The CydDC family of transporters

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1. Overview

The CydDC family of ABC transporters export the low molecular weight thiols glutathione and cysteine to the periplasm of a variety of bacterial species. The CydDC complex has previously been shown to be important for disulfide folding, motility, respiration, and tolerance to nitric oxide and antibiotics. In addition, CydDC is thus far unique amongst ABC transporters in that it binds a haem cofactor that appears to modulate ATPase activity. CydDC has a diverse impact upon bacterial metabolism, growth, and virulence, and is of interest to those working on membrane transport mechanisms, redox biology, aerobic respiration, and stress sensing/tolerance during infection.

2. Regulation and assembly of the ABC transporter CydDC

2.1 Identification and cloning of the *cydDC* genes

By the early 1990s, the group of Bob Gennis had established by classical genetic techniques the presence and location on the *E. coli* chromosome of two structural genes encoding the cytochrome *bd*-type terminal oxidase [1], now often referred to as cytochrome *bd*-I following the discovery of a second *bd*-type oxidase in *E. coli* [2]. The *bd*-I encoding genes were named *cydA* and *cydB*, constituting an operon at 16.6 min (physical map position 785-788 bp [3]) on the chromosomal map. It is important to note that for many years the cytochrome *bd*-I complex was considered to be a two-subunit complex, but later sequencing studies and the crystal structure reveal a third small subunit [4]. A further gene, *cydC*$^{+}$, annotated as involved in 'cytochrome *d* assembly' was also identified, remote from the *cydAB* locus at 19.2 min (938-943 bp) [5]. In brief, a strain lacking cytochrome *bo′* was mutagenized and screened for mutants incapable of aerobic growth on non-fermentable substrates, other than those with mutations in *cydAB*. The strain (CG03) was used to map the newly identified gene, first by F-

prime conjugation and then by P1 co-transductions. Reintroducing *cyo* genes on an F-prime episome carrying the *cydAB* operon failed to complement a cytochrome *bd*-I-deficient mutant, suggesting a new locus. The mutant showed no visible absorbance bands attributable to cytochrome *bd*-I but the spectrum was returned to wild-type characteristics by introducing *cydC*$^+$ on an episome. Importantly, *in vitro* transcription/translation experiments and Western immunoblotting of *cydC* strain membranes showed the CydA and CydB subunits to be diminished in *cydC* membranes compared to the isogenic *cydC*$^+$ strain [5]. Expression from a multicopy plasmid of the oxidase subunits in a *cydC* strain demonstrated that the b_{595} and b_{558} haems were over-produced but the haem *d* component was absent. This led to the hypothesis that a likely role for CydC was biosynthesis of haem *d* and that, in the absence of this haem, the oxidase subunits are destabilised and therefore largely absent [5].

The *cydC* gene was independently described by Siegele et al. and named *surB*, its product being required for *E. coli* cells to exit (i.e. survive) stationary phase at 37 ^{o}C under aerobic conditions [6]. It was established that the severe temperature-sensitive defect in exiting stationary phase of *surB1* mutants is due to the absence of terminal cytochrome oxidase activity and specifically to the presence of a defective cytochrome *bd*-I oxidase [7, 8].

Shortly after, we described a fourth *cyd* locus identified by an entirely different strategy. Allan Downie had screened survivors of nitrosoguanidine mutagenesis for loss of spectroscopically detectable cytochrome *bd*-I using the laborious method of individually examining colonies for the 630 nm band of cytochrome *d* in a Keilin-type hand spectroscope at 77 K. The mutation in one such mutant was mapped genetically and shown to be highly co-transducible with *aroA* near 20 min on the *E. coli* chromosome [9]. Further mapping by F-primes, co-transduction data and three-point crosses clearly established the following gene order: *poxB* – (*zbj*::*Tn*10) – *cydC* – *cydD* at 19.3 min. Furthermore, a gene implicated in the

ability to survive at elevated temperatures, *htrD*, was shown to be closely linked to *cydC* and *cydD* [10] and was later shown to be identical to *cydD* after correction for a missing G in the Delaney sequence [11].

Resulting from this work was a useful selective medium, incorporating zinc and azide ions, to distinguish between Cyd^+ and Cyd^- strains [9]. This was used during the first cloning of the $cydC^+$ and $cydD^+$ genes using a cosmid library and subsequent sub-cloning to give plasmids capable of complementing *cydC* and *cydD* mutants [12], demonstrating their joint presence on plasmids pRP33 and pRP39; restriction maps showed that the cloned fragment of the latter lies within the 19 min region of the Kohara map, consistent with earlier P1 mapping [9]. *In vivo* protein synthesis using these plasmids as templates demonstrated the formation of CydD and CydC with estimated molecular masses of 61 kDa and 59 kDa, respectively [12], whereas the actual molecular weights calculated from the primary sequences (Section 2.2) are 65 kDa, and 63 kDa, respectively.

The complete nucleotide sequence of the insert was obtained by manual Sanger sequencing [12]. The deduced amino acid sequences of CydD and CydC have 50% similarity and 27% identity. Hydropathy profiles showed both to be similar membrane proteins, the N-terminal halves each forming six transmembrane helices. As detailed below, the C-terminal hydrophilic portions each contain an ATP-binding site. Thus CydDC was identified as the first known example of a bacterial heterodimeric ABC transporter. The structurally analogous HlyB protein, involved in export of haemolysin from *E. coli*, is a homodimer [13].

Note that in certain bacteria the *cydDC* and *cydAB* genes are not well separated on the genome, as described here in *E. coli*, but are found as a single operon and expressed as a single transcript, as in *Bacillus subtilis* [14]. Furthermore, certain bacteria have oxidases resembling cytochrome *bd*-I at the level of gene sequence yet have no spectrally detectable haem *d*. It is therefore interesting to consider possible CydDC proteins in such cases. For

example, in *Campylobacter jejuni*, two genes (cj0081, cj0082), encoding proteins similar to those of the CydAB complex in *E. coli*, were identified from genome sequences [15]. However, spectroscopic studies of this oxidase, named CioAB (for cyanide-insensitive oxidase) [16], showed that the high-spin haems b_{558}, b_{595} and the *d*-type haem, which are typical of *bd*-type oxidases, could not be detected in *C. jejuni*, suggesting that the enzyme was distinct. Furthermore, there are no obvious homologues of *cydDC* in *C. jejuni*. Although we cannot exclude the possibility of another ABC type transporter fulfilling CydDC functions, the apparent correlation between the lack of cytochrome *d* and a cognate ABC transporter is intriguing. Note that *C. jejuni* does not synthesise glutathione, an identified substrate of the *E. coli* CydDC system (Section 3.1). Other bacteria such as *P. aeruginosa* also possess a *bd*-type oxidase that lack the characteristic spectral features associated with the *bd* haems [17], and these constitute the CioAB (cyanide-insensitive oxidase) or "short Q-loop" sub-family [18].

Neither *cydD* nor *cydC* are essential genes as evidenced by the manners in which the mutants described above were isolated and used. Curiously, Eng *et al.* [19] describe *E. coli cydC* as an essential gene, without apparent justification, and, interestingly, a specific point mutation in an aspartic acid residue at the cytoplasm/membrane interface on helix II (*cydC*-D86V) restored production of a jet-fuel candidate, D-limonene. This study also suggested that cytochrome *bd*-I (i.e. CydABX) and CydDC do not always co-occur: computational analyses using the MicrobesOnline server [20] on 1965 genomes showed that highly conserved *cydC* genes are found in 407 species, but only 53 of these species also contained a *cydB* homologue. The presence of CydDC genes in so many genomes that do not encode cytochrome *bd*-I is surprising, especially since an earlier study by Kranz and coworkers did not detect any species that encoded CydDC but lacked cytochrome *bd*-I (from 33 bacterial genomes analysed) [21]. Furthermore, Kranz *et al.* highlighted six species that

encode cytochrome *bd*-I but not CydDC: *Rickettsia prowazekii*, *Chlamidia tracomatis*, *Campylobacter jejuni*, *Porphyromonas ginigivalis*, *Aquifex aeolicus*, and *Thermotoga maritima*: all of these species were reported to lack both CydDC and cytochrome *bd*-I in Eng *et al.* [19]. This suggests an issue with detection of cytochrome *bd*-I homologues in the genomics analysis by Eng *et al.*, which is supported by the observation that none of the *Geobacillus* nor *Mycobacterium* species were found by Eng *et al.* to encode cytochrome *bd*-I, yet several papers report functional/structural studies on cytochrome *bd*-I from these organisms [4, 22, 23]. This perhaps reflects the challenging nature of assigning functional homologues using high-throughput bioinformatics pipelines, and highlights the need for caution when interpreting an apparent *absence* of genes from a given genome.

2.2 Membrane topology of the CydDC complex

A hydrophobicity analysis of the CydD (65 kDa) and CydC (63 kDa) proteins predicted that both subunits would have six transmembrane helices, with the C-terminal half of each polypeptide being hydrophilic and containing an ATP binding site [12]: this study was the first to recognise that CydDC was a likely ABC transporter. A significant proportion (c. 5%) of the *E. coli* genome has been reported to be occupied by ABC transporters, which have been categorised into 10 subfamilies [24]. This phylogenetic study placed CydD and CydC in group 6, along with the homodimeric MsbA lipid flippase involved in membrane biogenesis [25, 26]. A subsequent study presented a membrane topology model for CydDC, which predicted both subunits to be quite similar with six transmembrane regions separated by two major cytoplasmic loops, and both ends of each polypeptide chain were suggested to be located in the cytoplasm [27]. Topography modelling in the current review is consistent with this previous work, and has been used to highlight the Walker A motif that binds ATP, the

Walker B motif that interacts with magnesium, and a conserved histidine, glutamine, and aspartic acid that are part of the H-loop, Q-loop, and D-loop, respectively (Figure 1).

2.3 Regulation of *cydDC* genes

It is well known that the structural genes for ABC transporters and terminal oxidases in most bacteria are subject to strict and complex regulation at the transcriptional level [28, 29]. The regulation of *cydDC* was therefore investigated using a chromosomal Φ(*cydD-lacZ*) fusion [30]. The operon is transcribed as a polycistronic message from a single transcriptional start site, independently of the upstream *trxB*. Transcription was independent of the carbon source for growth under aerobic conditions but declined as the oxygen transfer to the culture decreased. This is the converse of the well-known elevation of *cydAB* expression at lowered oxygen availability [28], an observation that is rationalised by the high oxygen affinity of the CydAB oxidase [31]. Anaerobic levels of Φ(*cydD-lacZ*) fusion activity were lower and independent of Arc and FNR, the major regulators of *cydAB* expression. However, the inclusion in medium of anoxic electron acceptors (nitrite, nitrate, fumarate) elevated *cydDC* transcription in a manner dependent on FNR and NarL.

Other studies have given apparently conflicting data: gene expression profiling showed a c. 2-fold increase under anoxic conditions in an *fnr* mutant [32] and potential FNR-binding sites were recognised in the promoter region of *cydDC* [30, 32]. However, we suggested that FNR repression is indirect and mediated at the post-transcriptional level [30]. In agreement with this view, Boysen *et al.* [33] showed a marked decrease in the 5′-end portion of *cydD* mRNA on short-term induction of FnrS expression (c. 3-fold). Thus transcription of the *cydDC* operon of *E. coli* is negatively regulated, albeit modestly, by FnrS and *cydDC* mRNA is a direct target of FnrS [33].

Structural genes encoding *bd*-type oxidases and the *cydDC* genes encoding the ABC transporter have been identified in numerous other bacteria but the regulation of the *cydDC* operon has rarely been described. In *Shewanella violacea*, the *bd*-type oxidase is evident in difference spectra but only in cells grown under high hydrostatic pressure, although *cydAB* was expressed at all pressures tested. In contrast, *cydC* is depressed at atmospheric pressures but up-regulated strongly by high hydrostatic pressures [34]. The nucleotide sequence upstream of *cydDC* was described in further detail and putative binding sites for the NarL protein (which is part of a two-component regulatory system also containing the sensor protein NarX) were found [35]. In *Brucella suis*, another bacterium in which four *cyd* genes constitute a single operon, *bd*-type oxidase expression was not detected under aerobic conditions, although a slight induction was observed under microaerobiosis. At both oxygen concentrations, disruption of *fnrN* increased *cydDCAB* transcription levels. These results indicate that in contrast to the cytochrome cbb_3-type oxidase in this organism, a *bd*-type oxidase is poorly expressed *in vitro* under microaerobic conditions and that *fnrN* regulator represses its expression [36].

In *Mycobacterium smegmatis*, *cydAB* and *cydDC* constitute two separate operons. Although *cydAB* is regulated by CRP and mutation of the identified CRP site abolishes *cydA-lacZ* expression in response to hypoxia, regulation of *cydDC* is CRP-independent and its regulation is not understood [37]. Also, all *cyd* genes in *Lactobacillus lactis*, which are required for the organism's haem-induced respiration-dependent mode of growth, appear to lack specific patterns of regulation [38].

2.4 Roles for CydDC in cytochrome assembly and haem metabolism

The expression of CydDC is clearly linked to the incorporation of haem cofactors into a variety of periplasmic cytochromes as well as the *bd*-type respiratory oxidases. Early genetic

studies and biochemical studies on *cydC* (Section 2.1) revealed that a *cydC* strain and its isogenic parent were each capable of catalysing the *in vitro* synthesis of the subunits encoded by *cydAB* genes encoding cytochrome *bd*-I. However, the levels of subunits I and II in membranes from a *cydC* strain were very low and no incorporation of the subunit into the membrane was observed in a *hemA* mutant unable to synthesise haem [5]. These low levels of subunits were unexpected given earlier work with a *hemA* mutant in which apocytochromes are incorporated into the membrane [39, 40]. It appeared that the CydAB subunits were absent when protoporphyrin IX synthesis is blocked. This issue was re-examined by Williams [41] who showed that subunit I of CydAB is clearly present after immunoblotting membranes from both a *cydD* mutant and its parent, albeit at slightly lower levels in the former. They also demonstrated that a *cydD* mutation did not prevent expression from a *cydA-lacZ* fusion, showing that CydDC is not obligatory for expression of the structural genes.

A possible role for CydDC is in haem *d* biosynthesis. Indeed, it has previously been suggested that export of the redox-active CydDC substrates may be required for the formation of the chlorin cofactor (i.e. haem *d*) in *bd*-type cytochromes [5, 42]. It is not known how the *d*-type haem in cytochrome *bd*-I is synthesised, although using hydroperoxidase catalase II from *E. coli* as a model it is likely to involve the oxidation of haem *b* to an oxoferryl species (FeIV=O) and formation of a porphyrin radical (compound I), followed by reduction to a ferric haem, hydroxylation of the C-ring, and cyclisation of the adjoining propionate [43]. It is conceivable that reducing power from GSH or cysteine may contribute to this process, although in the absence of insights into any enzymatic or autocatalytic mechanisms for incorporation of haem *d* into cytochrome *bd*-I it would be premature to speculate further on this topic.

Other links with haem metabolism and processing have, however, been established. First, the ATPase activity of purified CydDC is modulated by haem (see Section 3.2).

Second, CydD is required for assembly of periplasmic cytochrome *c*, as well as cytochrome *bd* in *E. coli*. Periplasmic fractions from anaerobically grown *E. coli* showed the clear spectral signature of cytochrome *c* (absorbance of the reduced form at 550 nm) in the wild-type strain but not the isogenic *cydD* mutant [44]. Since thioredoxin reductase is encoded by a gene (*trxB*) adjacent to *cydDC*, and TrxB is required for periplasmic cytochrome *c* assembly in certain bacteria, its role in cytochrome *c* formation in *E. coli* was checked, but cytochrome *c* levels in a *trxB cydD*$^{+}$ strain were at wild-type levels [44]. Finally, cytochrome b_{562} is not assembled in a *cydD* mutant [45].

Given the finding that both cytochromes *bd* and *c* are affected in a *cydD* mutant, a reasonable hypothesis is that the CydDC transporter might be involved in export of haems to the periplasm or outer membrane face. Transmembrane haem movements are likely to be slow in the absence of a transporter [46] but direct measurements of haem transport are frustrated by the self-aggregation of haem and non-specific interactions with cell components. We [47] used [^{14}C] haemin solubilised in alkali and in the presence of bovine serum albumin (BSA) as a high-affinity carrier. Since sequence homologies suggest that CydDC is an exporter in the intact cell, everted, energised membranes were used, which were hypothesised to take up haemin *in vitro*. In membranes from both *cydD* and wild-type strains, uptake of haemin (or its binding to membrane vesicles) were independent of the presence of ATP or an energising substrate (lactate) and thus do not support an ABC-driven uptake mechanism in cells. Chase experiments with unlabelled haemin-BSA failed to displace label suggesting that the labelled haem was either specifically bound to membrane protein or had entered the lumen of the vesicle [47].

During attempts to clone *cydDC,* candidate plasmids were assessed for their ability to restore assembly of a *bd*-type oxidase in a *cydD* mutant, and a variability in the effectiveness of the cloned genes to restore wild type oxidase levels was noted [12]. Moreover, in addition

to the elevated levels of cytochrome *d*, we noted an unusual and distinctive absorbance signal that appeared as a shoulder to the 560 nm peak of the *b*-type cytochromes. This species was variable in level and unstable. This component ('P-574' named for the visible absorbance maximum) was more evident in anaerobically grown cells, rendered cells reddish in colour, suggestive of the formation of haem compound(s), and was formed even when *bd*-type oxidase assembly was abolished by *cydAB* mutations [48]. The lability of P-574 was exploited to determine its spectrum in the absence of other stable haem compounds. The freeze-thaw-labile P-574 had distinctive maxima at 574 and 448 nm in reduced *minus* oxidised difference spectra. Despite showing that P-574 formation is dependent upon haem synthesis, its chemical nature and significance for CydDC function remain unclear [48]. The identity of P-574 remains unclear, but one possibility is that it represents the association of haem with over-expressed CydDC subunits, but not oxidase subunits.

This proposal has an interesting parallel with assembly of certain nitrous oxide reductase (Nos proteins) in bacteria such as *Paracoccus denitrificans*. Here, a tricistronic *nosD, -F, -Y* gene arrangement is thought to encode an ABC transporter complex. NosD is transported by the Sec system and NosD-L fusion proteins have been reported [49]. It is speculated that NosD acts as a scaffold for copper centre assembly, dependent on keeping the copper atom in a reduced state [50]. Might CydDC act as a scaffold for haem *d* or cytochrome *bd*-I assembly?

In summary, it seems likely that cytoplasmic ATP hydrolysis is used to drive an unknown periplasmic process, similar to the essential role of the Ccm complex in periplasmic cytochrome *c* biogenesis [51]. The precise role of CydDC requires further research.

3. Reductant export, kinetic modulation, and structural insights

3.1 Export of reduced glutathione and cysteine by CydDC

The demonstration that the periplasm of an *E. coli cydDC* knockout mutant is over-oxidising [45] provided the first robust evidence that this complex exported reductants. This finding was recently verified by demonstrating that loss of CydDC diminished the extracytoplasmic thiol content, and overexpression of CydDC decreased the reduced thiol pool in the cytoplasm [52]. These observations complemented earlier *in vitro* studies that demonstrated CydDC-mediated export of cysteine via import of ^{35}*S*-labelled cysteine into everted membrane vesicles prepared from wild type and *cydDC E. coli* strains [53]. Addition of the ATP transition state analogue sodium orthovanadate abolished transport of cysteine [53], providing good evidence that this process was energy dependent. Note that *E. coli* possesses other L-Cys exporters; an inducible L-Cys/L-Cys shuttle system plays an important part in oxidative stress tolerance through provision of a reducing equivalent to the periplasm [54].

Since substrate promiscuity is a well-known phenomenon amongst ABC transporters, it was hypothesised that CydDC might also transport the tripeptide glutathione (L-γ-glutamylcysteinylglycine, GSH), a major regulator of cellular redox poise [55]. Uptake of [^{35}S]-labelled GSH into everted membrane vesicles was shown to be dependent upon both CydDC and ATP, confirming this tripeptide as a substrate for CydDC. The transport rate for GSH by CydDC was five-fold higher than that of cysteine and, given the abundance of GSH in the bacterial cytoplasm, it is likely that GSH is the main substrate for CydDC. Later work reported that the addition of GSH and cysteine both stimulated the ATPase activity of purified CydDC [56], further supporting a role in reductant export. However, while robust evidence exists for GSH as a CydDC substrate, other data point to the complexity of the process. Eser (ref 42) examined the effects of mutating *cydD, ggt* (encoding periplasmic γ-

glutamyl transpeptidase) and *mdhA* (encoding a multidrug resistant-like ABC transporter) that might influence the concentration of periplasmic GSH pools. However, none of these mutations affected the ability of glutaredoxin 3 (GrxCp) to promote disulfide bond formation [57]. These data suggest the existence of more than one route for GSH export in *E. coli*.

Several studies link transmembrane GSH fluxes with extracellular oxidative stress. GSH efflux from *E. coli* is promoted by extracellular superoxide anion but this process is not dependent on CydDC [58]. Note that GSH plays crucial roles in oxidative stress protection [59] and so the export of this compound by CydDC is of interest.

3.2 Modulation of ATPase kinetics by reductants and haem

Over two decades ago, *cydDC* strains of *E. coli* were shown to accumulate a novel haem compound P-574 in the periplasm and membrane fractions [48] (Section 2.4). Together with the previously identified role in haem incorporation into cytochrome *bd*-I [5], this suggested that haem might be a substrate for CydDC export, although later experiments with everted vesicles could not demonstrate a haem export function for CydDC [47]. Later work subsequently showed that purified CydDC displayed an absorption peak at 410-412nm, and pyridine haemochrome analyses indicated the presence of a bound *b*-type haem with a CydDC:haem ratio of 5:1 [56]. This bound haem could be reduced and oxidised, and a CO ligand was shown to bind to the reduced CydDC:haem complex. Addition of both haemin and GSH/cysteine had synergistic stimulatory effects upon the ATPase activity of the complex, indicating a functional role for the haem cofactor. The purified CydDC complex showed a basal ATPase activity of 100 nmol Pi/min/mg protein, and addition of GSH and cysteine elicited a 3-4-fold increase in activity. The addition of up to 1 µM haemin elicited a 4-fold increase in ATPase rate, and co-incubation of 1 µM haemin and 5 mM GSH, for example, produced a synergistic rate stimulation of 8-fold.

In addition to GSH and cysteine, a variety of reduced thiol compounds were also shown to activate CydDC ATPase activity, including DTT, β-mercaptoethanol, homocysteine, and methionine. Control experiments with *S*-substituted/non-thiol analogues and protoporphyrin (i.e. haem lacking the central iron) did not elicit rate stimulation, and inclusion of non-thiol reductants actually decreased the ATPase rate. This strongly suggested the need for thiol groups or an iron-containing tetrapyrrole for rate stimulation to occur. One intriguing exception to this was that histidine elicited a 2-fold increase in ATPase rate, which was elevated to an eight-fold rate enhancement by inclusion of 1 μM haemin. Given that both histidine and reduced thiol compounds can act as axial ligands to haem, this might suggest that the haem-ligating capacity of reduced thiols is a contributory factor to the rate enhancements observed for GSH and cysteine in Yamashita *et al* [56].

3.3 Structural investigations into the CydDC complex

The CydDC complex was first purified in 2014 using a DDM-solubilisation approach to solubilise his-tagged CydDC for subsequent affinity chromatography [56]. This provided the purified sample that enabled the identification of bound haem (Section 3.2), with the purified protein having a deep brown colour. Subsequent gel-filtration of this sample resolved two main oligomeric states, with a larger complex eluting with a 26:1 ratio of CydDC:haem, and a smaller complex with a 5:1 ratio of CydDC:haem. This suggests that haem binding causes a disaggregation of CydDC.

Purified CydDC was reconstituted into *E. coli* lipids and two dimensional crystals were obtained for cryo-electron microscopy (cryo-EM) analyses. This work revealed arrays of dimeric units in ‘up’ and ‘down’ orientations, reflecting electron densities of CydDC heterodimers in the crystal lattice. With regard to the native oligomeric state in the membrane, it is worth considering that CydDC may associate with other membrane

complexes. Indeed, a study on *Francisella tularensis* membranes has reported that the CydC subunit co-migrates with the CydA and CydB subunits of cytochrome *bd*-I using Blue Native SDS-PAGE [60], and the authors suggest that this provides evidence to support the existence of a 235 kDa CydABCD complex. This remains the only evidence for such a complex, and biochemical and structural work in this area will hopefully reveal further insights. While cryo-EM and Blue Native PAGE have provided useful insights into the overall structure of the CydDC complex and potential interaction partners, a crystal structure remains elusive, so herein we call upon structural modelling approaches to discuss the role of individual residues in catalysis and cofactor binding.

Early random mutagenesis studies on cytochrome *bd*-I yielded the first mutant allele of CydD, named *cydD1*, that was suggested to be non-functional due to an inability to support the assembly of the cytochrome *bd*-I respiratory oxidase [9]. The two point mutations in *cydD1* were later identified as G319D and G429E [27], and the crystal structure of the lipid A flippase MsbA from *Vibrio cholera* [61], a close structural homologue of CydD, was used to provide structural insights into why mutation of residues in the Walker A and B motifs in CydD resulted in loss of function [27]. The G429 residue was found to be close to a conserved aspartate, so it was postulated that substitution for a glutamate (also positively charged) could cause structural disruptions in the Walker A motif. The G319 residue was found to be buried at the bottom of the hydrophobic pocket, and it was postulated that mutation of this residue may impact upon conformational changes during the catalytic cycle. Indeed, dramatic conformational changes are key to catalytic mechanism of ABC transporters, and the CydDC complex has only previously been modelled in an ‘outward open’ conformation [62]. Hence, it was of interest to investigate structural models for CydDC in different conformations.

The alternating access model for the transport of substrates across membranes by transporter complexes [63] describes a mechanism involving initial formation of a substrate:transporter complex, which then triggers a conformational change within the transporter that exposes the substrate binding site at the other side of the membrane. The bound substrate is then able to dissociate from the transporter complex into the second environment in a controlled fashion, never freely permitting substrate movement through the membrane, as allowed through channel proteins. It was proposed that there are three conformational states including 'inwardly open', a 'midpoint' whereby the binding site is unable to access or interact with either side of the membrane, and finally an 'outward open' state that permits substrate release from the binding pocket [64]. Hence, it was of interest to investigate CydDC in different conformational states using modelling approaches.

The CydDC complex has been modelled previously [62] using the SAV1866 multidrug ABC transporter from *Staphylococcus aureus* (PDBid = 2HYD, [65]) as a template. However, with this approach it was possible to model only an outwardly open conformation. Hence, in an attempt to model multiple conformations, polypeptide sequences for CydC and CydD were submitted to the Phyre2 server [66] for structural modelling, and an outward open conformation was produced using the human ABCB8 transporter (PDBid = 5OCH, chain H) that has a slightly higher % sequence identity with CydD (23%) compared to 2HYD (22%). The model for CydD was superposed onto chain H (of 5OCH) and the model for CydC was superposed onto chain G (of 5OCH), and bound ADP from the 5OCH structure was shown to fit into the nucleotide-binding domains (NBDs) of the CydDC model adjacent to the Walker A motifs (Figure 2A, left hand panel). Using a similar approach, a structural model for an inward-open conformation was also generated using the TM287/TM288 transporter from *Thermotoga maritima* (3QF4) as a template [67] (Figure 2A, right hand panel). One of the most important structural questions to be asked pertains to where haem

may interact with CydDC. Since this bound cofactor has the spectral characteristics of a *b*-type haem [56], and is therefore ligated by a histidine residue, it was of interest to identify potential ligands on the CydDC models. Hence, all histidine sidechains in CydDC are shown in Figure 2A and key residues in the periplasm and nucleotide-binding domains are labelled in the right-hand model.

It has previously been postulated that His51 on CydD may be a potential ligand for haem due to the periplasmic location (for a potential redox-sensing role) and the favourable hydrostatic environment for haem binding [62]. However, recent work in the Shepherd lab has demonstrated that purified CydDC H51A^{CydD} still retains a significant amount of bound haem [68], discounting the involvement of this residue in the axial ligation of haem. There is, however, another periplasmic histidine in CydD at position 284 (Figure 2A) that remains a potential ligand to participate in a periplasmic redox sensing role. However, the jury is still out on the location of haem ligation, as the full-length CydDC sequences contain 22 fully-conserved histidine residues across the four closest homologues (*E. coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Shigella sonnei*, *Citrobacter portucolensis*). Hence, all the histidine residues shown on Figure 2A are conserved and are therefore potential haem ligands, which will lead to an exciting mutagenesis study in future. Given the profound effects that haem binding has upon ATP hydrolysis [56], the location of histidine residues in the NBDs have been annotated in detail on Figure 2B, which may allow a productive focus in the first instance. Aside from the residues H542^{CydD} and H531^{CydC}, which are part of the conserved H-loops involved in ATP hydrolysis, the remaining histidine residues annotated in Figure 2B seem like plausible candidates for haem ligation.

4. Physiological impact of CydDC

4.1 Protein disulfide folding

It has been hypothesised that the pleiotropic phenotype of *cydDC* strains results largely from the disruption of disulfide folding in the periplasm [55]. Indeed, sensitivity of the *cydDC* mutant to benzylpenicillin was attributed to misfolding of the disulfide-containing Penicillin-Binding Protein 4, and lack of motility was suggested to result from a defective P-ring motor protein (also disulfide-containing) [53], especially since exogenous addition of cystine (oxidised cysteine) had previously been shown to correct a motility defect in a *dsbB* mutant [69]. Complementation of *cydDC* strains with exogenous reductant largely complemented those phenotypes that are associated with defective disulfide folding [55].

Disulfide bond formation plays a critical role in the stability of numerous proteins, especially those that are secreted (for an overview, see[70]). In the bacterial periplasm, there are two main pathways for disulfide bond formation: disulfide oxidation and disulfide isomerisation. Disulfide oxidation is catalysed by the DsbAB system [71] and disulfide isomerisation is catalysed by the DsbCD system [72]. It has previously been hypothesised that reduced thiol-containing CydDC substrates can contribute to the reduction of protein disulfides [53, 55], and that electrons evolved during subsequent DsbAB-mediated disulfide oxidation can be channelled into the terminal oxidases of the respiratory chain [73]. This is particularly interesting as loss of CydDC function was first described to abolish the assembly of the terminal oxidase cytochrome *bd*-I [5], and exogenous addition of the CydDC-substrates GSH and cysteine was later shown to be restore haem incorporation into cytochrome *bd*-I in a *cydD* strain [52]. Together, these observations indicate that CydDC-mediated respiratory oxidase assembly can provide an electron sink for Dsb-mediated disulfide oxidation, and the CydDC substrates GSH and cysteine may also have a more direct role in disulfide isomerisation (reviewed in [74]).

4.2 Roles of CydDC and cytochrome *bd* in virulence

Since *cydDC* mutants do not synthesise cytochrome *bd*, and both *cydDC* and *cydAB* mutants display diverse and sometimes overlapping phenotypes, it is important but difficult to differentiate between oxidase deficiency *per se* and the additional defects in *cydDC* mutants. These problems are discussed in [29]. Thus, while all *cydAB* mutants (to our knowledge) lack spectroscopically detectable cytochrome *bd*, fail to survive as robustly as wild-type cells in stationary phase [6, 7], and are sensitive to various inhibitors (cyanide, azide, Zn(II) ions) and oxidative stress, *cydDC* mutants additionally show low levels of other cytochromes, particularly periplasmic haemproteins, and have a more oxidised periplasm.

As discussed in Section 3.1, *cydDC* mutants fail to export glutathione and cysteine to the periplasm and this results in a more oxidised periplasm. However, what is not clear is why *bd*-type oxidase assembly is inhibited by these defects. Among plausible hypotheses are the following:

- CydDC exports to the periplasm haem(s) to be immediately assembled onto outward-facing CydAB subunit regions (although we have failed to detect bulk transmembrane movements of haem; [47]). However, the finding that haem stimulates the ATPase activity of purified CydDC as well as reductants is intriguing (Section 3.2).
- CydDC positions haems at periplasmic sites such as chaperones for oxidase assembly.
- The oxidised periplasm is incompatible with haem-handling and –assembling processes.
- CydDC exports to the periplasm components thus far unidentified, in addition to the reductants glutathione and cysteine.

It has been frequently reported that the presence of *bd*-type oxidases is correlated with bacterial virulence. For example, growth of *Mycobacterium* sp. at low oxygen tensions thought to enhance expression of a *bd*-type oxidase also enhances invasion [75]. Furthermore, a positive correlation has been shown between *bd*-type oxidase expression and *Shigella*

flexneri virulence [76]. Because *cydDC* mutants share all phenotypes of *cydAB* mutants, but have additional defects, the abrogation of bacterial virulence and pathogenicity evident in *cydAB* mutants would be expected to be shared by *cydDC* mutants, although this has been less frequently tested. Mutations in *aarD*, a *cydD* homologue in *Providencia stuartii*, caused a 32-fold increase in resistance to gentamycin [77]. Interestingly, mutations in *aarE*, a *ubiA* homologue and thus involved in respiratory ubiquinone biosynthesis, also led to high-level aminoglycoside resistance [78] consistent with the requirement for respiratory chain function in aminoglycoside uptake [79]. A *Brucella abortus cydC* mutant created by a Tn*5* transposon insertion was virtually incapable of intracellular replication in murine macrophages and a HeLa cell line and also exhibited impaired virulence in BALB/c mice [80]. A further study of a virulent strain used unmarked knockout mutants in *cydD* and *cydC*. Mutation in either gene caused sensitivity to metal ions, oxidative stress and low pH and, significantly, a growth defect in RAW264.7 cells and attenuation in mice [81]. Both *cydC* and *cydD* mutants exhibited significant attenuation of virulence when assayed in murine macrophages or in BALB/c mice and were readily cleared from spleens by 4 weeks post-inoculation. Inoculated mice showed no splenomegaly, which indicates that the mutants are highly attenuated [82]. In contrast, a *cydC* mutant of *Moraxella catarrhalis*, while being defective in growth rates and oxidative stress tolerance, did not display a difference in a murine pulmonary clearance model . The mutation in the mutant studied may however merely alter transcription levels of *cydC* [83].

4.3 Perturbations resulting from loss of CydDC

The loss of a functional CydDC transporter yields a pleiotropic phenotype, including loss of haem insertion in *bd*-type cytochromes as described earlier [5, 9, 41], and periplasmic cytochromes *c* and b_{562} are also less abundant in *cydDC* mutants [44, 45]. In addition, the

periplasm of cells lacking CydDC were found to be over-oxidised [8], consistent with a later study where CydDC overexpression diminished the cytoplasmic reduced thiol content [52], although the cytoplasm of cells lacking CydDC were not 'over-reducing'. Strains lacking CydDC have also been shown to exhibit sensitivity to benzylpenicillin and loss of motility [53], which was hypothesised to be due to defective disulfide folding of penicillin binding protein 4 and the P-ring flagellar protein, respectively.

To further characterise the pleiotropic *cydDC* phenotype, transcriptomics was used to demonstrate a dramatic up-regulation of a range of transcripts in a *cydD* strain involved in protein degradation pathways, protein chaperones, β-oxidation of fatty acids, and transcripts involved in nitrate/nitrite reduction [52]. The upregulation of protein degradation pathways and fatty acid β-oxidation were proposed to be involved in the re-balancing of metabolic flux into the Krebs cycle through elevation of succinyl CoA and pyruvate production, respectively. The up-regulation of nitrate reductase *nap* genes in the *cydD* strain was also interesting, as these systems are usually induced only during anaerobic conditions. However, the expression of CydDC has previously been shown to be induced by nitrate/nitrate under the control of the transcription factors NarL, NarP, and FNR [30], and in a *cydD* mutant that cannot assemble *bd*-type oxidases it seems logical that the Nap system is up-regulated to compensate for an inability to oxidise reduced ubiquinol via *bd*-type respiratory oxidases.

4.4 Tolerance to nitric oxide

CydDC has recently been shown to protect *E. coli* against nitric oxide (NO) [52], a toxic free radical produced by the host immune system in response to bacterial infection. Since CydDC expression is required for the assembly of cytochrome *bd*-I [5, 9], which was known to promote growth and survival in the presence of NO [52, 84], it was anticipated that CydDC expression would promote NO tolerance indirectly via facilitating cytochrome *bd*-I assembly.

In addition, as the reduced thiols that are exported by CydDC may react with incoming NO (to form *S*-nitrosothiols), it was hypothesised that CydDC would provide protection from NO beyond the involvement of cytochrome *bd*-I. This hypothesis was tested via growth of knockout strains in the presence of NO-donor compounds, and CydDC was confirmed to provide protection against NO beyond the contribution to assembly of cytochrome *bd*-I. This has led to the generation of a model for the role of CydDC in NO tolerance, as well as disulphide folding and respiratory oxidase assembly (Figure 3). These insights highlight the potential importance of CydDC-mediated reductant export in stress tolerance of pathogenic *E. coli* strains during infection.

5. Conclusions

The CydDC ABC-type transporter of *Escherichia coli* was the first heterodimeric exporter of this class to be described in prokaryotes. The *cydDC* genes are widely distributed in bacteria but their precise functions are still unresolved. The genes are sometimes found in an operon with *cydABX* that encodes a *bd*-type terminal oxidase and this, coupled with the loss of *bd*-type oxidase assembly and function in CydDC mutants, identifies CydDC as crucial to this respiratory complex. Current hypotheses favour the view that the CydDC system transports reducing molecules to the periplasm, notably glutathione and cysteine; these, together with other reduced thiol compounds, also stimulate the ATPase activity of the isolated CydDC complex. We lack unambiguous evidence that CydDC may transport haem outwards for periplasmic cytochrome assembly, but there are tantalising clues to suggest a direct involvement of haem with CydDC function. Given the importance of cytochrome *bd*-I assembly, and of direct CydDC-catalysed reactions with nitric oxide, for bacterial virulence and pathogenesis, future studies should aim to elucidate fully the function of CydDC.

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Figure Legends

Figure 1 – Membrane topology modelling for CydD and CydC.

Membrane topology models for CydD (A) and CydC (B) were produced using the Protter online tool [85]. Transmembrane helices are numbered sequentially with Roman numerals, and the conserved motifs in the nucleotide-binding domains are colour coded and annotated on the figure.

Figure 2 – Structural modelling of the CydDC complex.

Polypeptide sequences for CydC and CydD were submitted to the Phyre2 server [66], and a structural model in an 'outward open' conformation (left hand side) was produced using the human ABCB8 transporter (PDBid = 5OCH, chain H) as a template. Using a similar approach, a structural model for an 'inward-open' conformation was also generated using the TM287/TM288 transporter from *Thermotoga maritima* (3QF4) as a template [67] (right hand side). All histidine sidechains are shown (they are potential ligands to haem – shown in centre of the figure panel) and key residues in the periplasm and nucleotide-binding domains are labelled in the right-hand panel. Bound ADP is shown as spheres and Walker A motifs are shown in red. B) The nucleotide-binding domain of the inward open model from panel A is shown, with conserved motifs highlighted in colour and annotated in the key. Abbreviations: ICLs, intracytoplasmic loops; NBDs, nucleotide-binding domains; TMDs, transmembrane domains.

Figure 3 – Model for the multiple roles for the CydDC complex.

CydDC activity is required for cofactor assembly in *bd*-type oxidases (red), which is probably mediated by the reduced substrates (GHS and cycteine) as their exogenous addition can restore *bd*-type oxidase assembly [52], although direct delivery of haem from CydDC cannot yet be ruled out (red dashed line). Haem cofactors are shown as red rhomboids with black dots. CydDC provides additional protection against NO beyond that provided by cytochrome

bd-I, and it is hypothesised that the reduced substrates form *S*-nitroso adducts (R-S-N=O) that sequester NO and diminish entry into the cytoplasm (blue). It is hypothesised that the reduced substrates may control gating of the CydDC complex through reduction of bound haem cofactors (cyan). Finally, has been hypothesised that CydDC provides a role in disulphide assembly [53] (purple), chiefly because loss of *cydDC* genes affects phenotypes that rely upon disulphide assembly (e.g. motility, penicillin sensitivity).

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Figure 1

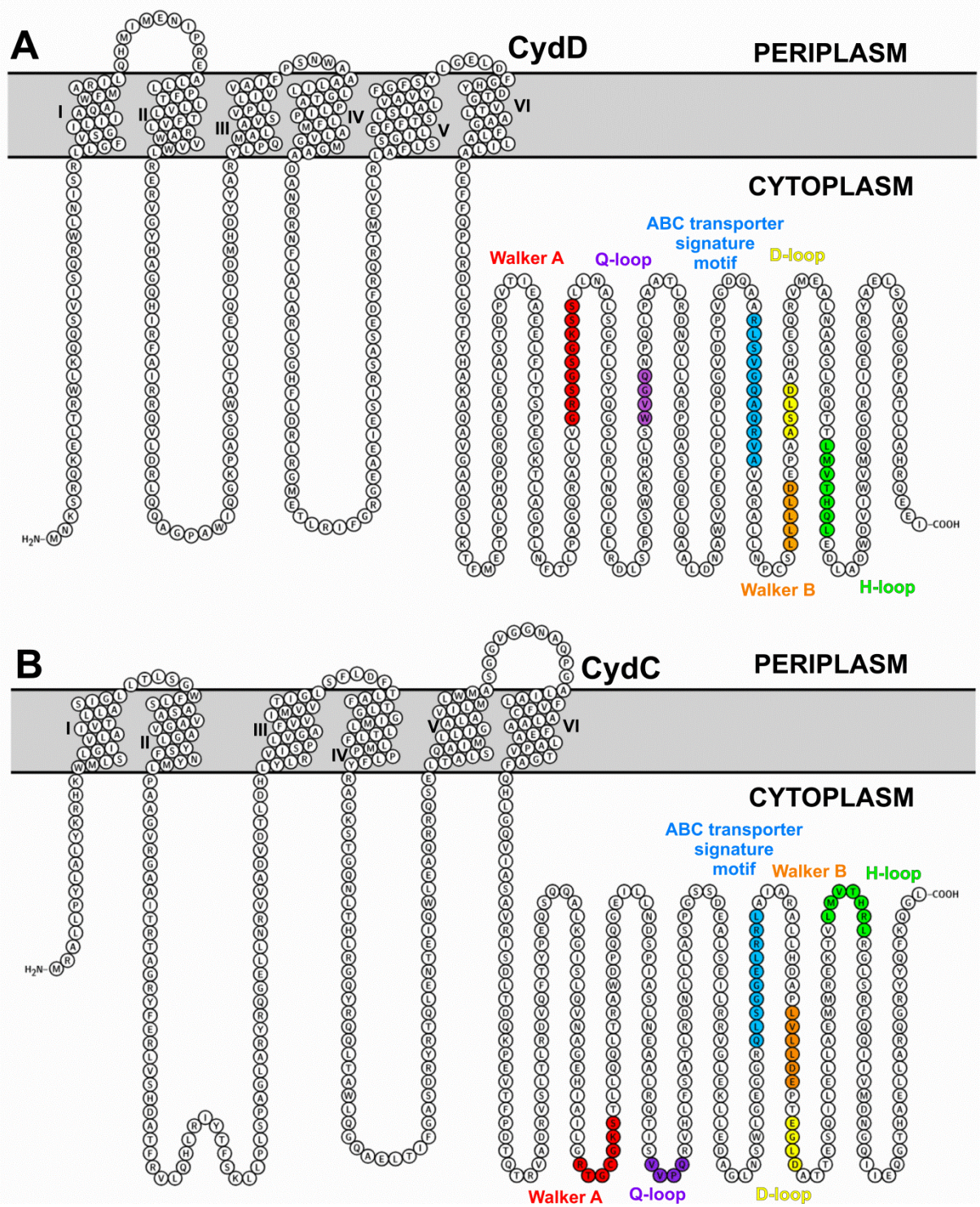

A
CydD
PERIPLASM
CYTOPLASM
I
II
III
IV
V
VI
ABC transporter signature motif
Walker A
Q-loop
D-loop
Walker B
H-loop
H2N-
-COOH
B
CydC
PERIPLASM
CYTOPLASM
I
II
III
IV
V
VI
ABC transporter signature motif
Walker B
H-loop
Walker A
Q-loop
D-loop
H2N-
-COOH

Figure 2

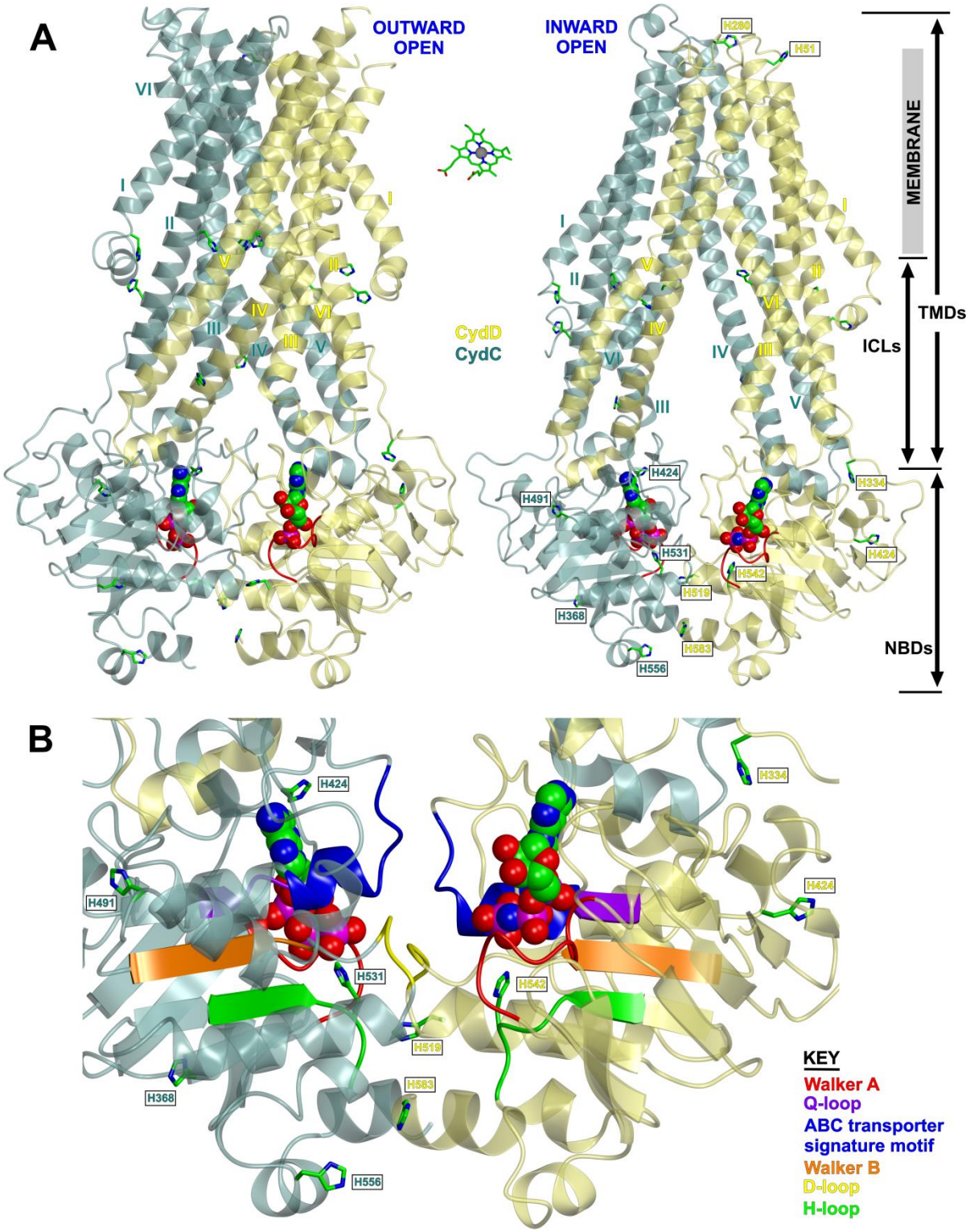
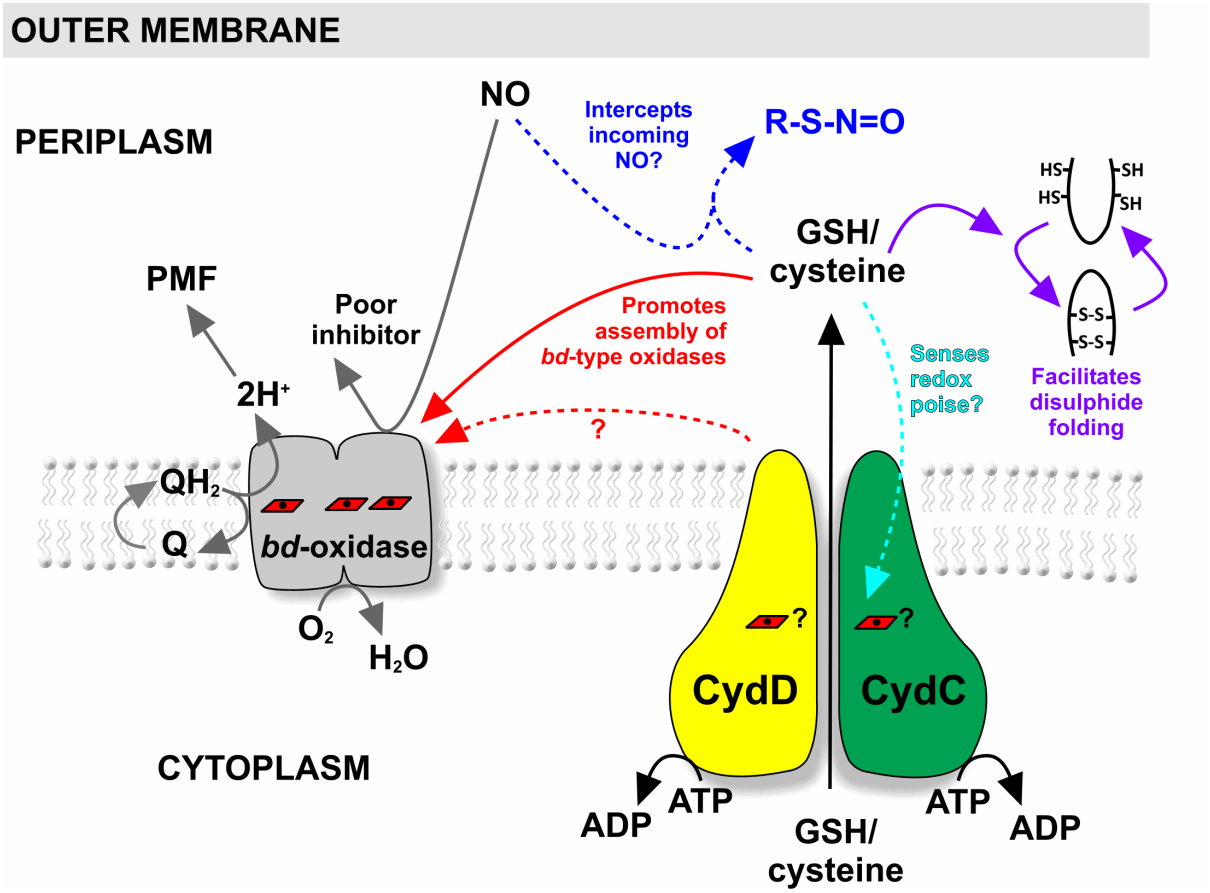

A
OUTWARD OPEN
INWARD OPEN
MEMBRANE
TMDs
ICLs
NBDs
CydD
CydC
H200
H51
H424
H491
H531
H542
H519
H368
H583
H556
H334
B
KEY
Walker A
Q-loop
ABC transporter signature motif
Walker B
D-loop
H-loop

Figure 3


OUTER MEMBRANE
PERIPLASM
NO
Intercepts incoming NO?
R-S-N=O
GSH/ cysteine
HS
HS
SH
SH
S-S
S-S
Facilitates disulphide folding
PMF
2H+
Poor inhibitor
Promotes assembly of bd-type oxidases
?
Senses redox poise?
QH2
Q
bd-oxidase
O2
H2O
?
?
CydD
CydC
CYTOPLASM
ADP
ATP
GSH/ cysteine
ATP
ADP