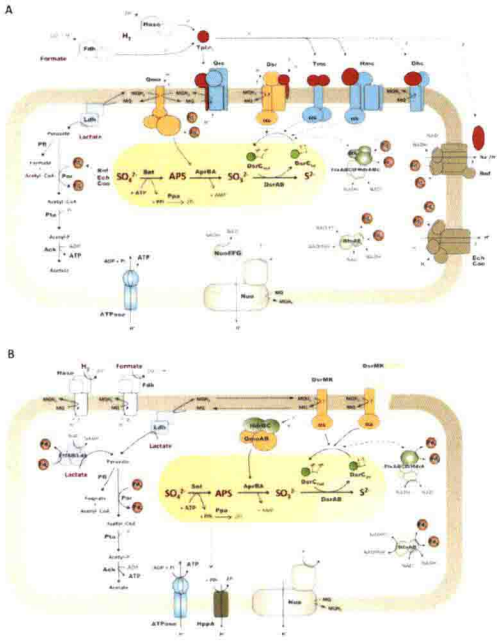
Advances in MICROBIAL PHYSIOLOGY

EDITED BY ROBERT K. POOLE



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ADVANCES IN MICROBIAL PHYSIOLOGY

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CONTENTS

CHAPTER ONE

The CydDC Family of Transporters and Their Roles in Oxidase Assembly and Homeostasis

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Abstract

The CydDC complex of *Escherichia coli* is a heterodimeric ATP-binding cassette type transporter (ABC transporter) that exports the thiol-containing redox-active molecules cysteine and glutathione. These reductants are thought to aid redox homeostasis of the periplasm, permitting correct disulphide folding of periplasmic and secreted proteins. Loss of CydDC results in the periplasm becoming more oxidising and abolishes the assembly of functional *bd*-type respiratory oxidases that couple the oxidation of ubiquinol to the reduction of oxygen to water. In addition, CydDC-mediated redox control is important for haem ligation during cytochrome *c* assembly. Given the diverse roles for CydDC in redox homeostasis, respiratory metabolism and the maturation of virulence factors, this ABC transporter is an intriguing system for researchers interested in both the physiology of redox perturbations and the role of low-molecular-weight thiols during infection.

ABBREVIATIONS

ABC transporter ATP-binding cassette type transporter
Ccm cytochrome *c* maturation
FNR fumarate and nitrate reductase
GSH reduced glutathione
GSSG glutathione disulphide
H_2O_2 hydrogen peroxide
NO nitric oxide
O_2^- superoxide
$ONOO^-$ peroxynitrite
PMF proton motive force
ROS reactive oxygen species
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis
UPEC uropathogenic *E. coli*
Δp_{Na}^+ transmembrane sodium potential
$\Delta\mu H^+$ transmembrane proton potential
$\Delta\Psi$ electrical potential

1. OVERVIEW

The bacterial periplasm is an oxidising environment that is suitable for the formation of disulphide bonds in periplasmic and secreted proteins, a process that does not occur in the more reducing location of the cytoplasm. As well as the role of CydDC in disulphide folding, of particular interest, is the requirement of this transporter for the correct assembly of various respiratory complexes, including periplasmic *b*- and *c*-type cytochromes and the

bd-type terminal oxidase complexes. This review will focus on the respiratory complexes that rely upon CydDC activity for their assembly, the structure and function of the CydDC ATP-binding cassette type transporter (ABC transporter) and the role of CydDC in general bacterial physiology and pathogenicity.

2. THE *ESCHERICHIA COLI* TERMINAL OXIDASES: ASSEMBLY AND FUNCTION

2.1 Cytochrome *bo′*

The *bo′*- and *bd*-type oxidases of *E. coli* both catalyse the two-electron oxidation of ubiquinol by molecular oxygen within the cytoplasmic membrane, concomitantly generating a proton gradient across the membrane that can be utilised by bacterial cells to produce ATP for use as an energy source. In addition to generating a proton gradient via the vectorial translocation of protons that is linked to quinol reduction and oxidation, cytochrome *bo′* is also able to directly pump protons across the membrane (Puustinen, Finel, Haltia, Gennis, & Wikstrom, 1991) and has an H^+:e^- ratio of 2. The site of oxygen reduction in cytochrome *bo′* is a haem–copper binuclear oxygen-reactive centre, making it a member of the haem–copper superfamily of terminal oxidases (Anraku, 1988) that has been extensively studied.

The *cyoABCDE* genes encode subunits I, II, III and IV of the cytochrome *bo′* complex and a protohaem farnesyltransferase (haem *o* synthase), respectively (Minghetti et al., 1992; Saiki, Mogi, & Anraku, 1992). The assembly pathway for cytochrome *bo′* is an ordered process wherein subunits III and IV assemble first, followed by subunit I and finally subunit II (Stenberg, von Heijne, & Daley, 2007). CyoABC is homologous to the core subunits of the aa_3-type cytochrome *c* oxidase (Lemieux, Calhoun, Thomas, Ingledew, & Gennis, 1992) both in terms of their primary sequence (Cotter, Chepuri, Gennis, & Gunsalus, 1990; Saraste, Sibbalda, & Wittinghoferb, 1988) and structure (Abramson et al., 2000; Gohlke, Warne, & Saraste, 1997). A crystal structure of the entire cytochrome *bo′* terminal oxidase complex has been determined at 3.5 Å resolution (Abramson et al., 2000) that reveals 25 transmembrane helices with a ubiquinone-binding site within the membrane domain of subunit I (Abramson et al., 2000). The entire complement of redox centres reside within subunit I, the largest of the four subunits. A low-spin haem *b* associated with a copper ion (Cu_B) (Puustinen & Wikstrom, 1991; Puustinen et al., 1991) is thought to act as an electron donor to reduce a binuclear centre composed of a high-spin haem *o* and

another copper ion (Cu_B): this is where oxygen reduction takes place (Salerno, Bolgiano, Poole, Gennis, & Ingledew, 1990).

2.2 Cytochrome *bd*-I

The first description of a spectrally distinctive haem protein in bacteria, with an absorbance in the reduced state near 630 nm was reported by Yaoi, Tamiya, Negelein and Gerischer in the 1920s and 1930s in *E. coli* and *Azotobacter* (for a fascinating historical overview, see Keilin, 1966). Other authors later confirmed the existence of such a pigment in numerous bacteria, but its identification as an oxidase was made only when Chance and colleagues applied Warburg's photochemical action spectrum method (Castor & Chance, 1959). The oxidase was called cytochrome a_2, to underline its distinction from cytochrome a_1 (absorbance in the reduced state near 590–595 nm) and cytochromes *a* and a_3 (absorbance in the reduced state near 600–605 nm), the last two being the CO-unreactive and -reactive components, respectively, of cytochrome *c* oxidase in mitochondria and certain bacteria. These spectral characteristics were later attributed to the terminal respiratory oxidase cytochrome *bd* in *E. coli*, which was later renamed to cytochrome *bd*-I following the discovery of another *bd*-type oxidase in this organism (Section 2.3).

Cytochrome *bd*-I is confined to the prokaryotic world and is well characterised in *E. coli*. Unlike cytochrome *bo*′, cytochrome *bd*-I does not contain copper and so is not a member of the haem–copper oxidase superfamily. Instead, cytochrome *bd*-I utilises an unusual di-haem oxygen-reactive site (Borisov et al., 2013; Junemann, 1997; Rothery & Ingledew, 1989). All known members of the *bd*-family use quinol as a substrate, receiving electrons commonly from either ubiquinol or menaquinol. With a three-dimensional structure yet to be elucidated, existing data show that cytochrome *bd*-I is a trimer of three membrane polypeptides, subunits I (CydA) and II (CydB) (Kita, Konishi, & Anraku, 1984) and CydX (Van Orsdel et al., 2013). Three haems are associated with the oxidase in a 1:1:1 stoichiometry peroxidase complex. Two high-spin haems (*d* and b_{595}) are thought to form the di-haem active site where oxygen is reduced (Arutyunyan et al., 2008; Borisov & Verkhovsky, 2013; Hill, Alben, & Gennis, 1993; Rappaport, Zhang, Vos, Gennis, & Borisov, 2010; Vos, Borisov, Liebl, Martin, & Konstantinov, 2000), and modelling of the excitonic interaction between haems *d* and b_{595} has led to an estimated intermolecular distance of 10 Å (Arutyunyan et al., 2008). Despite the

proximity and evidence for functional cooperation (Vos et al., 2000), doubt has been cast upon the existence of a di-haem active site as no spin coupling has been observed between the haems (Junemann, 1997). The third haem cofactor is a hexacoordinate low-spin haem b_{558} located within subunit I that is responsible for quinol oxidation that supplies electrons to the di-haem site for the reduction of molecular oxygen to water.

Cytochrome *bd*-I contributes to the proton motive force (PMF) via the vectorial translocation of protons that is linked to quinol reduction and oxidation (Calhoun, Oden, Gennis, Demattos, & Neijssel, 1993), but unlike cytochrome *bo′* is unable to directly pump protons (Puustinen et al., 1991) and is therefore considered less efficient in bioenergetic terms (H^+:e^- ratio = 1). Despite having a reduced contribution to the PMF, cytochrome *bd*-I does facilitate aerobic respiration under conditions of low oxygen due to a very high affinity for oxygen: *bd*-I has a K_m of 3 ± 8 nM (D'mello, Hill, & Poole, 1996) compared to a K_m of 0.016–0.35 μM for cytochrome *bo′* (D'mello, Hill, & Poole, 1995). It is likely that the di-haem active site plays a role in this high affinity for oxygen (Borisov et al., 2002), promoting growth in microaerobic environments.

2.2.1 *The* cydABX *Genes*

Until recently, cytochrome *bd*-I was generally believed to be comprised of two subunits encoded by *cydA* (subunit I: 57 kDa) and *cydB* (subunit II: 43 kDa) (Calhoun, Newton, & Gennis, 1991; Green et al., 1988; Kranz & Gennis, 1983) located at 16.6 min on the *E. coli* genetic map (Bachmann, 1990; Calhoun et al., 1991). The molecular weights of subunits I and II determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Miller & Gennis, 1983) are consistent with predicted masses calculated from the protein sequences (Green et al., 1988).

Two open-reading frames, *ybgE* and *ybgT* are found at the 3′ end of the *cydAB* genes which together are thought to form an operon; *cydABET* (Fig. 1; Muller & Webster, 1997). The 4-kDa YbgT protein was shown to co-purify with CydAB and is believed to be a part of the complex (Van Orsdel et al., 2013), and *ybgT* has since been renamed *cydX*. Cells

Figure 1 The *cydABX* locus of *E. coli*. The cytochrome *bd*-I terminal oxidase is encoded by the *cydA-X* genes, but no role in cytochrome *bd*-I assembly/function has been identified for *ybgE*.

lacking CydX exhibit diminished oxidase activity which can be restored by the addition of *cydX* on a plasmid (Van Orsdel et al., 2013) and silver staining shows that CydX is present in stoichiometric amounts with CydA and CydB (Hoeser, Hong, Gehmann, Gennis, & Friedrich, 2014), confirming CydX as a third subunit of cytochrome *bd*-I. CydX is required for either the insertion or the stability of haem *d* and b_{595} thought to make up the di-haem active site of cytochrome *bd*-I; UV–visible difference spectroscopy revealed that the signals of haem *d* and b_{595} are lost when CydA and CydB are not accompanied by CydX. However, no role in cytochrome *bd*-I assembly/function has been identified for *ybgE*, with its deletion having no effect on phenotypes typically observed for *cydA* or *cydB* mutants (Van Orsdel et al., 2013).

Beyond *cydABX*, two additional genes *cydC* and *cydD* together form an operon to encode a heterodimeric ABC transporter. This transporter is essential for assembly of functional cytochrome *bd*-I (Bebbington & Williams, 1993; Georgiou, Hong, & Gennis, 1987; Poole, Gibson, & Wu, 1994; Poole et al., 1993). In its absence, CydA and CydB are still synthesised and inserted into the membrane, but the oxidase lacks haem groups essential for function (Georgiou et al., 1987). CydDC exports two low-molecular-weight thiols, glutathione and cysteine, from the cytoplasm to the periplasmic space (Pittman, Robinson, & Poole, 2005; Pittman et al., 2002). However, exogenous addition of either of these thiols to a strain lacking CydDC does not restore cytochrome *bd*-I assembly, so the molecular mechanism via which CydDC contributes to cytochrome *bd*-I assembly remains unclear.

2.2.2 Spectral Characteristics

Cytochromes display a variety of spectral characteristics that depend upon the local environment of the haem-binding site within the protein as well as the structural attributes of the haem cofactor itself. Reduced/ferrous cytochromes produce three main absorbance peaks in the visible UV spectrum termed the α, β and γ (or Soret) peaks, and the wavelength of the α-region is usually used to classify these cofactors. The α- and β-bands of the reduced haem b_{558} of cytochrome *bd*-I exhibit maxima at 560–562 and 531–532 nm, respectively (Bloch, Borisov, Mogi, & Verkhovsky, 2009; Koland, Miller, & Gennis, 1984; Lorence, Koland, & Gennis, 1986). Reduced haem *d* of cytochrome *bd*-I in whole cells has an α-band peak at 628–630 nm. However, due to a high oxygen affinity, the *d*-type haem usually exists in the stable oxygenated ferrous form, which is characterised by an absorption band with a maximum at 647–650 nm in the absolute absorption spectrum (Poole,

Kumar, Salmon, & Chance, 1983). The high-spin haem b_{595} was first annotated as cytochrome a_1 due to a similarity with the absorbance characteristics of cytochrome a_1 of other bacteria (Castor & Chance, 1959). Reduced *minus* oxidised difference spectra later showed an α-band at 595 nm, a strong β-band near 560 nm and a trough near 645 nm. This difference spectrum was similar to that of protohaem IX (haem *b*) from cytochrome *c* peroxidase, suggesting the presence of an additional *b*-type haem within cytochrome *bd*-I, which explains why the a_1 cofactor was renamed to cytochrome b_{595} (Lorence et al., 1986).

Cytochrome *bd*-I has a complex Soret region with a contribution from all three haems. In reduced *minus* oxidised difference spectra, the Soret band spectra for haem b_{558} has a maximum and minimum of 429.5 and 413 nm, respectively, haem b_{595} has a maximum and minimum of 439 and 400 nm, respectively (Vos et al., 2000), and haem *d* exhibits maxima and minima at 430 and 405 nm, respectively. The spectral contribution of haem *d* to the Soret band is smaller than its contribution in the α-band and smaller than the contribution of either of the *b*-type haems.

2.2.3 Membrane Topology and Cofactor Binding

Despite the absence of an X-ray structure of cytochrome *bd*-I, it is known that all three subunits of cytochrome *bd*-I are integral membrane proteins. Secondary structure prediction models suggest that CydA contains nine membrane-spanning helices, CydB contains eight membrane-spanning helices (Osborne & Gennis, 1999) and CydX consists of just one membrane-spanning helix (Fig. 2). A large periplasmic loop between helices six and seven of subunit I is involved in quinol binding and is consequently known as the Q loop (Dueweke & Gennis, 1991; Matsumoto et al., 2006; Mogi et al., 2006). Some cytochrome *bd*-I oxidases such as those found in *E. coli* and *Azotobacter vinelandii* contain an insert in the C-terminus of the Q loop, but in a majority of *bd*-type oxidases this insert is not present (Osborne & Gennis, 1999; Sakamoto et al., 1999). As of yet, the significance of this insertion is unclear. Site-directed mutagenesis has revealed that two residues within the Q loop, Lysine-252 and Glutamate-257 are required for cytochrome *bd*-I oxidase activity (Mogi et al., 2006) and are thought to play a role in quinol binding. Shifts in 'reduced *minus* oxidised' spectra following Glu257 mutation indicate a close proximity of this residue to haem b_{558}, suggesting that Glu257 not only binds quinols but also participates in electron transfer from the quinol to haem b_{558} (Mogi et al., 2006).

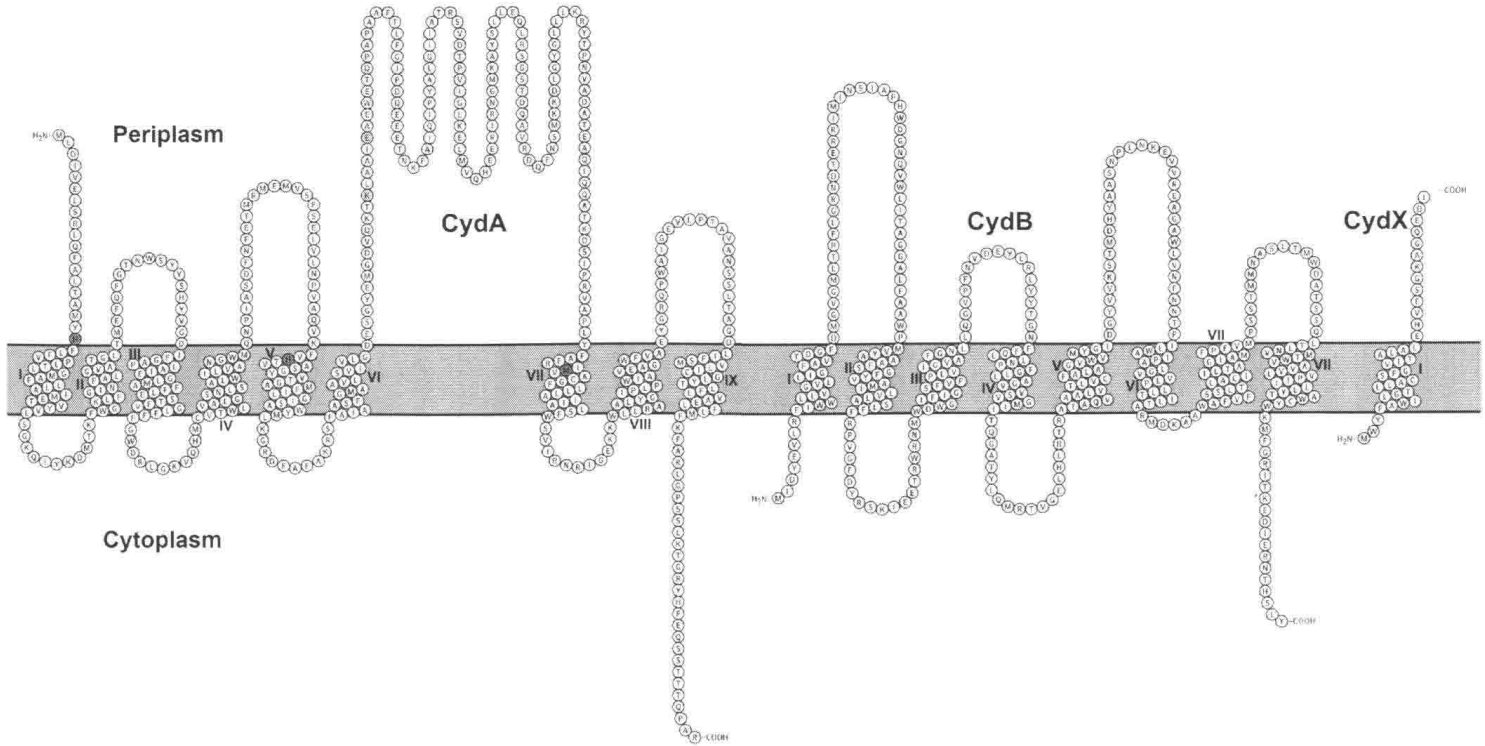

Figure 2 Secondary structure prediction of CydA, CydB and CydX. Secondary structure prediction was performed using the Protter online tool (Omasits, Ahrens, Mueller, & Wollscheid, 2014). Transmembrane helices are numbered sequentially, the haem b_{595} ligand (His19) is shown in blue (grey in the print version), the haem b_{558} ligands (His186 and Met393) are shown in red (dark grey in the print version) and the likely sites of quinol binding (Lys252 and Glu257) are shown in green (light grey in the print version).

Site-directed mutagenesis studies have been used alongside spectroscopic methods to reveal that highly conserved Histidine-186 and Methionine-393 of CydA are axial ligands of b_{558} (Fang, Lin, & Gennis, 1989; Kaysser, Ghaim, Georgiou, & Gennis, 1995; Spinner et al., 1995). The positive charge of the conserved Arginine-391 residue has a role in stabilising the reduced form of haem b_{558} and is required for oxidase activity (Zhang, Hellwig, Osborne, & Gennis, 2004), and Histidine-19 of CydA provides the essential axial ligand for b_{595} (Sun et al., 1996). An E99L mutation within CydA abolishes the haem *d* spectral signals (Bloch et al., 2009), supporting the idea that Glutamate-99 could be the axial ligand to haem *d* (Mogi et al., 2006). All three haem cofactors appear to be located on the periplasmic side of cytochrome *bd*-I (Zhang et al., 2004), which presents the problem of how protons are translocated across the membrane from the cytoplasm to the site of oxygen reduction which occurs on the periplasmic side. As the translocation of protons is unlikely to involve inter-haem transfer, focussing instead on conserved amino acid residues that can be reversibly protonated has implicated glutamates 99 and 107 within transmembrane helix III of CydA in proton translocation (Osborne & Gennis, 1999).

In the absence of CydB, CydA is still integrated into the cytoplasmic membrane, and haem b_{558} is still incorporated but the high-spin haems *d* and b_{595} are absent from the cytochrome subunits (Newton & Gennis, 1991). In an attempt explain this loss of haem groups, it has been suggested that the two high-spin haems are located at the interface between CydA and CydB subunits. Loss of *cydDC* abolishes haem cofactor incorporation into cytochrome *bd*-I but the CydAB polypeptides are still inserted into the membrane (Georgiou et al., 1987). Synthesis of this apocytochrome *bd*-I is also observed in haem-deficient cells (Calhoun et al., 1991), implying that haem insertion is the last step of cytochrome *bd*-I assembly.

2.3 Cytochrome *bd*-II

2.3.1 *The* appBC *Genes*

In the years between the discovery of cytochrome a_2 (later called cytochrome *d*; for references, see Poole, 1983), there appeared to be no suspicion that a second type of cytochrome *d* might exist: numerous biochemical and physiological studies were made (reviewed in Poole, 1983) on the assumption that cytochrome *d* was functionally, structurally and genetically homogenous. That complacency was shattered in 1991 when an operon was discovered (Dassa et al., 1991) comprising three genes upstream of *appA*, an acid phosphatase gene that is regulated positively in response to oxygen