The CydDC ABC transporter of *Escherichia coli*: new roles for a reductant efflux pump

Supervisor: Dr. Mark Shepherd

A thesis submitted to the University of Kent Faculty of Sciences for the degree of MSc in Microbiology.

Faculty of Sciences, School of Biosciences,

The University of Kent

James Yorke

2016

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.

James Yorke

5th August 2016

Abstract

CydDC is an ATP-binding Cassette (ABC) transporter found within *Escherichia coli* (*E. coli*), previously demonstrated to be important for the maintenance of the redox poise of the periplasm through the active transport of cysteine and glutathione. *cydDC* knockout strains have previously been reported to be unable to assemble *bd*-type respiratory oxidases, lack motility, and are sensitive to benzylpenicillin and nitrosative stress. Previous work has identified a unique haem bound to the heterodimeric CydDC complex, hypothesised to bind to CydD at amino acid residues Arginine 47 and Histidine 51. In the current work, UV spectroscopic analysis of purified CydDC confirmed the presence of bound haem and tryptophan fluorescence titrations revealed a high affinity of CydDC for haem with a K_d of 0.32 μM. A CydDC expression vector was modified to encode a H51A variant of CydDC, but this mutation resulted in loss of assembly of the CydDC complex, indicating an important role for Histidine 51 of CydD in the complex assembly. Finally, disk diffusion assays were consistent with a role of CydDC in exporting glutathione:metal conjugates, reflecting a potentially novel role for CydDC in metal tolerance.

Acknowledgements

Firstly, I'd like to thank Dr. Mark Shepherd for offering the opportunity to undertake this research project for my MSc and all the guidance, knowledge and support he has offered throughout the year.

I would also like to thank all within the Shepherd, Robinson and Blomfield laboratories for their training, advice and council throughout the year, more specifically Cláudia Riberio for her great friendship and tolerance.

Finally, I would like to acknowledge my late grandfather and my grandmother who help support me throughout my MSc emotionally and financially. Without the help of the many friends and family this would not have been possible.

Abbreviations

ATP Binding Cassette	ABC
Adenosine Diphodphate	ADP
Adenosine Triphosphate	ATP
Base pair	bp
Dithiothreitol	DTT
Escherichia coli	*E. coli*
Glutathione	GSH
Histidine 51 Alanine	His51Ala
Histidine 51	His51
Isopropyl β-D-1-thiogalactopyradinoside	IPTG
Kilo Dalton	kDa
Liquid broth	LB
Nanometre	Nm
Nucleotide binding domain	NBD
Penicillin-binding protein	PBP
Polymerase chain reaction	PCR
Ultra violet visible spectroscopy	UV/vis spectroscopy
Wild type	WT

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SUPPLEMENTARY DATA

Chapter I:

INTRODUCTION

The periplasm of *Escherichia coli* (*E. coli*) is the assembly site for essential proteins involved in the processes of pathogenicity, respiration and resistance mechanisms (Holyoake, Poole and Shepherd 2015). Protein assembly is made possible through maintenance of the redox poise, created through efflux of thiol-containing molecules like cysteine and glutathione (GSH), which are exported via the ATP-binding cassette (ABC) transporter, CydDC (Pittman *et al.* 2002; Pittman, Robinson and Poole 2005).

CydDC is closely related to cytochrome *bd*-I terminal oxidase, CydAB. Within the inner membrane two integral membrane terminal oxidases are present, cytochromes -*bo'*, –*bd*-type oxidases (*bd*-I and *bd*-II), and are essential for respiration. The two terminal oxidases of *E. coli* are regulated differentially by oxygen tension (Georgiou *et al*. 1987). Investigations into the terminal oxidases have shown that under aerobic conditions, expression favours the assembly cytochrome *bo*. However, under micro-aerobic conditions expression is favoured towards cytochrome *bd-type* oxidases. Furthermore, it has been shown that CydDC is essential for correct assembly of *bd-type* terminal oxidases, with *cydDC* strains exhibiting phenotypes similar to the *dsbAB* strains. *i.e.* decreased motility, regained susceptibility to benzylpenicillins and incorrect assembly of *bd*-type terminal oxidases (Georgiou, Fang and Gennis 1987). The link between CydDC and *bd*-type cytochromes, suggest CydDC is likely to play a role in to resistance to nitric oxide, through aiding the correct folding of cytochrome *bd*-I and through the active efflux of cysteine and GSH, which are known to form *S*-nitrosothiols (Shepherd 2015).

1.1. Regulation and interactions of the *cydDC* operon

In *E. coli*, the *trxB* gene is located upstream the *cydDC* operon (**Figure 1.1**). This gene encodes for a thioredoxin reductase, an enzyme involved in facilitation of reducing power to the periplasmic disulphide folding machinery. *E. coli cydDC* strains exhibit over-oxidised periplasms, supporting the hypothesis that CydDC maintains the redox homeostasis of the periplasm (Cook, Membrillo-Hernandez and Poole 1997). Primer extension of *cydD* mRNA identified a transcriptional start site 68 bp upstream of the *cydDC* operon. Further to this, northern blot analysis revealed that *cydDC* transcription is polycistronic; both proteins are encoded by a single strand of mRNA (Cook, Membrillo-Hernandez and Poole 1997). This study also characterised expression levels of *cydDC* under a variety of environmental conditions. Under aerobic conditions *cydD* transcription was 2.5-fold higher in the stationary phase. However, under anaerobic conditions *cydD* expression levels were five-fold lower, suggesting that *cydDC* transcription is regulated by oxygen tension (Cook, Membrillo-Hernandez and Poole 1997). *cydDC* transcription was also shown to be independent of

cydAB transcription (Tseng, Albrecht and Gunsalus 1996). Moreover, Cook *etl al* (1997) showed that under microareobic conditions, *cydDC* expression in *E. coli* is elevated in the presence of electron acceptors, such as nitrates and fumarates and failed to assembly both *c*-type cytochromes and *bd*-type terminal oxidases (Cook, Membrillo-Hernandez and Poole 1997). Due to elevated expression of *cydDC* in the presence of nitrates and fumarates, effects of mutations lead to investigations into the two-component regulatory system *narL* and *narP*. *narL* and *narP* are transcription factors that are regulated in response to nitrate and nitrite (Darwin *et al.* 1997). It was shown that mutations within both *narL* and *narP* together and *narL* alone, significantly decreased expression of *cydDC*. Whereas mutations just within *narP* elicited no effect on gene expression of *cydDC* (Cook, Membrillo-Hernandez and Poole 1997). Moreover, analysis of the *cydDC* promoter region revealed a binding site closely resembling the consensus sequence of *narL* (**Figure 1.1.**) (Cook *et al.* 1997). These results suggest the role of CydDC is to assist in the active growth in varying oxygen levels.

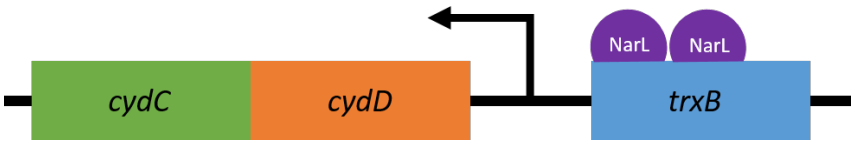


Figure 1.1. The *cydDC* operon and its proximity to *trx*, adapted from (Holyoake, 2015).

1.2. Membrane topology of CydDC

The CydDC complex is a heterodimeric ABC transporter consisting of proteins CydC (63kDA) and CydD (65kDa), that exhibit 27% homology (Shepherd 2015). The role in which CydDC complexes are thought to contribute to the regulation and maintenance of the redox poise of the periplasm remains widely unknown. The *cydAB* operon encodes for the terminal oxidases *–bd,* however *cydDC* mutant strains of *E. coli* show loss of *–bd* cytochromes as well as loss of cytochromes type *b* and *c* (Poole, Gibson and Wu 1994; Goldman, Gabbert and Kranz 1996). Therefore, it was hypothesised that CydDC imported haem into the periplasm for integration into *c*- type cytochromes (Pittman *et al.* 2002).

To investigate the hypothesis, *cydDC* knockout strains of *E. coli* were investigated though whole cell spectra. Cook *et al* (2002) discovered that *cydDC* strains accumulated haem within the periplasm, equally within overexpressed *cydDC* strains addition of ATP revealed no increase in haem accumulation within the periplasm. Therefore, it was hypothesised that

haem transport into the periplasm occurs via one of two methods: (i) Passive binding and crossing of the membrane, or (ii) transportation via an unknown transporter. The mechanisms of haem translocation to the periplasm are still unknown.

1.3. Structural investigations of CydDC

The identification of CydDC's heterodimeric topology was supported through cytochrome *bd-I* knockouts, producing no phenotypic differences within the periplasm on GSH or cysteine transport. This suggests that transcription of cytochrome *bd-I* and *cydDC* are independent of one another (Georgiou *et al*., 1987). Earlier investigations involving phylogenetic studies and hydrophobicity plots hypothesized CydDC to contain six membrane-spanning helices, with C and N terminal domains facing the cytoplasm (**Figure 1.2**). Each subunit of the CydDC complex was hypothesised to contain nucleotide binding domains (NBDs) (Cook, Membrillo-Hernandez and Poole 1997). ABC transporters are known to possess highly conserved NBDs, that are used in the hydrolysis of Adenosine triphosphate (ATP) to Adenosine diphosphate (ADP). These domains are Walker A and B motifs Blue and green, respectively (**Figure 1.2.**). The energy from the hydrolysis of ATP to ADP is utilised by ABC transporters, such as CydDC, to transport substrates.

Purification of wild type CydDC over expressed in BL21 cells revealed, co-purification of a *b*-type haem. Two dimensional crystallisation studies of CydDC, analysed through electron microscopy and circular dichroism (CD), revealed two domains of differing densities, providing strong evidence that CydDC is a heterodimeric ABC transporter, composed of mainly alpha helices (Yamashita *et al.* 2014).

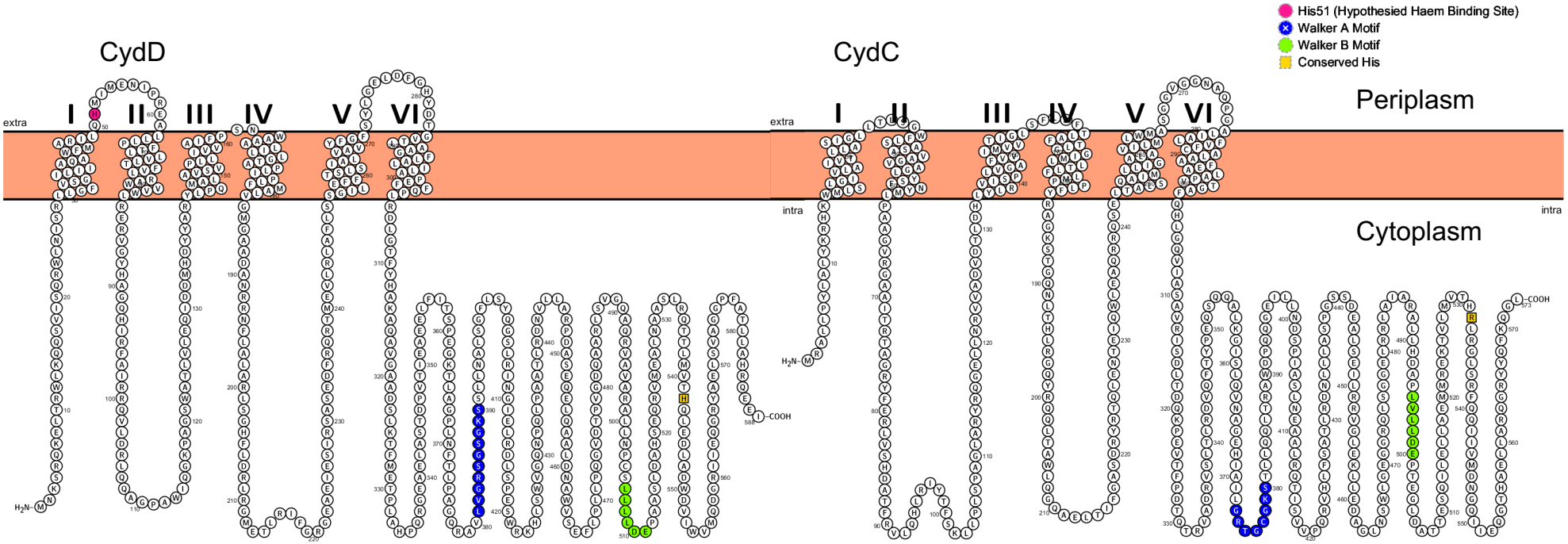


Figure 1.2. – Hypothesised topology of membrane bound wild type CydDC complex, in *E. coli*. Walker A (blue) and Walker B (green), associated with phosphate binding are located within the cytoplasm for ATP hydrolysis, fuelling the transportation of thiols from the cytoplasm to the periplasm for maintenance of the redox poise. Hypothesised haem binding domain, Histidine 51 (Red) is located on the periplasmic loop of CydD and is thought to attract bind the central iron core of haem, whilst the proximal Arginine is thought to stabilised haem binding through stabilisation of the carboxyl group of b-type haem. (Shepherd 2015).

1.4. P-574, a novel haem compound

Overexpression of CydDC complexes in anaerobic environments, revealed a peak at 574 nm in absorbance spectra studies; likely to be haem. Whole cell fragmentations showed decreases levels of P-574 with increased peaks at 560 nm. Overexpression was additionally shown to recover cytochrome *b* and *c* biosynthesis, as well as revealing P-574 was membrane bound (Cook *et al.* 2002), suggesting P-574 is involved in the expression of CydDC and cytochrome biogenesis.

It is hypothesised that the redox environment in the periplasm is important for cytochrome biogenesis, tolerance to oxidative and nitrosative stress, and folding of disulphide bonds, which are present in many proteins involved *in E. coli* pathogenesis, including flagellar P-ring motor proteins and type III secretion systems. Hence, CydDC has an important role for it maintains the oxidative environment of the periplasms that allows the correct folding of these proteins (Shepherd 2015).

CydDC purification revealed a heterodimeric protein on SDS-PAGE gel analysis, around 55 kDa; lower than the predicted molecular weight. Further UV spectral analysis revealed ~~a~~ peaks at 280 nm, the CydDC complex, and 415 nm, suggesting CydDC is purified with a covalent bound haem (Yamashita *et al.* 2014). The haem, thought to be a cofactor, is hypothesised to be bind at amino acid residue histidine (His51) and arginine (Arg47), located on the initial periplasmic loop of CydD (**Figure 1.3.**). Analysis of adjacent amino acids revealed a charged central pore in which His51 resides surrounded by a hydrophobic opening; a common feature of haem binding sites (Shepherd 2015). Hence, His51 is a primary candidate for haem binding on CydD.

It is also thought that haem has an important role in the regulation of the redox poise of the periplasm, and tolerance to oxidative and nitrosative stress, due to oxygen binding capacity of haem (Cook *et al.* 2002). Relating back to CydDC role in oxygen tension it may reveal that haem plays an important role in the activity of CydDC during various aerobic environments.

1.5. Interactions of CydDC with haem

Purified WT CydDC complexes have previously been subjected to ATPase activity assays in the presence of haem, this research concluded that haem interacts with CydDC. Resulting in increased turnover of ATPase activity of the protein, with maximal activity reached at a haem:protein ratio of 5:1. Further to this excess concentrations of haem exhibited inhibitory

effects on ATPase activity(Yamashita, 2015). Addition of GSH to the assays of haem:protein ratios over 5:1 alleviated inhibitory affects, suggesting strong synergistic stimulation of ATPase activity between haem and GSH. However, the effect of thiols is only to increase the turnover rate of ATPase cycle and not the binding of haem to CydDC. Concurrently, it was also shown that the binding of haem enhanced the transport of low molecular weight thiols across the inner membrane into the periplasm. Knowing that GSH molecules interact with cysteine amino acid residues of haem, it is hypothesised that GSH and thiol stimulation of haem enhances CydDC transport activity, increasing transition to a reducing environment; facilitating disulphide bond formation and correct isomerisation.

Further investigations into the binding of haem *b* showed tight interactions between CydDC and haem. Attempts to substitute haem *b* with haemin failed, suggesting tight interaction within the complex. It is therefore hypothesised that CydDC conformation may result from unknown factors other than haem binding (Yamashita *et al.* 2014).

These observations raised important questions: (i) is haem co-transported with thiol compounds by CydDC, or does haem binding facilitate enhanced transportation of thiol compounds into the periplasm? (ii) is haem important in the regulation of activity of CydDC efflux?

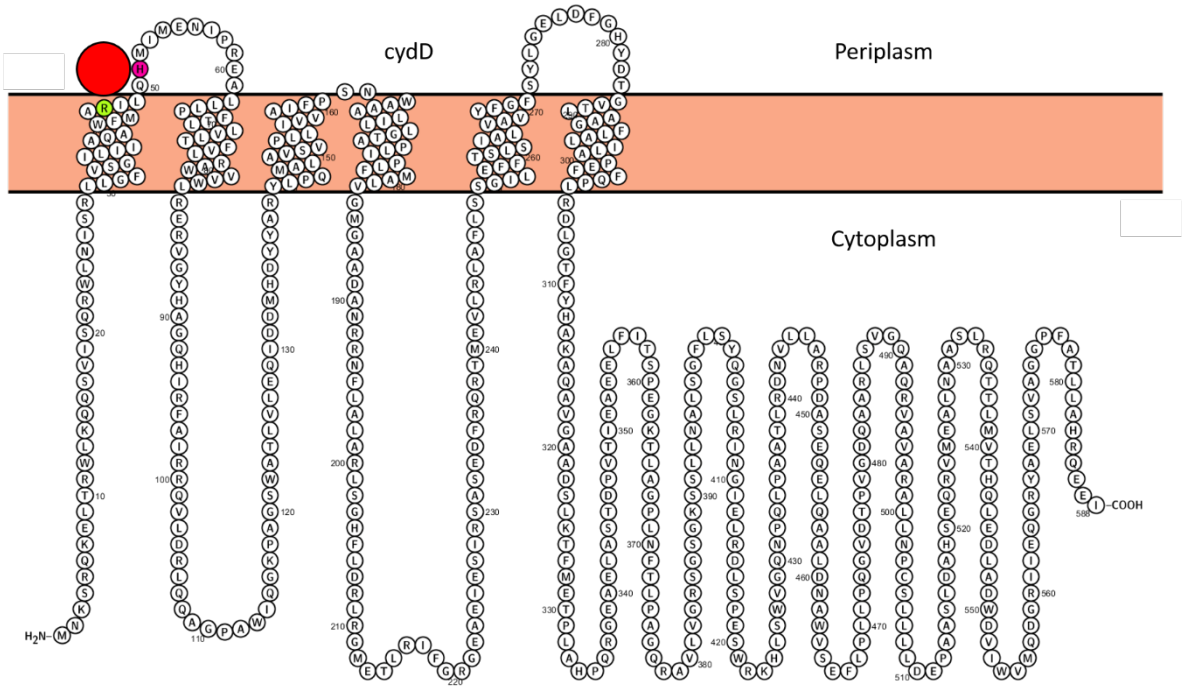


Figure 1.3. The hypothesized haem (Red) interaction located on the initial periplasmic loop of cydD, on His51 (Pink) and Arg47 (Green), (Shepherd 2015).

1.6. Reductant export

1.6.1. Cysteine transport

CydDC-mediated uptake of cysteine into the periplasm was demonstrated using CydD knockout strains within an *orf299* background (Pittman *et al.* 2002). o*rf299* encodes for a transporter of the major facilitator superfamily, a group of proteins known to export and import small molecules, and has subsequently been shown to play a role in cysteine export (Daßler *et al.* 2000). The addition of ATP showed a significant increase in uptake of cysteine into the periplasm, and while the main aim of the study was to identify the substrate exported by CydDC, they discovered the translocation of cysteine into the periplasm via CydDC (Pittman *et al.* 2002), which is now hypothesised to assist in the homeostasis of the redox microenvironment (**Figure 1.4**).

Given the close interplay of CydDC and Dsb proteins (**Figure 1.5**), mutations within both *cydDC* and *dsb* genes share similar phenotypes from: hypersensitivity to benzylpenicillins and DTT, and failure to assemble cytochrome *b* and *c* (Pittman, Robinson and Poole 2005). This suggests that CydDC provides the power to produce a reducing environment to facilitate disulphide bond formation within proteins and cytochrome assembly. Loss of CydDC also displays an inability to assemble cytochrome *bd,* the electron transfer protein to facilitate DsbB-mediated re-oxidation of DsbA within the disulphide bond formation pathway (**Figure 1.5**) (Pittman, Robinson and Poole 2005). The redox microenvironment of the periplasm was also thought to play an important role in the correct folding of disulphide bonds within the periplasm through the DsbA pathway, subsequently reducing sensitivity to antibiotics like benzylpenicillin (Pittman *et al.* 2002; Pittman, Robinson and Poole 2005).

Reduction in sensitivity to benzylpenicillins can be explained through the structure of penicillin-binding protein (PBP) 4 (Pittman *et al.* 2002; Pittman, Robinson and Poole 2005). PBP4 has the potential to form two disulphide bonds within its active site when folded incorrectly, increasing affinity to penicillin (Missiakas and Raina 1997). Binding of penicillins to PBPs inactivates transpeptidation of peptidoglycan amino acid side chains, and activation of autolysins. Subsequently activation of autolysins causes degradation of peptidoglycan layers and lysis of bacteria due to differential osmotic pressure. Incorrect disulphide bond formation, controlled and regulated through the Dsb pathway, and influenced greatly by the efflux of thiols via CydDC, explains the increase in susceptibility of benzylpenicillin seen within *cydDC* and *dsb* mutants.

Therefore, to test the effects of *cydDC* mutants on antibiotic resistance, the WT strain (AN2342) was compared to the *cydDC* strain (AN2343). Both strains were plated in agar with 20μg ml^{-1} and in the presence or absence of 2mM cysteine. In the presence of cysteine the *cydDC* mutants showed a recovery against benzylpenicillin, suggesting that the role of CydDC complexes in *E. coli* play an important role in the resistance and recovery against antibiotics.

1.6.2. Glutathione transport

Reduced glutathione (GSH) is thought to play an important role within the disulphide folding pathways of DsbA, DsbB and DsbD, and cytochrome *c* biogenesis (Pittman *et al*. 2005, Fabianek *et al*., 2000). The first obervation that GSH plays a role in the tolerance to oxidative stress and xenobiotics came from the study of GSH deficient strains of *E. coli* that were hypersensitive to hypochlorous acids (Chesney, Eaton and Mahoney 1996). Using *cydDC* mutant strains (AN2343) and WT strains (AN2342), GSH uptake was measured both with and without the addition of ATP. After addition of the ATP, the uptake of GSH was rapid, reaching maximal uptake within 5 min of addition (Pittman, Robinson and Poole 2005). With cysteine also being exported via CydDC, as well as GSH (**Figure 1.4**), it was hypothesized that external GSH may also play a role in recovering the resistance to penicillin's in *cydDC* mutant strains of *E. coli.* In the presence of GSH, the *cydDC* mutants showed a recovery against penicillin-G, supporting the hypothesis that CydDC plays an important role in antibiotic resistance in *E. coli.*

1.6.3. Interaction of Export Products

As previously mentioned, disulphide bond proteins (Dsb) are extremely important in the role of cytochrome biogenesis. Coupled with the Dsbs are cytochrome maturation proteins (Ccm), such as cytochrome *c* maturation proteins (Ccm A - G). The cytochrome maturation proteins' key functions are involved in the formation of correctly folded proteins containing disulphide bonds through reduction of disulphide bonds, through unknown mechanisms (Stevens, Gordon and Ferguson 2004). Evidence provided by Stevens (2004) indicates that over production of Ccm proteins restores the maturation of cytochrome *c* in DsbD knock out strains.

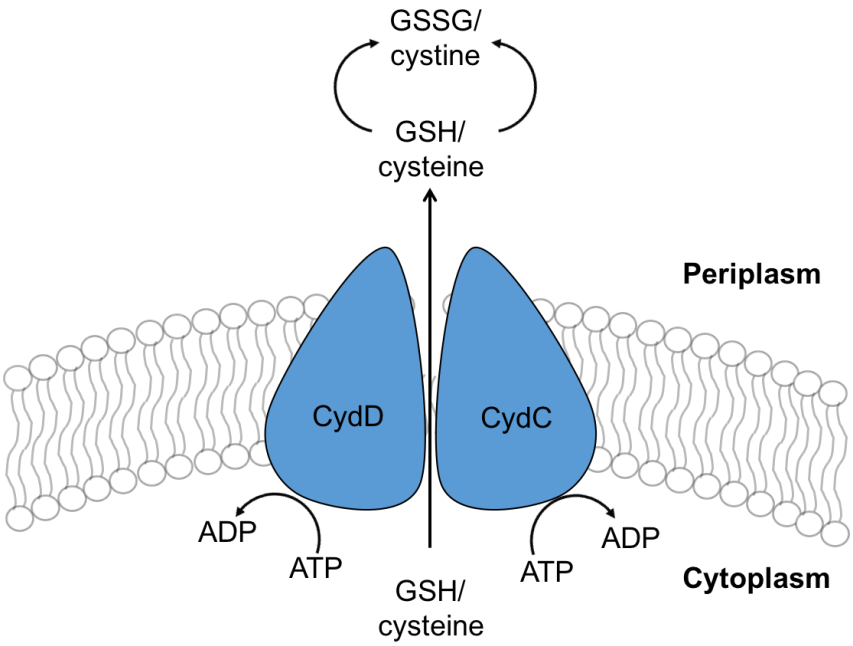


Figure 1.4. CydDC exports glutathione and cysteine from the cytoplasm to the periplasm, to maintain the redox environment of the periplasmic space, important to facilitate disulphide bond formation in essential proteins.

1.7. Physiological impact of CydDC

1.7.1. Protein disulphide folding

Protein disulphide folding is catalysed by Dsb proteins (Inaba 2009), and it is important for the correct folding and function of proteins involved in virulence, motility, and survival of *E. coli* such as, type III secretory systems, heat stable proteins, pili, flagella P-ring motors and adhesion factors (Yu and Kroll 1999). All of the Dsb proteins are located within the inner membrane or periplasm of *E. coli* and all contain Cysteine – X – X – Cysteine amino acid motifs (Yu and Kroll 1999). Dsb proteins have also been shown to play crucial roles in the maintenance of the redox poise, with mutations in genes encoding Dsb proteins, resulting in increased sensitivity to reducing agents (Missiakas and Raina 1997).The Dsb machinery can be split into two pathways: the oxidative pathway and the isomerisation pathway (**Figure 1.5**).

DsbA catalyses oxidative protein folding, with oxidised DsbA being regenerated via donating electrons to DsbB, which subsequently reduces the respiratory electron carrier coenzyme Q. From here, reduced Coenzyme Q can donate electrons to *bd*-type respiratory oxidases, which intriguingly, require CydDC for their assembly (Inaba 2009).

The isomerisation pathway involved in disulphide folding occurs through reduction of disulphide bonds via DsbC. This pathway is essential for the restoration of correct disulphide bonds within misfolded proteins, which can occur in proteins containing more than one disulphide bond during the disulphide formation pathway involving DsbA. The isomerisation pathway consists of two major Dsb proteins: DsbC and DsbD (Inaba 2009). DsbC contains a Cys – X – Cys active site and only exerts isomerase activity when in a reduced state, which is maintained by DsbD. DsbD, an inner membrane protein, ~~that~~ maintains DsbC activity through an electron transport pathway involving transport of electrons from Trx A to DsbD and finally to DsbC (Missiakas and Raina 1997). DsbD also supports the activity of DsbE/CcmG, which reduces apocytochromes and plays a vital role in cytochrome maturation (Fabianek, Hennecke and Thony-Meyer 1998). Maintenance of the reduced state of DsbC occurs from another Dsb protein, DsbG. DsbG was hypothesised to be an essential protein in *E. coli*. However, recent studies indicate that DsbG is not essential for growth, but primarily acts upon the maintenance of DsbC in the correct redox state (Bessette *et al.* 1999). This pathway offers a restoration pathway for proteins that have been folded into non-native conditions allowing for the re-formation of viable proteins.

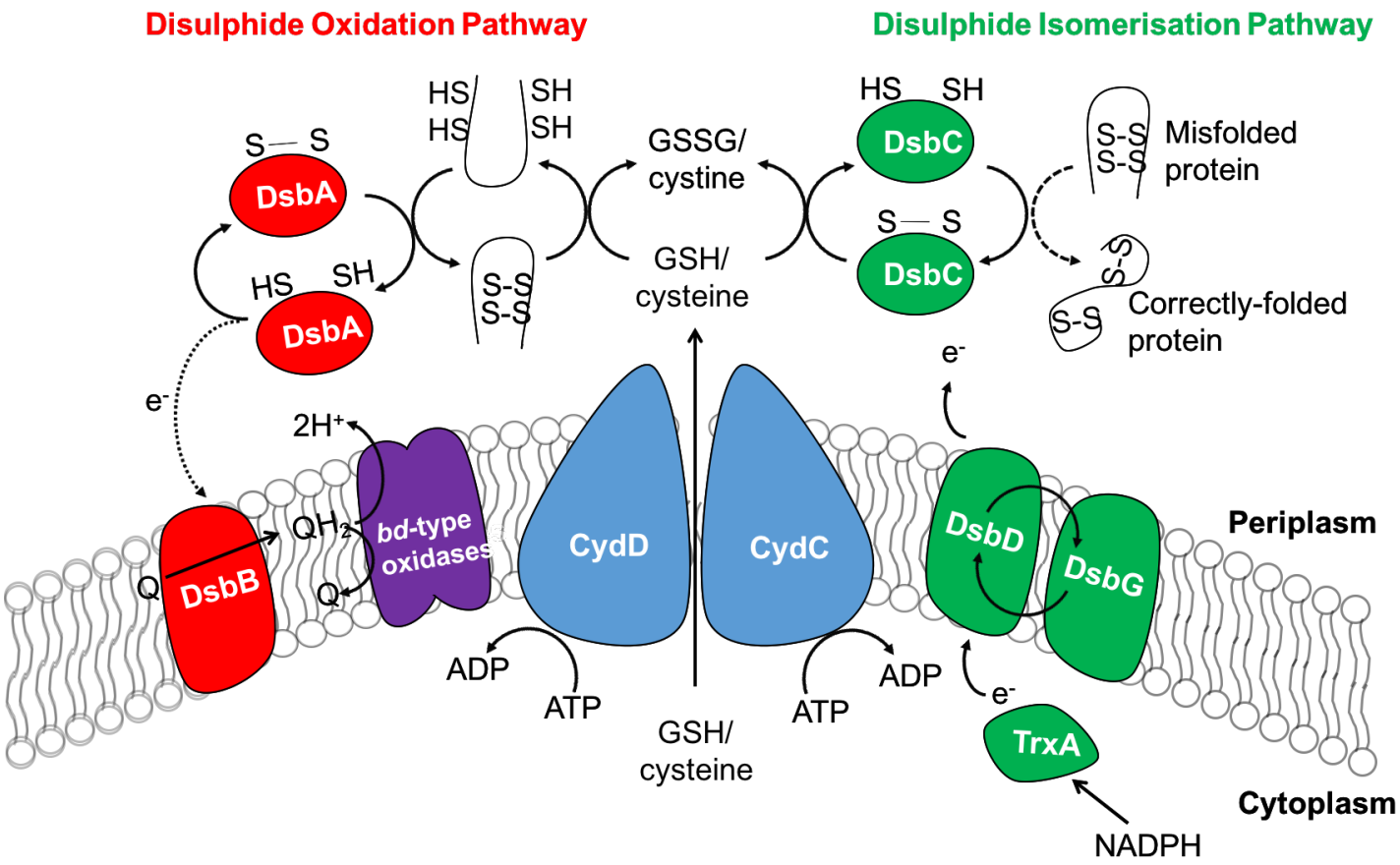


Figure 1.5. The active efflux of cystine and glutathione enables the correct formation of disflphide bonds through the Dsb pathway, faciliting both the formation of disluphide bonds (DsbAB pathway) and the correct isomerisation of dislphuide bonds within proteins (DsbCD pathway).

1.8. Metal tolerance in *E. coli*

Metals can be essential for bacteria in low concenctrations. However, in high concentration metal cations offer a wide range of destructive affects, *i.e.* the removal of strucutal metals, destruction of Fe-S clusters and the oxidation of redox active sites, allowing for the production of reactive oxygen speieces (ROS), thiol depletion and interference with nurtient uptake (Lemire, Harrison and Turner 2013). Originally resistance was though to be aquired through plasmids, simillarly to antibiotic resistance. Nonetheless, analysis of bacterial genomes revealled genomic proteins belonging to three major classes of efflux pumps, Resistacne-Nondulation-cell Division (RND) protein family, the Cation Diffusion Facillitator (CDF) family and P-type ATPase family (Lemire, Harrison and Turner 2013). Production of these classes of proteins offer methods to reduced intake of metal cations, and also active and passive efflux of cations based on chemiosmotic potential. Recent work has identified many alternative resistance mechanisms, including alteration of metabolic pathways by bacteria, production of chapereons, production of antioxidants, and expression of enzymes responsible for restore disulphide bond restoration (Harrison *et al.* 2004; Harrison *et al.* 2009; Lemire, Harrison and Turner 2013). To sequesteration of metals within the periplasm and exportation into extracellular environments within siderophores through unknown biochemical pathways (Macomber, Rensing and Imlay 2007).

1.9. Investigations in the novel functions and haem binding site of CydDC

This study aims to identity the relationship between CydDC and haem, as well as to characterise the novel haem co-factor identified in previous studies, as described in. **Section 1.2**. It had been previously hypothesised that the haem binding domain resides on the periplasmic loop of CydD at residue His51.Therefore, it is hypothesised that site directed mutagenesis of His51 will remove the haem binding domain and purification of mutated CydDC will no co-purify with haem.

Additionally, this study aims to assess the functionality of CydDC in the transportation of GSH-metal conjugates as a novel biochemical method for exerting metal tolerance within *E. coli*. **Section 1.8**. describes the importance of metal resistance in bacteria and the sequestration of metal ions within the periplasm through unknown biochemical pathways. It is known that GSH can form metal-GSH conjugates and evidence indicating strains of yeast and Leishmania can form GSH metal conjugates (Li *et al.* 1996; Dey *et al.* 1996). It is therefore hypothesised that *E. coli* can form GSH-metal conjugates which are transported

via CydDC into the periplasm for either diffusion across the membrane or sequestration within the periplasm as a form of metal tolerance.

Chapter II:

MATERIALS AND METHODS

2.1. Bacteriological Methods

All materials used within this thesis were obtained from Fisher Scientific or Sigma Aldrich unless specified. Yeast extract, Tryptone and nutrient agar were purchased from Melford. Sterilisation of growth media was performed through autoclaving at 121 ^{o}C, 15 psi, for 20 minutes.

2.1.1. Bacterial Strains

Table 2.1. *E. coli* bacterial strains used in this work.

Strain ID	*Description*	*Source*
MS2	K-12 MG1655	Bachmann 1996
MS8	BL21 (DE3) pLysS	Bachmann 1996
MS14	MG1655 *cydDC*::Cm	Shepherd Laboratory
MS48	BL21 (DE3) pLysS and pX100	Yamashita, 2015
MS85	MG1655 *cydDC*::Cm pRKP1602	Holyoake, 2015
MS462	BL21 (DE3) pLysS and His51Ala pHX100	This work

2.1.2. Plasmids

Table 2.2. List of plasmids used in this work

PLASMID ID	**FEATURES**	**REFERENCE**
pHX100	LacI, Amp^R, CydDC-His_6 Tag	Yamashita, 2015
pLyss	p15A_origin, Cam^R, Tet^R	Bachmann 1996
pHX100-H51A	LacI, Amp^R, CydDC-H51A-His_6 Tag	This work
pRKP1602	CydDC, Amp^R	Pittman 2005

2.1.3. Oligonucleotide sequences

Oligonucleotides **Table 2.3.** were designed in Vector NTI™ in accordance to specifications for desired kits, or protocols outlined by manufactures or outsources companies.

Table 2.3. Oligonucleotides used in this work were produced from MWG – Eurofins.

DATABASE #	PRIMER ID	SEQUENCE (5' – 3')	SOURCE
174	CydDC_Cloning_F	CCCAGGCCTGGTTCATGGCGCGTATTCTGCAAGCTATGATTATGGAGAATATTCCCCGTG	This work
175	CydDC_Cloning_R	CCCAAGCTTCTATTAATGGTGATGGTGATGGTG	This work
167	CydDC_Seq_R	TCATGTTTGACAGCTTATCATCGAC	This work
166	CydDC_Seq_F	TCATGTTTGACAGCTTATCATCGAC	This work
45	CydDC Seq2	TACTGCGCGCATGGGTGGTC	Holyoake, 2015
44	CydDC Seq1	ATGAATAAATCTCGTCAAAAAGA	Holyoake, 2015

2.1.4. Growth media

2.1.4.1. Luria Bertani (LB) media

LB media contained 10 g Tryptone, 10 g NaCl, 5 g Yeast extract per litre of distilled-deionised water.

2.1.4.2. M9 minimal media

One litre of 20x M9 salts contained; 140 g Na_2HPO_4, 60 g KH_2PO_4, 10 g NaCl and 20 g NH_4Cl. M9 media contained 50 mL of M9 salts, 2 mL 0.1M $CaCl_2$, 4 mL 50% glycerol, 2 mL 1 M $MgSO_4$ and 20 mL 10% (w/v) casamino acids.

2.1.4.3. SOC medium

SOC media was prepared by adding 20 g Tryptone, 5 g Yeast extract, 0.584 g NaCl and 0.186 g KCl within 970 mL of distilled-deionised water and autoclaved. Once cooled 10 mL of Mg^{2+} solution (20.33 g $MgCl_2$, 25.65 g $MgSO_4$, dissolved in 100 mL of distilled-deionised water and filter sterilised), and 20 mL 1 M glucose (filter sterilised).

2.1.5. Media supplements

2.1.5.1. Antibiotics

Growth media were supplemented, where indicated, within working concentrations of antibiotic, Table 2.4.

Table 2.4. Stock and working concentrations of antibiotics used throughout this project, listing the liquid used to solubilise the antibiotics in the far column.

Antibiotic	Stock concentration	Working concentration	Solubilised with
Ampicillin	125 mg/mL	100 µg/mL	Water
Chloramphenicol	35 mg/mL	25 µg/mL	Ethanol

2.1.5.2. Nutrient agar

One litre of LB medium, 15 g of dried nutrient agar.

2.1.5.3. Casamino acids

A 5% (w/v) stock solution of casamino acids was prepared by dissolving 5 g of dried casamino acids in 100 mL of distilled deionised water, then autoclaved as predefined sterilisation settings and left to cool.

2.1.5.4. Isopropyl β-D-1-thiogalactopyranoside (IPTG)

To 10 mL of sterile MilliQ water, 2.83 g of IPTG was added and whirlmixed till dissolved to create a 1 M solution. The 1 M IPTG solution was then filter sterilised using 0.2 µ filters and aliquoted into 1 mL samples, and stored at -20 ^{o}C until needed.

2.1.6. Bacterial growth conditions

Bacteria were grown in LB-media or on LB-agar, supplemented with appropriate working concentrations of antibiotics, where necessary. Growth was carried out at 37 ^{o}C, stationary or at 180 rpm, for solid cultures, and liquid cultures respectively.

2.1.7. Metal efflux disc diffusion assay

Four morphologically similar colonies where used to inoculate 5 mL of LB-media containing appropriate antibiotics, and incubated at 37 ^{o}C and 180 rpm until a the visual turbidity was equal or greater than that of a 0.5 McFarland Standard (Andrews 2001). When necessary, density was adjusted to that of the standard using sterile milliQ water.

Cell suspension was diluted 1:100 in sterile Milliq® water, and the inoculum was spread evenly over the entire surface of a LB-agar plate with the aid of a sterile cotton swab. Filter paper discs, 5 mm in diameter created from filter paper (Whatman™ 1001-185), were inoculated with 5 µL of metal solution then applied directly to the agar. Plates were incubated at 37 ^{o}C overnight. Zones of inhibition were measure, with the diameter of the disc subtracted from the total diameter of the zone of inhibition.

2.1.7.1. Zinc ion solution

A 1 Molar Zinc Sulphate solution contained 1.61 g dissolved within 10 mL of distilled deionised water. 100 mM, 50 mM and 10 mM solutions were created through diluting stock 1 M zinc solution with required distilled deionised water, all solutions were filter sterilised using a 0.22 µm Millipore filter.

2.1.7.2. Cadmium ion solution

A 1 Molar cadmium sulphate solution contained 2.08 g dissolved within 10 mL of distilled deionised water. 100 mM, 50 mM and 10 mM solutions were created through diluting

stock 1 M zinc solution with required distilled deionised water, all solutions were filter sterilised using a 0.22 μm Millipore filter.

2.1.8. Bacterial growth for CydDC expression

An overnight in M9 media of strain MS48 or MS462, was used to inoculate 50 mL of fresh M9 media supplemented with 0.1% casamino acids and the appropriate antibiotics (Table X) in a 1:100 ratio. The new culture was incubated at 37 ^{o}C, 180 rpm until Optical Density (OD) at 600 nm was approximately 0.600. When desired OD was reached, 1 mL samples were aspirated and pelleted, and 0.6 mM IPTG was added. The bacterial culture was further incubated at 30 oC, 180 rpm for 4 hours. After 4 hours, 1 mL samples were aspirated and pelleted.

2.1.9. Chemically competent cells

An overnight of desired strain was used to inoculate 100 mL of fresh LB-medium, supplemented with appropriate antibiotics, at a 1:100 ratio and grown at 37 ^{o}C, 180 rpm until OD 660nm reached 0.4 ABS. When desired OD was achieved, cells were chilled on ice for 10 minutes. Cells were then harvested at 3,000 rpm, 4 ^{o}C for 8 minutes before being re-suspended in 25 mL 100 mM $CaCl_2$, then returned to chill for 10 minutes on ice. Cells were then re-harvested at 3,000 rpm, 4 ^{o}C for 8 minutes and re-suspended in 2 mL $CaCl_2$ and 30% (v/v) glycerol. Afterwards cells were aliquoted into 100 μL samples for single time use and stored at -80 ^{o}C.

2.1.10. Plasmid DNA transformation

Chemically competent cells, prepared as per **Section 2.1.9.** were thawed on ice before 10 μL of plasmid DNA was aspirated onto the cells. Cells were then left to incubate on ice for 30 minutes. Upon end of incubation, cells were heat-shocked at 42 ^{o}C for 90 seconds precisely before returning to ice for incubation for 2 minutes. 900 μL of SOC media (**Section 2.1.4.3**) was aspirated onto the cells, and they were further incubated at 37 ^{o}C, 180 rpm for 1 hour. Upon end of incubation cells were pelleted and re-suspended in 100 μL SOC media, the whole reaction was then plated on Nutrient agar (**Section 2.1.5.2.**) supplemented with appropriate antibiotics.

2.2. Genetic Methods

2.2.1. Plasmid DNA isolation

A 10 mL overnight of strains containing desire plasmid was subjected to plasmid extraction using the QiaPREP spin miniprep kit (Qiagen) according to manufacturer's instructions.

2.2.2. Gel electrophoresis

Gel electrophoresis was performed using a 1% (w/v) agarose gel, submerged in a 1x TAE buffer (40 mM Tris acetate, 1 mM EDTA). DNA was mixed in a 5x loading buffer at a DNA:Buffer ratio of 1:5, before loading. The gel was run 150 v, 300 mA, for 25 mins, using Hyperladder I (Bioline) as a marker. Gel was stained with 5 µL ethidium bromide (10 mg/mL stock) (Sigma Aldrich) in 100 mL distilled deionised water for 30 minutes before visualisation on a Sysgene G box.

2.2.3. Site directed mutagenesis of fragment

Extracted pHX100, as per section **2.2.1** was subjected to a mutagenic PCR reaction using primers 174 and 175, **Table 2.3**. PCR reaction was set up as a 25 µL total reaction consisting of: 12.5 µL 2x Q5 master mix, 1 µL pHX100 (10 ng/ L), 1.2 µL 174 primer (10x stock), 1.2 µL 175 primer (10x stock), 9 µL milliQ water, then run on the following programme settings **Table 2.5.**

Table 2.5. PCR programme for amplification of H51A *cydDC* fragment, with use of Q5 polymerase (New England Biolabs).

Segment	**Cycles**	**Temperature**	**Time**
1	1	95 ^{o}C	1 minute
2	35	95 ^{o}C	15 seconds
		72 ^{o}C	15 seconds
		72 ^{o}C	8 min
3	1	72 ^{o}C	2 min

The PCR fragment was then quantified via gel electrophoresis, **Section 2.2.2.** The remaining PCR product was then cleaned using a PCR purification kit (Qiagen) according to the manufacturer's instructions.

2.2.4. Molecular cloning

The extracted pHX100, as per section **2.2.1**, and mutated PCR fragment, **Section 2.2.3** were digested using HindIII (Promega), as per manufacturer's instructions. Digested DNA samples were cleaned using a PCR purification kit (Qiagen) according to the manufacturer's instructions and then digested a second time with StuI (New England Biolabs). After performing the second digests, the whole reaction of pHX100 was processed by gel electrophoresis, **Section 2.2.2,** the 4500 bp vector was gel extracted using the Gel Extraction Kit (Qiagen), and the mutated PCR fragment was PCR cleaned using the PCR purification kit (Qiagen).

Ligation reactions were set up using T4 DNA ligase (Promega) with a ratio of 3:1 of fragment:vector and incubated overnight at room temperature. Ligated plasmids were then transformed, **Section 2.1.10** into BL21 (DE3) competent cells, **Section 2.1.9.**

2.2.5. Colony PCR

Briefly, a colony was re-suspended in 50 µL of miliQ water and boiled for 10 minutes at 95 ^{o}C. 2 µL of boiled supernatant was added to 12.5 µL *taq* master mix red (PCR BIO), 1.25 µL primer 166 (10x working stock), 1.25 µL primer 167 (10x working stock), 9 µL milliQ water, then run on the following programme settings **Table 2.6.**

Table 2.6. PCR programme for colony PCR of H51A vartiation *cydDC* fragment in pHX100.

Segment	**Cycles**	**Temperature**	**Time**
1	1	95 ^{o}C	4 minute
2	35	95 ^{o}C	15 seconds
		55 ^{o}C	15 seconds
		72 ^{o}C	15 sec/kb
3	1	72 ^{o}C	2 min

PCR products were then analysed by gel electrophoresis, **Section 2.2.2.**

2.2.6. Digestion assay and sequencing

Positive colonies from colony PCR (**Section 2.2.5.**) were grown overnight in 10 mL LB-media at 37 ^{o}C and underwent plasmid extraction, **Section 2.2.1**. Extracted plasmid was then digested with HindIII (Promega, R6401) to confirm plasmid size through visualisation, before digestion with NdeI (Promega, R6801). Colonies with a single cut were then prepared for sequencing with Genewiz™ as per company guidelines.

2.3. Biochemical Methods

2.3.1. Purification of the CydDC complex

An overnight of strain MS48 or MS462 in LB medium was used to inoculate fresh LB media, supplemented with the appropriate antibiotics, in a 1:50 ratio and incubated at 37 ^{o}C, shaking 200 rpm. This overnight culture was then used to inoculate 1 L of M9 media, supplemented with 0.2% casamino acids and appropriate antibiotics, in a 1:100 ratio. Incubation was carried out at 37 ^{o}C, 220 rpm until OD600nm reached 0.6, at which 0.6 mM IPTG was added to induce expression of CydDC. After addition of IPTG, incubation was continued at 30 ^{o}C, 220 rpm for a further 4 hours.

Cells were then harvested by centrifuging for 20 min at 4,000 rpm, 4 ^{o}C. The pellet was re-suspended in 120 mL of Tris-HCl (pH 7.4), 2 mM MgCl2 and 1 mM EGTA. Re-suspended cells were sonicated at 15μ for 6x30 seconds on ice, centrifuged for 15 min at 8,000 rpm, 15 ^{o}C. Supernatant was then subjected to ultra-centrifugation for 1 h at 41,000 rpm, 4 ^{o}C to pellet membranes. Membrane pellets were re-suspended in 4mL Tris-HCl (pH7.4), 2 mM MgCl2 and 1 mM EGTA. 22.3 mL membrane extraction buffer (Tris-HCl pH 8.0, 20% glycerol, 300 mM NaCl, 10 mM Imidazole, and 1% DDM) was added to the re-suspended membrane pellets and incubated on ice, gently shaking for 1 h. Insoluble membranes were pelleted by ultra-centrifuging for 1 h at 37,000 rpm, 4 ^{o}C. Supernatant was then mixed with 1.4 mL equilibration buffer (Tris-HCl pH 8.0, 20% glycerol, 5 mM Imidazole, 500 mM KCl and 0.1% DDM).

5mL Ni-NTA was incubated with supernatant at 4^{o}C for 2 h with constant mixing. Resin was then cast in a polypropylene column to give a bed of 2.5 mL. Supernatant and equilibration buffer solution were then passed through the column, and the flow through collected as 1 mL samples. The column was washed with 10 column volumes of purification buffer (Tris-HCl pH 8.0, 20% glycerol, 500 mM KCl, 5 mM Imidazole and 0.1% DDM) with flow though collected. The column was then washed with 10 column volumes of purification buffer (Tris-HCl pH 8.0, 20% glycerol, 500 mM KCl, 10 mM Imidazole and 0.1% DDM) with flow though collected in 1 mL samples. Column was then washed with 10 column volumes of purification buffer (Tris-HCl pH 8.0, 20% glycerol, 500 mM KCl, 20 mM Imidazole and 0.1% DDM) with flow though collected. Protein was then eluted from

the column using elution buffer (Tris-HCl pH 8.0, 20% glycerol, 500 mM KCl, 300 mM Imidazole, and 0.02% DDM).

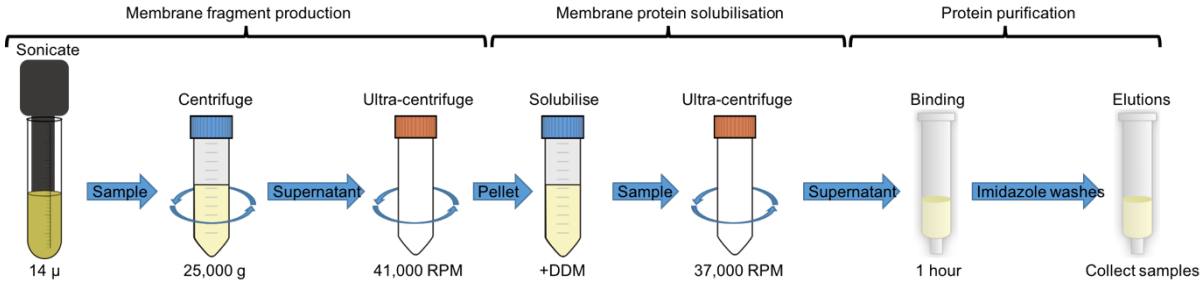


Figure 2.1. Overview of CydDC purification process adapted from Yamashita *et al* (2015).

2.3.2. SDS-PAGE gel analysis

Purified proteins and plasmid induction tests were analysed by SDS-PAGE gels. A 4% stacking gel was cast on top of a set 10% resolving gel, recipe in **Table 2.6.** Cast gels were used on day of creation. Gel apparatus was submerged within SDS running buffer (25 mM Tris, 250 mM Glycine, 0.1% SDS, at pH 8.3).

Table 2.II.7. Recipe for 10% SDS-PAGE gel used in this work.

Chemical	**10% Resolving Gel**	**4% Stacking Gel**
Water	3.8 mL	3.1 mL
40% Bis-Acrylamide	2 mL	0.5 mL
10% SDS	0.08 mL	0.05 mL
10% APS	0.08 mL	0.05 mL
TEMED	0.008 mL	0.005 mL
Resolving Buffer (1.5 M Tris-HCl, pH 8.8)	2 mL	0 mL
Stacking Buffer (0.5 M Tris-HCl, pH 6.8)	0 mL	1.25 mL

Samples were mixed in a 1:1 ratio with SDS loading buffer (50 mM Tris, 2% SDS, 0.1% Bromophenol blue, 10% glycerol and 100 mM DTT) and boiled at 95 ^{o}C for 5 minutes. Once boiled, samples were loaded onto gel, with a BIO RAD Precision Protein marker, and run at 165 V for 40 minutes. Gels were then removed from apparatus and stained with InstantBlue™ for 1 hour.

2.3.3. PVDF transfer and western blot

Section 2.3.2. was followed until gel was removed from apparatus, at which point the separated proteins were transferred to a (methanol –activated) PVDF membrane (BIO RAD) using a wet transfer apparatus, at 100V for 1 hour. Membranes were blocked for 1 hour in Blotto (137 mM Sodium chloride, 10 mM Tris, 5% Skimmed milk powder, 0.1% Sodium azide, at pH 7.4), followed by 1 hour incubation with 2 µL of His6 Tag Antibody (ABCam) in 10 mL of fresh Blotto solution. Membrane was washed in 1x TBST (137 mM Sodium chloride, 10 mM Tris, 0.1% Tween 20, at pH 7.4) for 5 minutes. This step was repeated a total of 5 times. Subsequently, the membrane was developed for 10 minutes using 1x BCIP/NBT (Sigma Aldrich), used as per manufacturer's instructions. To stop the development, the membrane was washed in distilled, deionized water for 5 minutes and then left to dry.

2.3.4. Protein buffer exchange and quantification

Eluted fractions containing CydDC were concentrated using a viva-spin concentrator (10 kDa cut off), at 6687 rpm for 15 mins. A PD10 desalting column (GE healthcare) was equilibrated with 30 mL of protein storage buffer (20 mM Tris-HCl (pH 8.0), 0.05% DDM). Concentrated CydDC was passed through the column, followed by 5 mL of protein storage buffer. Eluted fractions were collected and analysed via UV-vis spectroscopy (Cary 60 UV-Vis, Agilent Technologies) in a quartz cuvette (240-700 nm at 600 nm/min).

Buffer-exchanged protein was then quantified using a Markwell assay as previously described (Markwell *et al.* 1978). Briefly, reagent A (20 g/L Na_2CO_3, 4 g/L NaOH, 1.6 g/L Sodium Tartrate and 10 g/L SDS) was mixed with reagent B (4% $CuSO_4.5H_2O$) in a 99:1 ratio respectively, to form reagent C. 3 mL reagent C was whirl-mixed with 1 mL of protein (diluted 1:10), sterile Milli-Q water and a range of known concentrations of BSA to form a standard curve, and left to stand for 1 hour at room temperature. 0.3 mL of Folin (50% v/v/)

reagent was whirl-mixed into protein/reagent C solution and left to incubate for 45 minutes. Absorbance readings were measured at 660 nm. Concentrations of proteins were calculated from the standard curve and adjusted using initial dilution factor before calculating the molar concentration using the molecular weight of the protein.

2.3.5. Tryptophan fluorescence titrations to analyse haem binding to CydDC

Haem stocks were created via solubilising 1 mg of haemin (Sigma) with 1 mL dimethyl sulfoxide (DMSO) (Fluka) and subsequent dilution with water. For quantification, pyridine haemochrome spectra were collected essentially as described previously (Berry and Trumpower 1987). Haem solutions were then diluted 1:1 with 4.2 M pyridine containing 0.4 M NaOH. This solution was then divided into two cuvettes with one containing a few grains of sodium dithionite and the other containing a few grains of potassium ferricyanide. Reduced-oxidised absorbance spectra was measured between 600 nm and 500 nm. Using the Beer-Lambert equation ($a = \varepsilon cl$) concentrations of haem were calculated using the differences between the peak (at 557 nm) and trough (at 541 nm) using the extinction coefficient $\varepsilon_{557\text{-}541} = 20.7\ mM^{-1}cm^{-1}$ (Falk and Smith 1975).

100 nM CydDC was prepared following quantification from with Markwell assay (**Section 2.2.4**). Fluorescence titrations were performed using 2 mL of 100 nM CydDC in a quartz cuvette. Quantified haemin solution in protein storage buffer was prepared at concentrations of 0.1 μM to 0.5 μM, and mixed thoroughly. Protein fluorescence was scanned between 310 nm to 400 nm at a speed of 250 nm/min, with initial excitation set to 280 nm, with emission and excitation slits set to 5 nm. Haemin was added to the cuvette in small volumes and the solution was left for 1 min before fluorescence emission scans were recorded. Titrations were carried out until the change in fluorescence had diminished significantly. Control titrations were performed under the same conditions with n-acetyl-tryptophanamide (NATA) replacing CydDC. The concentration of NATA was adjusted accordingly to match the initial maximum fluorescence of 100 nM CydDC with 0 μM haemin.

Chapter III:

RESULTS

3.1. Site-directed mutagenesis of the putative haem-binding residue His51 in CydD

3.1.1 Mutagenesis of pHX100 and preliminary plasmid analysis

As introduced in Section 1.5, His51 of CydD is a potential haem-binding residue. In order to generate a plasmid vector to express this variant in *E. coli*, the pHX100 plasmid (**Figure 3.1A**) encoding wild type CydDC (with a C-terminal His6-tag on CydC) was subjected to mutagenesis using the PCR and conventional cloning method detailed in **Section 2.2.3**. In brief, mutagenic primers were used to amplify a modified *cydDC* fragment using pHX100 as a template (**Figure 3.1B**). This purified PCR product was then cut using HindIII and StuI. The pHX100 plasmid was then digested with HindIII and StuI and the 4.5 kbp fragment was gel extracted (**Figure 3.1C**), and the cut vector and insert were quantified on an agarose gel using marker bands of known size and concentration (**Figure 3.1D**) (**Section 2.2.4**).

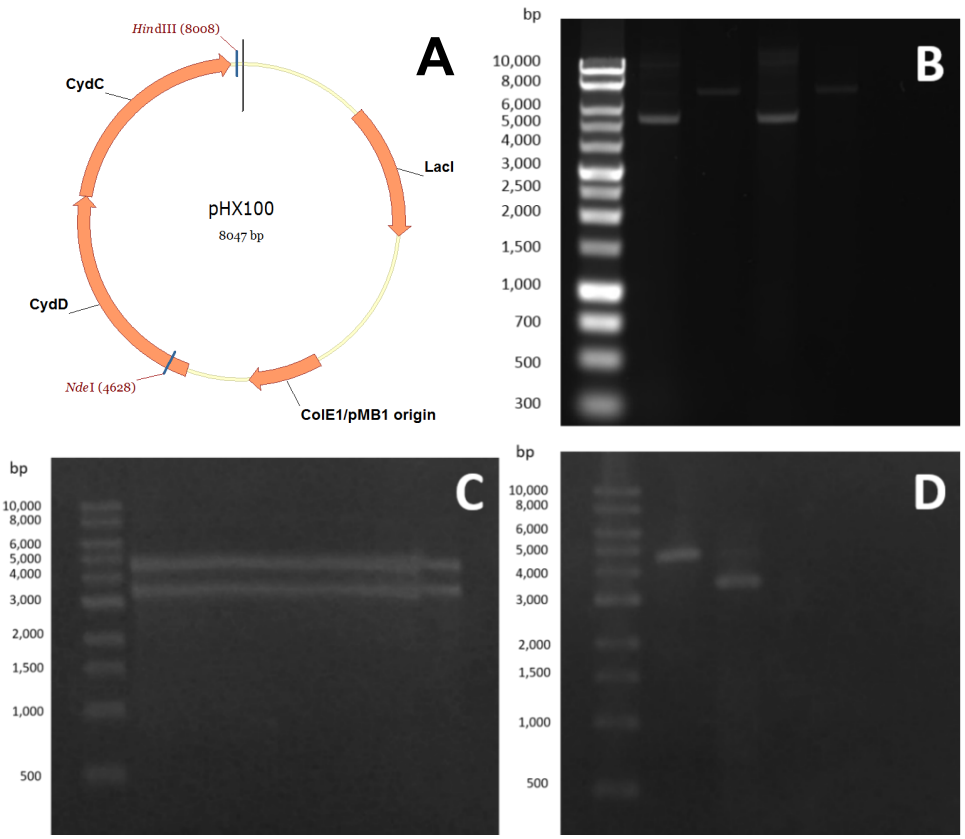


Figure 3.1. Mutagenic PCR of pHX100 to form H51A variant. A) Plasmid map of WT pHX100. B) Mutagenic PCR product following alongside cut pHX100 plasmid for digest with StuI. C) Double digest of WT pHX100 plasmid in preparation of fragment insertion. D) Quantification of prepared vector and mutagenic fragment using Hyperladder I (BIO LINE) in preparation of ligation.

Ligations were then performed (**Section 2.2.4**) and competent BL21 (DE3) cells were transformed with the ligation mixture (**Section 2.1.10**). Colony PCR (**Section 2.2.5.**) was used to identify positive colonies, namely colonies 8, 18 and 19 as containing a pHX100 plasmid (**Figure 3.2**). Plasmid DNA of these colonies was mini-prepped (see section X) and digested with HindIII and NdeI, to determine if the H51A mutation was present in the *cydD* gene (**Figure 3.3.**). The mutation primers were designed in such a way that if the plasmid encodes the desired mutation, digestion with HindIII and NdeI will yield a single fragment of 8.05 kbp, whereas the wild type plasmid will yield fragments of 3.38 kbp and 4.67 kbp (i.e. mutation removes the NdeI site). The restriction digest on plasmid isolated from colony 8 produced a 8.05 kbp fragment when digested with HindIII alone or a HindIII/NdeI double digest, revealing the absence of NdeI site associated with the H51A mutation. A single digest with NdeI failed to linearize the plasmid, further suggesting the presence of the desired H51A mutation.

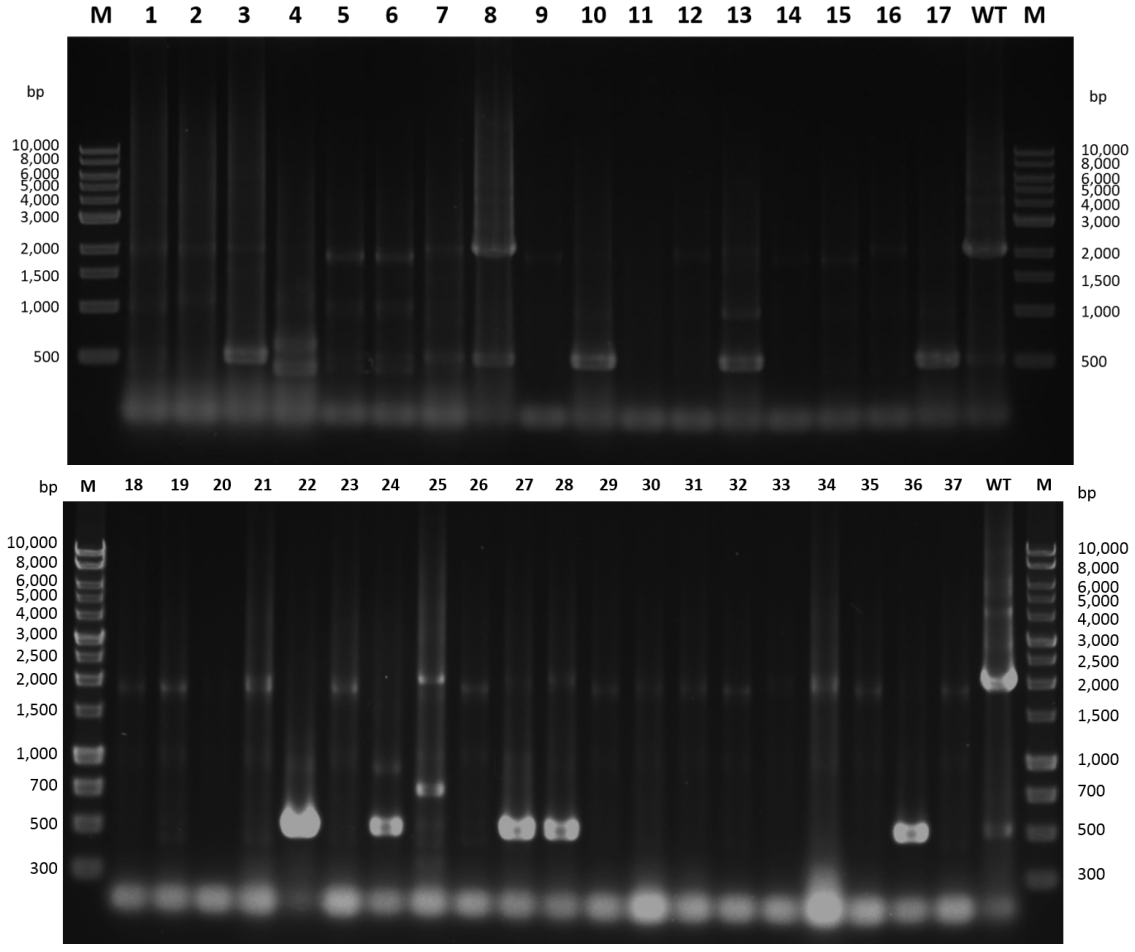


Figure 3.2. Colony PCR screening of transformants for the presence of pHX100. Ligation transformant colonies were subjected to colony PCR using primers that specifically amplify a 2000 bp fragment of pHX100.

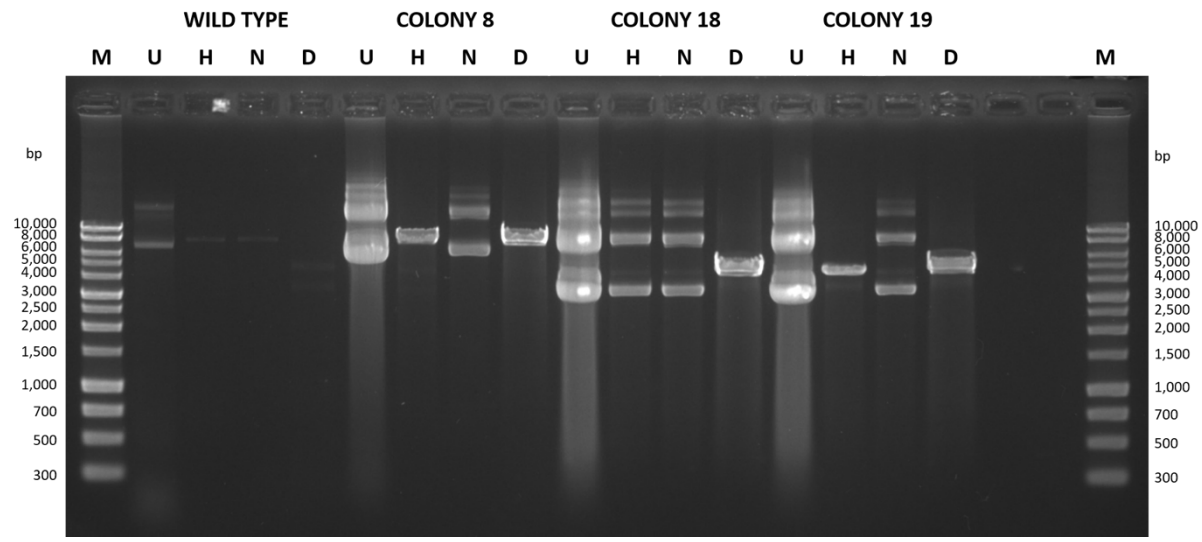


Figure 3.3. Restriction digest screening for the pHX100 plasmid encoding the *cydDC* H51A variant. M= Marker, U= Undigested, H= HindIII digest, N= NdeI Digest, D= Double Digest with HindIII and NdeI. Colony 8 lacks the NdeI site and is therefore likely to encode the desired H51A mutation.

3.1.2. Sequencing of the H51A mutation in pHX100

Plasmid isolated from colony 8 (**Section 2.2.1**) was sequenced via Sanger sequencing at Genewiz™. Data was then analysed through slignment with Bioedit v7.2.5, revealing that the *cydD* codon 51 (CAT) encoding histidine had indeed been changed to GCT, which encodes alanine upon (**Figure 3.4A**). Analysis of the translated sequence using the Snapgene program revealed that the mutant H51A codon was in frame with the start codon, with a mutation, equivalent to the WT gene mutation of H51A (**Figure 3.4B**).

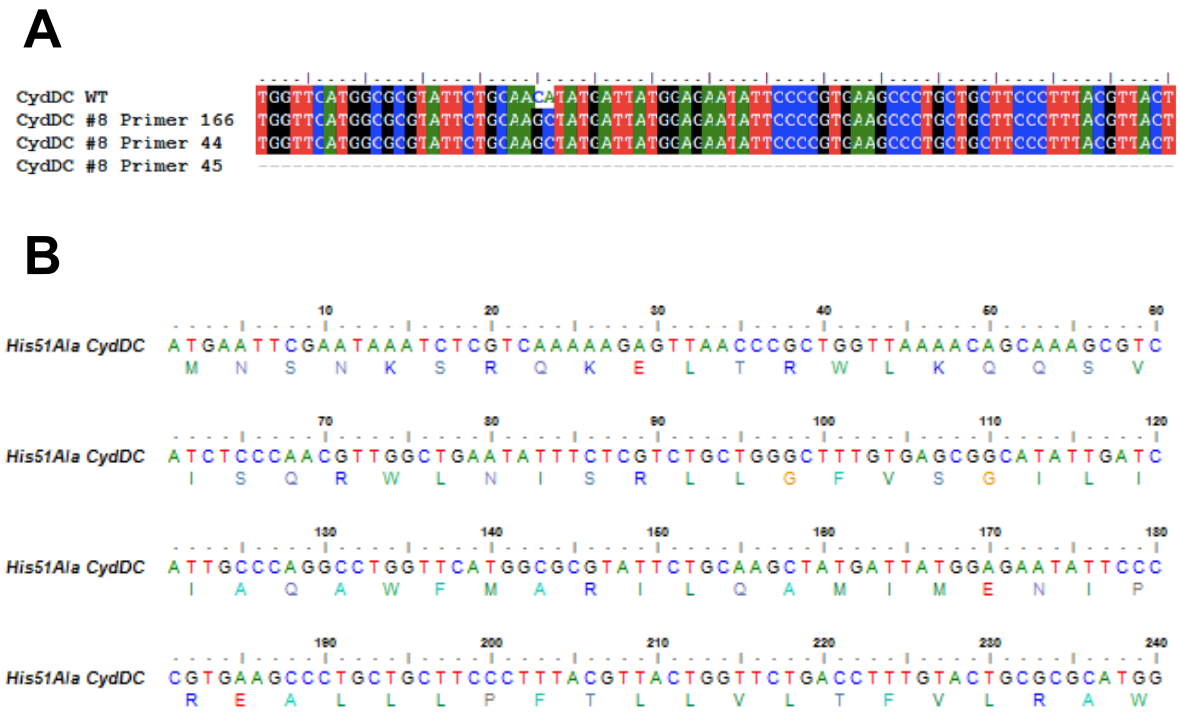


Figure 3.4. Sequencing data for the pHX100 *cydDC* H51A plasmid. Plasmid from colony 8 was sequenced using primers 166, 44 and 45. A) Sequencing results revealed C426G and A427C mutation after start codon. B) Translation of the sequence data demonstrated the desired H51A mutation is encoded in frame with the start codon.

3.2. Isolation and analysis of purified CydDC

3.2.1 Overexpression and purification of wild type CydDC

E. coli CydDC, containing a C-terminal His_6-tag, was overexpressed in *E. coli* BL21 cells and purified by affinity chromatography (**Section 2.3.1)**. Purity of CydDC was analysed using a 10% SDS-PAGE gel (**Section 2.3.2**). Results obtained show high purity of CydDC, shown within the purified protein lane by the presence of two proteins migrating at a rate corresponding to approximately 55 kDa. This suggests the presence of CydDC (**Figure 3.5A**): the calculated molecular weight CydD and CydC are 65 kDa and 63 kDa, respectively, and these membrane proteins have previously been shown to migrate at a slightly faster rate compared to soluble proteins of the same size (Yamashita *et al.* 2014). To confirm the identity of CydC on the gel, a Western blot was performed (**Section 2.3.3**) using an anti-His_6-tag antibody from AbCam (**Figure 3.5B**). Results from the Western blot revealed His_6-tagged protein in both the supernatant and the purified protein lanes, at a molecular weight corresponding to CydC.

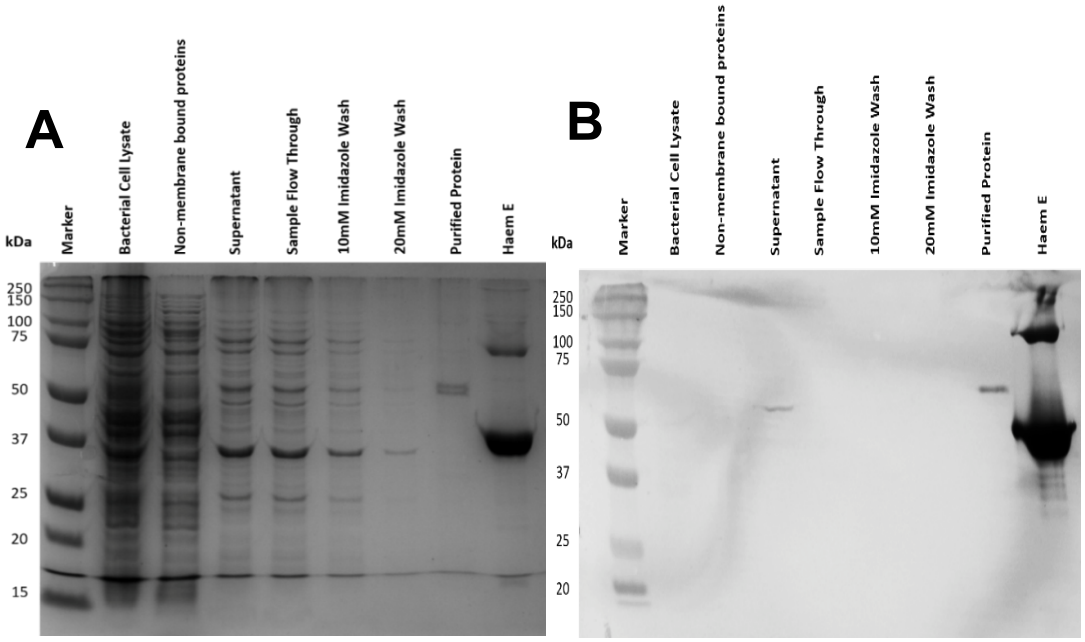


Figure 3.5. **SDS-PAGE and Western blot analysis of CydDC purification.** A) Samples collected during CydDC protein purification were resolved on a 10% SDS-PAGE gel. The presence of two proteins migrating at a rate corresponding to approximately 55 kDa in the purified protein lane suggests the presence of CydDC. B) Western blot analysis of protein purification samples probed with anti-His_6-tag antibody (Abcam) detected His_6-tagged CydC protein in supernatant and purified protein fractions. As a positive control, a previously characterised His_6-tagged HemE protein was used.

3.2.2 Attempts to overexpress and purify H51A CydDC

Plasmid induction tests were performed within BL21 (DE3) pLysS strains harbouring pHX100 plasmids encoding either wild type CydDC or the or H51A mutant CydDC. Cells were grown as described in **Section 2.1.8** and whole cells were loaded onto 10% SDS-PAGE gels, transferred to PVDF membrane and probed with antibody specific to His_6- tagged protein (**Section 2.3.2**). The Western blot shows that induction with IPTG for 4 hours induced expression of wild type CydC (**Figure 3.6A**), whereas a similar induction for the H51A mutant CydDC complex did not result in the expression of CydC (**Figure 3.6B**). Nevertheless, protein purifications were attempted in order to isolate CydDC H51A mutant, but no purified CydDC could be detected using SDS-PAGE nor Western Blotting.

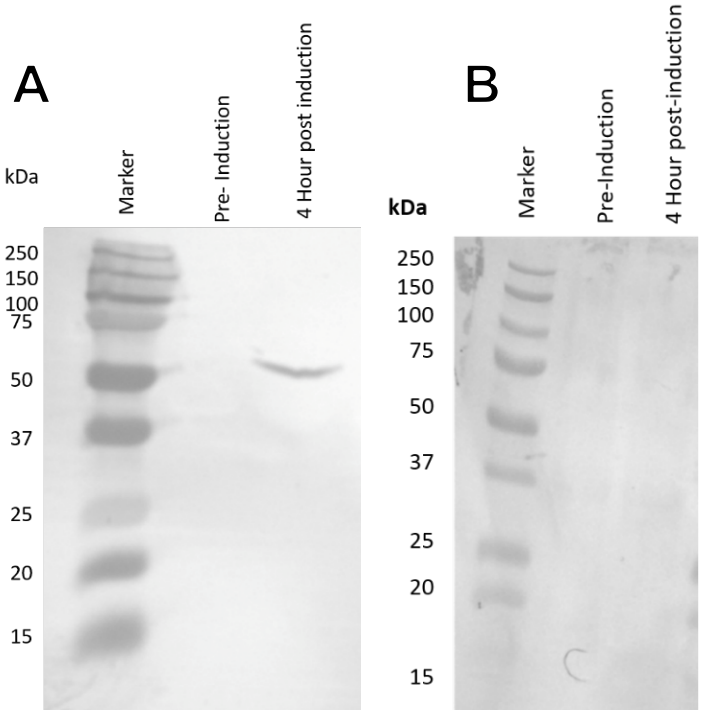


Figure 3.6. Induction tests to monitor CydC expression via Western blotting. A) Induction of wild type CydDC (A) and H51A CydDC (B) with IPTG followed by detection of CydC using anti His_6-tag antibodies.

3.2.3. Spectroscopic analysis of purified CydDC

UV/vis spectroscopic analysis of purified wild type *E. coli* CydDC revealed absorbance peaks at 280 nm and 415 nm (**Figure 3.7**), corresponding to CydDC protein and bound haem as previously reported (Yamashita *et al.* 2014). A Markwell assay (**Section 2.3.4**) revealed the concentration of CydDC obtained from the purification to be 430 µg/mL.

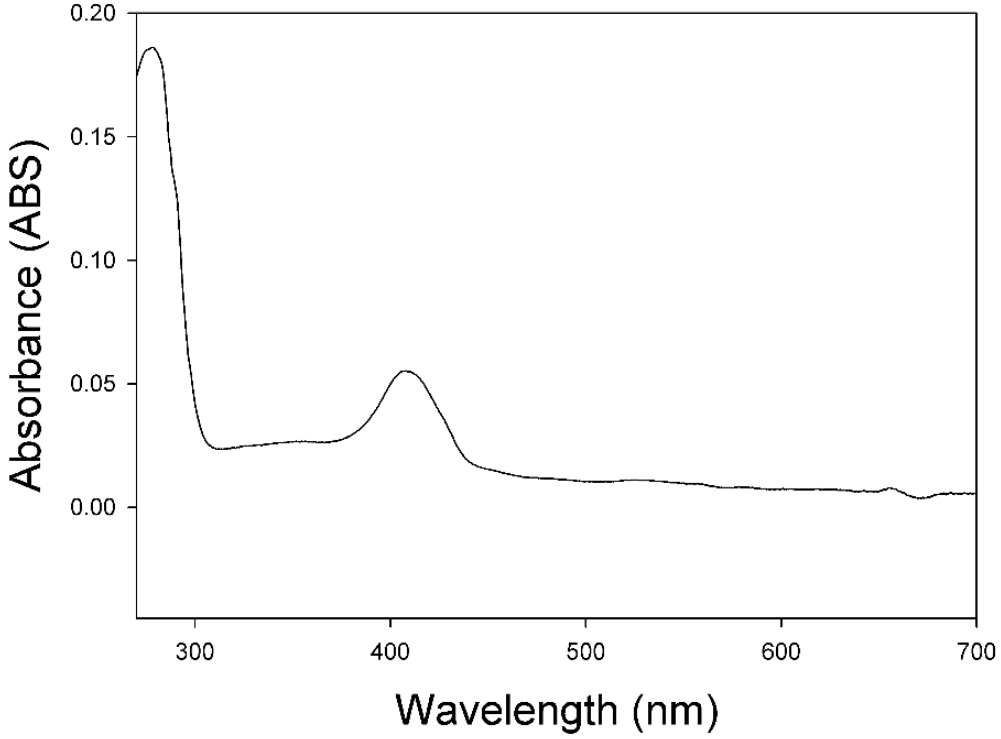


Figure 3.7. UV/vis spectrum of purified wild type CydDC. Peaks at 280 nm and 415 nm are due to absorbance of protein and haem, respectively.

3.3. Haem affinity measurements for CydDC

Haem has previously been shown to bind to CydDC, so it was of interest to measure the affinity of this interaction. CydDC tryptophan fluorescence titrations were performed as described in **Section 2.3.5** along with control titrations with the fluorescent small molecule N-acetyl-tryptophanamide (NATA). Fluorescence emission spectra demonstrated quenching in the 430 nm peak upon titration of CydDC with haemin (**Figure 3.8A**), and the control experiment with NATA also exhibited quenching (Spectra shown in Appendix C), presumably due to the absorbance of fluorescence emission by the haem ligand. The magnitudes of these peaks are plotted on the same graph (**Figure 3.8B**), which suggested a linear response for the NATA control whereas the CydDC titration appeared to have a hyperbolic component. The linear control data was subtracted from the CydDC data to reveal a hyperbolic transition that was solely due to haemin binding to CydDC (**Figure 3.8C**). To estimate a K_d value, these data were fitted to **equation 1** using nonlinear regression (Sigmaplot), which yielded a dissociation constant of 0.32 ± 0.11 μM.

Equation 1. [L] is equal to haemin (ligand) concentration (i.e. X-axis values) and [E] is equal to concentration of CydDC (0.1 μM), F0 is the intercept (i.e. fluorescence at [haemin] = 0), F_{max} is the total magnitude of fluorescence change, and K_d is the dissociation constant.

$$F_{obs} = F_0 + F_{max}\frac{[L]_T + [E]_T + K_d - \sqrt{([L]_T + [E]_T + K_d)^2 - 4[L]_T[E]_T}}{2[E]_T}$$

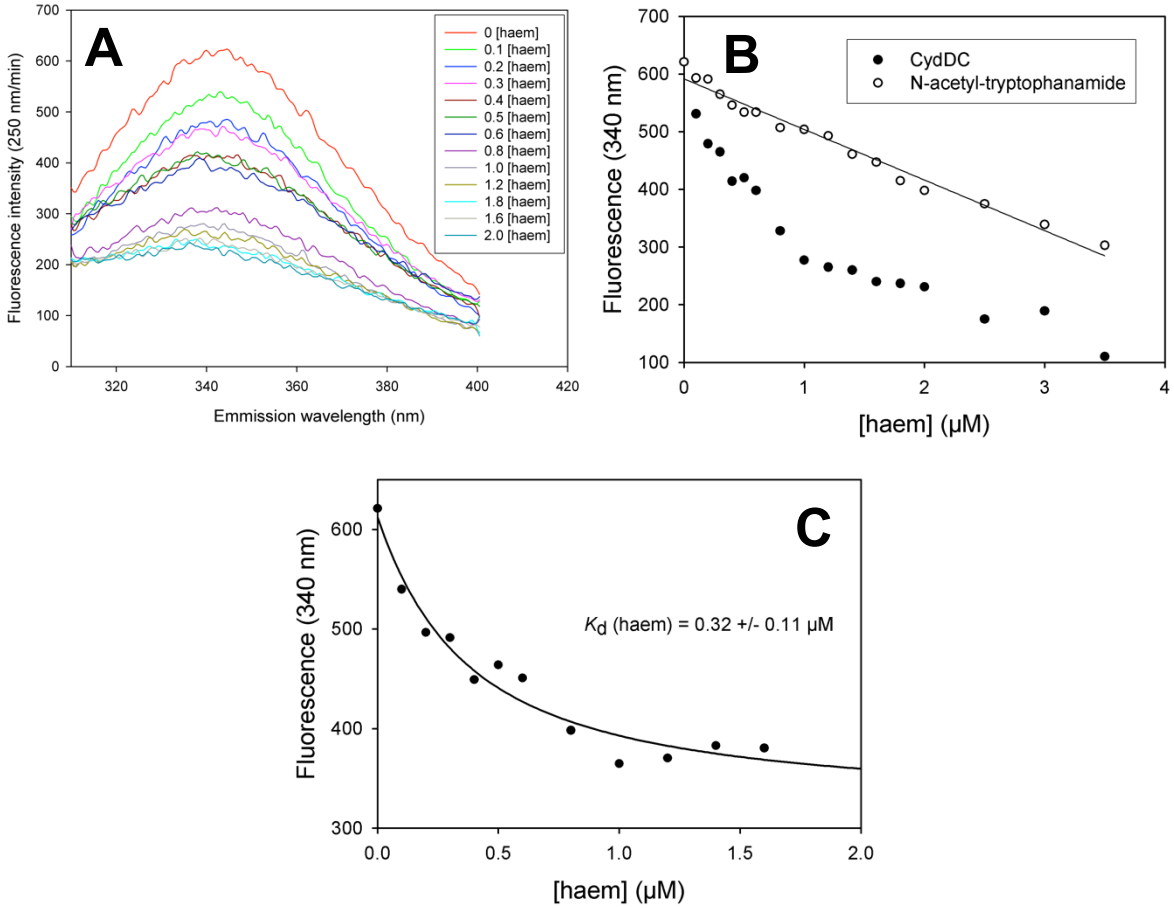


Figure 3.8. Tryptophan fluorescence quenching to monitor haem hinding to CydDC. A) Change in fluorescence emission spectra upon haem binding to CydDC. Data recorded using excitation at 280 nm and emission scans at speed of 250 nm/min, with emission and excitations slits set to 5 nm. B) Change in fluorescence at 340 nm upon haem binding for both 100 nM wild type CydDC and N-acetyl-tryptophanamide (NATA). C) 100 nM wild type CydDC titrated with haemin, with data normalised via subtraction of F data for NATA. The fitted binding curve predicts a K_d value for the CydDC/haem interaction as 0.32 μM.

3.4. CydDC transports metal-bound Glutathione across the periplasmic membrane.

To further investigate CydDC functionality *in vivo*, the contribution of this complex to metal tolerance was investigated. The CydDC substrate reduced glutathione (GSH) is well-known to bind to a variety of metal ions (Rubino *et al.* 2004; Rubino *et al.* 2006; Burford, Eelman and Groom 2005), so it was hypothesised that CydDC might provide protection to *E. coli* against metal toxicity via the export of GSH:metal conjugates. There is a precedent for this, as cadmium and aresenite have been reported to be exported as GSH conjugates in yeast (Li *et al.* 1996) and Leishmania (Dey *et al.* 1996), respectively. Disc diffusion assays (**Section 2.1.7.**) were used to test the ability of CydDC to confer tolerance of *E. coli* to zinc and cadmium ions (**Figure 3.9**). An *E. coli cydDC* knockout strain showed a significant increase in zone of inhibition size to these metals compared to a wild type strains and a *cydDC* mutant strain complemented with the pRKP1602 CydDC expression vector. The complemented strain shows recovery to wild type susceptibility, and surprisingly no additional metal tolerance is provided by the presence of the *cydDC* locus on a multi-copy plasmid.

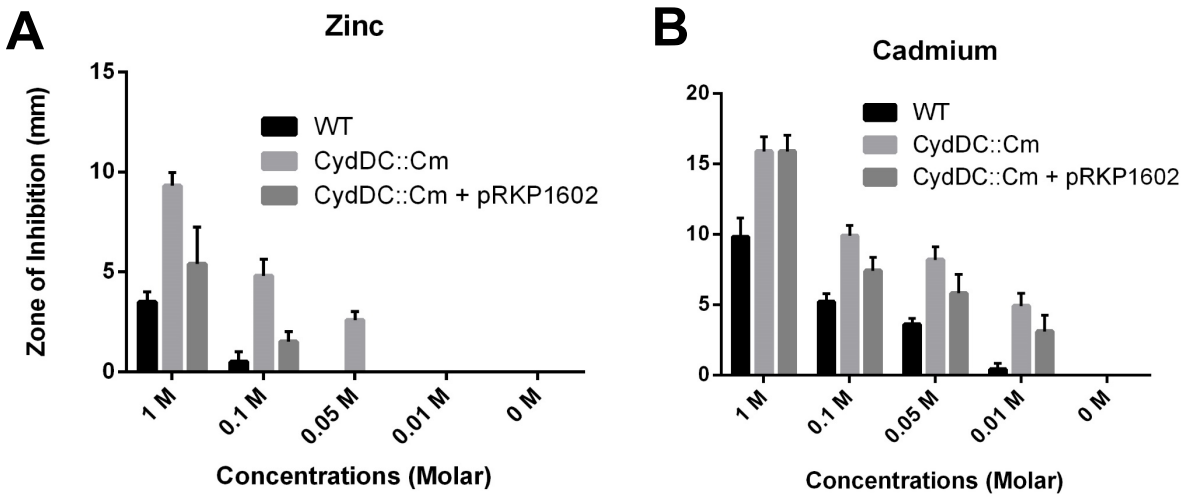


Figure 3.9. Disc diffusion analysis of CydDC expression and metal tolerance. Wild type (WT), *cydDC* knockout mutant (CydDC::Cm), and complemented mutant (CydDC::Cm pRKP1602) *E. coli* strains were tested for tolerance to zinc (A) and cadmium (B).

Chapter IV:

DISCUSSION

4.1. Engineering a plasmid to express a H51A variant of CydDC

It has recently been shown that the CydDC complex binds haem (Yamashita *et al.* 2014) and a good candidate for haem binding is the His51 residue on CydD (Shepherd 2015) It was therefore of interest to test the hypothesis that His51 binds haem by generating an expression construct for this mutant CydDC complex. This was successfully achieved via mutagenic PCR and conventional cloning in the expression vector pHX100 (**Figures 3.1A-D, 3.2 and 3.3**), and the desired mutation was confirmed by Sanger sequencing (**Figures 3.4, Appendix B**). Prior to this, extensive efforts to introduce this mutation were made using the Q5 site directed mutagenesis kit (NEB), but after multiple failed attempts the aforementioned PCR/cloning approach yielded the desired vector.

4.2. Expression, purification and antibody detection of the CydDC complex

Expression of wild type CydDC was successfully achieved through the use of the expression vector pHX100, a high copy number plasmid containing a P_{tac} promoter upstream of the *cydD* gene (**Appendix A**). The P_{tac} promoter is a hybrid of the *trpB* and *lac* promoters, which offers high yield and tight regulation of transcriptional units. Cell growth was performed in M9 minimal media to reduce the chance of P_{tac} activation from Tryptone found in some growth media. Indeed, no expression of wild type CydDC was detected before the addition of the inducer IPTG (**Figure 3.6**), demonstrating that this promoter is not leaky when cells are grown in M9 medium.

Analysis of the CydDC purification process was performed using SDS-PAGE, which revealed two proteins migrating at a rate corresponding to proteins of a lower molecular weight than the predicted 63 and 65 kDa that were expected. This has been observed before for CydDC (Yamashita *et al.* 2014), and it is not uncommon for membrane proteins to migrate at unusual rates on SDS-PAGE gels: the lower than expected molecular weight can be explained from the solubilisation and stabilisation of CydDC within micelles causing anomalous migration (Rath *et al.* 2009). Western blot analysis using an anti-His_6-tag antibody confirmed the presence of CydDC in the supernatant and protein elution lanes, providing validation that the purified complex is indeed CydDC (**Figure 3.5**).

Unfortunately, attempts to overexpress and purify the H51A variant of CydDC were unsuccessful. Given the location of His51, within the periplasmic loop of CydD (Shepherd 2015), it was anticipated that a H51A mutation would not perturb incorporation of CydDC in the cell membrane. However, the absence of CydDC H51A in expression and purification attempts analysed by SDS-PAGE and Western blotting confirm the absence of the CydDC complex. It is unlikely that the promoters and expression systems have been affected from the mutagenesis and cloning, as sequencing revealed no mutations within the P_{tac} promoter. Hence, the lack of detectable protein suggests that His51 provides an important role in CydDC assembly.

4.3. CydDC has a high affinity for haem

The original aim of this project was to purify wild type and H51A variant of CydDC for subsequent haem-binding analyses. Unfortunately, the mutant protein was not assembled in *E. coli* so haem-binding studies could only be performed on the wild type protein. Analysis of purified CydDC via UV-visible spectrophotometry revealed a broad peak centred around 415 nm (**Figure 3.7**), indicating the presence of bound haem. This is consistent with previous reports that CydDC is co-purified from *E. coli* with haem *b* (Yamashita *et al.* 2014) Comparison between protein peak (280 nm) and the haem peak (~400 nm) indicates a ratio of 4:1 of protein:haem. This substoichiometric binding may be explained by a limited availability of haem in the cell.

To further investigate this interaction, tryptophan fluorescence titrations were conducted with purified wild type CydDC and haemin, which revealed a K_d value of 0.32 μM +/- 0.11 μM (**Figure 3.8C**). This confirms that CydDC has a high affinity to haem, and the co-purification is unlikely to be due to nonspecific interactions. This study is consistent with previous studies of CydDC structural models which identified histidine and arginine residues on CydD that could potentially provide specific interactions with haem (Shepherd, 2015): hydrogen bonds may be formed between Arg47 and the carboxyl groups of *b*-type haem, along with an axial coordination of the central haem iron by His51. The high affinity between CydDC and haem suggests that haem is likely to play an important role within the functionality of CydDC in its reductant efflux. Given that the haem cofactor is redox active (Yamashita *et al.* 2014) and that CydDC exports redox active substrates (i.e GSH and cysteine) it is therefore proposed that haem may regulate the activity of CydDC through

sensing changes in redox poise, although further investigations are required to test this hypothesis.

4.4. New roles of CydDC in metal tolerance

To investigate the ability of CydDC to export GSH:metal conjugates, disk diffusion assays were performed with zinc and cadmium. It was hypothesised that cells expressing CydDC would be better able to export these metals, and therefore be able to grow in the presence of higher external metal concentrations. The data in **Figure 3.9** shows that *E. coli cydDC* strains exhibited a significant loss of tolerance to metal ions, in comparison to wild type and complemented strains. This data is consistent with the hypothesis that CydDC can export both GSH-Zn^{2+} and GSH-Cd^{2+}, and are consistent with previous observations that ABC transporters can transport metal conjugates in other organisms, (Potter, Trappetti and Paton 2012).

4.5. Future work

H51A CydDC expression may be affected from varying environmental factors such as growth medium, IPTG concentration and total viable cell mass. Therefore, protein expression needs to be investigated under varying conditions and analysed via Western blot using a His_6 antibody as described in **Section 2.3.3**. If H51A CydDC variant protein is viable, further analysis through UV/vis spectroscopy and Tryptophan fluorescent titrations could confirm that His51 is the location of haem binding. It has also been shown that CydDC is required for cytochrome *bd*-I assembly (Shepherd 2015). CO difference spectras and reduced *minus* oxidised difference spectras of H51A mutants would offer an insight into the effect of bound haem in cytochrome *bd*-I assembly and accumulation of the unknown haem compound P-574.

Previously shown in literature, CydDC is co-purified with bound haem at a haem:CydDC ratio of 1:4 (Yamashita *et al.* 2014). Current hypothesis suggest this substoichiometric ratio is due to bioavailability, and it would be interest to see if haem loading of CydDC can occur during the purification process, to achieve a protein:haem ratio of 1:1. This could in-turn be used to maximise redox potential within the periplasm for disulphide bond formation, that could be adapted to enhance recombinant protein production in *E. coli.*

Finally, this work suggests a novel role for CydDC as a GSH:metal conjugate transporter, but further research with different metal ions is required to further support this hypothesis. Alongside future work into CydDC role in metal sequestration in the periplasm, investigations in the ATPase activity in the presence of GSH:metal conjugate could offer insight into the exportation process of conjugates into the periplasm. As well as the effects of GSH synthesis into CydDC-mediated tolerance of metals, which can be undertaken through the use of mutant *gshA* and *gshA cydDC E. coli* strains in disc diffusion assays outlined within **Section 2.1.7.**

Chapter V:

REFERENCES

Andrews, J.M. (2001). The development of the BSAC standardized method of disc diffusion testing. *The Journal of antimicrobial chemotherapy* **48 Suppl 1**:29–42.

Berry, E.A. and Trumpower, B.L. (1987). Simultaneous determination of hemes a, b, and c from pyridine hemochrome spectra. *Analytical Biochemistry* **161**:1–15.

Bessette, P.H. *et al.* (1999). In vivo and in vitro function of the Escherichia coli periplasmic cysteine oxidoreductase DsbG. *Journal of Biological Chemistry* **274**:7784–7792.

Burford, N., Eelman, M.D. and Groom, K. (2005). Identification of complexes containing glutathione with As(III), Sb(III), Cd(II), Hg(II), Tl(I), Pb(II) or Bi(III) by electrospray ionization mass spectrometry. *Journal of Inorganic Biochemistry* **99**:1992–1997.

Chesney, J.A., Eaton, J.W. and Mahoney, J.R. (1996). Bacterial glutathione: A sacrificial defense against chlorine compounds. *Journal of Bacteriology* **178**:2131–2135.

Cook, G.M. *et al.* (2002). A novel haem compound accumulated in Escherichia coli overexpressing the cydDC operon, encoding an ABC-type transporter required for cytochrome assembly. *Archives of Microbiology* **178**:358–369.

Cook, G.M., Membrillo-Hernandez, J. and Poole, R.K. (1997). Transcriptional Regulation of the [i]cydDC[/i] Operon, Encoding a Heterodimeric ABC Transporter Required for Assembly of Cytochromes [i]c[/i] and [i]bd[/i] in [i]Escherichia coli[/i] K-12: Regulation by Oxygen and Alternative Electron Acceptors. *Journal of Bacteriology* **179**:6525–6530.

Darwin, a J. *et al.* (1997). Differential regulation by the homologous response regulators NarL and NarP of Escherichia coli K-12 depends on DNA binding site arrangement. *Molecular microbiology* **25**:583–595.

Daßler, T. *et al.* (2000). Identification of a major facilitator protein from Escherichia coli involved in efflux of metabolites of the cysteine pathway. *Molecular Microbiology* **36**:1101–1112.

Dey, S. *et al.* (1996). An ATP-dependent As(III)-glutathione transport system in membrane vesicles of Leishmania tarentolae. *Medical Sciences* **93**:2192–2197.

Fabianek, R.A., Hennecke, H. and Thony-Meyer, L. (1998). The active-site cysteines of the periplasmic thioredoxin-like protein CcmG of Escherichia coli are important but

not essential for cytochrome c maturation in vivo. *Journal of Bacteriology* **180**:1947–1950.

Falk, J.E. and Smith, K.M. (1975). *Porphyrins and Metalloporphyrins*. New York: Elsevier.

Georgiou, C.D., Fang, H. and Gennis, R.B. (1987). Identification of the cydC locus required for expression of the functional form of the cytochrome d terminal oxidase complex in Escherichia coli. *Journal of Bacteriology* **169**:2107–2112.

Goldman, B.S., Gabbert, K.K. and Kranz, R.G. (1996). Use of heme reporters for studies of cytochrome biosynthesis and heme transport. *Journal of Bacteriology* **178**:6338–6347.

Harrison, J.J. *et al.* (2004). Biofilm susceptibility to metal toxicity. *Environmental Microbiology* **6**:1220–1227.

Harrison, J.J. *et al.* (2009). Chromosomal antioxidant genes have metal ion-specific roles as determinants of bacterial metal tolerance. *Environmental Microbiology* **11**:2491–2509.

Holyoake, L. V., Poole, R.K. and Shepherd, M. (2015). *The CydDC Family of Transporters and Their Roles in Oxidase Assembly and Homeostasis*. Vol. 66.

Inaba, K. (2009). Disulfide bond formation system in Escherichia coli. *Journal of Biochemistry* **146**:591–597.

Lemire, J. a, Harrison, J.J. and Turner, R.J. (2013). Antimicrobial activity of metals: mechanisms, molecular targets and applications. *Nature reviews. Microbiology* **11**:371–84.

Li, Z.S. *et al.* (1996). The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. *Journal of Biological Chemistry* **271**:6509–6517.

Macomber, L., Rensing, C. and Imlay, J.A. (2007). Intracellular copper does not catalyze the formation of oxidative DNA damage in Escherichia coli. *Journal of Bacteriology* **189**:1616–1626.

Markwell, M.A.K. *et al.* (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry* **87**:206–210.

Missiakas, D. and Raina, S. (1997). Protein folding in the bacterial periplasm. *Journal of Bacteriology* **179**:2465–2471.

Pittman, M.S. *et al.* (2002). Cysteine is exported from the Escherichia coli cytoplasm by CydDC, an ATP-binding cassette-type transporter required for cytochrome assembly. *Journal of Biological Chemistry* **277**:49841–49849.

Pittman, M.S., Robinson, H.C. and Poole, R.K. (2005). A bacterial glutathione transporter (Escherichia coli CydDC) exports reductant to the periplasm. *Journal of Biological Chemistry* **280**:32254–32261.

Poole, R.K., Gibson, F. and Wu, G. (1994). The cydD gene product, component of a heterodimeric ABC transporter, is required for assembly of periplasmic cytochrome c and of cytochrome bd in Escherichia coli. *FEMS microbiology letters* **117**:217–23.

Potter, A.J., Trappetti, C. and Paton, J.C. (2012). Streptococcus pneumoniae uses glutathione to defend against oxidative stress and metal ion toxicity. *Journal of Bacteriology* **194**:6248–6254.

Rath, A. *et al.* (2009). Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proceedings of the National Academy of Sciences of the United States of America* **106**:1760–5.

Rubino, F.M. *et al.* (2004). Characterization of the disulfides of bio-thiols by electrospray ionization and triple-quadrupole tandem mass spectrometry. In: *Journal of Mass Spectrometry*. pp. 1408–1416.

Rubino, F.M. *et al.* (2006). Electrospray Ionization and Triple Quadrupole Tandem Mass Spectrometry Study of Some Biologically Relevant Homo- and Heterodimeric Cadmium Thiolate Conjugates. *Journal of the American Society for Mass Spectrometry* **17**:1442–1455.

Shepherd, M. (2015). The CydDC ABC transporter of Escherichia coli: new roles for a reductant efflux pump. *Biochemical Society Transactions* **43**:908–912.

Stevens, J.M., Gordon, E.H. and Ferguson, S.J. (2004). Overproduction of CcmABCDEFGH restores cytochrome c maturation in a DsbD deletion strain of E. coli: Another route for reductant? *FEBS Letters* **576**:81–85.

Tseng, C.P., Albrecht, J. and Gunsalus, R.P. (1996). Effect of microaerophilic cell growth

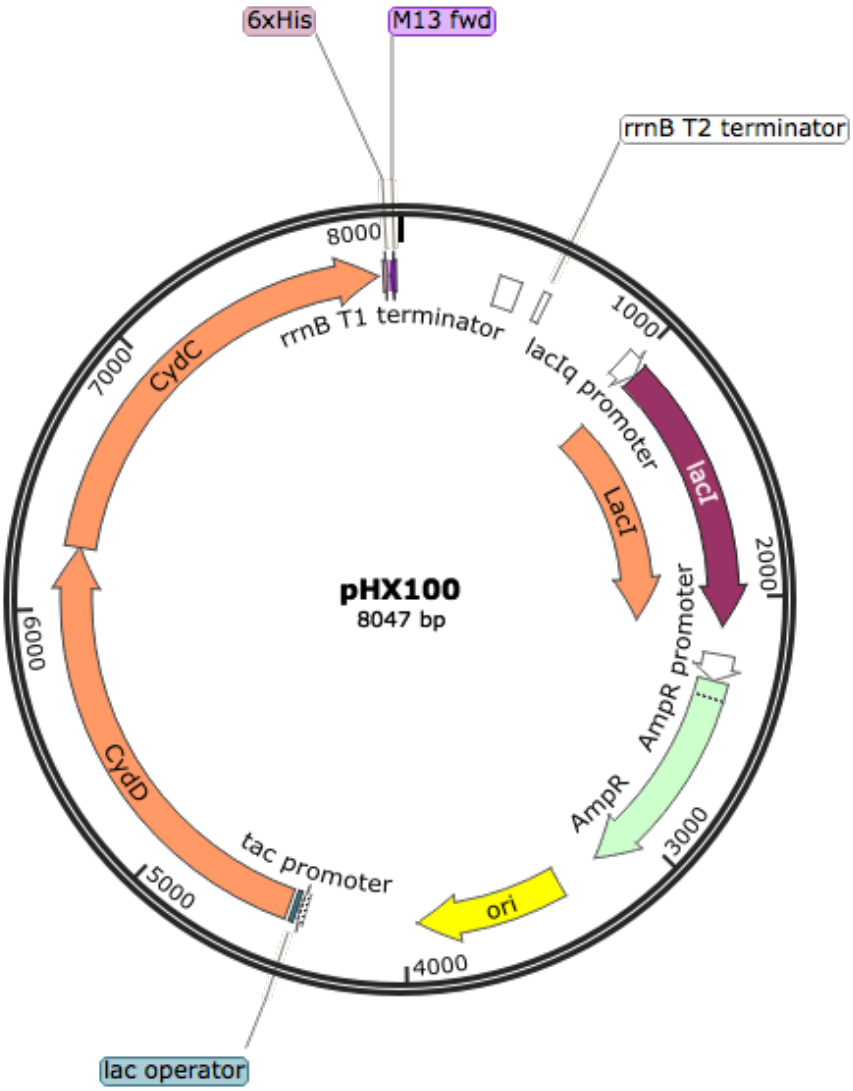
conditions on expression of the aerobic (\emph{cyoABCDE} and \emph{cydAB}) and anaerobic (\emph{narGHJI}, \emph{frdABCD}, and \emph{dmsABC}) respiratory pathway genes in \emph{Escherichia coli}. *J Bacteriol* **178**:1094–1098.

Yamashita, M. *et al.* (2014). Structure and function of the bacterial heterodimeric ABC transporter CydDC: Stimulation of atpase activity by thiol and heme compounds. *Journal of Biological Chemistry* **289**:23177–23188.

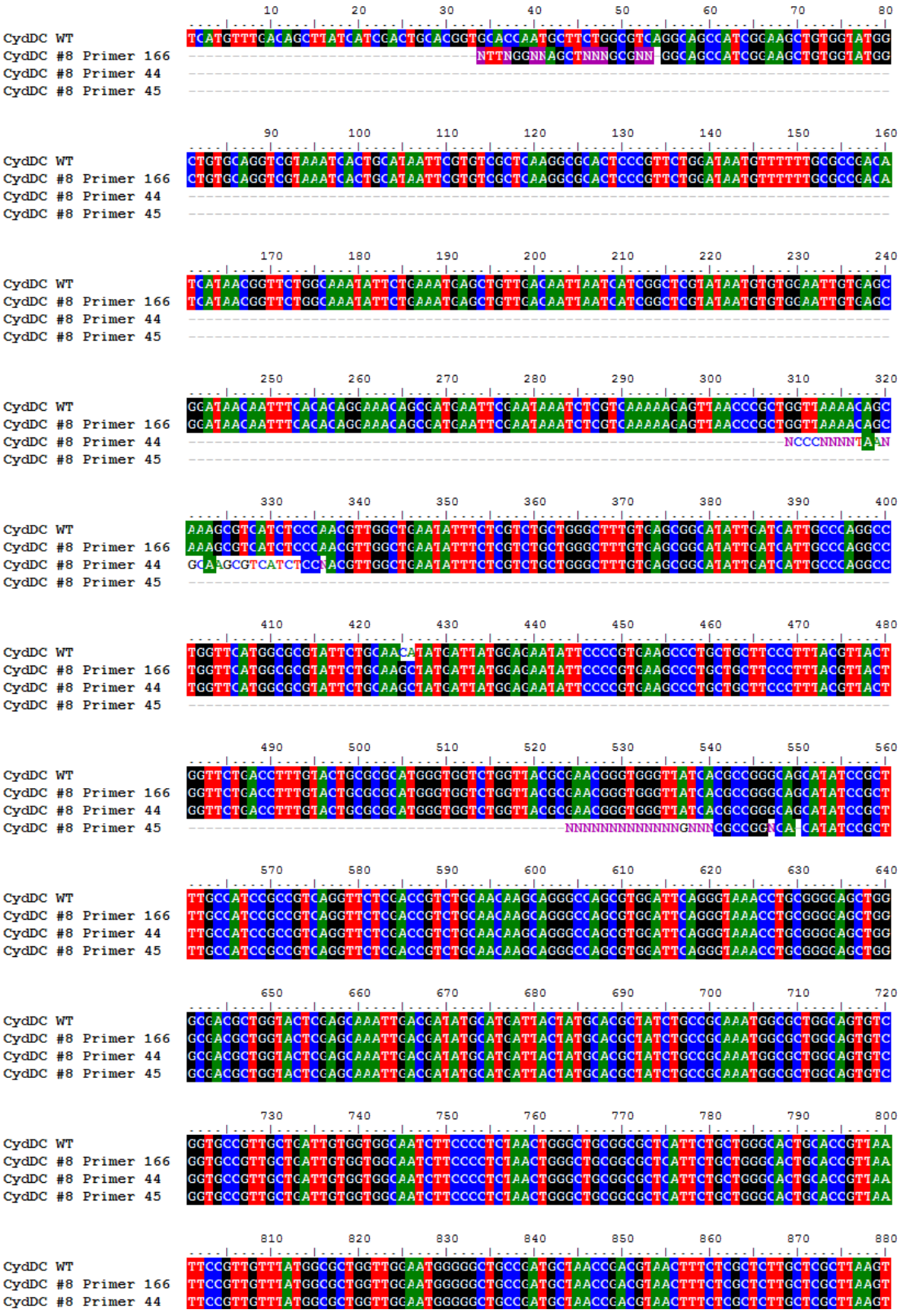
Yu, J. and Kroll, J.S. (1999). DsbA: A protein-folding catalyst contributing to bacterial virulence. *Microbes and Infection* **1**:1221–1228.

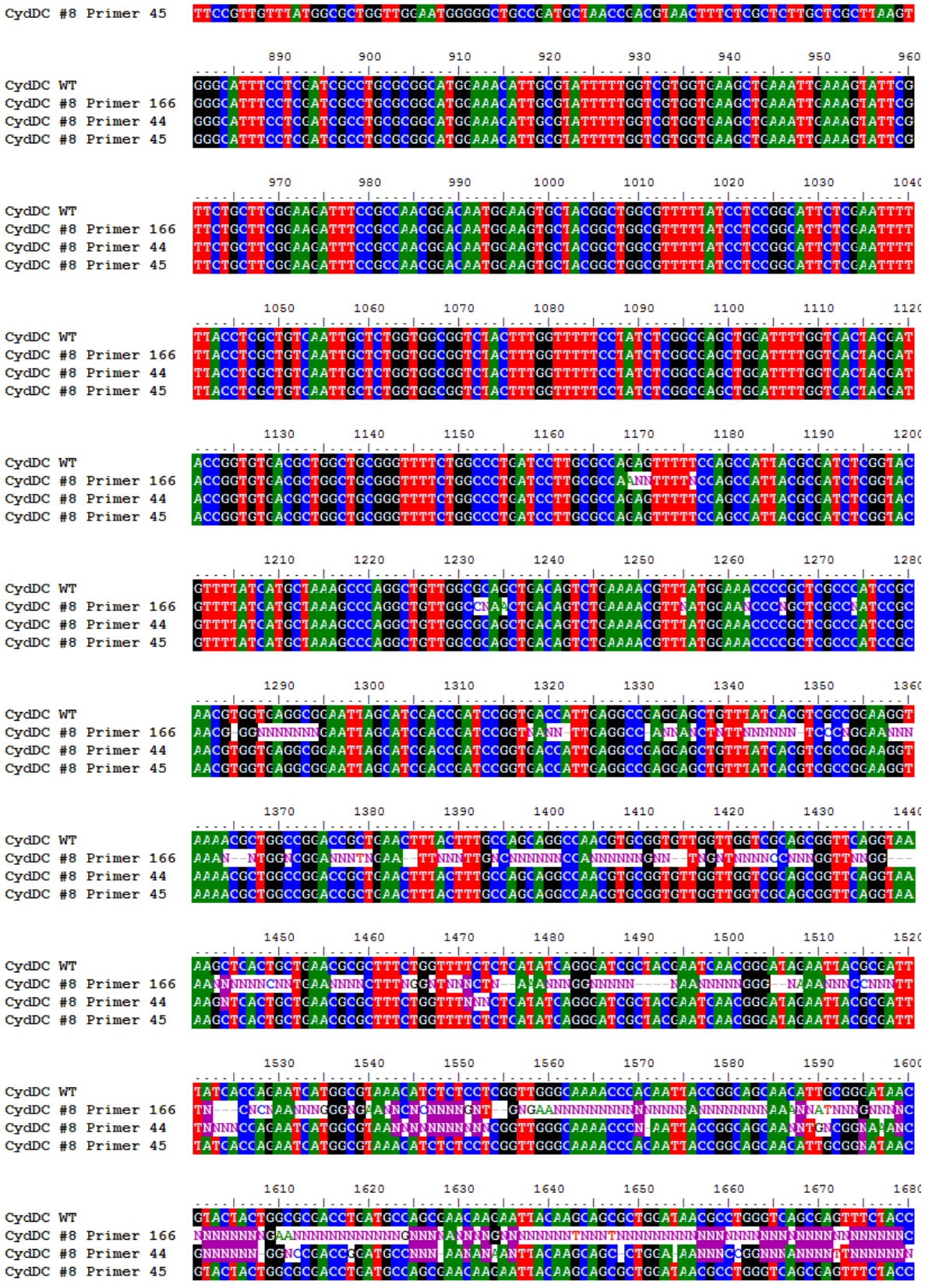
Chapter VI:

APPENDICES

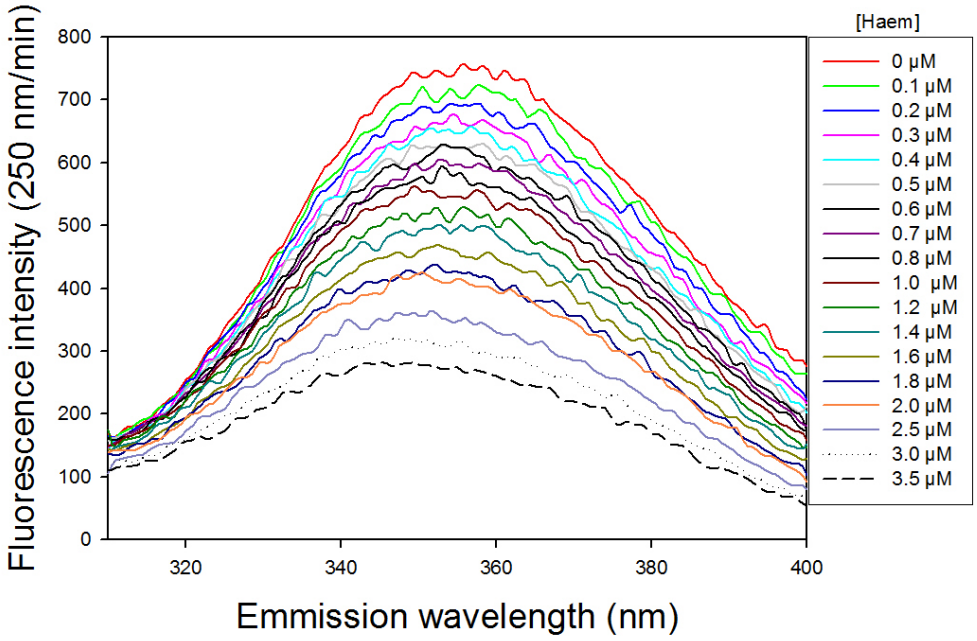


Appendix A. Plasmid map of PHX100 - a wild type CydDC expression vector.


CydDC WT
CydDC #8 Primer 166
CydDC #8 Primer 44
CydDC #8 Primer 45



Appendix B. WT CydDC and H51A mutant sequencing data alignment, using Bioedit v7.2.5 software. Sequencing results revealed C426G and A427C mutation after start codon (ATG) located at base 296, with a Shine-Dalgarno (AGGA) sequence located at 258 bp, suggesting sequence.



Appendix C. Change in fluorescence emission spectra upon haem addition to N-acetyl-tryptophanamide (NATA). Data recorded using excitation at 280 nm and emission scans at speed of 250 nm/min, with emission and excitations slits set to 5 nm.