. 2009). AutoDock Vina uses several atoms types for the force field calculations such as having a separate atom type for aromatic and aliphatic carbons (Morris et al. 2009). Rotatable bonds are assigned to give the ligands flexibility (Morris et al. 2009). The python script prepare_ligand4.py (Appendix A.7) used in the current study sets carbon-carbon backbone bonds to be flexible whilst amide and guanidinium bonds are non-rotatable (Morris et al. 2009). Finally, the number of torsional degrees of freedom, which is the number of rotatable bonds, is specified which is used to analyse the conformational entropy (Forli et al. 2016).

The *E. coli* cytochrome *bd-I* models produced using either Phyre2 in intensive mode or AlphaFold 2 in .pdb format were used as the input structures for the molecular docking. The cytochrome *bd-I* quinol binding site was selected as the first target for repurposing of known FDA-approved ligands. The modelled structures had the polar hydrogens added in PyMOL before conversion to .pdbqt format using AutoDockTools part of the MGLTools suite (Morris et al. 2009). AutoDock Vina uses a docking box to define the area of the protein structure in which to search for binding modes for each ligand (Jaghoori et al. 2016). Using AutoDockTools, the ligand docking site box coordinates were defined to include just the quinol binding site. FDA-ligands with file size above 10 KB were removed (31 in total) as these are very unlikely to bind to the quinol binding site due to their large size. Ligands with large numbers of flexible bonds also greatly reduce the docking accuracy and slow down the docking process (Trott,O., Olson 2010). All of the remaining FDA-approved ligands were then docked to the quinol binding site using a Bash shell script for automation (Appendix

A.8). The results were analysed by predicted binding affinity (kcal/mol) with another python script (Appendix A.9) used to order the ligands by affinity. The top 70 ligands, ordered by affinity, were manually analysed in PyMOL/LIGPLOT (Wallace et al. 1995; Seeliger and de Groot 2010) to identify interactions with the key residues in the quinol binding site. MolLogP values as a measure of hydrophobicity were also taken into account as cytochrome *bd-I* is located within the inner membrane of *E. coli*. Finally, information about known drug function, purchase cost and pharmacokinetic data was taken into consideration before deciding on which drugs to purchase for laboratory screening.

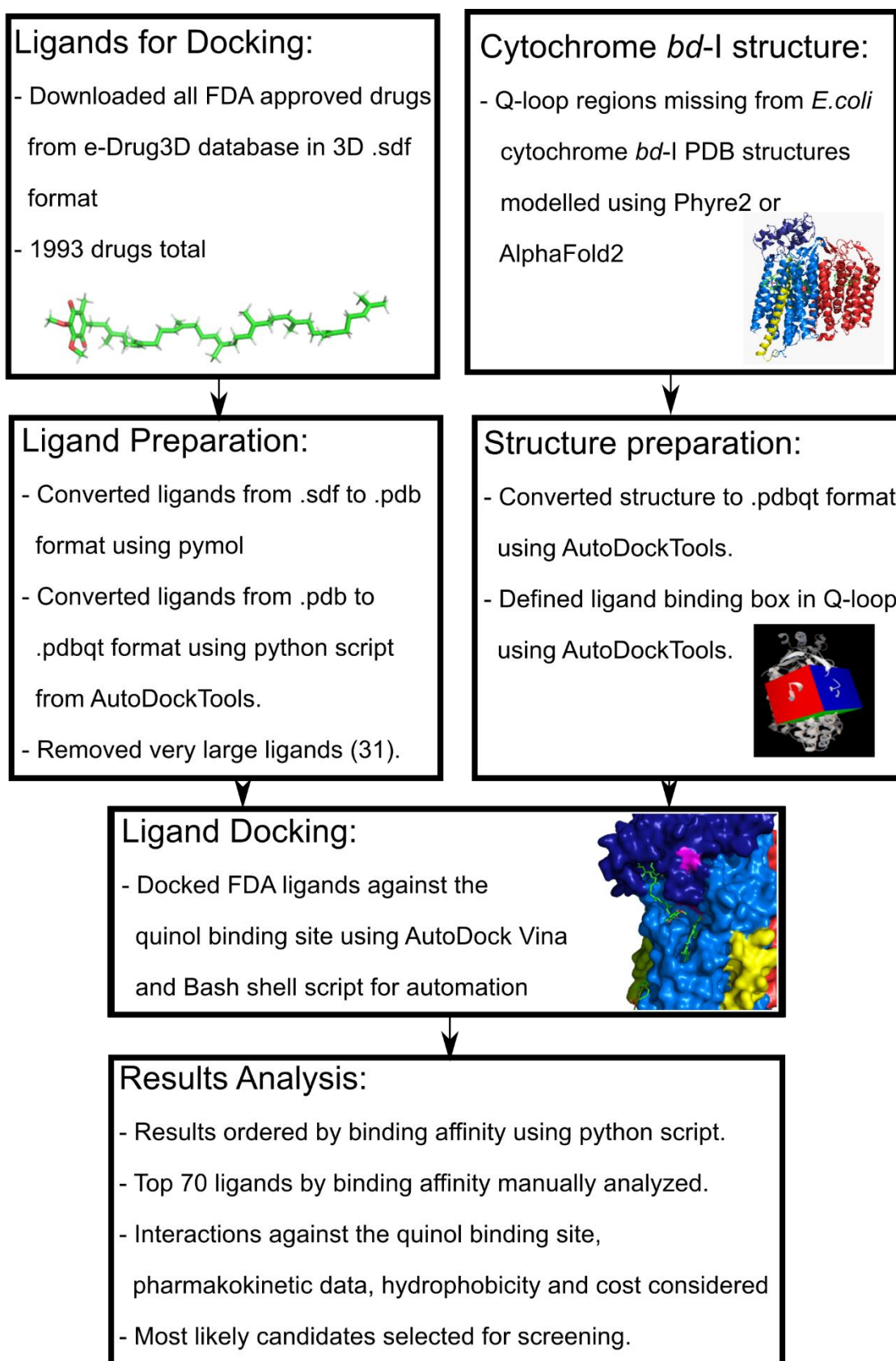


Figure 5.19 - Flow chart for docking FDA drugs to *E. coli* cytochrome *bd-I* quinol binding site.

5.3.7. Docking of known inhibitors of *E. coli* cytochrome *bd-I* to the quinol binding site.

Docking of known cytochrome *bd-I* quinol binding site inhibitors was undertaken as a validation test for the docking protocol (see section 5.3.6). The inhibitors were docked to both the *E. coli* cytochrome *bd-I* structural models and the *E. coli* cytochrome *bd-II* structure (Fig. 5.20). Several docking runs were performed to determine the ideal docking box position and size for the quinol binding site (see section 5.3.6). Interestingly, the *E. coli* cytochrome *bd-II* cryo-EM structure already contained the inhibitor aurachin D bound (Grauel et al. 2021). When AutoDock Vina was used to predict the docking of aurachin D, the conformation is very similar to the published aurachin D position with the quinolinone head groups overlapping (Fig. 5.20). The affinity values predicted by AutoDock Vina for the known ligands were compared to the experimentally determined values (Table. 5.2.), showing a positive trend of increasing predicted binding affinity with increased biochemically derived binding affinity. However, the lack of published affinity measurements ($IC_{10}/IC_{50}/IC_{90}$) precluded a statistical comparison being undertaken between the docking and experimental results.

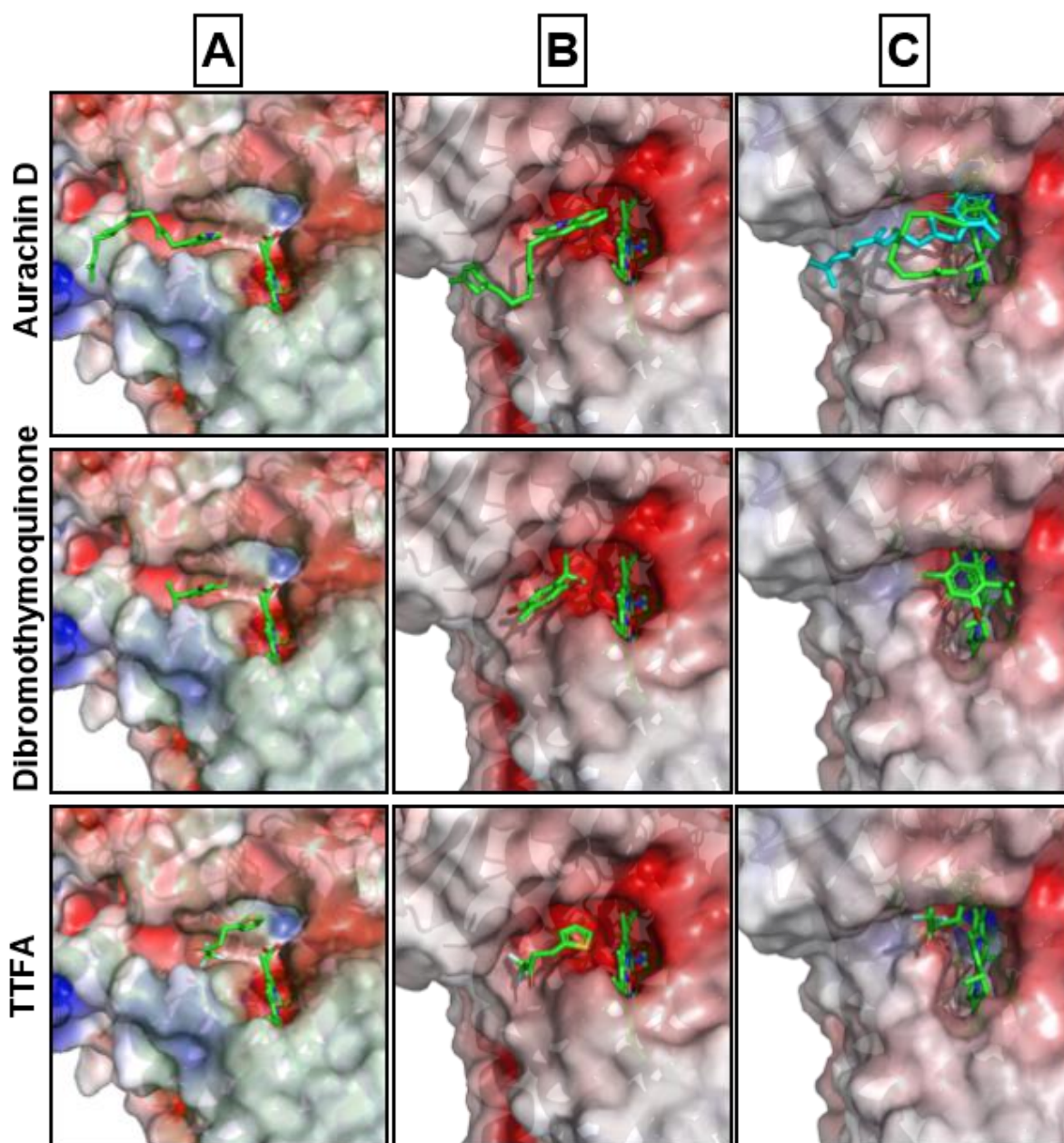


Figure 5.20 - Known quinol binding site inhibitors aurachin D, dibromothymoquinone and 2-thenoyltrifluoroacetone (TTFA) docked to haem b_{558} quinol-binding site of *E. coli* cytochrome *bd-I* and *bd-II*. Known inhibitors docked to (A) *E. coli* cytochrome *bd-I* Phyre2 Intensive model, (B) AlphaFold2 produced cytochrome *bd-I* model or (C) cytochrome *bd-II* cryo-EM structure 7ose. Aurachin D contained in cryo-EM structure 7ose shown in cyan for the top right panel.

Table 5.2 - Known cytochrome *bd*-I inhibitors predicted compared to experimental binding affinity.

Inhibitor	Predicted affinity to <i>E. coli</i> cytochrome <i>bd</i> -I Phyre2 model (kcal/mol)	Predicted affinity to <i>E. coli</i> cytochrome <i>bd</i> -I AlphaFold 2 model (kcal/mol)	Estimated K_d <i>E. coli</i> cytochrome <i>bd</i> -I AlphaFold 2 model (μ M)	Published cytochrome <i>bd</i> -I % inhibition ¹	Experimental IC_{50} or K_i
Aurachin A	-10.3	-8.1	1.14	27% at 700 μ M	-
Aurachin C	-8.7	-8	1.35	90% at 214nM	-
Funiculosin	-8.4	-7.7	2.24	10% at 60 μ M	-
Aurachin D	-9.1	-7.4	3.71	93% at 400nM 98% at 5 μ M	-
Piericidin A	-9.1	-7	7.30	70% at 100 μ M	$IC_{50} = 15\mu M^3$
MOA-stilbene	-8.0	-6.6	14.35	13% at 100 μ M	-
Antimycin A	-8.7	-6.6	14.35	80% at 50 μ M	-
Stigmatellin	-8.1	-6.5	16.99	14% at 200 μ M	-
Myxothiazol	-7.5	-6.4	20.12	0% at 125 μ M	-
HOQNO	-8.2	-6.2	28.20	90% at 50 μ M	$IC_{50} = 7\mu M^3$
UHDBT	-7.1	-5.4	108.99	0% at 2 μ M 18% at 20 μ M	-
Dibromothym o-quinone	-7.1	-5.4	108.99	38% at 100 μ M	-
TTFA	-6.4	-5.2	152.81	35% at 1mM ²	-
2,6-Dimethyl-p-benzoquinone	-6.3	-4.6	421.15	-	$IC_{50} = 65\mu M^4$
Pentachloro-phenol	-6.1	-4.3	699.16	-	$K_i = 200\mu M^5$
p-benzoquinone	-5.1	-3.8	1627.38	-	$IC_{50} = 120\mu M^4$

1) % Inhibition of oxygen consumption at stated concentration of 'cytochrome *bd*-I only' containing membranes from *E. coli* in the presence of 1.5 mM duroquinol (Sakamoto et al. 1999) (Meunier et al. 1995) apart from TTFA².

2) % inhibition at 1mM TTFA for purified *E. coli* cytochrome *bd*-I ubiquinol-1 oxidase activity (Miller and Gennis 1983).

3) IC_{50} for purified *E. coli* cytochrome *bd*-I ubiquinol-1 oxidase activity (Kita et al. 1984).

4) IC_{50} for purified *B. stearothermophilus* cytochrome *bd*-I ubiquinol-1 oxidase activity (Sakamoto et al. 1999).

5) Inhibition constant (K_i) for purified *E. coli* cytochrome *bd*-I ubiquinol-1 oxidase activity (Borisov 1996)

5.3.8. Docking of e-Drug3D library to *E. coli* cytochrome *bd-I* quinol binding site.

The FDA approved drugs from e-Drug3D library were docked to the quinol binding site for both the Phyre2 and AlphaFold 2 *E. coli* cytochrome *bd-I* structural models. Autodock Vina predicts up to 9 different binding modes for each ligand which are outputted into .pdbqt results files. A python script (Appendix A.9) was used to extract the predicted binding affinity associated with the strongest binding mode for each ligand, rank the ligands according to binding affinity, and extract the results into an Excel table (Table 5.3 + 5.4). The top predicted binding mode was used for each drug (Autodock Vina predicts 9 binding modes for each drug). Initially, only the *E. coli* cytochrome *bd-I* Phyre2 model results were available and so these were used to select drugs to test. Once available, the *E. coli* cytochrome *bd-I* AlphaFold 2 model was used to check the docking results and would be the preferred option for future drug screening studies due to the improved accuracy of the Q-loop region modelling. Drugs in the top 70 by predicted affinity were manually selected for purchase following consideration of binding affinity, hydrophobicity (LogP), modelled interactions with CydA, cost, known pharmacokinetics, and potential off-target effects.

Table 5.3 - Top 70 potential *E. coli* cytochrome *bd-I* inhibitors ranked by predicted affinity to the quinol binding site of the Phyre2 modelled structure. Drugs of particular interest in bold were tested experimentally using the oxygen consumption assay on *E. coli* EC958 '*bd-I* only' membranes.

Ranking by predicted affinity	Drug Name	eDrug3D Database Number	Predicted Affinity (kcal/mol)	Estimated K_d (nM)
1	LUMACAFTOR	1782	-11.9	2
2	ERGOTAMINE	70	-11.4	4
3	QUINESTROL	383	-11.3	5
4	CONIVAPTAN	927	-11.3	5
5	VENETOCLAX	1822	-11.3	5
6	LOMITAPIDE	1572	-11	8
7	TADALAFIL	893	-11	8
8	ENTRECTINIB	1969	-10.9	10
9	LEDIPASVIR	1731	-10.9	10
10	M ENTRECTINIB	1970	-10.9	10
11	PANOBINOSTAT	1744	-10.8	12
12	APREPITANT	915	-10.8	12

13	DUTASTERIDE	883	-10.8	12
14	HEXAFLUORENIUM	1336	-10.7	14
15	ELTROMBOPAG	1450	-10.6	17
16	ROLAPITANT	1801	-10.6	17
17	TUBOCURARINE	25	-10.6	17
18	DIHYDROERGOTAMINE	36	-10.6	17
19	BEXAROTENE	847	-10.6	17
20	TROGLITAZONE	790	-10.6	17
21	LORLATINIB	1917	-10.5	20
22	ESTRADIOL CYPIONATE	20	-10.5	20
23	NILOTINIB	1310	-10.5	20
24	ATOVAQUONE	710	-10.5	20
25	EXATECAN	1993	-10.5	20
26	M MIDOSTAURIN	1830	-10.4	23
27	ADAPALENE	718	-10.4	23
28	M ENZALUTAMIDE	1591	-10.4	23
29	RUCAPARIB	1826	-10.3	28
30	RIMEGEPANT	1971	-10.3	28
31	TELMISARTAN	814	-10.3	28
32	NETUPITANT	1726	-10.3	28
33	PEXIDARTINIB	1951	-10.3	28
34	VILAZODONE	1511	-10.2	33
35	M MIDOSTAURIN	1829	-10.2	33
36	M MIDOSTAURIN	1852	-10.2	33
37	BUTENAFINE	747	-10.2	33
38	ERGOLOID	1213	-10.1	39
39	MIDOSTAURIN	1828	-10.1	39
40	CAPMATINIB	1983	-10.1	39
41	MESTRANOL	213	-10.1	39
42	BROMOCRIPTINE	470	-10.1	39
43	IRBESARTAN	796	-10.1	39
44	DESOGESTREL	676	-10.1	39
45	PIMOZIDE	424	-10.1	39
46	ENASIDENIB	1837	-10	46
47	CARBENICILLIN INDANYL	1447	-10	46
48	BICTEGRAVIR	1879	-10	46
49	SULINDAC	468	-10	46
50	M OZANIMOD	1937	-10	46
51	LIFITEGRAST	1824	-10	46
52	M RIFAPENTINE	1652	-10	46
53	M CARBAMAZEPINE	1752	-10	46
54	PEMETREXED	1297	-9.9	54
55	TUCATINIB	1982	-9.9	54

56	ABAMETAPIR	1326	-9.9	54
57	M OZANIMOD	1938	-9.9	54
58	NORETHYNODREL	214	-9.9	54
59	BETA CAROTENE	1380	-9.9	54
60	PRAZIQUANTEL	536	-9.9	54
61	PARITAPREVIR	1734	-9.9	54
62	CINACALCET	926	-9.9	54
63	PIMAVANSERIN	1802	-9.9	54
64	BENZHYDROCODONE	1862	-9.8	64
65	TRETINOIN	395	-9.8	64
66	ETHINYL ESTRADIOL	18	-9.8	64
67	APOMORPHINE	873	-9.8	64
68	ACITRETIN	637	-9.8	64
69	ABIRATERONE ACETATE	1519	-9.8	64
70	RIFAMPIN	1004	-9.8	64

Table 5.4 - Top 70 potential *E. coli* cytochrome *bd*-I inhibitors ranked by predicted affinity to the quinol binding site of the AlphaFold 2 modelled structure. Drugs of particular interest in bold were tested experimentally using the oxygen consumption assay on cytochrome *bd*-I only containing *E. coli* EC958 membranes. Mestranol and ethinyl estradiol also included for reference.

Ranking by predicted affinity	Drug Name	eDrug3D Database Number	Predicted Affinity (kcal/mol)	Estimated K_d (nM)
1	LOMITAPIDE	1572	-9.8	64
2	LEDIPASVIR	1731	-9.8	64
3	ERGOTAMINE	70	-9.6	90
4	IRINOTECAN	755	-9.4	127
5	NILOTINIB	1310	-9.4	127
6	CALCIPOTRIENE	1412	-9.4	127
7	DOXERCALCIFEROL	1417	-9.4	127
8	LUMACAFTOR	1782	-9.4	127
9	DROSPIRENONE	855	-9.3	150
10	ERGOCALCIFEROL	10	-9.2	177
11	ADAPALENE	718	-9.1	210
12	ALECTINIB	1819	-9.1	210
13	LIFITEGRAST	1824	-9.1	210
14	DIHYDROERGOTAMINE	36	-9	249
15	CYPROHEPTADINE	303	-9	249
16	DUTASTERIDE	883	-9	249
17	M DOXERCALCIFEROL	1556	-9	249

18	MIDOSTAURIN	1828	-9	249
19	M REVEFENACIN	1910	-9	249
20	ESTRADIOL CYPIONATE	20	-8.9	294
21	BROMOCRIPTINE	470	-8.9	294
22	BEXAROTENE	847	-8.9	294
23	CONIVAPTAN	927	-8.9	294
24	BETA CAROTENE	1380	-8.9	294
25	CALCITRIOL	480	-8.8	349
26	SIROLIMUS	853	-8.8	349
27	CANDICIDIN	1079	-8.8	349
28	PARITAPREVIR	1734	-8.8	349
29	M MIDOSTAURIN	1829	-8.8	349
30	M MIDOSTAURIN	1852	-8.8	349
31	MOXIDECTIN	1916	-8.8	349
32	ENTRECTINIB	1969	-8.8	349
33	HEXAFLUORENIUM	1336	-8.7	413
34	ELTROMBOPAG	1450	-8.7	413
35	SIMEPREVIR	1628	-8.7	413
36	VENETOCLAX	1822	-8.7	413
37	M ENTRECTINIB	1970	-8.7	413
38	CAPMATINIB	1983	-8.7	413
39	QUINESTROL	383	-8.6	489
40	MARAVIROC	958	-8.6	489
41	NYSTATIN	990	-8.6	489
42	ERGOLOID	1213	-8.6	489
43	CALCIFEDIOL	1390	-8.6	489
44	ABIRATERONE ACETATE	1519	-8.6	489
45	PONATINIB	1593	-8.6	489
46	NETUPITANT	1726	-8.6	489
47	TUCATINIB	1982	-8.6	489
48	LURBINECTEDIN	1986	-8.6	489
49	EXATECAN	1993	-8.6	489
50	PIMOZIDE	424	-8.5	579
51	DANAZOL	435	-8.5	579
52	TELMISARTAN	814	-8.5	579
53	HYDROCORTISONE CYPIONATE	1327	-8.5	579
54	VORAPAXAR	1710	-8.5	579
55	DASABUVIR	1735	-8.5	579
56	ELBASVIR	1815	-8.5	579
57	GLECAPREVIR	1850	-8.5	579

58	AVATROMBOPAG	1878	-8.5	579
59	BALOXAVIR	1914	-8.5	579
60	TRIFAROTENE	1944	-8.5	579
61	AVAPRITINIB	1968	-8.5	579
62	LOPERAMIDE	450	-8.4	685
63	ITRACONAZOLE	678	-8.4	685
64	RISPERIDONE	713	-8.4	685
65	EXEMESTANE	795	-8.4	685
66	CHOLECALCIFEROL	824	-8.4	685
67	DACTINOMYCIN	1058	-8.4	685
68	TESTOSTERONE CYPIONATE	1335	-8.4	685
69	ERIBULIN	1505	-8.4	685
70	RILPIVIRINE	1516	-8.4	685
94	ATOVAQUONE	710	-8.2	961
164	MESTRANOL	213	-7.9	1595
203	ETHINYL ESTRADIOL	18	-7.8	1889

5.3.9. Development of a laboratory assay for validation of cytochrome *bd-I* inhibitors

In order to validate candidate compounds as bona fide cytochrome *bd-I* inhibitors, an oxygen electrode assay was developed to measure cytochrome *bd-I* activity. This assay used cytochrome *bd-I* only containing membranes from the uropathogenic *E. coli* EC958 $\Delta cyoA/\Delta appCB$ mutant strain and was adapted from (Beard et al. 1995). Oxygen consumption of the membranes was measured in a Clarke type oxygen electrode on addition of succinate (8 mM) as the substrate for the aerobic electron transport chain (Fig. 5.21). 2-Thenoyltrifluoroacetone (TTFA) was selected as a known inhibitor of cytochrome *bd-I* for optimisation of the assay. TTFA has previously been shown to be a weak inhibitor of cytochrome *bd-I* with 35% inhibition shown at 1 mM (Miller and Gennis 1983). TTFA is a known quinol binding site inhibitor (Kluckova et al. 2015) so molecular docking was used to dock it to the *E. coli* cytochrome *bd-I* quinol binding site (Fig. 5.22). TTFA shows weak binding affinity to the quinol binding site of cytochrome *bd-I* (-5.2 kcal/mol/ Estimated K_d = 152.81 μ M) which is reflected in the previous experimental derived value (35% inhibition at 1 mM) (Miller and Gennis 1983). TTFA was selected as a positive control quinol binding site inhibitor due to its known cytochrome *bd-I* inhibition, low purchase cost and hydrophobic nature (logP = 1.46 (Hansch et al. 1995)) to promote membrane access.

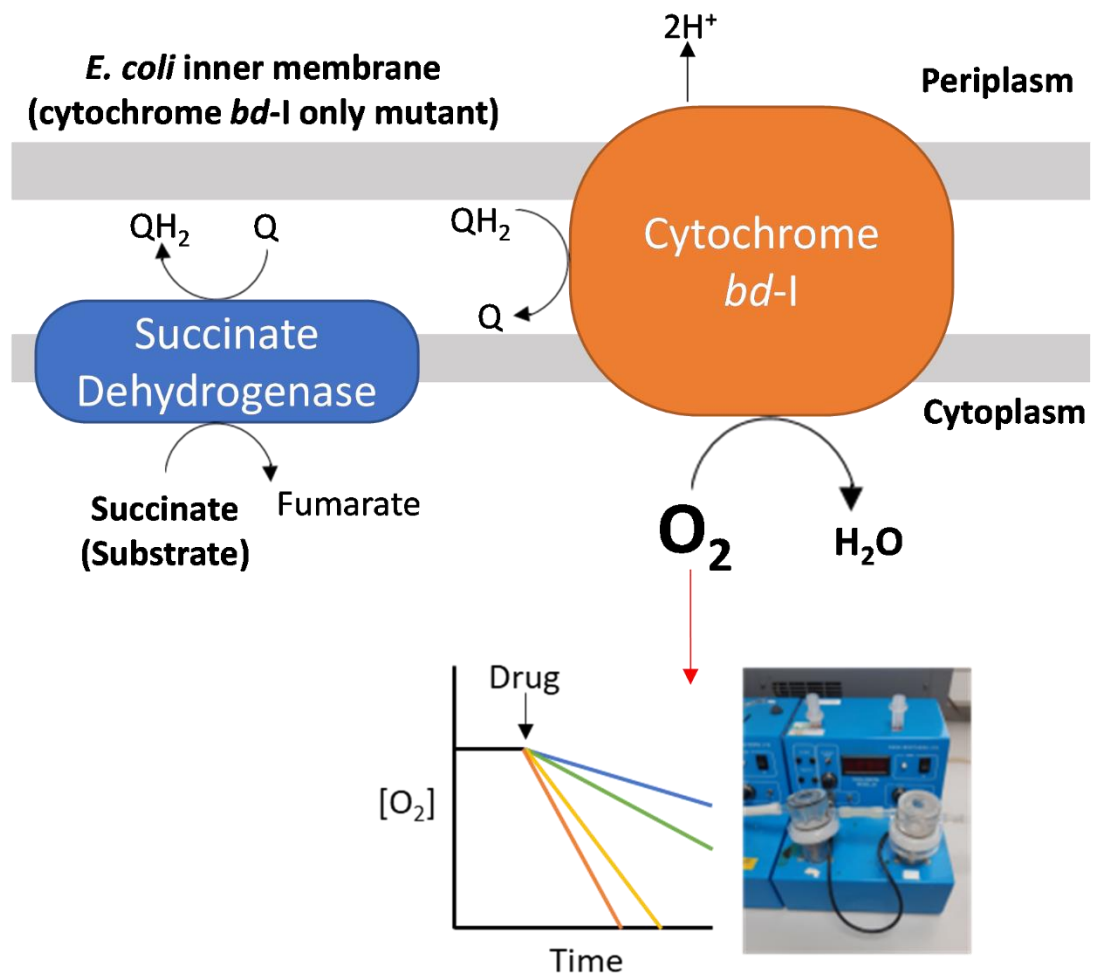


Figure. 5.21 - Membrane based oxygen consumption assay to screen cytochrome *bd-I* inhibitors. Succinate dehydrogenase provides a source of reduced quinone for cytochrome *bd-I* to oxidise. Cytochrome *bd-I* activity is measured by monitoring the rate of oxygen consumption with an oxygen electrode.

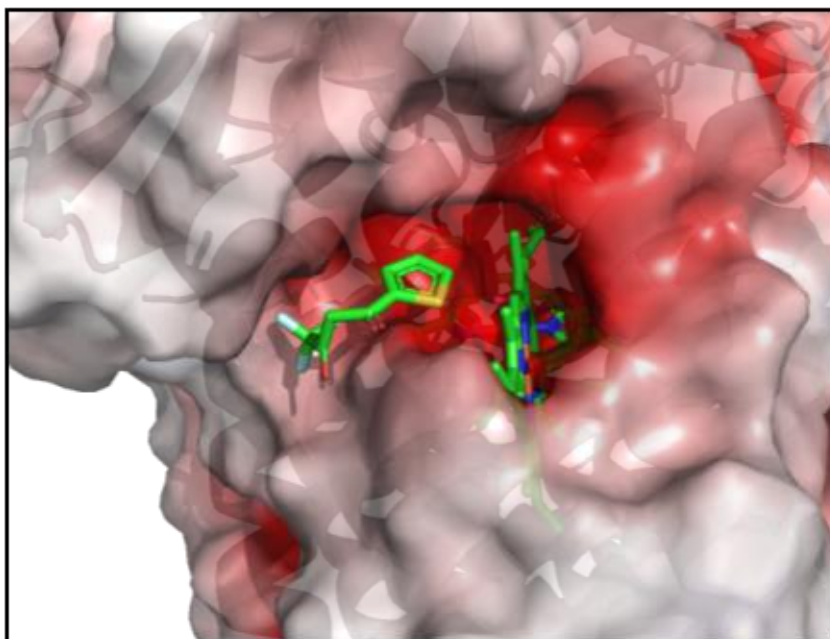


Figure 5.22 - Known quinol binding site inhibitor 2-thenoyltrifluoroacetone (TTFA) docked to haem b_{558} quinol-binding site of *E. coli* AlphaFold 2 cytochrome *bd-I* model.

The oxygen electrode assay discussed above was carried out using varying concentrations of TTFA (Fig. 5.23). Data are expressed as % activity relative to a positive control without inhibitor (2.5% ethanol), as ethanol was added to a final concentration of 2.5% v/v due to TTFA being solubilised in ethanol. The IC_{50} value of 1.36 mM is consistent with previously published data that report 35% inhibition at 1 mM (Miller and Gennis 1983). Several controls were carried out for the oxygen electrode assay, including the addition of milliQ H_2O instead of substrate (8 mM succinate) that resulted in no oxygen consumption. Similarly, 50 mM HEPES buffer and succinate (8mM) without the addition of *E. coli* membranes resulted in no oxygen being consumed. These results have provided verification of the oxygen electrode inhibition assay for detection of cytochrome *bd-I* inhibitors using succinate as the substrate.

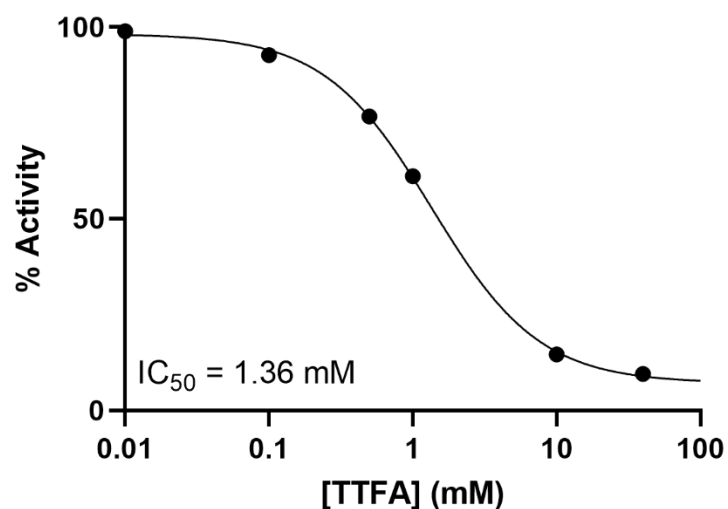


Figure 5.23 - TTFA inhibition of cytochrome *bd*-I only membranes. *E. coli* EC958 ST131 cytochrome *bd*-I only membranes were added to the oxygen electrode at the final concentration of 0.5 mg/mL in 50 mM HEPES pH 7.5 with varying concentrations of TTFA in ethanol. Reactions were started with the addition of 8 mM succinate and the oxygen consumption was measured at 37 °C. Values are reported as % activity relative to a positive control without TTFA (i.e. just ethanol was added to a final concentration of 2.5% v/v).

The oxygen electron inhibition assay was also tested on ‘*bo*’ only’ membranes isolated from the *E. coli* EC958 $\Delta cydAB/\Delta appCB$ mutant (Fig. 5.24). TTFA also shows inhibition against ‘*bo*’ only’ membranes with an IC_{50} of 0.164 mM. This suggests that some inhibitors that target the cytochrome *bd*-I quinol binding site may also target the quinol binding site of cytochrome *bo*’.

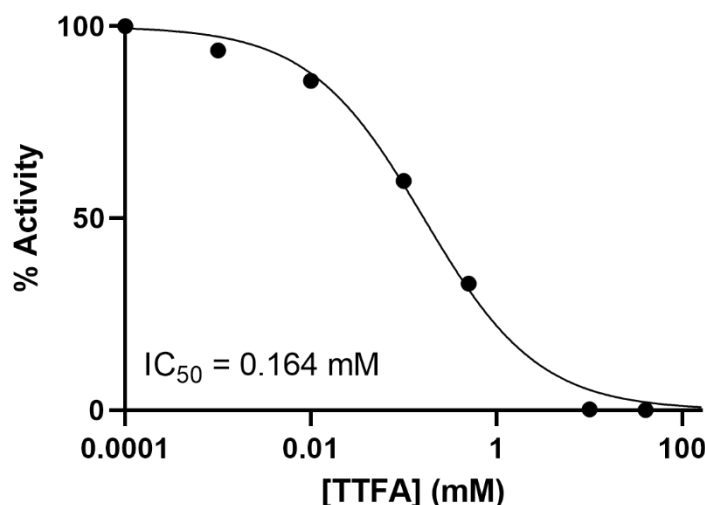


Figure 5.24 - TTFA inhibition of 'bo' only' membranes. *E. coli* EC958 ST131 cytochrome *bo'* only membranes were added to the oxygen electrode at the final concentration of 0.5 mg/mL in 50 mM HEPES pH 7.5 with varying concentrations of TTFA in ethanol. The reactions were started via addition of 8 mM succinate and the oxygen consumption was measured at 37 °C. Data are reported as % activity relative to a 2.5% ethanol control.

To further validate the targeting of *E. coli* cytochrome *bd-I* as an effective antibacterial strategy, whole cell growth inhibition assays were conducted, with the known inhibitor TTFA being used as a positive control to validate this approach. This was achieved via plate reader assays of *E. coli* EC958 WT, '*bd-I* only' and '*bo'* only' strains, with TTFA added during mid-exponential growth (4.8×10^8 CFU/mL) (Fig. 5.25). TTFA elicited growth inhibition for all three strains to a similar degree, with the '*bd-I* only' strain showing the greatest sensitivity ($IC_{50} = 0.02142$ mM). This demonstrates that cytochrome *bd-I* inhibitors can directly inhibit aerobic respiratory complexes in pathogenic *E. coli*. Interestingly, the observed inhibition is greater by around 100-fold in the whole cell assay compared to the membrane electrode assay by way of IC_{50} values (Figures 5.23 and 5.25).

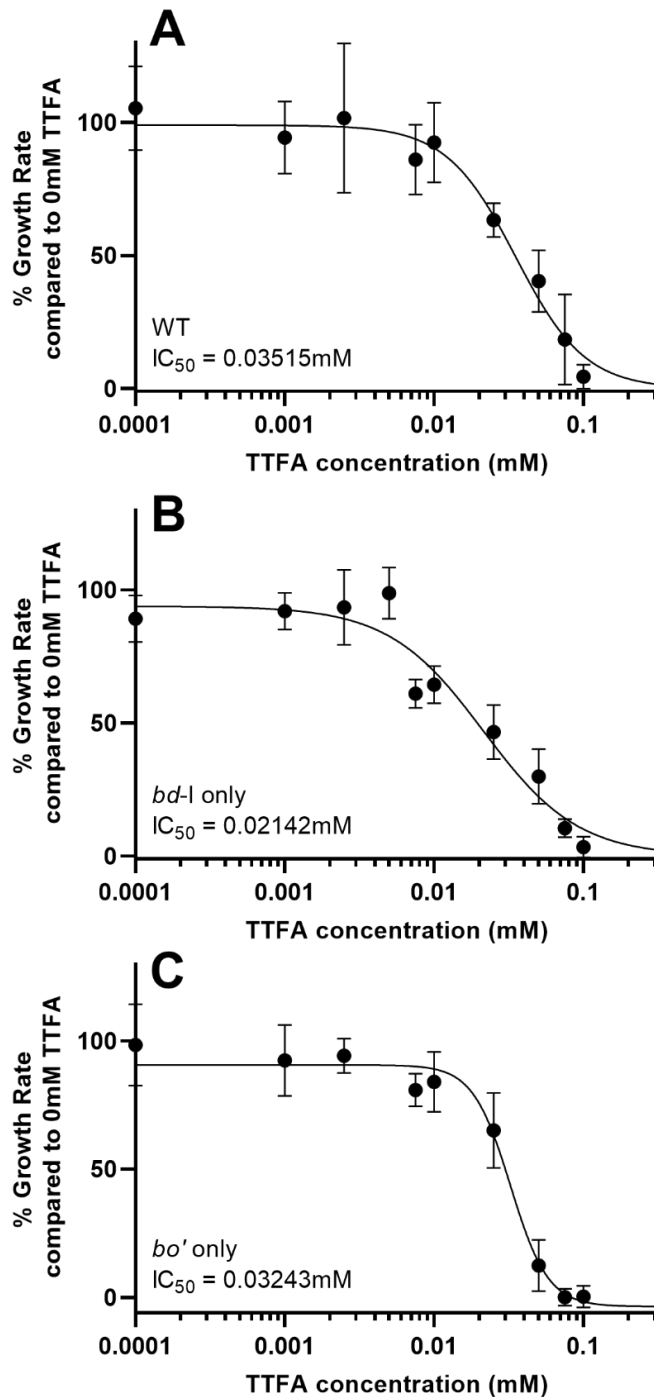


Figure 5.25 - *E. coli* growth inhibition assays with the cytochrome *bd-I* inhibitor TTFA. *E. coli* EC958 A) wild-type, B) '*bd-I* only' and C) '*bo'* only' cells were grown aerobically in M9 minimal media supplemented with 0.1 % casamino acids and glycerol as the carbon source. *E. coli* cultures were grown until mid-exponential phase before being diluted in fresh M9 to 8.0×10^7 CFU/mL and exposed to varying TTFA concentrations. The growth rate was measured during a time window 1.5-4 h after TTFA addition (exponential phase). Data are expressed as % growth rate relative to the uninhibited rate (ethanol control). Two biological repeats each with three technical repeats completed with the error bars showing the standard deviation.

5.3.10. Atovaquone and 2-hydroxy-1,4-naphthoquinone

From the molecular docking results atovaquone was selected as the first drug to test with a predicted binding affinity of -10.5 kcal/mol (Table. 5.3.) to the quinol binding site of the Phyre2 produced model. Atovaquone also docks into the quinol binding site of the AlphaFold 2 model with high affinity (-8.2 kcal/mol) (Table. 5.4. and Fig. 5.26). Atovaquone was primarily chosen due its known mode of action being a quinol binding site inhibitor (Michael R. Bronsert et al. 2005). Atovaquone is a hydroxy-1,4-naphthoquinone used to treat malaria (Osei-Akoto et al. 2005) and *Pneumocystis carinii* pneumonia (PCP) (Comley et al. 1995). Atovaquone is a ubiquinone analogue and functions as a competitive inhibitor of ubiquinol targeting the mitochondrial electron transport chain cytochrome *bc*₁ complex of *P. falciparum* (Nixon et al. 2013). Atovaquone displays a very low IC₅₀ of between 1 to 3.5 nM against susceptible *P. falciparum* (Nixon et al. 2013), although it does target the human mitochondrial cytochrome *bc*₁ complex with sub-micromolar affinity resulting in a human/*P. falciparum* selectivity index of 230 (Nilsen et al. 2013). The X-ray crystal structure of *Saccharomyces cerevisiae* cytochrome *bc*₁ complex with atovaquone bound to the Q₀ catalytic site has been published (Birth et al. 2014). A polarized H-bond is formed between the ionized hydroxyl group of atovaquone and His181 of the Rieske protein of cytochrome *bc*₁ (Birth et al. 2014). Additional hydrophobic interactions are present between the naphthoquinone group and conserved residues in cytochrome *b* along with other non-polar interactions between less conserved residues in cytochrome *b* and the cyclohexyl–chlorophenyl tail of atovaquone (Birth et al. 2014). This binding mode is believed to be similar to that in *P. falciparum* and *Pneumocystis carinii* (Birth et al. 2014). The molecular docking results for atovaquone into the quinol binding site of cytochrome *bd-I* predict multiple hydrophobic interactions with the quinol binding pocket (Fig. 5.26). Residues involved include Ile295, Phe390 and Arg391. Following consideration of both the known mode of action (i.e. targets quinol-binding sites) and the predicted tight binding to *E. coli* cytochrome *bd-I* (Fig. 5.26), atovaquone was selected as the first compound to test in the oxygen consumption assay (Fig. 5.27). A concentration of 50 µg/mL (136 µM) was selected for initial *E. coli* cytochrome *bd-I* oxygen consumption trials with plasma atovaquone levels of >35 µg/mL shown to clear *P. carinii* infection in mice (Comley et al. 1995). However, only

a small amount of inhibition was seen for '*bd-I* only' membranes (Fig. 5.27). Additionally, no inhibition was observed for whole *E. coli* cells at this concentration. Due to the poor affinity which would lead to selectivity problems with the human *bc₁* complex and lack of inhibition for *E. coli* cells, no further work was undertaken on atovaquone. However, the 2-hydroxy-1,4-napthoquinone head group of Atovaquone remained an interesting scaffold for the following reasons. 2-hydroxy-1,4-napthoquinone, also known as lawsone, is present in the leaves of *Lawsonia inermis* and has known antimicrobial activities against a range of pathogens (Yang and Lee 2015). Various derivatives of 2-hydroxy-1,4-napthoquinone have been synthesized and recently tested against *E. coli* cytochrome *bo'* and *bd-I* (Elamri et al. 2020) with some designed particularly against the *E. coli* cytochrome *bo'* structure. Some of these new derivatives show selective inhibition to either cytochrome *bo'* or *bd-I* (Elamri et al. 2020). Other derivatives show no inhibition to either oxidase (Elamri et al. 2020) showing the importance of the tail group to selectivity. Another lawsone derivative has shown inhibition to MRSA (Song et al. 2020). In the current work, the 2-hydroxy-1,4-napthoquinone molecule docks in a similar position to Atovaquone (Fig. 5.26) but with much lower predicted affinity (-5.4 kcal/mol) and estimated K_d (109 mM). There is the possibility that the bulky cyclohexyl–chlorophenyl tail of atovaquone fits poorly into the *E. coli* cytochrome *bd-I* quinol binding cleft, especially if flexibility of the Q-loop is taken into account. 2-hydroxy-1,4-napthoquinone is more hydrophilic than atovaquone resulting in greater solubility in HEPES buffer for the oxygen consumption assay allowing a full dose response to be undertaken (Fig. 5.28), although the affinity is again quite poor with an IC_{50} of 825.5 μ g/mL (4.74 mM)(Fig. 5.28).

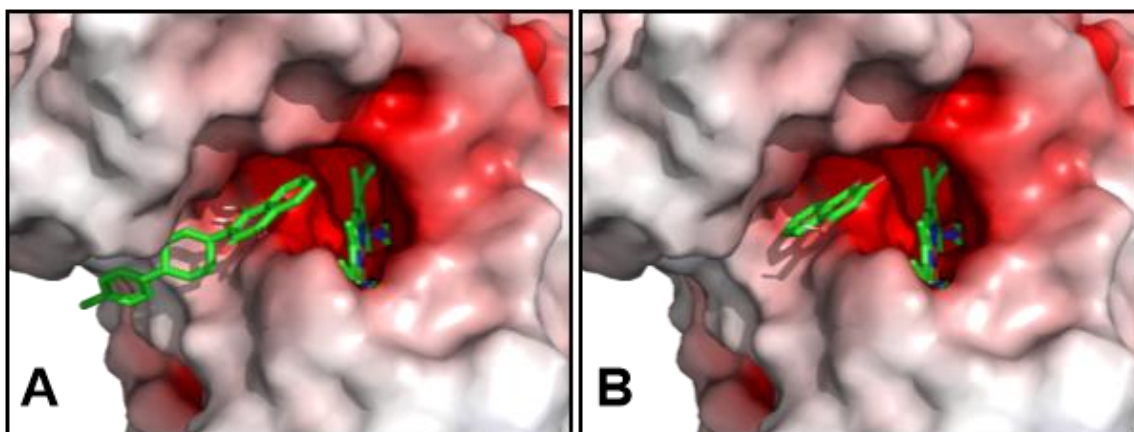


Figure 5.26 - Docked structures of (A) Atovaquone and (B) 2-hydroxy-1,4-naphthoquinone into the *E. coli* AlphaFold 2 cytochrome *bd-I* model quinol binding site.

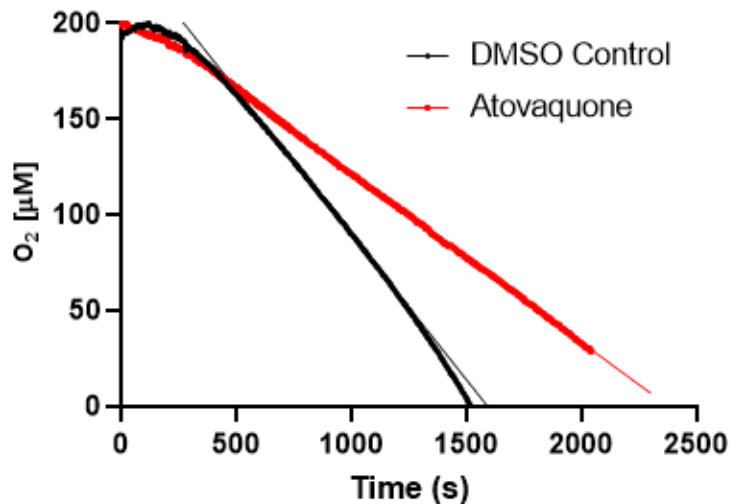


Figure 5.27 - Activity of *bd-I* only membranes in response to 50 µg/mL atovaquone. Representative traces shown +/- Atovaquone. 3 biological repeats carried out for Atovaquone and DMSO control. Atovaquone produced a mean of 34% inhibition (+/- 3.23%) to the oxygen consumption rate of '*bd-I* only' membranes at 50 µg/mL.

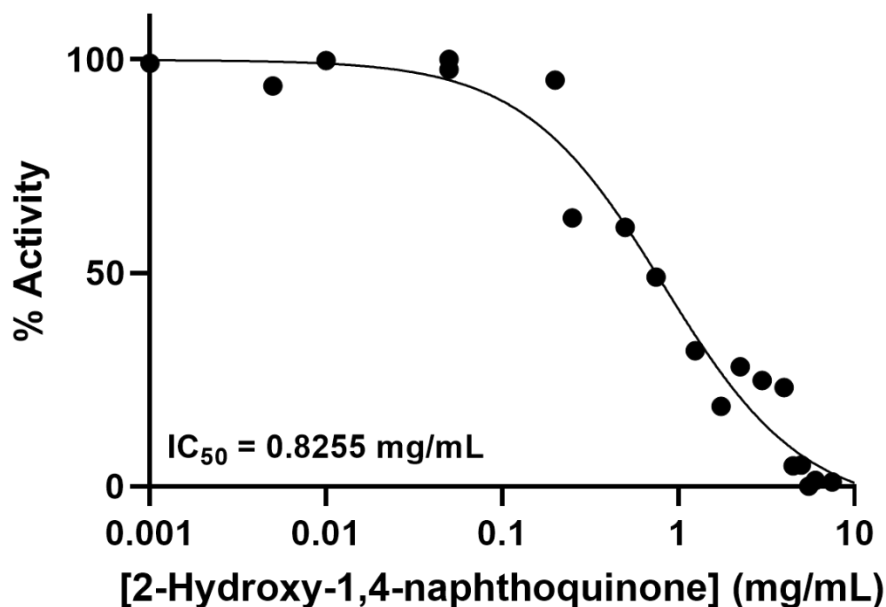


Figure. 5.28 - 2-hydroxy-1,4-naphthoquinone dose response. % activity refers to % oxygen consumption rate of EC958 '*bd-I* only' membranes relative to a DMSO control.

5.3.11. Quinestrol

Several steroid drugs feature in the ranked list above (Table 5.3). These include mestranol, quinestrol and ethinyl estradiol (the active metabolite of the prodrug quinestrol). Quinestrol, mestranol and ethinyl estradiol all have the same steroid backbone structure and dock in a similar configuration in the quinol binding site of cytochrome *bd-I* (Fig. 5.29). They all dock with high affinity to the quinol binding site (Tables 5.3 + 5.4) and are hydrophobic, which is a useful property for membrane access. Hence, these were selected for laboratory screening. Quinestrol showed the highest predicted binding affinity to the alphafold 2 modelled structure (-8.6 kcal/mol) and estimated K_d (488 nM). An initial oxygen consumption screen was conducted using 100 μM of quinestrol, mestranol and ethinyl estradiol (Fig. 5.30). Quinestrol displayed 76% inhibition and ethinyl estradiol 23% inhibition to the oxygen consumption of *E. coli* EC958 cytochrome *bd-I* only membranes (Fig. 5.30), whereas mestranol displayed zero inhibition.

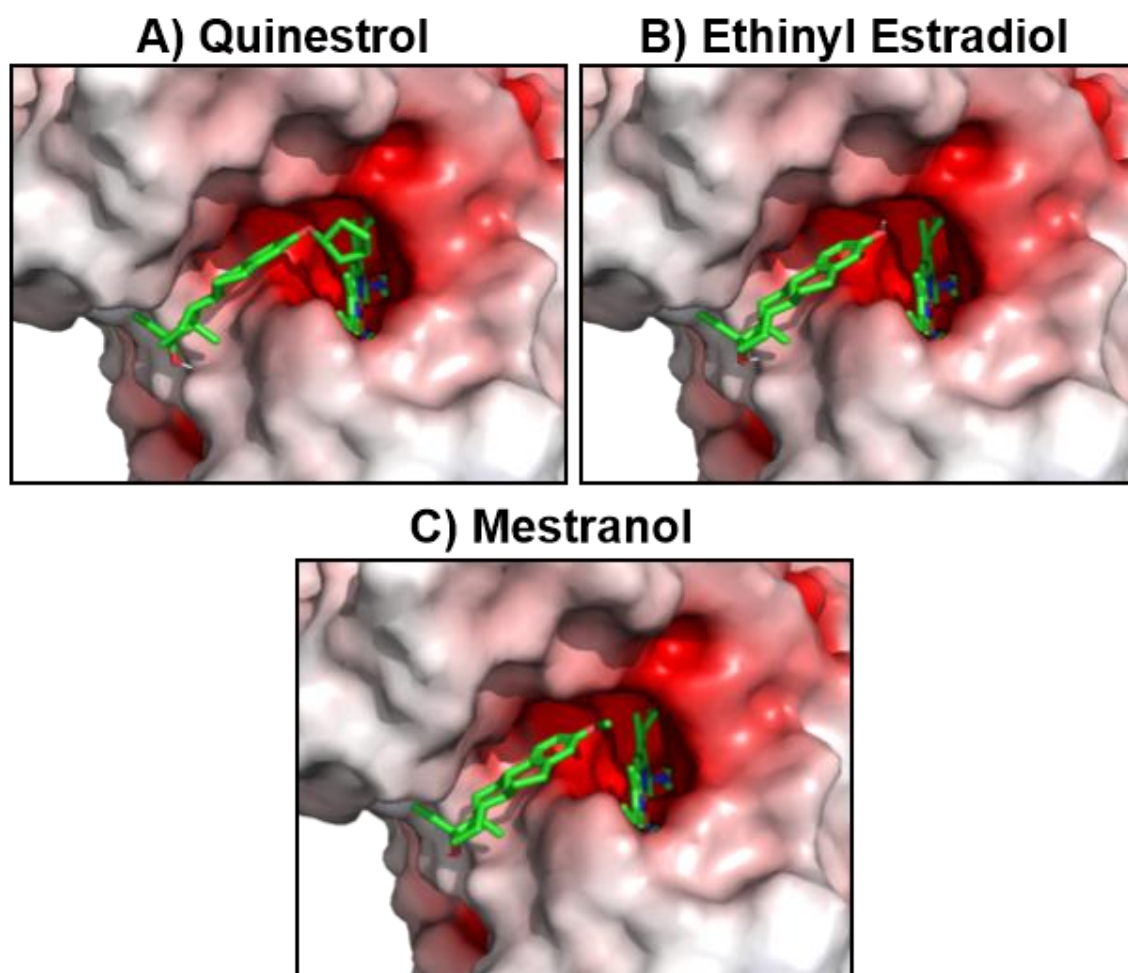


Figure 5.29 - Docked structure of steroid drugs A) quinestrol, B) ethinyl estradiol, C) mestranol into the AlphaFold 2 *E. coli* cytochrome *bd-I* model quinol binding site.

Quinestrol was selected for purchase in larger quantity and further testing due to it possessing the highest predicted binding affinity (Tables 5.3 + 5.4), displaying the highest level of inhibition in the initial drug screen (Fig. 5.30), and lower purchase cost compared to ethinyl estradiol. A full dose response was conducted for quinestrol against EC958 '*bd-I* only' membranes (Fig. 5.31) and EC958 whole cells (Fig. 5.32).

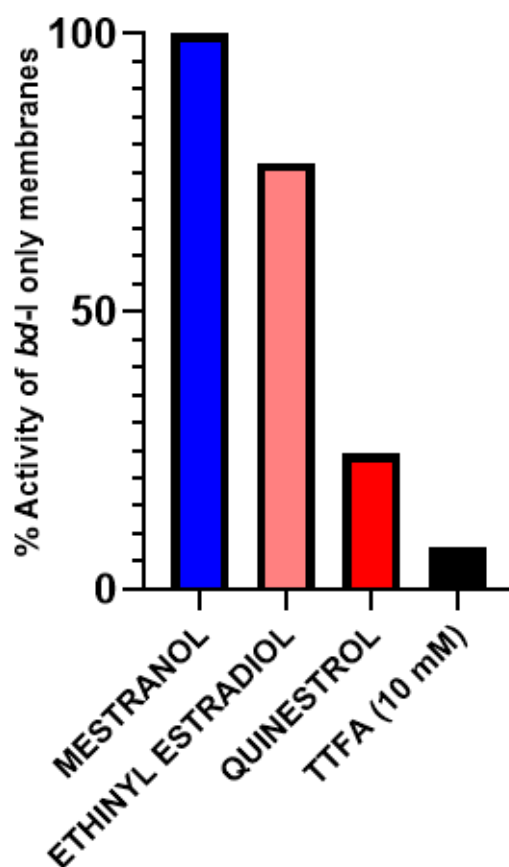


Figure 5.30 - Initial screen of steroid drugs. % activity refers to % oxygen consumption rate of cytochrome *bd-I* only membranes relative to a DMSO control. Drugs were screened at 100 μ M concentration apart from TTFA positive control (10 mM).

Quinestrol is an estrogen drug used for hormone replacement therapy and as a contraceptive (Odell and Molitch 1974). Quinestrol is a pro-drug stored in fat tissue and metabolized slowly into ethinyl estradiol the active metabolite (Odell and Molitch 1974). Quinestrol has also been used to treat prostate and breast cancer but is not commonly used for this purpose (Tang et al. 2012). The oxygen consumption assay for quinestrol against *bd-I* only membranes from *E. coli* EC958 (Fig. 5.31) showed an IC_{50} of 0.018 μ g/mL (49.4 nM), which is much tighter than atovaquone or 2-hydroxy-1,4-napthoquinone. Interestingly, in a whole cell screen of FDA approved compounds against the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*) quinestrol inhibited growth of the two Gram-positive species (Waleed Younis et al. 2017). The minimum inhibitory concentration (MIC) for quinestrol was determined to be 16 μ M for *E. faecium* ATCC 700221 and >16 μ M

for *Staphylococcus aureus* NRS384 (Waleed Younis et al. 2017). Quinestrol did not show any inhibition to the Gram-negative ESKAPE pathogens (Waleed Younis et al. 2017). A residual activity is present at quinestrol concentrations higher than 5 µg/mL (Fig. 5.31).

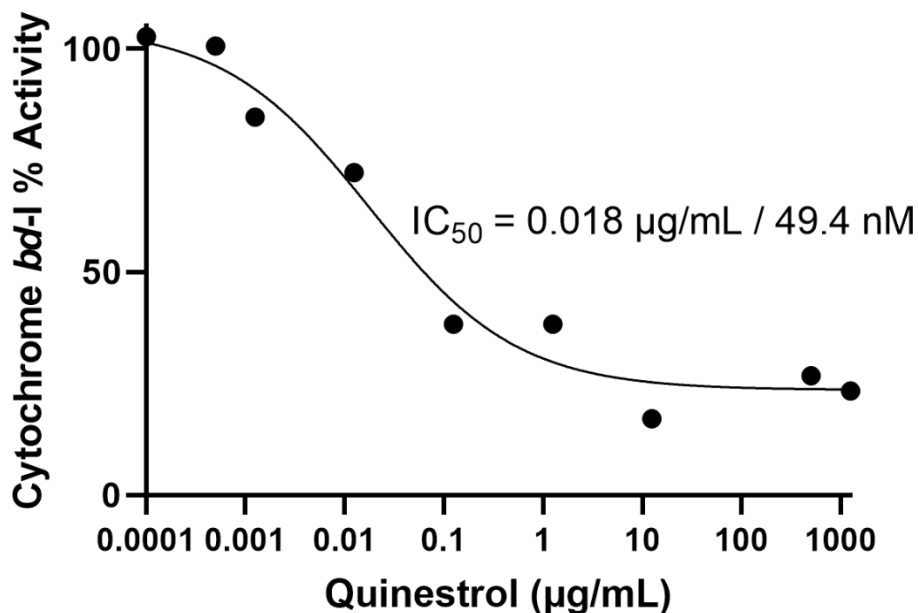


Figure 5.31 - Quinestrol Dose response. % activity refers to the oxygen consumption rate of EC958 '*bd-I* only' membranes relative to a DMSO control.

In the whole cell growth rate assay (Fig. 5.32), quinestrol showed similar inhibition does responses for *E. coli* EC958 '*bd-I* only' cell growth and the oxygen consumption assays with EC958 '*bd-I* only' membranes (Fig 5.31). *E. coli* EC958 '*bo*' only' and WT cells also showed a similar degree of inhibition to the growth rate (Fig. 5.32). This would suggest that quinestrol targets the cytochrome *bo*' quinol binding pocket as well as cytochrome *bd-I*.

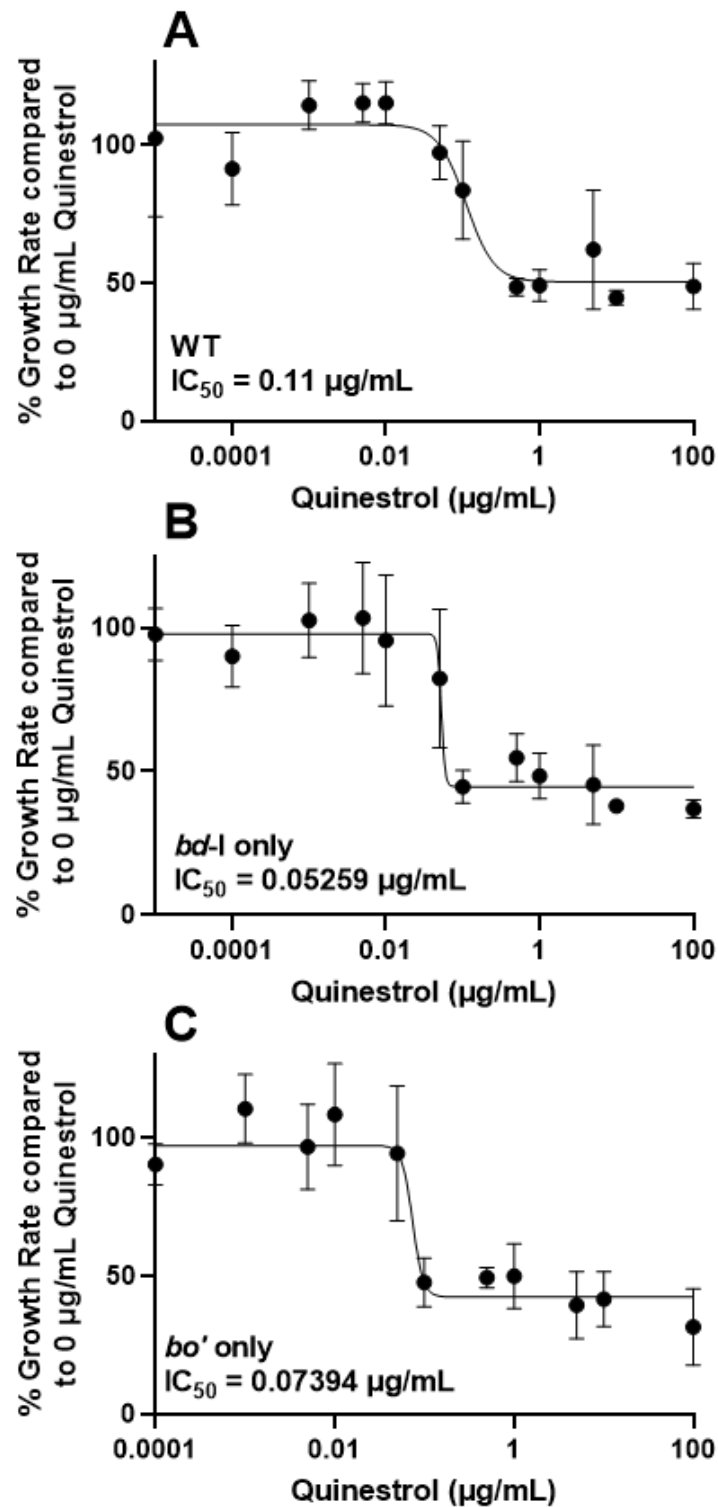


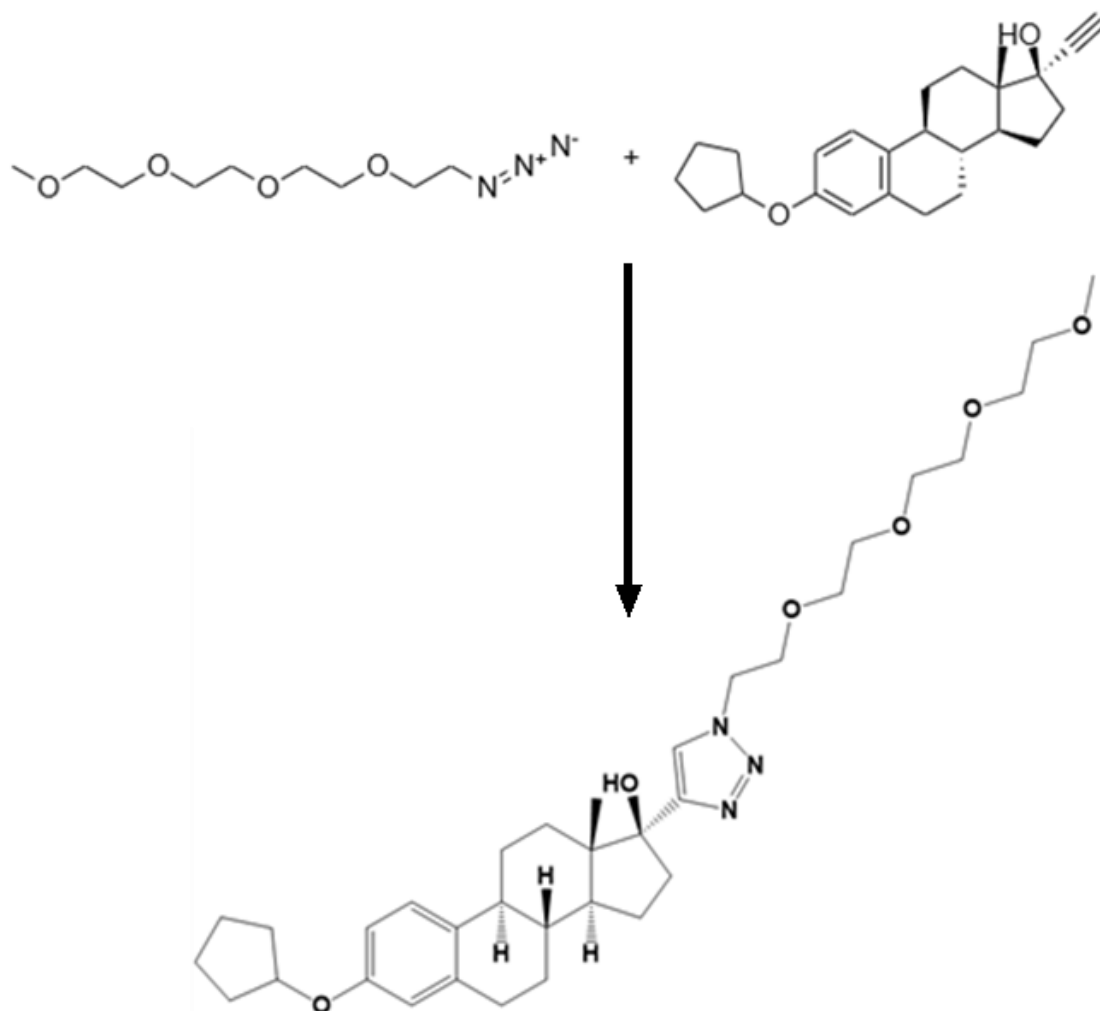
Figure 5.32 - Effect of quineestrol on *E. coli* growth. *E. coli* EC958 A) wild-type, B) cytochrome *bd-I* only and C) cytochrome *bo'* only were grown aerobically in M9 minimal media supplemented with 0.1 % casamino acids and glycerol as the carbon source until mid-exponential phase. *E. coli* cultures then diluted in fresh M9 to 8.0×10^7 CFU/mL and exposed to varying quineestrol concentrations. Growth rate measured between 1.5-4 h s after quineestrol addition (exponential phase) and reported as % growth rate compared to the 2.5 % DMSO control.

5.3.12. Quinestrol Derivatives

Synthesizing derivatives of known drugs is an important process in drug discovery in order to increase binding affinity, overcome resistance or improve the drug safety profile (Wohlleben et al. 2016). The chemistry of quinestrol was investigated to determine if any useful derivatives could be formed that may increase the inhibitory effect on cytochrome *bd-I*. The click chemistry reaction Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between azides and alkynes (Kacprzak et al. 2016) can be used on quinestrol due to the presence of the alkyne group (Fig 5.33). The alkyne group is pointing away from the quinol binding site in the docking results (Fig. 5.29) and so represents an interesting target for derivatization to increase interactions with the Q-loop. A 200 Da PEG (Polyethylene glycol) azide was chosen to react with quinestrol (Fig. 5.33). It was hoped that the PEG group may function in a similar way to the tail of ubiquinol-8 in binding to the cytochrome *bd-I* quinol binding site. This reaction was selected as the first derivatising attempt of quinestrol due to the simplicity of the reaction and stability of product (Kacprzak et al. 2016). The resultant quinestrol PEG derivative was docked to the cytochrome *bd-I* AF2 model (Fig. 5.34). The docking conformation for the steroid group as the same as for quinestrol (Fig. 5.29). The top predicted docking mode shown on the left (Fig. 5.34) has a binding affinity of -6.7 kcal/mol and estimated K_d of 12.1 μ M which is slightly lower than for quinestrol (-8.6 kcal/mol, estimated K_d = 489 nM). Experimental testing of the PEG derivative is needed to determine whether it is a better inhibitor of cytochrome *bd-I* than quinestrol.

PEG-azide

Quinestrol



Quinestrol-PEG derivative

Figure 5.33 - Click chemistry reaction to generate quinestrol PEG derivative from quinestrol and PEG azide.

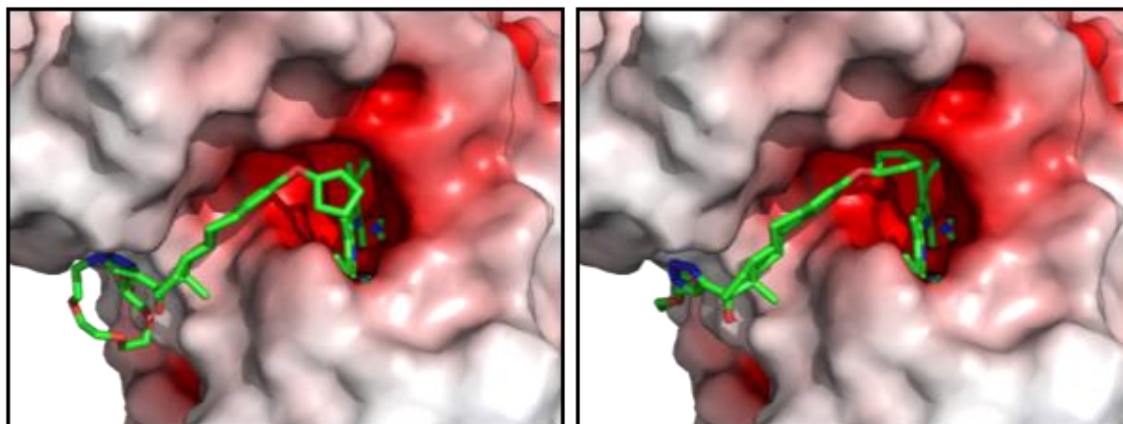


Figure 5.34 - Quinestrol PEG derivative docked into the *E. coli* AlphaFold 2 cytochrome *bd-I* model quinol binding site. Two different predicted docking conformations are shown.

5.3.13. Drug screen additional results

Other drugs were also screened with the drugs selected by binding affinity, hydrophobicity, modelled interactions with CydA, availability, cost, known pharmacokinetics and FDA approved target (Table. 5.5). Drugs selected were in the top 70 by binding affinity to the Phyre2 cytochrome *bd-I* model (Table 5.3) apart from sorafenib (~90th by binding affinity). 11 compounds that dock to the CydA quinol binding site that have not already been discussed were also selected for screening (Table 5.5). Drugs were not selected that bound to the poorly modelled part of the Q-loop. Three additional drugs (Fig. 5.35) show promising results.

Table 5.5 - FDA approved drugs selected for screening.

Drug	Molecular weight (g/mol)	MolLogP	Predicted affinity (kcal/mol)	Estimated K_d (nM)
DUTASTERIDE	528.53	6.58	-10.8	12
APREPITANT	534.44	4.95	-10.8	12
BEXAROTENE	347.46	4.77	-10.6	17
ENZALUTAMIDE	450.42	3.73	-10.4	23
TELMISARTAN	513.6	5.93	-10.3	28
MESTRANOL	310.54	3.92	-10.1	39
CARBAMAZEPINE	252.27	3.03	-10	46
SULINDAC	355.4	3.03	-10	46
PRAZIQUANTEL	312.4	2.53	-9.9	54
PEMETREXED	425.4	-2	-9.9	54
ETHINYL ESTRADIOL	296.39	3.61	-9.8	64
SORAFENIB	464.4	5.55	-9.6	90

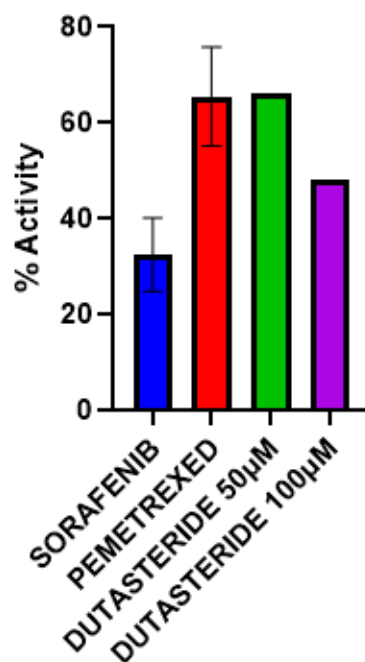


Figure 5.35 - Additional Drug screening. % activity refers to % oxygen consumption rate of EC958 '*bd-I* only' membranes relative to a DMSO control. Drugs were screened at 50 µM except dutasteride (both 50 and 100 µM). Two biological repeats were performed for sorafenib and pemetrexed.

5.3.14. Cloning of cytochrome *bd-I* and *bo'* for future mutagenesis studies.

For future studies on cytochrome *bd-I* inhibitors and kinetic studies on this complex, *E. coli* EC958 cytochrome *bd-I* oxidase was cloned into a plasmid vector. The plasmid was designed to produce a 6*His Tag on the C-terminus of CydX to allow for purification by affinity chromatography. The backbone of the pSU2718G vector was amplified using primers (2F_New_pSU2718 (431) and 2R_New_pSU2718 (432)) from an *E. coli* TOP10 strain containing the pSU2718G plasmid with a known insert (MS402, Table 2.1, Shepherd lab). The *cydABX* operon was amplified from *E. coli* EC958 genomic DNA using a forward primer (*CydABX_F_EC958*) that adds a 28 bp overlap region to the plasmid pSU2718G plasmid and a reverse primer (1RNew_pSU2718) that adds DNA encoding a 6*His Tag to the 3' end of *cydX* and 25 bp overlap to pSU2718G backbone (Fig 5.36). The amplified *cydABX*-6*HIS fragment was cloned into the pSU2718G vector downstream of the IPTG-inducible pLac

promoter using Gibson assembly and chemically transformed into *E. coli* DH5 α . Colony PCR was used to select colonies likely to have the correct recombinant pSU2718G-*cydABX*-6*His plasmid (Fig 5.37.A) using a forward primer that binds to the vector backbone (pSU2718seq_200_F) and a reverse primer that binds to the 6*His Tag (RvSc1_*cydABX*_His (416)). Transformant colonies 5 and 7 (C5 + C7) showed bands of approximately the correct size (expected 3114 bp). The plasmids from these colonies were extracted by miniprep and the joining regions between the pSU2718G vector backbone and *cydABX*-6*His operon sequenced by Sanger sequencing. Colony 7 was correct with the partially sequenced map displayed in Figure 5.37.B).

The cytochrome *bo'* operon *cyoABCD* was also cloned into pSU2718G with additional 7*His Tag on the C-terminus of *cyoD* using the same method as for *cydABX*-6*His operon (Fig 5.36 + Fig 5.38). The His Tag contains an extra residue due to the *cyoD* gene ending in a histidine codon. Attempts were made to clone the cytochrome *bd-II* operon *appCBX* into a vector with additional 6*His Tag (Fig 5.36) but these were unsuccessful due to non-specific binding of the amplification primers.

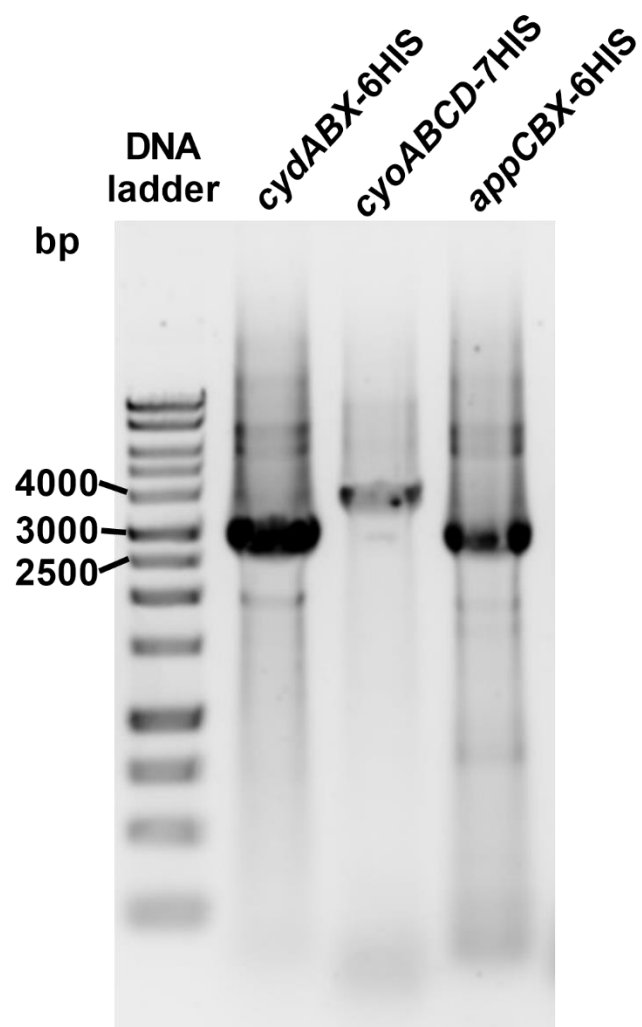


Figure 5.36 - PCR amplification of *E. coli* terminal respiratory oxidases with attached His Tag from genomic DNA. Amplified cytochrome *bd*-I *cydABX*-6HIS operon (expected size: 2926 bp), cytochrome *bo'* *cyoABCD*-7HIS (expected size: 3965 bp) and cytochrome *bd*-II *appCBX*-6HIS (expected size: 2869 bp).

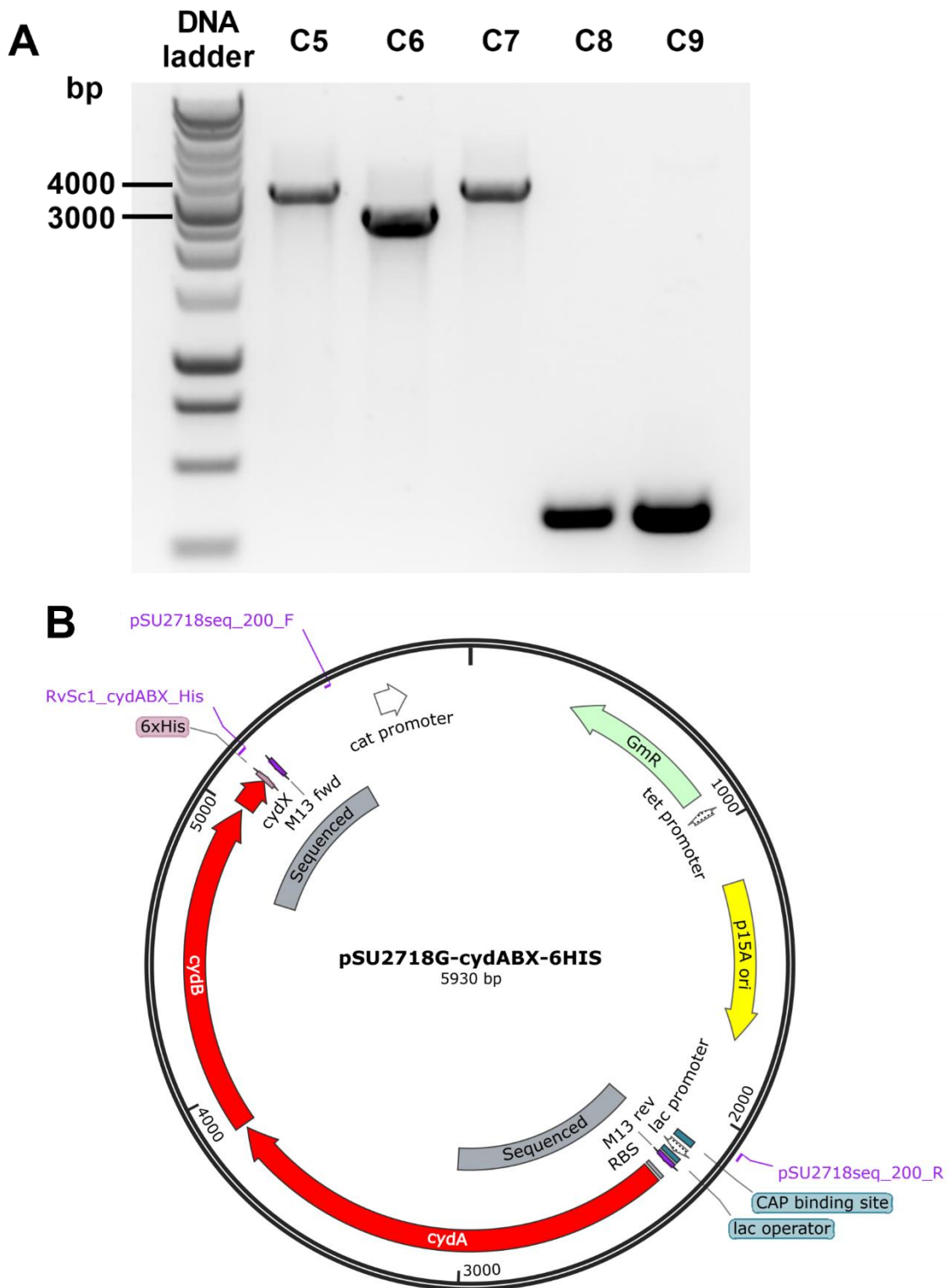


Figure 5.37 - Cloning of *E. coli* *cydABX*-6*HIS operon. (A) Colony PCR of 5 transformants from the Gibson reaction (C5-C9). PCR reaction used primers pSU2718seq_200_F and RvSc1_cydABX_His (expected fragment size: 3114 bp). (B) Genetic map of plasmid pSU2718G containing cloned *E. coli* *cydABX*-6*HIS operon. Primers pSU2718seq_200_R

and pSU2718seq_200_F were used to sequence the regions shown between the pSU2718G backbone and *cydABX-6*HIS* operon.

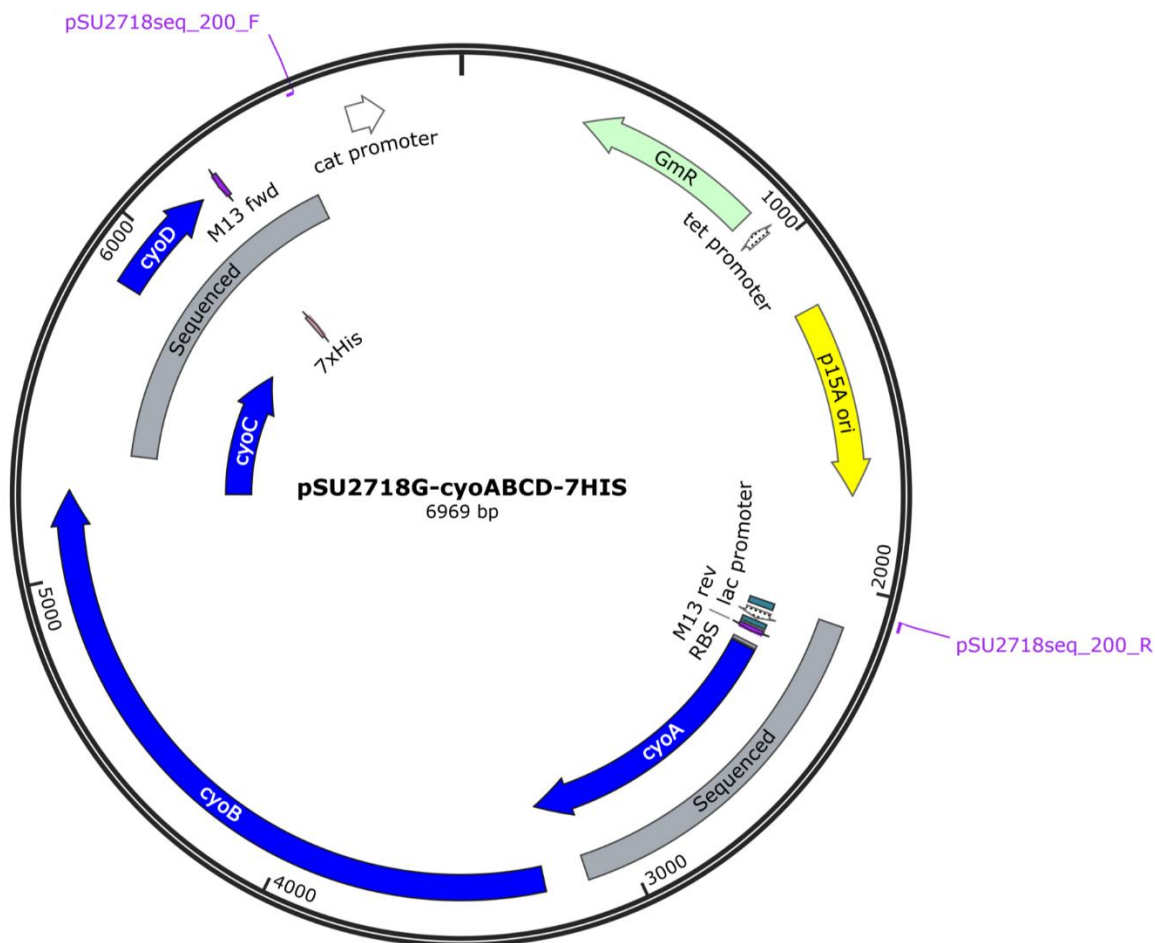


Figure 5.38 - Cloning of *E. coli* *cyoABCD-7*HIS* operon. Map of plasmid pSU2718G containing cloned *E. coli* *cyoABCD-7*HIS* operon. Primers pSU2718seq_200_R and pSU2718seq_200_F used to sequence the regions shown between the pSU2718G backbone and *cydABCD-7*HIS* operon.

5.4. Discussion

5.4.1. Development of experimental tools to screen cytochrome *bd* inhibitors

Cytochrome *bd* is a good drug target for new antimicrobials due to its role in infection for numerous pathogenic bacteria including strains of *E. coli* (Mason et al. 2009), its unique structure to prokaryotes (Borisov et al. 2011a) and known published protein

structures (Safarian et al. 2019; Theßeling et al. 2019). In order to target cytochrome *bd* in pathogenic *E. coli* a number of experimental tools were developed. *E. coli* has the 3 terminal respiratory oxidases of cytochrome *bd-I*, *bd-II* and *bo'*. In order to target *bd-I* the other two terminal oxidases had to be deleted. The multidrug-resistant uropathogenic strain of *E. coli* EC958 was selected for Lambda Red mutagenesis, and mutant strains were created that express either cytochrome *bd-I* (*bd-I* only) or cytochrome *bo'* (*bo'* only) as the sole terminal oxidase. Unfortunately, several attempts to make a strain expressing cytochrome *bd-II* (*bd-II* only) as the sole terminal oxidase all failed. The '*bd-I* only' strain was used for the development of membrane-based oxygen consumption assays to test potential inhibitors on cytochrome *bd-I*. Some known quinol binding site inhibitors of cytochrome *bd-I*, such as TTFA, are also known to target cytochrome *bo'*. Hence, the '*bo'* only' mutant allowed specificity to be investigated. An oxygen electrode was used to test potential inhibitors against cytochrome *bd-I* using succinate as the electron donor. This is a time-consuming assay and could potentially be replaced by a high-throughput fluorescence plate reader assay in the future using a fluorescent dye that is quenched by oxygen for the screening of a higher number of compounds.

5.4.2. Development of an *in silico* molecular docking pathway for drug repurposing to target cytochrome *bd*

An *in silico* molecular docking pipeline was developed to target the quinol binding pocket of *E. coli* cytochrome *bd-I* for drug re-purposing. Firstly, structural modelling was used to fill in gaps in cytochrome *bd-I* structures where resolution was poor/undefined. An FDA approved drug library from e-Drug3D (Pihan et al. 2012) was selected to be screened against cytochrome *bd-I* for aim of drug repurposing. The use of an FDA approved library has the advantages of reduced risk of toxicity and faster development times compared to *de novo* developed antimicrobials (Douguet 2018; Pushpakom et al. 2018; Farha and Brown 2019). The AutoDock Vina (Trott,O., Olson 2010) molecular docking software allowed fast automated screening of multiple ligands against the quinol binding site producing a list of results ordered by predicted binding energy. The top results by binding energy were then further analysed by hydrophobicity, specific interactions to the quinol binding site and

known mode of action in order to narrow the number of candidates down for experimental screening.

This molecular docking pathway using Autodock Vina has the potential to be up-scaled to larger drug libraries, used for derivatives of hits, to target other sites in *E. coli* cytochrome *bd-I* and cytochrome *bd* from other pathogenic bacteria (or indeed any protein). The python scripts used for automation and results analysis can be easily modified to accommodate targeting new sites of cytochrome *bd*.

5.4.3. New potential cytochrome *bd-I* inhibitors

Several interesting new inhibitors were identified for *E. coli* cytochrome *bd-I*. These include atovaquone and quineestrol. The antimalarial atovaquone (Osei-Akoto et al. 2005) showed 40% inhibition to cytochrome *bd-I* at 50 µg/mL (136 µM)(Fig. 5.27). Atovaquone is a known quinol binding site inhibitor that is a competitive inhibitor of ubiquinol for the cytochrome *bc₁* complex of *P. falciparum* (Nixon et al. 2013). Atovaquone displays a very low IC₅₀ of 1 - 3.5 nM against *P. falciparum* (Nixon et al. 2013). Atovaquone does target the human mitochondrial cytochrome *bc₁* complex with sub-micromolar affinity resulting in a human/*P. falciparum* selectivity index of 230 (Nilsen et al. 2013) allowing it to be used for malaria treatment. The main problem with using Atovaquone as a cytochrome *bd-I* inhibitor is that the affinity is too low to avoid problems with targeting human mitochondrial cytochrome *bc₁*.

The head group of atovaquone, 2-hydroxy-1,4-napthoquinone (lawsone), also showed inhibition towards cytochrome *bd-I* with an IC₅₀ of 825.5 µg/mL (4.74 mM). This inhibition is weak and 2-hydroxy-1,4-napthoquinone is not an FDA approved drug. However, this could be used as a scaffold for future derivatisation. Some recent papers have synthesised derivatives of 2-hydroxy-1,4-napthoquinone (Yang and Lee 2015) and then tested their efficacy against methicillin-resistant *S. aureus* (Song et al. 2020) and *E. coli* cytochrome *bo'* (Elamri et al. 2020). Some of the lawsone derivatives showed inhibition against *E. coli* cytochrome *bd-I* as well as *bo'* such as 2-Hydroxy-3-tridecyl-1,4-napthoquinone (HNQ-1), whilst others showed no inhibition such as 2-Hydroxy-3-[(3,5-

dihydroxybenzyl)methyl]-1,4-naphthoquinone (HNQ-10) (Elamri et al. 2020). The tail group is clearly important for both affinity and selectivity. The published cryo-EM structures along with the AlphaFold2 model of *E. coli* cytochrome *bd-I* should allow for more structure-based drug design of lawsone derivatives to target the quinol binding pocket. Clearly 2-hydroxy-1,4-naphthoquinone derived molecules are important new antimicrobials that still require optimisation to target cytochrome *bd-I*.

Several FDA approved steroid drugs including mestranol, quinestrol and ethinyl estradiol, all with similar structures, were identified as promising cytochrome *bd-I* inhibitors using the docking screen. The most promising of these, quinestrol, is a prodrug used in birth control and prostate/breast cancer treatment and displayed an IC_{50} of 0.018 $\mu\text{g/mL}$ (49.4 nM) when tested against *E. coli* EC958 '*bd-I* only' membranes (Fig. 5.31). This is significantly more potent than either atovaquone or 2-hydroxy-1,4-naphthoquinone. Quinestrol also showed activity against EC958 WT cells and the '*bd-I* only' and '*bo*' only' strains. Inhibition of the '*bo*' only' mutant indicates that quinestrol targets both cytochromes *bo*' and *bd-I*, suggesting a similar quinol binding pocket for these terminal oxidases. This is consistent with previous studies where a large number of known quinol binding site inhibitors for cytochrome *bd-I* do also inhibit cytochrome *bo*' to varying degrees, including TTFA (Meunier et al. 1995) used in this work as a positive control.

Residual growth is again observed above 1 $\mu\text{g/mL}$ quinestrol in all of the *E. coli* strains. This is unusual as you would expect the activity/growth rate to fall to 0% in the cytochrome *bd-I* only and cytochrome *bo*' only cells at high quinestrol concentrations if quinestrol was targeting cytochrome *bd-I* and *bo*' during aerobic growth. This is the case with TTFA which targets both cytochrome *bd-I* and *bo*' (Fig. 5.21, 5.22 + 5.23). The mechanism of quinestrol inhibition therefore needs to be determined in the future. Quinestrol could alternatively be targeting the quinol binding site of succinate dehydrogenase as succinate was the substrate used in the oxygen consumption assay (Fig. 5.21). Alternatively, quinestrol could be disrupting the inner membrane but again this would not explain the residual activity/growth.

Only one mention of quinestrol having antibacterial activity can be found in the literature (Waleed Younis et al. 2017). This was in an *in vitro* screen of FDA compounds

against the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*) (Waleed Younis et al. 2017). Quinestrol showed activity against the Gram-positive *E. faecium* ATCC 700221 (MIC of 16 μ M) and *Staphylococcus aureus* NRS384 (MIC >16 μ M) (Waleed Younis et al. 2017). No inhibition was seen against the Gram-negative ESKAPE pathogens (Waleed Younis et al. 2017). This is therefore the first reporting of antibacterial activity for quinestrol against *E. coli* or any other Gram-negative bacteria.

Mestranol, which shares the same core structure as quinestrol but lacks the cyclopentane ring, shows no inhibition to cytochrome *bd-I*. This suggests that the cyclopentane ring of quinestrol is required for binding to the quinol site of cytochrome *bd-I*, which is supported by the molecular docking (Fig. 5.29). According to the docking conformation for quinestrol (Fig. 5.29) this cyclopentane group binds deep inside the quinol binding cleft next to haem *b*₅₅₈. The cyclopentane group of quinestrol is predicted to form hydrophobic interactions with the haem ligand and conserved aspartate 239. Ethinyl estradiol is the active metabolite of quinestrol, again lacking the cyclopentane ring, and only shows weak inhibition of cytochrome *bd-I* (Fig. 5.30). Quinestrol is stored in body fat and is slowly metabolized to ethinyl estradiol (Tang et al. 2012), and will therefore be present for an extended period of time after administration.

A few other FDA approved drugs also showed promising inhibition of membranes containing cytochrome *bd-I* as the sole terminal oxidase. These include dutasteride, sorafenib and pemetrexed (Fig. 5.35). Only preliminary data was gathered for these compounds. More work will be needed to investigate these drugs, especially as pemetrexed is a weak amphipathic acid and so could potentially be disrupting the membranes rather than targeting cytochrome *bd-I*. Additionally, a larger screen with more biological repeats would need to be completed based on the AlphaFold2 cytochrome *bd-I* model docking results.

5.4.4. Future Directions

For further studies on the mode of action for quinestrol-mediated inhibition of cytochromes *bd-I* and *bo'* would be beneficial, especially to explain the residual oxygen consumption seen when a high concentration of quinestrol is present (Fig. 5.31 + 5.32). Furthermore, generating a cryo-EM structure of quinestrol bound to *E. coli* cytochrome *bd-I* would be helpful in determining the binding mode and for future derivatisation. This has previously been reported for the inhibitor aurachin C/D and *E. coli* cytochrome *bd-II* (Grauel et al. 2021). Molecular dynamics is another tool that could be used to study binding of inhibitors to the cytochrome *bd-I* quinol binding site. Binding of known inhibitors such as aurachin D could be studied by molecular dynamics to give insights into any structural changes involved in ligand binding to the quinol binding site. The Q-loop which includes part of the quinol binding site, is predicted to have some flexibility that renders sections of the Q-loop poorly resolved in published structures (Safarian et al. 2019; Theßeling et al. 2019). Molecular dynamics could provide information useful for drug derivatisation purposes and improved structure-based drug design which has previously been performed for antimalarial cytochrome *bc₁* inhibitors (Gangwal et al. 2013). The binding of quinestrol to cytochrome *bd-I* could also be studied using molecular dynamics, and the parameter files have already been built for the all-atom Amber ff14SB force field for this respiratory oxidase (Ahn et al. 2021).

The experimental system used to screen the potential inhibitors used succinate as the substrate with electrons flowing into the electron transport chain via succinate dehydrogenase (Borisov and Verkhovsky 2015). Therefore, there was the possibility of the potential cytochrome *bd-I* inhibitors may be also targeting the quinol binding site of succinate dehydrogenase. However, since writing this thesis a succinate dehydrogenase assay has been implemented in the laboratory and quinestrol has been shown to be ineffective against succinate dehydrogenase (data not shown).

In order to improve drug efficacy, drug entry or delivery derivatisation can be undertaken (Wohlleben et al. 2016). Further derivatives of the 2-hydroxy-1,4-naphthoquinone head group of Atovaquone could be designed to target the cytochrome *bd-I* quinol binding site. Indeed, a successful attempt has already been made to do this with

the cytochrome *bo'* quinol binding site in *E. coli* (Elamri et al. 2020). Also, quineestrol can be easily derivatized due to the alkyne group using the click chemistry reaction Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition between azides and alkynes (Kacprzak et al. 2016). A 200 Da PEG (Polyethylene glycol) derivative has been synthesised by collaborators with the next step being to test for affinity to cytochrome *bd-I* (data not shown). Further derivatization is also possible using for example using Sonogashira coupling (Bacsa et al. 2017). Sonogashira coupling is another commonly used reaction in steroid chemistry (Bacsa et al. 2017) which could be used to introduce an aryl group onto the alkyne of quineestrol (Bacsa et al. 2017).

The cloning work undertaken in section 5.3.13 will allow future mutagenesis work to be undertaken to analyse inhibitor binding and to investigate how potential mutations might lead to drug resistance. Interesting residues around the quinol binding site include Leu253, Ile295, Phe269 and Phe390. The mutation Leu253A has previously been shown to abolish cytochrome *bd* activity, probably due to its role in coordinating the haem *b*₅₅₈ (Safarian et al. 2019). However, other non-essential residues in the quinol pocket could be mutated to investigate whether specific sidechains affects drug binding. In addition, *bd-I* only cells could be exposed to low concentrations of drug for extended periods to investigate the potential for the emergence of resistance through natural selection.

Several other potential sites in cytochrome *bd-I* were identified in chapter 4 that are promising for the development of new inhibitors to cytochrome *bd-I*. These include the oxygen entry channel and proton channels. Mutagenesis work could also be undertaken to further confirm the predicted proton/oxygen channels and investigate what effect on the oxygen consumption rate of cytochrome *bd-I* blocking these channels would have. A similar *in silico* screen starting with FDA drugs could be used to target these potential inhibition sites. The experimental system developed for the quinol site inhibitors would also work with inhibitors targeted to other sites of cytochrome *bd-I*. Now that the cytochrome *bd-II* structure is also available for *E. coli* (Grauel et al. 2021; Grund et al. 2021) any identified inhibitors for cytochrome *bd-I* could also be tested against cytochrome *bd-II* as cytochrome *bd-II* has a very similar structure.

Chapter 6

Final Discussion

The overarching theme of this PhD is the link between drug susceptibility and respiratory activity. The aim of this final chapter is to briefly summarise the main outcomes of this work and to illustrate how the chapters fit together to improve our overall understanding of this broad topic.

6.1. Role of PMF vs ROS in aminoglycoside toxicity

Respiratory activity is well-known to promote the lethality of many antibiotics via the generation of ROS (Stokes et al. 2019). Regarding the aminoglycoside class of antibiotics, the maintenance of the PMF provides an alternative route to potentiate the lethality of the antibiotics (Taber et al. 1987). It was decided to investigate the relative contributions between the ROS and PMF hypothesis using similar conditions to assess the underpinning mechanism for gentamicin lethality in a pathogenic *E. coli* strain. Firstly, lethal doses of gentamicin did not lead to significantly increased ROS production. Secondly exposure to the PMF uncoupler 2,4-DNP (1 mM) resulted in significantly increased survival to gentamicin in *E. coli* EC958. As expected, 1 mM 2,4-DNP also disrupted the electrical potential of the *E. coli* inner membrane. The NO-donor GSNO previously shown to reduce gentamicin lethality (Ribeiro et al. 2021) also showed a significant dissipation of the membrane electrical potential. Previous transcriptomics studies using *E. coli* was exposed to GSNO showed no perturbations in ROS stress responses detected (Flatley et al. 2005). This supports the hypothesis that GSNO diminishes the lethality of gentamicin through dissipation of the PMF and not alleviation of ROS generation. This is consistent with previous work on *Salmonella* which showed that diminished NO mediated respiratory inhibition diminished aminoglycoside toxicity by a mechanism involving diminished gentamicin uptake (McCollister et al. 2011). Furthermore, DNP did not show significantly altered oxygen consumption in *E. coli* EC958. Finally, as aminoglycoside antibiotics work on *E. coli* grown under anaerobic conditions, it was hypothesised that DNP will also diminish the lethality of gentamicin under anaerobic conditions. This was indeed the case with DNP abolishing gentamicin lethality under conditions that exclude ROS generation (anaerobic) by the respiratory chain. Taken together this supports the hypothesis that DNP diminishes the lethality of gentamicin through dissipation of the PMF and not reduction of ROS generation by the respiratory chain. Overall, the data suggests that maintenance of the PMF is the

dominant mechanism for the respiratory chain in potentiating the toxic effects of aminoglycosides.

6.2. *E. coli* cytochrome *bd*-I phylogenetics and investigation as a good drug target

Antibiotic resistance is an increasing problem coupled with the slowdown in antibiotic discovery in the last few decades leads to the pressing need for new antimicrobials (Coates et al. 2011). Cytochrome *bd* has been identified as a potential new drug target for a number of pathogenic bacteria (Friedrich et al. 2022). Herein, phylogenetic analysis linked to structural analysis with an emphasis of potential drug target sites was conducted. A hhsearch pathway was developed for the detection of cytochrome *bd* oxidases in bacteria and archaea that allows remote homologs to be detected for phylogenetic analysis. The present or absence of a residue at *E. coli* *bd*-I leucine 101 position was investigated as a potential tool for haem ligand arrangement characterization. The oxygen entry channel was shown to be a useful new drug target in cytochrome *bd* whilst the second quinol binding site in *E. coli* cytochrome *bd*-I was predicted to have a structural function with very tight ubiquinone-8 binding leading to it being hypothesized as a poor drug target. Conservation in the proton channels was identified leading to the conclusion that these channels may also be good targets for drugs to act on multiple cytochrome *bd* oxidases although there is redundancy in the proton channels with 2 channels being present per oxidase. Further work is needed on the oxygen and proton channels to access their suitability as drug targets. Molecular docking experiments could be completed on the oxygen entry site to test for potential inhibitors.

6.3. Drug Screening for *E. coli* cytochrome *bd*-I

An *in silico* molecular docking-based screening pipeline was developed to target the quinol binding site for *E. coli* cytochrome *bd*-I. A previous study found a novel *Mycobacterium smegmatis* cytochrome *bd* quinol binding site inhibitor in the micromolar

range called MQL-H₂ using a similar molecular docking approach using modelled *Mycobacterium tuberculosis* cytochrome *bd* (Harikishore et al. 2020). This study provided evidence of the potential for structure-based drug docking to target the quinol binding site of cytochrome *bd* and generate novel inhibitors (Harikishore et al. 2020). For this study an FDA approved drug library was selected for the aim of drug-repurposing to target *E. coli* cytochrome *bd*-I. Drug repurposing can have several advantages including faster development times, reduced cost and risk of toxicity compared to de novo drug development (Douguet 2018; Pushpakom et al. 2018; Farha and Brown 2019). Alongside the *in silico* screen an experimental assay was developed for the testing of potential drug candidates against cytochrome *bd*-I from pathogenic *E. coli*. An overview of how the *in silico* work ties into the laboratory screening is provided in Figure 6.1. A number of interesting new inhibitors of *E. coli* cytochrome *bd*-I were found including the antimalarial atovaquone (Osei-Akoto et al. 2005) and the prodrug quineestrol using in birth control (Tang et al. 2012). Quineestrol was the most promising candidate with IC₅₀ of 0.018 µg/mL/49.4 nM against *E. coli* membranes containing *bd*-I as the sole terminal oxidase and whole cell activity against both *E. coli* EC958 expressing cytochrome *bd*-I as the sole terminal oxidase and WT. Quineestrol represents an exciting new antimicrobial candidate with future work needed to determine the method of inhibition alongside testing against other pathogenic bacteria. Derivatisation of quineestrol also represents an interesting option to try and increase the binding affinity.

This cytochrome *bd* targeting drug development pipeline is readily transferrable to the other potential inhibitor sites of cytochrome *bd* such as the oxygen entry site as well as other cytochrome *bd* oxidases from a range of pathogenic bacteria. This is especially true for *Mycobacterium tuberculosis* as the structure has now been published (Wang et al. 2021; Safarian et al. 2021).

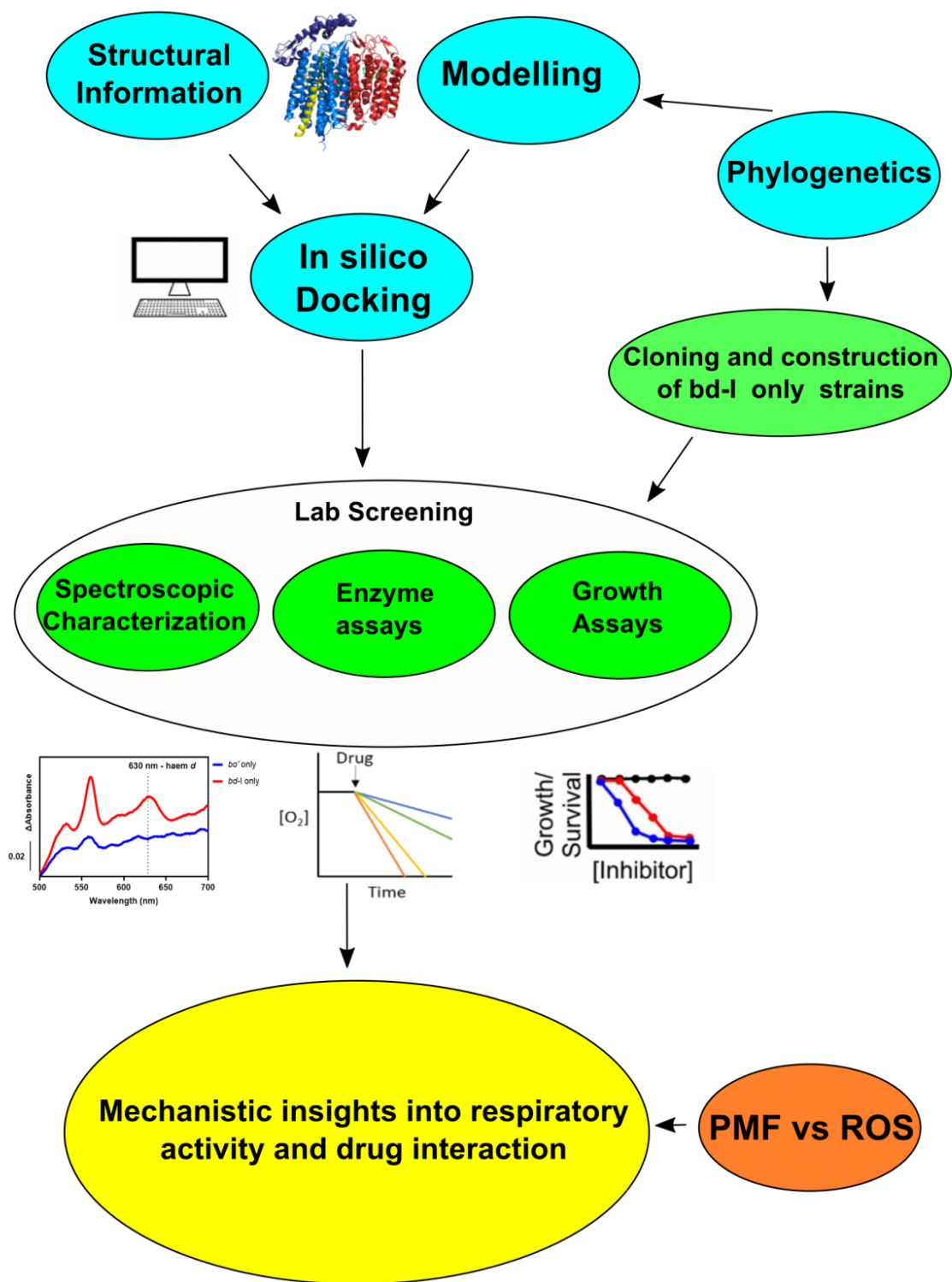


Figure 6.1 – Overview of in silico and laboratory screening.

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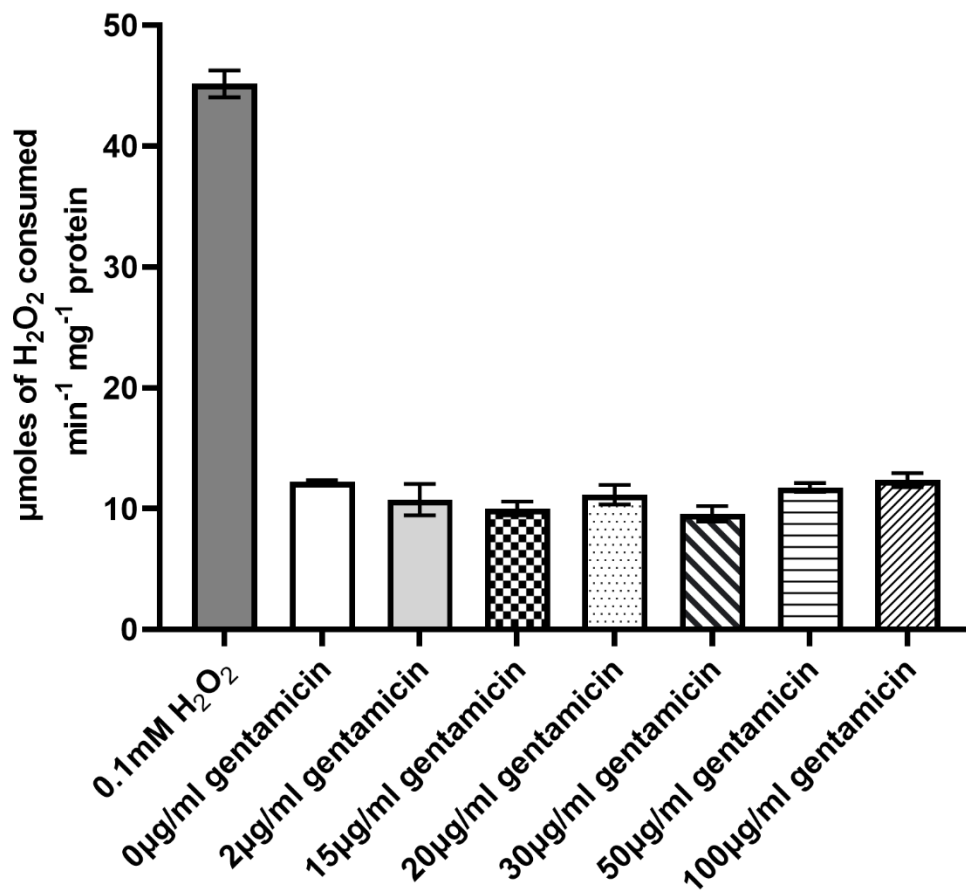
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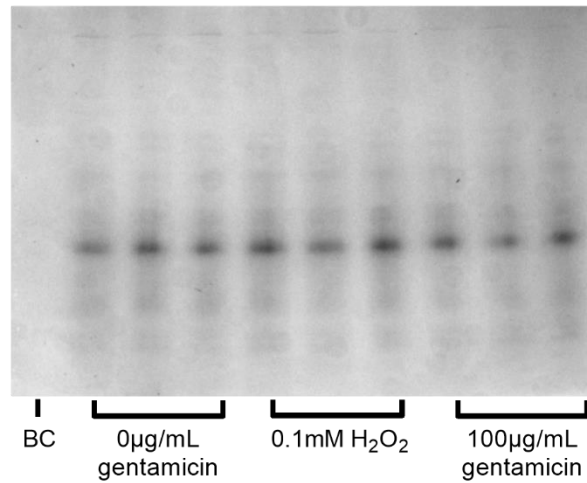
Appendix

Appendix A.1 Varying concentrations of gentamicin elicit little difference in catalase

activity in aerobically grown *E. coli*. (A) Suspensions of 1.6×10^8 cells/ml of *E. coli* EC958 grown in M9 minimal medium supplemented with 0.1% casamino acids were exposed to a sub-lethal level of hydrogen peroxide (0.1 mM) or varying concentrations of gentamicin for 30 min. Cells were then washed, sonicated, cell debris was removed and catalase activity was measured in the cell-free extract with three technical repeats completed for each condition.

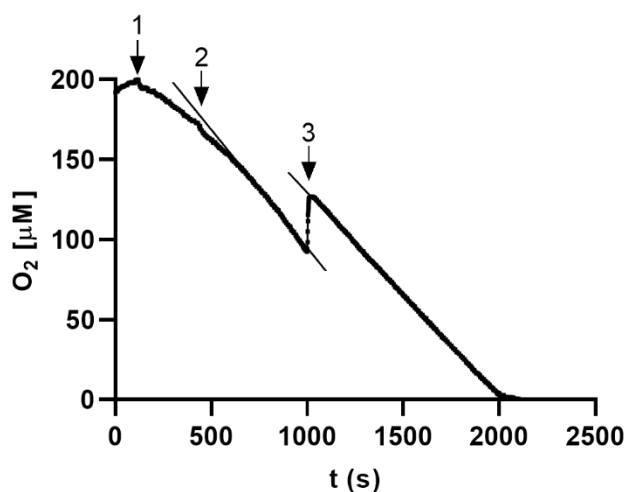


Appendix A.2. Coomassie stained Native-PAGE gel. *E. coli* EC958 cells exposed and extract prepared as in Figure 3.1A. 10 µg of each cell extract was loaded from cells exposed to hydrogen peroxide (0.1 mM), 0 µg/mL gentamicin or 100 µg/ml gentamicin. 10 units of bovine catalase (BC) loaded as a positive control.



Appendix A.3. Measurement of respiratory oxygen consumption example trace.

Suspensions of 4.8×10^8 CFU/mL of *E. coli* EC958 were diluted into fresh M9 minimal medium supplemented with 0.1 % casamino acids to 8.0×10^7 CFU/mL in a Rank Brothers oxygen electrode chamber after a baseline of 5 min was recorded (1). Voltage data was collected for 8 min followed by the addition of the plunger to exclude external oxygen (2) and data were recorded for another 10 min. Injection of 1 mM DNP (dissolved in ethanol) was performed to 5 % of the total volume (3). Conversion from voltage to nM oxygen and nM/s used $\Delta V = 200 \mu\text{M O}_2$.



Appendix A.4. List of archaeal strains in which a CydA or CydB homologue was found using the BLASTP search to *E. coli* cytochrome *bd-I*.

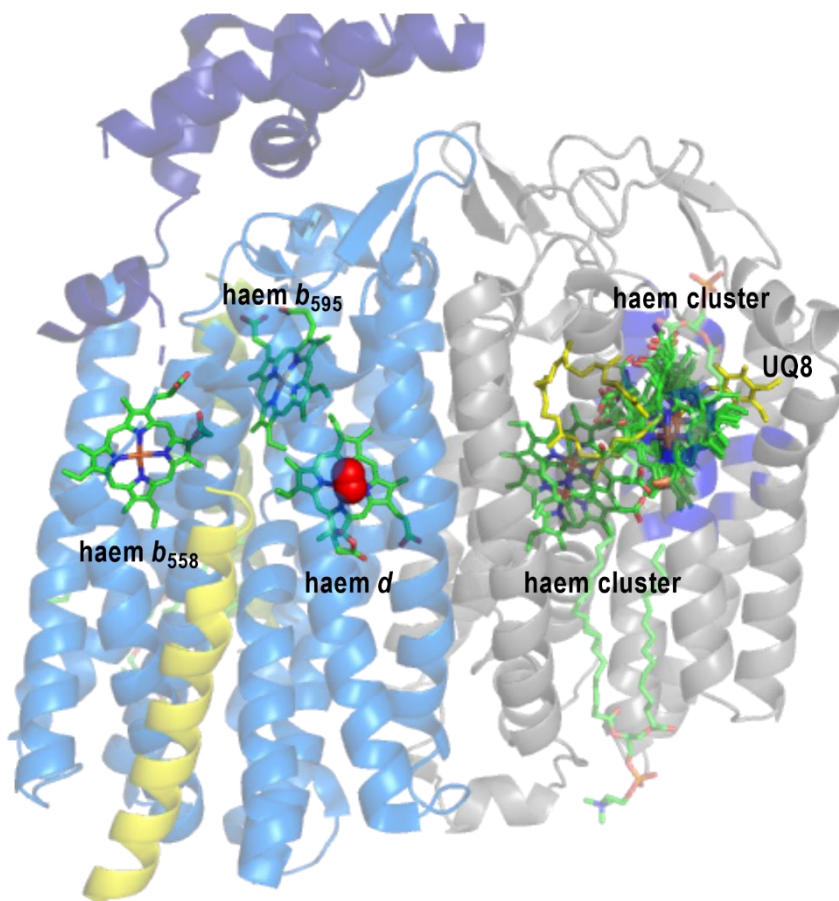
Species	Phyla	<i>E. coli</i> cytochrome <i>bd-I</i> Homologue	<i>E. coli</i> cytochrome CydB <i>bd-I</i> Homologue
<i>Thermococcus profundus</i>	Euryarchaeota	Yes	Yes
Thermococcus sp. AM4	Euryarchaeota	Yes	Yes
<i>Methanosarcina siciliae</i> C2J	Euryarchaeota	Yes	Yes
<i>Methanolobus profundus</i>	Euryarchaeota	Yes	Yes
<i>Halobacterium salinarum</i> (strain ATCC	Euryarchaeota	Yes	Yes

700922 / JCM 11081 / NRC-1)			
<i>Haloterrigena turkmenica</i>	Euryarchaeota	Yes	Yes
<i>Haloarcula hispanica</i> (strain ATCC 33960 / DSM 4426 / JCM 8911 / NBRC 102182 / NCIMB 2187 / VKM B-1755)	Euryarchaeota	Yes	Yes
<i>Natrialba magadii</i> (strain ATCC 43099 / DSM 3394 / CIP 104546 / JCM 8861/ NBRC 102185 / NCIMB 2190 / MS3)	Euryarchaeota	Yes	Yes
<i>Korarchaeum cryptofilum</i>	Proteoarchaeota	Yes - 2 homologues in operon (Kcr1583/1584)	No
<i>Archaeoglobus fulgidus</i> (strain ATCC 49558 / VC-16 / DSM 4304 / JCM 9628 / NBRC 100126)	Euryarchaeota	Yes 2 homologues in operon (CydA1/CydA2)	No
<i>Halorhabdus tiamatea</i> SARL4B	Euryarchaeota	Yes	Yes
<i>Thermococcus gammatolerans</i> (strain DSM 15229 / JCM 11827 / EJ3)	Euryarchaeota	Yes	Yes
<i>Methanosarcina acetivorans</i> (strain ATCC 35395 / DSM 2834 / JCM 12185 / C2A)	Euryarchaeota	Yes	Yes
<i>Methanosarcina barkeri</i> (strain Fusaro / DSM 804)	Euryarchaeota	Yes	Yes
<i>Haladaptatus paucihalophilus</i> DX253	Euryarchaeota	Yes	Yes
<i>Halalkalicoccus jeotgali</i> (strain DSM 18796 / CECT 7217 / JCM 14584 / KCTC 4019 / B3)	Euryarchaeota	Yes	Yes
<i>Halomicrobium mukohataei</i> (strain ATCC 700874 / DSM	Euryarchaeota	Yes	Yes

12286 / JCM 9738 / NCIMB 13541)			
<i>Natronobacterium gregoryi</i> (strain ATCC 43098 / DSM 3393 / CCM 3738 / CIP 104747 / JCM 8860 / NBRC 102187 / NCIMB 2189 / SP2)	Euryarchaeota	Yes	Yes
<i>Halonotius</i> sp. J07HN4	Euryarchaeota	Yes	Yes
<i>Acidianus hospitalis</i> (strain W1)	TACK Group	Yes	Yes
<i>Candidatus Parvarchaeum acidiphilum</i> ARMAN-4	Parvarchaeota	Yes	Yes

Appendix A.5. 3D Ligand Site results for *E. coli* cytochrome *bd-I* CydB ubiquinol-8 (UQ8) cleft.

Predicted haem clusters shown in ubiquinol-8 cleft of CydB (grey).



Appendix A.6. Sanger sequencing result of double terminal respiratory mutants $\Delta cyoA$ *appCB::Cm* (*bd-I* only) and $\Delta cydAB$ *appCB::Cm* (*bo'* only) displaying *appCB* deleted region.

Homology regions (yellow) are the regions flanking the *appCB* locus used for lambda red mutagenesis. Cm^R cassette consisting of the Cm^R gene (green), cat promoter (grey) and associated FRT sites (blue) have been inserted in place of the *appCB* operon.

GGCATTACGCTGTACGGACACCTTAAAAGGCCCGTTACTGGAAAGTTATGAAATCTGCCCAATACCGGA
AGTGGTGCTGGCAGCGCCAGAAGATTTGGTCGACTCTGCGCAGCGGCTTAGCGAGGTATGCCAGTGGCT
GGCGGAAGGTGCACCGACGTAAAAAGACGGTAAGTATCGCTTTCAGGCTTATGAATATCGCAATCGGCG
AATACCTCTGGTCGTAGAGTTTCAGGATAAAGAGGGAGATCTACCATTAACGGGTATTCTCTCTCTCTCG
CCTACAGGAGTGCGCGTGTAGGCTGGAGCTGCTTGAAGTTCCTATACTCTCTAGAGAATAGGAACTTCG
GAATAGGAACTTCATTAAATGGCGCGCCTTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATTC
ATTAAGCATTCTGCCGACATGGAAGCCATCACAAACGGCATGATGAACCTGAATCGCCAGCGGCATCAGC
ACCTTGTCGCTTGCGTATAATATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCCA
CGTTTAAATCAAACTGGTGAACTCACCCAGGATTGGCTGAGACGAAAAACATATTCTCAATAAACCC
TTAGGGAAATAGGCCAGGTTTTACCGTAACACGCCACATCTTGCGAATATATGTGTAGAACTGCCGG
AAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCATGGAAAACGGTGTAACAAG
GGTGAACACTATCCCATATCACCAGCTACCGTCTTTCATTGCCATACGTAATCCGGATGAGCATTATCA
GGCGGGCAAGAATGTGAATAAAGGCCGGATAAACTGTGCTTATTTTCTTACGGTCTTAAAAAGGC
CGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTGACTGAAATGCCTCAAAATGTTCTT
TACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTCTCCATTTTAGCTTCCTTAGCTC
CTGAAAATCTCGACAACTCAAAAATACGCCCGGTAGTGATCTTATTTTATTATGGTGAAAGTTGGAACCTC
TTACGTGCCGATCAACGTCTCATTTTCGCCAAAAGTTGGCCCAGGGCTTCCCGGTATCAACAGGGACACCA
GGATTTATTTATTCTGCGAAGTGATCTCCGTACAGGTAGGCGCGCCGAAGTTCCTATACTTTCTAGAGA
ATAGGAACTTCGGAATAGGAACTAAGGAGGATATTCATATGGGAGCAGGAACAATGTGGTATTTACTTT
GGTTCGTCGGCATTTTGTGATGTGTTGCTCTCCACCTTGTATCAGGCAATCAATAATGTCGGATATGA
AAAGCGGAAACATATCGATGAAAGCGATCTTGATCCCATTCTTATCTCTCTGATTCCGTAAACACCGAAA
TCTGCATTGCTCAGAGTGAGCCGGAGCTGAAGCTGGAAAGTGTTGGTATTGTCAGTCGTACGGTGTGC
GTGCACCAACCAAGGCCACGCAACTGATGCAGGATGTACCCCTGACGCATGGCCAACCTGGCCGGTAAA
ACTGGGTGGCTGACACCGCGCGGTGGTGAGCTAATCGCTTATCTCGGACATTACCAACGCCA

Appendix A.7. Ligand preparation python script for running AutodockVina.

```
#!/usr/bin/env python
#
#
#
# $Header:
# /mnt/raid/services/cvs/python/packages/share1.5/AutoDockTools/Utilities24/prepare_lig
# and4.py,v 1.10.4.1 2015/08/26 22:45:31 sanner Exp $
import os

from MolKit import Read

from AutoDockTools.MoleculePreparation import AD4LigandPreparation

if __name__ == '__main__':
    import sys
    import getopt

    def usage():
        "Print helpful, accurate usage statement to stdout."
        print "Usage: prepare_ligand4.py -l filename"
        print
        print "  Description of command..."
        print "    -l  ligand_filename (.pdb or .mol2 or .pdbq format)"
        print "  Optional parameters:"
        print "    [-v]  verbose output"
        print "    [-o pdbqt_filename] (default output filename is ligand_filename_stem +
        .pdbqt)"
        print "    [-d]  dictionary to write types list and number of active torsions "

        print "    [-A]  type(s) of repairs to make:\n\t\t bonds_hydrogens, bonds,
        hydrogens (default is to do no repairs)"
        print "    [-C]  do not add charges (default is to add gasteiger charges)"
        print "    [-p]  preserve input charges on an atom type, eg -p Zn"
        print "          (default is not to preserve charges on any specific atom type)"
        print "    [-U]  cleanup type:\n\t\t nphs_lps, nphs, lps, " (default is 'nphs_lps') "
        print "    [-B]  type(s) of bonds to allow to rotate "
        print "          (default sets 'backbone' rotatable and 'amide' + 'guanidinium' non-
        rotatable)"
        print "    [-R]  index for root"
```

```

    print "    [-F]  check for and use largest non-bonded fragment (default is not to do
this)"
    print "    [-M]  interactive (default is automatic output)"
    print "    [-I]  string of bonds to inactivate composed of "
    print "           of zero-based atom indices eg 5_13_2_10 "
    print "           will inactivate atoms[5]-atoms[13] bond "
    print "           and atoms[2]-atoms[10] bond "
    print "           (default is not to inactivate any specific bonds)"
    print "    [-Z]  inactivate all active torsions "
    print "           (default is leave all rotatable active except amide and
guanidinium)"
    print "    [-g]  attach all nonbonded fragments "
    print "    [-s]  attach all nonbonded singletons: "
    print "           NB: sets attach all nonbonded fragments too"
    print "           (default is not to do this)"
    print "    [-w]  assign each ligand atom a unique name: newname is original name
plus its index(1-based)"

# process command arguments
try:
    opt_list, args = getopt.getopt(sys.argv[1:], 'l:vo:d:A:Cp:U:B:R:MFI:Zgsw')
except getopt.GetoptError, msg:
    print 'prepare_ligand4.py: %s' %msg
    usage()
    sys.exit(2)

# initialize required parameters
#-l: ligand
ligand_filename = None
# optional parameters
verbose = None
add_bonds = False
#-A: repairs to make: add bonds and/or hydrogens
repairs = ""
#-C default: add gasteiger charges
charges_to_add = 'gasteiger'
#-p preserve charges on specific atom types
preserve_charge_types=""
#-U: cleanup by merging nphs_lps, nphs, lps
cleanup = "nphs_lps"
#-B named rotatable bond type(s) to allow to rotate
#allowed_bonds = ""
allowed_bonds = "backbone"
#-r root
root = 'auto'
#-o outputfilename

```

```

outputfilename = None
#-F check_for_fragments
check_for_fragments = False
#-l bonds_to_inactivate
bonds_to_inactivate = ""
#-Z inactivate_all_torsions
inactivate_all_torsions = False
#-g attach_nonbonded_fragments
attach_nonbonded_fragments = False
#-s attach_nonbonded_singletons
attach_singletons = False
#-w assign_unique_names
assign_unique_names = False
#-m mode
mode = 'automatic'
#-d dictionary
dict = None

#l:vo:d:A:CKU:B:R:MFI:Zgs'
for o, a in opt_list:
    #print "o=", o, " a=", a
    if o in ('-l', '--l'):
        #ligand_filename = a
        ligand_filename = os.path.basename(a)
        if verbose: print 'set ligand_filename to ', a
        ligand_ext = os.path.splitext(os.path.basename(a))[1] # eg .mol2
    if o in ('-v', '--v'):
        verbose = True
        if verbose: print 'set verbose to ', True
    if o in ('-o', '--o'):
        outputfilename = a
        if verbose: print 'set outputfilename to ', a
    if o in ('-d', '--d'):
        dict = a
        if verbose: print 'set dict to ', a
    if o in ('-A', '--A'):
        repairs = a
        if verbose: print 'set repairs to ', a
    if o in ('-C', '--C'):
        charges_to_add = None
        if verbose: print 'do not add charges'
    if o in ('-p', '--p'):
        # case1: single character
        # examples of 1 character element AND 1 character autodock element:
        B,C,N,O,F,P,S
        # examples of 2 character autodock elements: NA, HD, OA, SA

```

```

#-----
# case2: 2 characters
# examples of 2 character element and two character autodock elements:
# case2a: Zn, Na
#ONLY VALID if ligand_ext in [".pdbqt", ".mol2"]
if ligand_ext in ['.mol2']:
    if preserve_charge_types == "":
        if a.find(',') == -1:
            preserve_charge_types = a
        else:
            preserve_charge_types = []
            for subT in a.split(','):
                preserve_charge_types.append(subT)
    if verbose: print 'preserve initial charges on ', preserve_charge_types
if o in ('-U', '--U'):
    cleanup = a
    if verbose: print 'set cleanup to merge ', a
if o in ('-B', '--B'):
    allowed_bonds = a
    if verbose: print 'allow ', a, 'bonds set to rotate'
if o in ('-R', '--R'):
    root = a
    if verbose: print 'set root to ', root
if o in ('-F', '--F'):
    check_for_fragments = True
    if verbose: print 'set check_for_fragments to True'
if o in ('-M', '--M'):
    mode = a
    if verbose: print 'set mode to ', a
if o in ('-I', '--I'):
    bonds_to_inactivate = a
    if verbose: print 'set bonds_to_inactivate to ', a
if o in ('-Z', '--Z'):
    inactivate_all_torsions = True
    if verbose: print 'set inactivate_all_torsions to ', inactivate_all_torsions
if o in ('-g', '--g'):
    attach_nonbonded_fragments = True
    if verbose: print 'set attach_nonbonded_fragments to ',
attach_nonbonded_fragments
if o in ('-s', '--s'):
    attach_singletons = True
    if verbose: print 'set attach_singletons to ', attach_singletons
if o in ('-w', '--w'):
    assign_unique_names = True
    if verbose: print 'set assign_unique_names to ', assign_unique_names, ' newname
is original name plus its index(1-based'

```

```

if o in ('-h', '--'):
    usage()
    sys.exit()

if not ligand_filename:
    print 'prepare_ligand4: ligand filename must be specified.'
    usage()
    sys.exit()

if attach_singletons:
    attach_nonbonded_fragments = True
    if verbose: print "using attach_singletons so attach_nonbonded_fragments also"

mols = Read(ligand_filename)
if verbose: print 'read ', ligand_filename
mol = mols[0]
if len(mols)>1:
    if verbose:
        print "more than one molecule in file"
        #use the one molecule with the most atoms
        ctr = 1
        for m in mols[1:]:
            ctr += 1
            if len(m.allAtoms)>len(mol.allAtoms):
                mol = m
                if verbose:
                    print "mol set to ", ctr, "th molecule with", len(mol.allAtoms), "atoms"
coord_dict = {}
for a in mol.allAtoms: coord_dict[a] = a.coords
if assign_unique_names: # added to simplify setting up covalent dockings 8/2014
    for at in mol.allAtoms:
        if mol.allAtoms.get(at.name) >1:
            at.name = at.name + str(at._uniqIndex +1)
    if verbose:
        print "renamed %d atoms: each newname is the original name of the atom plus its
(1-based) uniqIndex" %(len(mol.allAtoms))

mol.buildBondsByDistance()
if charges_to_add is not None:
    preserved = {}
    preserved_types = preserve_charge_types.split(',')
    for t in preserved_types:
        if not len(t): continue
        try:
            ats = mol.allAtoms.get(lambda x: x.autodock_element==t)

```

```

        for a in ats:
            if a.chargeSet is not None:
                preserved[a] = [a.chargeSet, a.charge]
    except AttributeError:
        ats = mol.allAtoms.get(lambda x: x.element==t)
        for a in ats:
            if a.chargeSet is not None:
                preserved[a] = [a.chargeSet, a.charge]
    print "preserved = ",
    for key, val in preserved.items():
        print "key=", key
        print "val =", val

if verbose:
    print "setting up LPO with mode=", mode,
    print "and outputfilename= ", outputfilename
    print "and check_for_fragments=", check_for_fragments
    print "and bonds_to_inactivate=", bonds_to_inactivate
    LPO = AD4LigandPreparation(mol, mode, repairs, charges_to_add,
                               cleanup, allowed_bonds, root,
                               outputfilename=outputfilename,
                               dict=dict, check_for_fragments=check_for_fragments,
                               bonds_to_inactivate=bonds_to_inactivate,
                               inactivate_all_torsions=inactivate_all_torsions,
                               attach_nonbonded_fragments=attach_nonbonded_fragments,
                               attach_singletons=attach_singletons)
    #do something about atoms with too many bonds (?)
    #FIX THIS: could be peptide ligand (???)
    #    ??use isPeptide to decide chargeSet??
    if charges_to_add is not None:
        #restore any previous charges
        for atom, chargeList in preserved.items():
            atom._charges[chargeList[0]] = chargeList[1]
            atom.chargeSet = chargeList[0]
            if verbose: print "set charge on ", atom.full_name(), " to ", atom.charge
    if verbose: print "returning ", mol.returnCode
    bad_list = []
    for a in mol.allAtoms:
        if a in coord_dict.keys() and a.coords!=coord_dict[a]:
            bad_list.append(a)
    if len(bad_list):
        print len(bad_list), ' atom coordinates changed!'
        for a in bad_list:
            print a.name, ":", coord_dict[a], ' -> ', a.coords
    else:

```

```

        if verbose: print "No change in atomic coordinates"
    if mol.returnCode!=0:
        sys.stderr.write(mol.returnMsg+"\n")
    sys.exit(mol.returnCode)

```

To execute this command type:
prepare_ligand4.py -l pdb_file -v

Appendix A.8. Automation bash script for running AutodockVina.

```

#!/bin/bash
for f in eDrug3D_*.pdbqt; do
b=`basename $f .pdbqt`
echo Processing ligand $b
mkdir -p $b
/c/Program\ Files\ \(x86\)/The\ Scripps\ Research\ Institute/Vina/vina.exe --config
ecoli_bdl_AF2_confo.txt --ligand $f --out ${b}/out$b.pdbqt --log ${b}/log$b.txt
Done

```

Appendix A.9. Results analysis python script for docking results.

```

import os, re

all_outputs = {}

# All 9
# for filename in os.listdir("outfiles/"):
#     flag = 0
#     all_outputs[filename] = []
#     with open("outfiles/" + filename) as filey:
#         for line in filey:

#             if line.startswith("Writing"):
#                 flag = 0
#             if line.startswith(" 1"):
#                 flag = 1
#             if flag == 1:

```

```

#                                     splice = line.split()
#                                     all_outputs[filename].append(splice[1])

# Top model
for filename in os.listdir("outfiles/"):
    if filename.startswith("logeDrug3D_"):
        all_outputs[filename] = []
        with open("outfiles/" + filename) as filey:
            for line in filey:
                if line.startswith(" 1"):
                    splice = line.split()
                    all_outputs[filename].append(splice[1])

print(all_outputs)

# Use to replace print when writing to a new file
newf = open("outfiles/results_file.csv", "w")
# newf.write()

for k, v in all_outputs.items():
    for affinity in v:
        newf.write(k.replace("logeDrug3D_", "") + "," + affinity + "\n")
newf.close()

```