Membrane topology and mutational analysis of *Escherichia coli* CydDC, an ABC-type cysteine exporter required for cytochrome assembly

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Cytochrome *bd* is a respiratory quinol oxidase in *Escherichia coli*. Besides the structural genes (*cydA* and *cydB*) encoding the oxidase complex, the *cydD* and *cydC* genes, encoding an ABC-type transporter, are required for assembly of this oxidase. Recently, cysteine has been identified as a substrate (allocrite) that is transported from the cytoplasm by CydDC, but the mechanism of cysteine export to the periplasm and its role there remain unknown. To initiate an understanding of structure–function relationships in CydDC, its membrane topography was analysed by generating protein fusions between random and selected residues in the two polypeptides with both alkaline phosphatase and β -galactosidase. CydD and CydC are experimentally shown each to have six transmembrane segments, two major cytoplasmic loops and three minor periplasmic loops; both termini of each protein face the cytoplasm. The *cydD1* allele is shown to have two point mutations (G319D, G429E) within the ATP-binding domain of CydD; either mutation alone is sufficient to cause loss or severe reduction of cytochrome *bd* assembly. A comparative sequence analysis prompted the targeting of residues in CydD for site-directed mutational analysis, which identified (i) the ‘start’ methionine residue, (ii) essential residues in the ATP-binding site (Walker sequence A) and (iii) a duplicated positively charged heptameric motif, R-G/T-L/M-X-T/V-L-R, in CydD cytoplasmic loop II. The replacement of arginines in these motifs with glycines resulted in Cyd[−] phenotypes; however, activity could be restored at these positions by replacing the glycine with lysine or histidine and hence returning the positive charge. The conservation of these charges in CydD-like proteins indicates functional importance. Evolutionary aspects of bacterial *cyd* genes are discussed.

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INTRODUCTION

Escherichia coli uses two membrane-bound terminal oxidases for aerobic respiration, cytochromes *bo'* and *bd* (Gennis & Stewart, 1996). The genes encoding the cytochrome *bd* quinol oxidase, *cydAB*, are expressed both aerobically and anaerobically, but expression is maximal during aerobic

stationary phase or in low oxygen environments (Cotter *et al.*, 1990; Poole & Cook, 2000). Consistent with this expression profile is the extraordinarily high affinity of cytochrome *bd* for O₂ (K_m = 3–8 nM; D'Mello *et al.*, 1996). Cytochrome *bd* comprises two integral membrane polypeptides, subunit I (CydA, 58 kDa) and subunit II (CydB, 43 kDa). Subunit I contains haem *b*₅₅₈ directly involved in ubiquinol oxidation whilst two further haems, *b*₅₉₅ and *d*, in a catalytic binuclear centre are shared by both subunits (Jünemann, 1997; Borisov *et al.*, 2001). All three haems are probably near the periplasmic side of the membrane (Osborne & Gennis, 1999). Loss of cytochrome *bd* causes a complex phenotype that extends beyond the lack of a functional oxidase. The Cyd[−] phenotype in *E. coli* includes (i) an inability to exit aerobically from stationary phase at 37 °C (Siegele *et al.*, 1996); (ii) sensitivity to high temperature (Delaney *et al.*, 1992), (iii) increased sensitivity to H₂O₂ (Delaney *et al.*, 1992); (iv) sensitivity to iron chelators (Cook *et al.*, 1998); and loss of motility and increased benzylpenicillin sensitivity (Pittman *et al.*, 2002).

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Abbreviations: ABC, ATP-binding cassette; AP, alkaline phosphatase; BG, β -galactosidase; CL, cytoplasmic loop; PL, periplasmic loop.

The need to understand cytochrome *bd* assembly is underlined by the importance of this oxidase in many bacteria. Thus, in *Azotobacter vinelandii* and *Klebsiella pneumoniae*, cytochrome *bd* deficiency severely impairs nitrogen-fixing ability under aerobic or microaerobic conditions (Kelly *et al.*, 1990; Juty *et al.*, 1997; Edwards *et al.*, 2000). Furthermore, in *Shigella flexneri* and *Brucella abortis*, the Cyd[−] phenotype includes attenuation of intracellular survival and virulence (Way *et al.*, 1999; Endley *et al.*, 2001).

In *E. coli*, two other genes, *cydD* and *cydC*, constituting an operon unlinked to *cydAB*, are necessary for cytochrome *bd* assembly (Georgiou *et al.*, 1987; Poole *et al.*, 1989, 1993; Bebbington & Williams, 1993; Delaney *et al.*, 1993). These genes encode the strikingly similar, but distinct, components of an ATP-binding cassette (ABC)-type transporter (Poole *et al.*, 1993), thought to be the first found in bacteria composed of only two non-identical subunits. ABC transporters are central to many physiological processes, from bacteria to man, being responsible for solute uptake and for secretion of toxins, drugs and substrates that range from small ions to large proteins (for a review, see Schmitt & Tampe, 2002). The genome of *E. coli* encodes some 80 ABC transporters (Linton & Higgins, 1998), many of which, like CydDC, are poorly characterized. It was hypothesized that the substrate ('allocrite', i.e. the translocated but not transformed molecule; Holland & Blight, 1999) of CydDC might be haem (Poole *et al.*, 1993, 1994). However, the assembly of haem into heterologous apoproteins (e.g. *Ascaris* haemoglobin) exported to the periplasm of *E. coli* does not require *cydC* (Goldman *et al.*, 1996a) and, critically, transport studies using inside-out membrane vesicles revealed no discernible differences between wild-type and *cydD* mutant strains (Cook & Poole, 2000). Intriguingly though, overexpression of CydDC results in synthesis of a novel haem pigment in anaerobically grown cells (Cook *et al.*, 2002), suggesting a link with haem metabolism.

An important clue to the function of CydDC was the finding (Goldman *et al.*, 1996a) that the periplasm of a *cydC* mutant is more oxidizing than that of a wild-type strain. This, and the observation that Cyd[−] defects can be corrected by extracellular provision of reductants (Goldman *et al.*, 1996b; Pittman *et al.*, 2002), suggests that CydDC exports a reducing molecule to the periplasm for redox maintenance. Recently, we have demonstrated, using everted membrane vesicles, that CydDC exports cysteine in an ATP-dependent, uncoupler-independent manner (Pittman *et al.*, 2002). The sensitivity of a *cydD* mutant to benzylpenicillin and dithiothreitol, and the loss of motility, were all reversed by exogenous cysteine. The amino acid also increases cytochrome *c* levels in the periplasm of a *cydD* mutant, but does not restore cytochrome *d*. Consistent with CydDC being a cysteine exporter, a *cydD* mutant is hypersensitive to high cysteine concentrations in the medium and accumulates higher cytoplasmic cysteine levels. CydDC overexpression confers resistance to high extracellular cysteine concentrations.

The structure of this novel cysteine transporter is unknown. In an effort to understand its organization within the cytoplasmic membrane, we have determined the topography of each of the subunits. We report here agreement between topological models for these proteins, derived largely from hydrophobicity considerations, with experimental results from protein fusion studies. We also report two mutations in the ATP-binding domain found in the *cydD1* chromosomal allele, the first to be isolated (Poole *et al.*, 1989). Site-directed mutagenesis reveals other essential residues in CydD including, in a cytoplasmic loop (CL), a duplicated positively charged heptameric domain in which the positive charge has a key functional role.

METHODS

Bacterial strains, plasmids and phages. The bacterial strains used were derivatives of *E. coli* K-12, namely AN2342 (F[−], referred to as wild-type), AN2343 (F[−] *cydD1*) (Poole *et al.*, 1989), XL-1 Blue (Stratagene), CC118 [*araD139* Δ(*ara*, *leu*)7697 Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE_{am} recA1*] (Manoil & Beckwith, 1985; Manoil, 1990) and TG1 [Δ(*lac-pro*), *supE*, *thi*, *hsdD5*(F' *traD36*, *proAB*⁺, *lacI*^q, *lacZDM15*)] (Amersham Biosciences). Plasmid pRP33 is a pBR328 derivative carrying the *trxB* and *cydDC* genes (Poole *et al.*, 1993). Plasmid pRKP1602 originated from a 2.53 kb *Csp45I* deletion (ablating the *trxB* and *cat* genes) of plasmid pRP33 (Cook *et al.*, 2002). pRKP1604 harbours most of the *cydD* gene and is the religated product of a 3.46 kb *Eco47III* deletion of pRKP1602. The protein fusion vector pRS414 contains the *lacZ'* gene (Simons *et al.*, 1987). The phagemid pBluescript (Stratagene) was used for cloning and single-stranded DNA preparation. The phages λTn*phoA*/in and λTn*lacZ*/in (Manoil & Bailey, 1997) were provided by C. Manoil (University of Washington).

Construction of fusion proteins. Insertions of Tn*phoA*/in and Tn*lacZ*/in were made as previously described by Manoil & Bailey (1997). Strain CC118 (Manoil, 1990), harbouring plasmid pRP33, was grown for 2–3 h (OD₆₀₀ = 1.0) and aliquots (0.2 ml) were infected with λTn*phoA*/in or λTn*lacZ*/in at a m.o.i. of 0.1–0.3. The mixture was incubated at 37 °C for 10 min without aeration followed by the addition of 0.8 ml LB medium (Miller, 1972). Cells were aerobically grown at 37 °C for 1–2 h followed by plating of dilutions on LB supplemented with tetracycline (10 µg ml^{−1}) and kanamycin (50 µg ml^{−1}). The resulting colonies were then replica-plated onto LB agar containing the chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (X-P; 40 µg ml^{−1}) or 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-G; 40 µg ml^{−1}), tetracycline, and an increased concentration of kanamycin (300 µg ml^{−1}) to select for transposition of Tn*phoA*/in or Tn*lacZ*/in into plasmids. Plasmid DNA was isolated by alkaline-SDS extraction of pooled lysogenic colonies that had grown after 1–2 days of incubation at 37 °C. The pooled plasmid DNA was used to transform strain CC118 and selection was on LB agar containing tetracycline, kanamycin (300 µg ml^{−1}) and X-P or X-G. Individual blue colonies were purified and plasmids were isolated from these clones. Transpositions were mapped with restriction digests and were localized precisely by DNA sequencing using the primers 'Tn*phoA*-I' or 'Tn*lacZ*-II' (Table 1). To construct specific *cydD*–*phoA* fusions, the *phoA* gene was amplified by PCR using λTn*phoA*/in as template (GenBank accession no. U25548) and primers RP253 and RP254, both including an *AgeI* site, or primers RP161 and RP162, both including an *MfeI* site (Table 1). Plasmid pRKP1602 was cut by *AgeI* restriction enzyme and ligated to the PCR-generated *phoA* gene to construct a protein fusion at residue G284. The 1.06 kb *MfeI* fragment of plasmids pRKP1602 and derivatives (R210G/R216G,

Table 1. Oligonucleotide sequences

Primer	Sequence	Restriction endonuclease site
Transposition mapping by DNA sequencing		
TnphoA-I	AATATCGCCCTGAGCA; Manoil & Bailey (1997)	
TnlacZ-II	CGGGATCCCCCTGGATGG; Manoil & Bailey (1997)	
PCR primers		
RP140	GGGATGTGCTGCAAGG	None
RP161	ACTACAATTGACTCTTATACACAAGTAGCGTCCTGGACGG	MfeI
RP162	ACTACAATTGTTTCAGCCCCAGAGCGGCTTTCATGG	MfeI
RP249	ACTAATGCATTCTGTTTACAACGTCGTGACTGGG	NsiI
RP250	ACTAATGCATTTTTTGACACCAGACCAACTGGTAATGGTAGCGACCGGG	NsiI
RP251	ACTACTTAAGTCGTTTACAACGTCGTGACTGGG	Bst98I
RP252	ACTACTTAAGTTTGTGACACCAGACCAACTGGTAATGGTAGCGACCGGG	Bst98I
RP253	ACTAACCGGTACTCTTATACACAAGTAGCGTCCTGGACGG	AgeI
RP254	ACTAACCGGTTTTTCAGCCCCAGAGCGGCTTTCATGG	AgeI
Mutagenic oligonucleotides for site-directed mutagenesis*		
M1L†	CTAAGCGTGCAcTGAATAAATCTCG	None
M45L†	GGCCTGGTTcTGGCGCGAATTCTG	None
G171V	CTCATTCTGCTGGtCACCGCACCGTTAATTC	+ BstEII
G274F	TTCCTATCTCttCGAaCTGGATTTCGGTCACTACGATACC	+ Csp45I
P142L	CACGCTATCTGcTGCAGATGGCGCTGGCA	+ PstI
G284A	GGTCACTACGATACCGcgGTGACGCTGGCTGCG	+ SacI
P151L	GGCAGTGTGCGGTGcTGTGCTGATTGTGG	– BanI
R210G/R216G	CCTCGATCGCCTagGCGGCATGGAACATTGGGTATTTT	+ StyI
R238G/R244G	TCCGCCAAaGGACAATGGAAGTGCTAgGGCTGGCGTTT	None
R210K/R216K	CCTCGATCGCCTAaaaGGCATGGAACATTGaaaATTTTTGGTGC	+ StyI
R238H/R244H	GATTTCCGCCAAcatACAATGGAAGTGCTAcatCTGGCGTTTTATCCT	None
G319D†	CCCAGGCTGTcGaCGCAGCTGAC	Sall
G388L/K389Q†	CAGCGGTTCActgcagAGCTCACTGC	PstI
G429E†	TCCTCGGTTGaGCAAAACCCA	None

*In mutagenic oligonucleotide sequences, changed nucleotides (lower case), altered codons (bold), restriction endonuclease recognition sequences (underlined) and newly introduced or ablated restriction sites, with plus or minus symbols, respectively, are shown.

†Mutations constructed using the M13-based Eckstein method.

R238G/R244G, R210K/R216K and R238H/R244H) was exchanged with a 1.38 kb PCR-generated *MfeI* fragment containing the *phoA* gene. The *cydD-phoA* constructs have an in-frame fusion after the I261 residue of CydD and the mature alkaline phosphatase (AP). The *lacZ* gene was amplified by PCR using pRS414 as template. Specific *cydD-lacZ* gene fusions were constructed in pRKP1602 as follows. The *lacZ* gene amplified with primers RP249 and RP250 (with *NsiI* sites) or RP251 and RP252 (with *Bst98I* sites; Table 1), was inserted either in *NsiI* or *Bst98I* unique sites of the plasmid to construct protein fusions at residues H134 and S202, respectively. The orientations of the fusions were determined by restriction analysis. Sequences and reading frames were checked by DNA sequencing, using primers 'TnphoA-I' (Manoil & Bailey, 1997) or RP140 (Table 1, for *cydD-lacZ* gene fusions). The constructs were transformed into *E. coli* CC118 and transformants were screened on nutrient agar containing ampicillin (100 µg ml⁻¹) and X-P or X-G as before.

Assays of enzymic reporter activity. AP activity was determined by measuring the hydrolysis at 37 °C of *p*-nitrophenylphosphate in CHCl₃- and SDS-permeabilized cells as described elsewhere (Michaelis *et al.*, 1983; Maloy *et al.*, 1996). Cell pellets were washed once in 1 M Tris/HCl, pH 8.0, resuspended in 5–10 ml of the same buffer and stored on ice. β-Galactosidase (BG) activity was measured

in CHCl₃- and SDS-permeabilized cells by monitoring the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside (Giacomini *et al.*, 1992). Cell pellets were washed once with PBS and resuspended in 5–10 ml of Z buffer (Miller, 1972). Each culture was assayed in triplicate. Absorbance (A) at 420, 550 and 660 nm was measured to express both reporter activities according to the following equation: $1000 \cdot [A_{420} - (1.75 \cdot A_{550}) / \text{time}(\text{min}) \cdot \text{volume}(\text{ml}) \cdot A_{600}]$ (Miller, 1972).

Analysis of the *cydD1* allele. The *cydD1* mutation was found by screening survivors of nitrosoguanidine mutagenesis for loss of spectroscopically detectable cytochrome *d* (Poole *et al.*, 1989). The mutation in strain AN801 was transduced to other strains as described by Poole *et al.* (1989). A mutant allele in strain AN2343 (*cydD1*) was obtained and amplified from chromosomal DNA by PCR using primers flanking the entire gene sequence. The double-stranded product was used as template for another series of reactions using each primer separately to generate single-stranded DNA. Single-stranded template was purified by agarose gel electrophoresis, phenol/chloroform and Centricon 100 (Amicon) microconcentration, prior to sequencing using the ABI protocol for PCR products.

Construction of mutant CydD proteins. Site-directed mutagenesis of the *cydD* gene was performed using the QuickChange method (Stratagene) or the M13-based Sculptor (Amersham) method.

Table 1 shows the details for QuickChange mutagenesis of pRKP1604, namely the residue substitutions, mutagenic oligonucleotide sequences, changed nucleotides, altered codons, restriction sequences and newly introduced or ablated restriction sites. pRKP1604 derivatives carrying the mutated sequences were amplified in *E. coli* XL-1 Blue. Mutations from these plasmids were transferred to pRKP1602 for complementation analysis by replacing restriction fragments containing the altered sequences.

For the M13-based Eckstein method (Olsen *et al.*, 1993), a 1·8 kb *DdeI* fragment was excised from pRP39 and cloned into the *SmaI* site of pBluescript SK. Single-stranded DNA was prepared by superinfection with helper phage M13K07 (Vieira & Messing, 1987) and annealed with the mutagenic primers shown and described in Table 1. A mutant heteroduplex was generated using Klenow polymerase and T4 DNA ligase and the non-mutant strand removed by virtue of incorporation of a thionucleotide in the mutant strand during *in vitro* synthesis. Homoduplex mutant plasmids were reconstructed *in vitro* according to the Amersham protocol and used to transform *E. coli* TG1. Mutated plasmids were detected by endonuclease restriction analysis or sequencing and all changes were confirmed by sequencing. Complementation of AN2343 (*cydD*) was performed using the constructs in pBluescript, or after cloning the mutated *cydD* gene into pBR328 by replacement of an *EcoRV*–*Bam*HI fragment.

Immunoblot analysis. Detection of the CydD protein in cell membrane fractions was performed by immunoblot analysis with an antibody raised to a peptide comprising amino acids Q302 to V318 in the cytoplasmic carboxy-terminus (Cook *et al.*, 2002). Micro-aerobic cultures were grown with shaking in Erlenmeyer flasks containing 40% of their volume of Luria medium supplemented with D-glucose (0·5%) and ampicillin (100 µg ml⁻¹) with shaking. Membrane fractions prepared from shear-disrupted cells (Poole & Haddock, 1974) were washed and resuspended in buffer (50 mM Tris/HCl pH 7·4, 2 mM MgCl₂, 1 mM EGTA). Protein was estimated by the method of Markwell *et al.* (1978) using bovine serum albumen as standard. After SDS-PAGE, gels were electroblotted onto Hybond-C nitrocellulose (Amersham Biosciences). Blots were developed by using anti-CydD serum (diluted 1:200), monoclonal anti-rabbit IgG peroxidase conjugate (Sigma, diluted 1:2000) and the ECL Western blotting detection system (Amersham Biosciences).

Assay of the *Cyd*⁻ phenotype. Strains were challenged on zinc-azide medium (ZnSO₄·NaN₃, 0·15 mM each) supplemented with Casamino acids (ZABC plates; Poole *et al.*, 1989) and on nutrient agar containing 0·1 mM of the metal-chelating agent ethylenediamine-di(*o*-hydroxyphenyl acetic acid) (EDDHA) (Cook *et al.*, 1998).

Spectrophotometric analysis and protein assays for determining the cytochrome *bd* levels of the mutants were performed on cells from microaerobic cultures, grown as described above. Cells were washed and resuspended in Tris/HCl buffer (pH 7·5). A few grains of sodium dithionite were added to reduce the sample, and a few grains of ammonium persulfate and potassium ferricyanide were used with shaking to oxidize the sample. The reduced sample was bubbled with a steady stream of CO gas for 2 min. The reduced *minus* oxidized and CO-reduced *minus* reduced spectra were recorded on a Johnson Research Foundation SDB4 dual-wavelength scanning spectrophotometer (Kalnenieks *et al.*, 1998).

The H₂O₂ sensitivity of strains was examined on solid and in liquid media. Bacterial growth inhibition was measured in a disk diffusion assay as follows. A sample (500 µl) of liquid culture was plated in top agar on NA plates and a sterile mini-disc containing 20 µl of a 3% (w/v) H₂O₂ solution was placed on top. Plates were incubated overnight at 37 °C and the diameter of growth inhibition was measured. Second, the viability method of Gort *et al.* (1999) was used. LB cultures with ampicillin (100 µg ml⁻¹) were inoculated at a

starting OD₆₀₀ ~0·02. After cultures reached mid-exponential phase (OD₆₀₀ ~0·6), aliquots were removed and diluted 1:10 000 in PBS, pH 7·4, before adding 2 mM H₂O₂. Surviving cells were enumerated by plating in top agar on NA plates with appropriate antibiotic, after overnight incubation at 37 °C.

All complementation tests were done by transforming strain AN2343 (*cydD1 recA*⁺) with plasmids bearing wild-type or mutant copies of *cydDC*. We have not formally ruled out the possibility of intragenic complementation, specifically the energization by ATPase activity in one polypeptide, of solute transport via the transmembrane segments of another polypeptide. However, we are not aware of such activity and, in the case of CydDC, mutations in one ABC region are sufficient to inactivate all cysteine transport by the heterodimer (Pittman *et al.*, 2002).

***In silico* analysis of CydDC sequences.** Sequence searches were performed with the BLAST 2.0 and WU-BLAST 2.0 programs accessible at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>), and the Genome Sequencing Center (<http://genome.wustl.edu/bast/client.pl>), respectively. Searches were also made using the *Bordetella* BLAST Server from the Sanger Centre (http://www.sanger.ac.uk/Projects/B_pertussis/bast_server.shtml). Protein sequences were obtained from GenBank, the Comprehensive Microbial Resource of the Institute for Genomic Research (<http://www.tigr.org>), the Colibri and SubtiList databases (<http://genolist.pasteur.fr/Colibri/> and <http://genolist.pasteur.fr/SubtiList/>, respectively). The program MEME was employed to detect conserved (repeated) motifs in protein sequences.

Fig. 1. Prediction of the topographies of the CydD (top) and CydC (bottom) proteins using TMHMM. For details see text. For each protein, the probability of regions being transmembrane (red), inside (i.e. cytoplasmic, blue) or outside (i.e. periplasmic, magenta) are plotted as a function of amino acid residue number.

To predict the topography of CydD and CydC, TMHMM 2.0 (Sonnhammer *et al.*, 1998; Krogh *et al.*, 2001) was used. Multiple sequence alignments and additional analyses of transmembrane segments in the CydDC sequences were performed using the program CLUSTALW and both the von Heijne Helix (transmembrane) and Kyte–Doolittle hydrophilicity algorithms in MacVector 7.0 software. Gap creation and extension penalties used were 3.0 and 1.0, respectively. In certain regions, the alignments had to be adjusted manually.

The sequence of CydDC was compared with the sequences of all known protein structures (the pdb sequence database at <http://www.ncbi.nlm.nih.gov>) using BLAST (Altschul *et al.*, 1997). Very significant similarities were found to the MsbA proteins from *Vibrio cholerae* and from *E. coli*. The former was the more significant and includes the whole sequence of CydDC except for the short N-terminal helix and the first transmembrane helix (25% sequence identity over amino acids 66–577), whilst the latter (26% identity, but only over residues 154–580) omits the first three transmembrane sequences. Threading using 3D-PSSM (Kelley *et al.*, 2000) also yields the *V. cholerae* MsbA as having the most significant similarity over the whole sequence range of CydD. The structure of *V. cholerae* MsbA (Chang, 2003) therefore

provides a good structural model for CydDC. The two point mutations were modelled and their immediate environments in the structure examined using TurboFrodo (Roussel & Cambillau, 1991).

RESULTS AND DISCUSSION

Determination of the start methionine of CydD

In earlier hydrophobicity plots and in the analyses shown in Figs 1 and 2, the start methionine residue of CydD suggested by Poole *et al.* (1993) was used (labelled M1 in Fig. 2a, see later). However, the amino acid sequence shows another methionine, M45, according to this assumption, which the hydrophobicity and topological analyses place within the membrane. Protein synthesis *in vivo* shows CydD to have an apparent molecular mass of 61 kDa (Poole *et al.*, 1993) and comparison with the value (64 kDa) from the assumed protein sequence does not allow the start methionine to be

Fig. 2. Topological model of the CydD (top) and CydC (bottom) polypeptides. In each, the six transmembrane segments are numbered I–VI, the periplasmic loops PL I–III and cytoplasmic loops CL I–II. Amino acid residues (one letter code) at the fusion junctions are indicated with white arrows for LacZ fusions and with black arrows for PhoA fusions. Table 2 gives the measured enzyme activity for each fusion protein. Residues altered by site-directed mutagenesis are indicated by bold black arrowheads. The two point mutations in the *cydD1* allele (G319, G429) are indicated by bold type. The white arrowhead points to the site of in-frame *cydD*–*phoA* fusions used for topology studies of CL II (see text). The number of positively and negatively charged residues within the periplasmic and CLs is indicated in boxes. The underlined sequences in the ATP-binding domain (C terminus) of both polypeptides show, respectively, the Walker sequence A, the linker peptide, the Walker sequence B and the conserved His residue common to ABC transporters (Linton & Higgins, 1998).

identified unambiguously. However, confirmation of M1 was provided by construction of an M1L mutation, which, when present on a plasmid, failed to complement the *cydD1* allele, while mutation of M45 was fully complementing when transformants were tested for the *Cyd*[−] phenotype (see Methods; results not shown).

Predictions and experimental determination of membrane topography of the CydDC proteins

The CydDC proteins each comprise an N-terminal hydrophobic domain and a C-terminal hydrophilic domain (the ATP-binding or ABC domain) (Poole *et al.*, 1993). Early hydrophobicity predictions of the CydD and CydC sequences revealed stretches of hydrophobic amino acids corresponding to six putative transmembrane helices (Poole *et al.*, 1993) each of sufficient length to span the membrane. A variety of newer tools to predict the topography of transmembrane proteins is now available, of which TMHMM is considered the best-performing (Moller *et al.*, 2001). This implements circular hidden Markov models and determines the most probable topography for the whole protein. The topographies for CydD and CydC are strikingly similar (Fig. 1); each protein is predicted to comprise six transmembrane regions, with both the N and C termini in the cytoplasm. However, in CydC, the probability of a sixth, C-terminus-proximal transmembrane region is lower than for other transmembrane regions. According to Boyd & Beckwith (1989), the distribution of charged residues may function as a topogenic determinant, since they play an important role during protein translocation and membrane protein assembly (Andersson & von Heijne, 1993). The overall amino acid composition and net charge of the N-terminal regions (residues 1–25 and 1–15 for CydD and CydC, respectively) is more similar to that of a typical cytoplasmic than a periplasmic region (Nakashima & Nishikawa, 1992). All proposed CLs possess a net positive charge, whereas the periplasmic loops (PLs) display a net negative (excluding CydD loop I) or no net charge at all, consistent with the 'positive-inside' rule (von Heijne, 1992). Finally, the cytoplasmic location of the ABC-bearing C termini is consistent with their putative function, namely ATP binding and hydrolysis.

However, experimental evidence in support of topological models has been lacking. To assess which of these regions actually span the membrane, fusions between random amino acid residues in CydD and CydC with both AP and BG proteins were generated using *TnphoA*/in and *TnlacZ*/in transposition into pRP33, resulting in plasmids bearing in-frame *cydD*–, *cydC*–*phoA* and *cydD*–, *cydC*–*lacZ* fusions. A total of 26 fusions were well distributed, covering both protein sequences (Fig. 2a, b; Table 2). To obtain each hydrophobic segment flanked by either *phoA* or *lacZ* fusions, we also constructed fusion proteins with specific residues in CydD (i.e. H134, S202 and G284) as described in Methods.

To determine the subcellular locations of the *PhoA*/*LacZ*

Table 2. Locations and measured enzyme activities of AP and BG fusions to CydD and CydC

Fusion number	Amino acid*	Enzyme activity (for units see text)		Location
		AP	BG	
CydD				
1	K7		2100	Cytoplasm
2	Q15		2000	Cytoplasm
3	A46	357		Periplasm
4	L49	428		Periplasm
5	M52	457		Periplasm
6	L75	41		TM II?
7	H134		780	Cytoplasm
8	L167	53		TM IV?
9	A188	4		Cytoplasm
10	R192	4·4		Cytoplasm
11	S202		920	Cytoplasm
12	L276	360		Periplasm
13	G284	290		Periplasm
14	A317		840	Cytoplasm
15	E353		1060	Cytoplasm
16	S465		1500	Cytoplasm
CydC				
17	L35	429		Periplasm
18	L38	422		Periplasm
19	L43	440		Periplasm
20	L135		820	Cytoplasm
21	L162	436		Periplasm
22	L166	366		Periplasm
23	E242		830	Cytoplasm
24	Q248		660	Cytoplasm
25	L282	327		Periplasm
26	T329		690	Cytoplasm
27	T429		563	Cytoplasm
28	L483		456	Cytoplasm
29	L559		1100	Cytoplasm

*Numbers correspond to the last amino acid in CydD or CydC before the fusion junction with AP or BG.

fusions, AP and BG activities of fusion proteins were measured (Table 2). AP has low activity in the cytoplasm (Manoil, 1990), whereas BG remains inactive in the periplasm (Lee *et al.*, 1989). The *LacZ* fusion proteins K7, Q15, H134, S202, A317, E353 and S465 for CydD, as well as L135, E242, Q248, T329, T429, L483 and L559 for CydC exhibited high levels of BG activity (>450 Miller units). These data show that the N and C termini of each protein are in the cytoplasm and also allowed tentative identification of two CLs for each protein, as shown in Figs 2(a) and (b). Furthermore, the AP fusion proteins A46, L49, M52, L276 and G284 for CydD, as well as L35, L38, L43, L162, L166 and L282 for CydC showed high AP activity (>290 units), consistent with periplasmic exposure of these residues in each protein. The AP fusions place two loops of each

polypeptide (labelled PLI and PLIII in each of Figs 2a, b) in the periplasm. Despite the lack of LacZ fusions within the CydC N terminus, the predicted location of the first transmembrane section (I) (Fig. 2b) and the active PhoA fusions L35, L38 and L43 (residues labelled 17 to 19 in Fig. 2b) allowed us to place the N terminus in the cytoplasm. In CydD, AP fusions L75 and L167 (residues labelled 6 and 8, respectively in Fig. 2a) gave low activity; both are predicted to lie within transmembrane regions. The BG fusions A188 and R192 displayed activity that, although corresponding to a cytoplasmic location, are not readily explained. In the CydD sequence, beyond residue 300, further possible transmembrane regions are predicted but at very low probability (see Fig. 1) but the high BG activities of the LacZ fusions E353 and S465 clearly place this region in the cytoplasm.

Problems can arise from the use of these fusions through instability of the fusion proteins. For example, when a LacZ fusion protein is cleaved during synthesis, it is released into the cytoplasm giving a false positive. However, PhoA fusion proteins must be translocated to the periplasm to be measurable, so that stability is of less concern. The results from the LacZ and PhoA fusions are complementary and internally consistent and have been used to construct the transmembrane topological models of CydD and CydC shown in Figs 2(a) and (b), respectively. Both polypeptides span the membrane six times (TM I–VI) and each protein has two large CLs (labelled CL I–II in Fig. 2a, b) and three small PLs (PL I–III in Fig. 2). The positions of the helices predicted from TMHMM lie within the regions defined by protein fusion activity. von Heijne transmembrane and Kyte–Doolittle hydrophobicity plots also reveal hydrophobic clusters in CydD and CydC sequences similar to those shown in Fig. 2 (results not shown). Although the cytoplasmic location of the ATPase domains cannot be deduced from the positive LacZ fusions obtained alone (see above), there is ample evidence for a cytoplasmic location for the nucleotide-binding domain in related proteins (e.g. MsbA; Chang & Roth, 2001) and this location is consistent with the exclusively intracellular availability of ATP. Our experimental findings are also consistent with the topography of other prokaryotic and eukaryotic ABC transporters. For example, the *Drosophila melanogaster* pigment precursor transporter is also a heterodimer, in which both the White and Brown subunits possess six transmembrane domains with both N and C termini in the cytoplasm (Ewart *et al.*, 1994). Also, in the crystal structure of *E. coli* MsbA – the lipid A flippase – the transmembrane region is composed of six tilted α -helices per monomer, with contacts between helices from different monomers (Chang & Roth, 2001).

Mutations in the *cydD1* allele and the nucleotide-binding domain

The first mutant allele of *cydD* was isolated after random mutagenesis and screening spectroscopically for loss of the distinctive absorbance properties of cytochrome *bd* (Poole *et al.*, 1989). Despite use of this strain over many years,

during which time the stability of this allele has been noted, the mutated residue(s) have not been determined. Here we report two point mutations, G319D and G429E in the *cydD1* mutant allele. Both are in the C-terminal, cytoplasmic portion of the CydD protein, flanking the Walker A-motif (Fig. 2a). To test the importance of each mutation in conferring a Cyd^- phenotype, we individually engineered these changes into the *cydD* gene cloned into pBluescript and tested the ability of the mutant constructs to complement the *cydD1* allele in strain AN2343. The G319D mutation alone was sufficient to eliminate complementation as judged by the absence of spectroscopically detectable cytochrome *bd*. The G429E mutation in pBluescript reduced cytochrome *d* content to less than one-third the level of that afforded by the wild-type *cydD*⁺ gene cloned into either pBluescript or pBR328. Thus, either mutation affects cytochrome *bd* assembly, perhaps explaining the failure to find spontaneous revertants at this locus. Fig. 3 shows a model of the CydD protein based on the solved structure of MsbA in *Vibrio cholera* (Chang, 2003). The cytoplasmic domain comprises the N terminus, two large CLs, and the C terminus bearing the ATP-binding cassette, including Walker motifs A and B, as demonstrated in the fusion analysis. The upper half of the structure in Fig. 3 comprises the six membrane-spanning helices and small PLs, again as demonstrated in this work. G429 (in *E. coli*; Ser423 in *Vibrio*) is close to a conserved Asp residue. Substitution with Glu, a large negatively charged residue, next to a conserved Asp (510 in *E. coli*; 505 in *Vibrio*) may be responsible for disruption of structure near the Walker B region. G319 is buried in a pocket and may make van der Waals contact with Leu104 at the extreme end of a transmembrane helix, although the reason for the Cyd^- phenotype is unclear at this time. Mutation of G319 might slow down conformational changes that are required during the catalytic cycle. This region of the ABC domain may serve an intramolecular signalling function coupling the hydrolysis of ATP with the membrane-spanning components (Ames & Lecar, 1992). Glycines are expected to effect transmission of conformational changes within proteins, since they lack a lateral chain and appear crucial in dynamic functionality. Glycine residues have been shown to interact with the phosphoryl groups of ATP. A G313A mutation within the G box of HPK was observed to diminish greatly the formation of a covalent adduct with ATP upon UV irradiation (Ninfa *et al.*, 1993).

The invariant amino acid doublet Gly-Lys of the Walker motif A has been proposed to be directly involved in binding of ATP (Parsonage *et al.*, 1987) and mutation of these residues in various members of the traffic ATPase family eliminates or severely compromises function (Ewart *et al.*, 1994 and references therein). To establish the significance of the proposed nucleotide-binding fold in CydD, we introduced the mutation G388L/K389Q in the Walker sequence A (Fig. 3). This change in CydD was sufficient to disrupt function, as assessed by failure to restore growth of a *cydD* mutant under selective conditions and total lack of cytochrome *d* spectral signals (not shown). Several studies of

Fig. 3. Model of the solved structure of one 'half-molecule' of MsbA in *V. cholerae* and the positions of residues of significance in CydD. The lower half of the structure is the cytoplasmic domain (N terminus, two large cytoplasmic loops and C terminus bearing the ATP-binding cassette, including Walker motifs A and B). The upper half comprises six membrane-spanning helices and small periplasmic loops. The mutated residues in the *cydD1* mutant allele (Cyd⁻ phenotype) are G429E and G319D. In green are the Walker motifs. For clarity, the positions of residues 429 and 319 are shown in a space-filling representation (magenta and red, respectively) and the nearby residues Asp 510/505 and Leu 104 are shown as ball-and-stick. Most of the heptameric repeat region (207–246) is disordered and not observed in either the *V. cholerae* or the *E. coli* MsbA structure. Its approximate location is indicated by a blue ellipse. Residues 238–246, which are observed, are coloured in blue. For other details, see text.

P-glycoprotein (the multidrug-resistance protein) indicate that the two ATP-binding sites cannot function independently and a strong cooperative interaction occurs between the two (Urbatsch *et al.*, 1995). The requirement for two ABC domains in CydDC is demonstrated by the fact that the directed G388K/K389Q mutation of CydD in the nucleotide-binding domain disrupts function. It has been previously reported that mutation of G135 and K136 to LQ within the nucleotide-binding fold motif of the *white* gene

of *D. melanogaster* is unable to complement eye colour in recipient transgenic flies with a defective *white* gene (Ewart *et al.*, 1994).

Sequence-informed targeting of residues for mutagenesis in the CydD protein

A search for similarities between two transmembrane regions of both CydDC proteins (TM III and TM IV) with other proteins in GenBank has shown numerous hits with mitochondrial *b*-type cytochrome sequences from both invertebrates and vertebrates (Cook *et al.*, 2002). Moreover, TM IV of CydD has a pair of glycines (G171 and G184; Fig. 2a) separated by 12 residues, which, with a similar pair in TM I of CydC (G21 and G34; Fig. 2b) resemble the quartet of conserved glycines in cytochromes *b* involved in configuration of the haem pocket (Esposti *et al.*, 1993). Inspection of putative CydD and CydC sequences in other bacteria is confounded by a lack of information on features that distinguish CydDC transporters (i.e. that export Cys and GSH) from other members of the ABC transporter family. However, CLUSTALW alignments show that the *Salmonella typhimurium* LT2 CydD and CydC proteins exhibit precisely the same quartet of glycines as in *E. coli*, and similar groupings of glycines occur in the presumed CydD and CydC proteins of other Gram-negative bacteria. Thus, in both *Vibrio vulnificus* YJ016 (accession NP_934247) and *Photobacterium profundum* (accession CAG19569) a pair of glycines is found in CydD, separated by 17 residues, the second of the pair aligning with *E. coli* G184. In *Brucella melitensis* 16M (accession NP_459933), the alignment and spacing are as in *E. coli* and *Salmonella*. In all these bacteria, glycine pairs also occur in CydC, the first of each pair aligning with *E. coli* G21: in *V. vulnificus*, there are 12 residues between the glycines, whereas in *Photobacterium profundum* and *Brucella melitensis*, 10 residues separate the glycines. Interestingly, no such glycine pairs occur in the putative CydD and CydC proteins of the Gram-positive bacteria *Mycobacterium tuberculosis*, *Bacillus cereus* or *Listeria monocytogenes*. Another glycine pair (G274 and G284) is seen in CydD of *E. coli*, *Salmonella* and *Photobacterium luminescens* (accession NP_928889). In this preliminary examination of the possible roles of these paired glycines, we targeted glycine residues G171, G274 and G284 in CydD TM IV, PL III and TM VI, respectively (Fig. 2a), for mutagenesis.

Statistical analysis of transmembrane segments of ion channels (Brandl & Deber, 1986) reveals a frequent distribution of prolines. Furthermore, in light of the recent demonstration that CydDC transports cysteine (Pittman *et al.*, 2002), it is notable that most prolines in the TMs of prokaryote amino acid transporters are highly conserved (Pi *et al.*, 1998). Therefore, we decided to change the proline residues in CydD TM III (P142 and P151, Fig. 2a) for leucines whose relatively large size may change the local structure. These two prolines are strongly conserved in bacterial CydD-like proteins (data not shown).

Bacteria	protein	residue		R-rich region																						residue																				
		No.			+		+																																No.							
<i>E. coli</i>	CydD	207	-	D	R	L	R	G	M	E	T	L	R	I	F	G	R	G	E	A	E	I	E	S	T	R	S	A	S	E	D	F	R	Q	R	T	M	E	V	L	R	L	A	-	246	
<i>S. typhimurium</i>	CydD	207	-	D	R	L	R	G	M	E	T	L	R	I	F	G	R	G	E	A	E	I	E	S	T	R	A	A	S	O	D	F	R	Q	R	T	M	E	V	L	R	L	A	-	246	
<i>P. stuartii</i>	AarD	207	-	D	R	L	R	G	L	D	T	L	R	L	F	F	R	E	K	A	E	V	Q	I	R	E	S	T	E	D	F	R	S	R	T	M	E	V	L	R	M	A	-	246		
<i>P. multocida</i>	CycD	207	-	D	R	L	R	G	L	E	T	L	R	L	F	N	R	T	E	E	Q	T	Q	H	I	E	K	N	T	E	D	F	R	E	T	T	M	D	V	L	K	M	A	-	246	
<i>V. cholerae</i>	CydD	207	-	D	R	L	Q	A	L	T	T	I	R	L	F	D	R	T	T	A	E	T	E	H	M	R	G	A	S	E	V	F	R	H	R	T	I	R	V	L	R	I	A	-	246	
<i>H. influenzae</i>	CydD	207	-	D	R	L	R	G	L	E	T	L	R	L	F	N	R	T	S	E	Q	T	E	H	I	E	N	A	T	E	D	F	R	E	T	T	M	D	V	L	K	L	A	-	246	
<i>S. violacea</i>	CydD	207	-	D	R	L	K	G	L	P	T	L	K	L	F	N	R	G	D	A	E	V	K	E	I	E	T	A	S	E	E	F	R	E	T	T	M	S	V	L	R	M	A	-	246	
<i>B. pertussis</i>	CydD	206	-	D	R	L	R	G	L	A	T	L	K	L	Y	G	R	A	E	A	E	E	Q	S	V	V	A	A	S	D	A	L	R	Q	R	T	M	A	V	L	R	I	A	-	245	
<i>S. coelicolor</i>	CydD*	202	-	D	V	V	A	G	L	P	T	L	K	V	F	G	R	A	K	A	Q	A	E	S	I	K	R	I	T	G	E	Y	R	Q	A	T	M	R	T	L	R	I	A	-	241	
<i>C. crescentus</i>	CydD	206	-	D	R	V	R	A	L	P	V	V	L	A	F	Q	A	E	G	A	T	T	V	A	L	T	R	S	A	S	N	L	A	E	R	T	I	R	V	L	R	V	A	-	245	
<i>B. subtilis</i>	CycD	197	-	D	S	L	R	G	L	E	T	L	R	F	L	G	L	S	K	K	S	H	S	K	N	I	F	Y	V	S	E	R	Y	R	K	A	T	M	S	T	L	R	V	A	-	236
<i>E. faecalis</i>	CycC	198	-	D	S	L	R	G	I	D	T	L	K	L	F	G	L	S	K	K	Y	A	G	S	I	Y	H	T	S	E	R	F	R	K	A	T	M	S	T	L	K	I	A	-	237	
<i>M. tuberculosis</i>	CydD	183	-	D	L	I	A	G	I	P	T	L	R	A	L	G	R	A	S	G	P	E	Q	R	I	A	E	L	S	A	D	H	R	R	S	A	M	A	T	L	R	I	A	-	222	
<i>M. smegmatis</i>	CydD	179	-	D	L	I	A	G	I	P	T	L	R	A	V	G	R	A	G	S	V	Q	R	I	A	E	L	S	A	S	H	R	R	S	T	M	A	T	L	R	I	S	-	218		
<i>L. lactis</i>	CycC	184	-	D	S	L	R	G	I	S	T	L	K	Y	F	G	L	S	K	D	Y	S	N	S	I	Y	K	T	S	E	D	F	R	K	E	T	M	G	A	L	R	I	A	-	223	
<i>S. aureus</i>	SA639	188	-	N	I	A	K	G	L	V	T	L	K	L	F	N	R	T	E	Q	T	E	K	H	I	Y	D	D	S	T	Q	F	R	T	L	T	M	R	I	L	R	S	A	-	227	
<i>B. halodurans</i>	CycC	180	-	D	T	L	Q	G	L	N	T	I	K	F	F	G	Q	T	R	Q	Q	R	S	R	I	K	E	K	S	L	E	F	R	E	A	T	M	S	V	L	K	I	A	-	219	
<i>S. pyogenes</i>	Spy1791	212	-	D	D	L	K	G	L	N	T	L	Y	S	Y	Q	A	T	E	R	Y	E	Q	E	F	S	G	K	A	E	Q	F	R	K	A	T	M	S	L	L	G	F	Q	-	251	
<i>P. mirabilis</i>	NrpA	206	-	E	Y	I	K	G	I	R	E	I	K	A	F	N	L	T	S	H	T	F	G	K	L	S	Q	S	I	N	S	V	K	W	I	K	N	V	K	A	S	-	245			

Fig. 4. Positively charged heptameric regions of CydD-like sequences in a multiple alignment. Residue numbers defining the limits of these regions in each protein are shown. Identical and similar residues are in dark grey or light grey boxes, respectively. Positive charges define the start and end points of each proposed heptameric region and are indicated above the aligned sequences. Gene accession, or sequence identifier, or translated base-pair numbers (in parentheses) for each sequence follow: *E. coli* K-12-MG1655 (2506099), *Salmonella enterica* serovar Typhimurium (g1421_saltyLT2), *Providencia stuartii* (939711), *Pasteurella multocida* PM70 (12720839), *V. cholerae* El TorN16961 (9655658), *Haemophilus influenzae* Rd KW20 (1574714), *Shewanella violacea* (1536829), *Bordetella pertussis* (translated sequences from base pair 270836 to 272530), *Streptomyces coelicolor* (*N-terminus of 'CydCD': 3928723), *Caulobacter crescentus* CB15 (13421998), *Bacillus subtilis* 168 (2829798), *Enterococcus faecalis* V583 (EF2059), *Mycobacterium tuberculosis* H37Rv (2113905), *Mycobacterium smegmatis* (6289096), *Lactococcus lactis* subsp. *lactis* IL1403 (12723617), *Staphylococcus aureus* subsp. *aureus* N315 (13700575), *Bacillus halodurans* C-125 (10176596), *Streptococcus pyogenes* M1 GAS strain SF370 (13622838), *Proteus mirabilis* (4097161).

When CL II of the CydD protein was analysed using the MEME program, we noticed two positively charged heptameric motifs separated by 21 residues. These are shown in Fig. 4; the first starts at residue 210 in *E. coli* CydD and the second at residue 238. Each of these is well conserved in CydD proteins of other species. In the first heptamer, the second position is G in 17 of the 19 cases examined, whereas the second position in the second heptamer is T in 17 of 19 cases. We therefore describe the 'consensus' at this position as G/T. The third residue in the first heptamer is usually L (12L/19) and the third residue in the second heptamer is usually M (17M/19). We therefore describe the 'consensus' at this position as L/M. Overall, we suggest that the consensus is R-G/T-L/M-X-T/V-L-R, where 'I' separates the most probable occupants of certain positions (Fig. 4). The R210, R216 and R244 residues are frequently preceded by another positively charged residue, i.e. lysine. Thus, we decided to introduce the R210G/R216G and R238G/R244G changes into the CydD protein to annul the positive charges of these regions. Fig. 3 shows the heptameric repeat region. Residues 207–246 are disordered in the structures of both the *V. cholerae* and the *E. coli* MsbA; the approximate location of this region is indicated by a blue ellipse. Residues 238–246, which are observed, are coloured in blue.

Phenotypic analysis of site-directed *cydD* mutations

Cyd[−] mutants have a complex phenotype, including sensitivity to: (i) zinc and azide, inhibitors of the remaining

cytochrome *o*-mediated respiratory chain (Poole *et al.*, 1989); (ii) iron chelators (Cook *et al.*, 1998) and (iii) H₂O₂ (Delaney *et al.*, 1992; Lindqvist *et al.*, 2000). We exploited these sensitivities to test strain AN2343 (*cydD1*) that had been transformed with pRKP1602 (bearing the wild-type *cydDC* genes) or site-directed *cydD* mutant derivatives, by plating on ZABC medium (containing Zn²⁺ and azide ions) or plates supplemented with EDDHA or H₂O₂. The non-conservative substitutions of the targeted glycine or proline residues (G171V, G274F, G284A, P142L and P151L) did not prevent complementation of strain AN2343 by the pRKP1602 derivatives. Furthermore, spectral analysis revealed that all transformants synthesized cytochrome *bd* (data not shown). Therefore, these residues do not play key roles in CydD function and do not lend support to the notion that cytochrome *b*-like sequences in CydD and CydC play essential roles in haem binding. The ability of these mutant constructs to form the enigmatic P-574 pigment (Cook *et al.*, 2002) has not been tested, but the identification of cysteine as an allocrite for CydDC (Pittman *et al.*, 2002) suggests that changes in haemoproteins in CydDC-overexpressing strains probably result from alterations in periplasmic redox status, rather than a direct involvement of CydDC in haem transport.

To assess the functional importance of the duplicated heptameric motif in CydD CL II, we screened arginine-to-glycine substitutions. pRKP1602 (carrying wild-type *cydD*⁺) complemented strain AN2343 (*cydD1*) as shown by the high level of cytochrome *d*, lack of inhibition by

Fig. 5. Cytochrome *d* levels in *cydD1* strain carrying different plasmids. pRKP1602 carries the wild-type *cydDC* operon and pBR328 is the vector control. The remaining four plasmids carry the site-directed mutations shown. Growth inhibition under stress conditions (EDDHA, zinc/azide), percentage survival, and diameter of inhibition halo in the presence of H₂O₂, are shown below. For details, see text. Cytochrome *d* levels were measured in at least two different cultures for each strain; variations in duplicate measurements were <2–5%. Survival counts and growth inhibition on plates were repeated three times with similar results.

EDDHA, zinc or azide, and small zones of inhibition around disks impregnated with H₂O₂ (Fig. 5). However, plasmids encoding the R210G/R216G and R238G/R244G mutant forms of CydD exhibited significantly lower levels of cytochrome *d* than the wild-type (2.3- and 1.4-fold less, respectively; Fig. 5) and slower growth when challenged with the above agents.

Studies of membrane insertion and topology of CydD mutants

We investigated the possibility that unsuccessful insertion of mutant proteins into the membrane was responsible for

Fig. 6. Immunoblot analysis of the CydD protein. Identical amounts of protein membrane fractions (20 µg) were loaded for 12% SDS-PAGE, and the CydD proteins were detected with anti-CydD serum. Lanes 1 and 2 correspond to *cydD1* AN2343 and wild-type AN2342 strains, respectively, transformed with pBR328. Lanes 3 and 4 are wild-type and *cydD1* strains, respectively, transformed with pRKP1602. Construct derivatives of pRKP1602, R210G/R216G (lane 5), R238G/R244G (lane 6), R210K/R216K (lane 7) and R238H/R244H (lane 8), were put into the *cydD1* strain. Molecular mass markers (in kDa) are indicated to the left of the blot.

lack of complementation. Membrane fractions were probed with the anti-CydD serum as described in Methods. As can be seen in Fig. 6, the G319D/G429E CydD protein was detected at similar levels to that of the wild-type protein. Thus, the AN2343 strain phenotype is not due to a defective insertion of the CydD subunit into the membrane. Additionally, we detected a stronger signal when the CydD overproducer plasmid pRKP1602 and its derivatives were present. This suggests that the mutations described here do not disrupt CydD protein localization.

The importance of the positively charged domains located in the CLs of CydD was investigated by site-directed mutagenesis. The charges in the highly conserved R-X-G-R-R domains of the glucose transporter Glut1 form local cytoplasmic anchor points, ensuring the correct membrane topography (Sato & Mueckler, 1999). The overall charge of the CydD CL II is also rather positive with 12 positively and 10 negatively charged residues (Fig. 2a). We asked whether the removal of two positive charges in the R210G/R216G and R238G/R244G substitutions might provoke the 'looping-out' of CL II into the periplasm. A series of in-frame *cydD*–*phoA* fusions after residue I261 (see Fig. 2a) for mutations R210G/R216G, R238G/R244G, R210K/R216K and R238H/R244H was constructed and transformed into strain CC118. Choice of residue I261 allowed use of a unique *MfeI* site for construction of such fusions. Aberrant translocation of CL II into the periplasm would yield high AP activity, but neither the wild-type *cydD*–*phoA* fusion nor the other mutated fusions gave a strong AP activity (9–18 Miller units). This demonstrates that CL II remains located in the cytoplasm and that the targeted positive charges are not essential determinants of membrane topography.

Arg to Gly mutations are overcome by replacement of positive charges

We constructed plasmids including the R210K/R216K and R238H/R244H changes in CydD, and introduced them into the *cydD1* background. These substitutions prevented the growth inhibition in challenging conditions, and the cytochrome *d* levels were increased close to those from the wild-type strain (Fig. 5). Thus, regardless of the different residue side-chains, lysine and histidine are functional substitutions for arginines in the duplicated heptameric motif of CydD CL II, and are able to restore the wild-type phenotype by the sole supply of a positive charge. The function of this region is unclear but it may be involved in cysteine binding.

Evidence from sequence analysis for conservation, but shuffling, of *cydDC* genes

In *E. coli*, the genes encoding the ATP transporter are denoted *cydDC* because the second gene of the operon was discovered first (Georgiou *et al.*, 1987; Poole *et al.*, 1989). Gene nomenclature in *Bacillus subtilis* follows an alphabetical order and, in contrast with *E. coli*, the genes are transcribed as a *cydABCD* polycistronic message (Winstedt

et al., 1998). The *E. coli* nomenclature has been used in mycobacteria as well as in Gram-negative bacteria, whereas the *Bacillus subtilis* nomenclature has been reserved for Gram-positive bacteria. A further 17 and 18 complete orthologue sequences for *cydD* and *cydC*, respectively, were analysed. Interestingly, the *cyd* locus of *Streptomyces coelicolor* A3(2) encodes three proteins, namely CydA, CydB and a protein (GI:3928723; 1172 amino acids) that has two ABC transporter family signatures, one at the middle ('ABC1') and the second at its C terminus ('ABC2'). This apparent fusion protein ('CydDC') resembles, therefore, some eukaryotic ABC transport systems where a tandemly duplicated molecule yields a single protein with two ABC domains. In this case, we considered separately the N-terminal 'ABC1' (575 residues, here named CydD) and the C-terminal 'ABC2' (remaining 597 residues, named CydC here) segments, and used these truncated proteins as supplementary sequences for CydD and CydC analyses. An alignment of the CydDC sequences was performed. Alignment and analysis of these sequences using the von Heijne Helix (transmembrane) and Kyte–Doolittle hydrophobicity algorithms revealed similar topological features, namely six TMs, two major CLs and three minor PLs with both N and C termini located in the cytoplasm. Almost all the CLs from CydDC sequences showed a net positive charge. We also investigated the organization in bacterial genomes of the *cyd* genes and found four different types of genetic organization. The *cydAB* and *cydDC* genes can constitute (i) independent clusters, (ii) neighbouring divergent clusters ($\leftarrow cydCD\ cydAB \rightarrow$), or show a tandem *cydABDC* arrangement encoding (iii) three or (iv) four proteins. In the first case, we traced the presence of the *trxB* gene that is upstream of the *E. coli cydD* gene. It appears that across short phylogenetic distances this gene organization is conserved but vanishes in organisms such as *Streptomyces violacea* and Gram-positive bacteria. A similar analysis of the *aat* gene downstream of *E. coli cydC* in these organisms showed no conservation. In this context, it is interesting to mention that the *Aeromonas jandaei* locus (GI:1903398) displays the *trxB* and *aat* genes separated not by the *cydDC* cluster but by a totally unrelated gene encoding a response regulator protein, RrpX. A search of the upstream genes of the *cydAB* clusters from a few genomes showed weak conservation of gene ordering. Intriguingly, *Lactococcus lactis* exhibits the *tra983B* gene encoding the transposase of IS983B. Immediately upstream of the *cydAB* operon of *Brucella melitensis* biovar Abortus, two genes encoding ABC-type transporters of 331 and 322 residues were found (Endley *et al.*, 2001). Tentatively, these genes were named *cydC* and *cydD* by the authors although no functional evidence was provided.

Conservation of the *cydD* and *cydC* genes in an operon possibly facilitates interactions of the encoded proteins (Dandekar *et al.*, 1998), may favour lateral gene transfer (Lawrence & Roth, 1996) or allow co-localization of the mRNAs in the same region of the cell (Danchin *et al.*, 2000). The conservation of gene order is obvious between closely

related species, but rapidly becomes less conspicuous among more distantly related organisms (Tamames, 2001). Nevertheless, *cydDC* gene ordering in *Staphylococcus aureus*, *Bacillus halodurans* or *Streptococcus pyogenes* resembles more the *E. coli* organization (except for the upstream *trxB* gene) than the corresponding one found in Gram-positive bacteria. On the basis of the presence of a small number of insertion sequences among 11 complete genome sequences, *Bacillus subtilis* was found to have the most stable genome retaining the ancestral genome structure (Itoh *et al.*, 1999). Gene translocation events may be responsible for loss of gene order in some bacteria, as Itoh *et al.* (1999) have proposed for the elimination of operons.

Conclusions

We propose a heterodimeric structure for the ABC-type transporter CydDC that is absolutely required for cytochrome *bd* assembly in *E. coli*. The two constituent polypeptides are strikingly similar and each is shown here to comprise six N-terminal transmembrane segments and a C-terminal, cytoplasmic ATP-binding cassette. The function of this transporter appears to be the export of reductant, specifically cysteine (Pittman *et al.*, 2002), to the periplasm. Although *cydD*- and *cydC*-like genes have been reported in most bacteria that synthesize cytochrome *bd*, it is possible that the heterodimeric structure proposed may not in all cases have the same transport function. Thus, in *D. melanogaster*, the White and Scarlet proteins that constitute a heterodimeric ABC transporter form a tryptophan transporter, whereas the White and Brown proteins form a guanine transporter (Tearle *et al.*, 1989; Mackenzie *et al.*, 1999). Different pairings of CydD- and/or CydC-like proteins might export diverse substrate molecules.

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REFERENCES

- Altschul, S. F., Madden, T. L., Schffer A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.
- Ames, G. F.-L. & Lecar, H. (1992). ATP-dependent bacterial transporters and cystic fibrosis: analogy between channels and transporters. *FASEB J* **6**, 2660–2666.
- Andersson, H. & von Heijne, G. (1993). Position-specific Asp-Lys pairing can affect signal sequence function and membrane protein topology. *J Biol Chem* **268**, 21389–21393.
- Bebbington, K. J. & Williams, H. D. (1993). Investigation of the role of the *cydD* gene product in production of a functional

cytochrome *d* oxidase in *Escherichia coli*. *FEMS Microbiol Lett* **112**, 19–24.

Borisov, V. B., Sedelnikova, S. E., Poole, R. K. & Konstantinov, A. A. J. (2001). Interaction of cytochrome *bd* with carbon monoxide at low and room temperatures: evidence that only a small fraction of heme *b*₅₉₅ reacts with CO. *J Biol Chem* **276**, 22095–22099.

Boyd, D. & Beckwith, J. (1989). Positively charged amino acid residues can act as topogenic determinants in membrane proteins. *Proc Natl Acad Sci U S A* **86**, 9446–9450.

Brandl, C. J. & Deber, C. M. (1986). Hypothesis about the function of membrane-buried proline residues in transport proteins. *Proc Natl Acad Sci U S A* **83**, 917–921.

Chang, G. (2003). Structure of MsbA from *Vibrio cholera*: a multidrug resistance ABC transporter homolog in a closed conformation. *J Mol Biol* **330**, 419–430.

Chang, G. & Roth, C. B. (2001). Structure of MsbA from *E. coli*: a homolog of the multidrug resistance ATP binding cassette (ABC) transporters. *Science* **293**, 1793–1800.

Cook, G. M. & Poole, R. K. (2000). Oxidase and periplasmic cytochrome assembly in *Escherichia coli* K-12: CydDC and CcmAB are not required for haem-membrane association. *Microbiology* **146**, 527–536.

Cook, G. M., Loder, C., Søballe, B., Stafford, G. P., Membrillo-Hernández, J. & Poole, R. K. (1998). A factor produced by *Escherichia coli* K-12 inhibits the growth of *E. coli* mutants defective in the cytochrome *bd* quinol oxidase complex: enterochelin rediscovered. *Microbiology* **144**, 3297–3308.

Cook, G. M., Cruz-Ramos, H., Moir, A. J. & Poole, R. K. (2002). A novel haem compound accumulated in *Escherichia coli* overexpressing the *cydDC* operon, encoding an ABC-type transporter required for cytochrome assembly. *Arch Microbiol* **178**, 358–369.

Cotter, P. A., Chepuri, V., Gennis, R. B. & Gunsalus, R. P. (1990). Cytochrome *o* (*cyoABCDE*) and *d* (*cydAB*) oxidase gene expression in *Escherichia coli* is regulated by oxygen, pH, and the *fnr* gene product. *J Bacteriol* **172**, 6333–6338.

Danchin, A., Guerdoux-Jamet, P., Moszer, I. & Nitschke, P. (2000). Mapping the bacterial cell architecture into the chromosome. *Philos Trans R Soc Lond B Biol Sci* **355**, 179–190.

Dandekar, T., Snel, B., Huynen, M. & Bork, P. (1998). Conservation of gene order: a fingerprint of proteins that physically interact. *Trends Biochem Sci* **23**, 324–328.

Delaney, J. M., Ang, D. & Georgopoulos, C. (1992). Isolation and characterization of the *Escherichia coli* *htrD* gene, whose product is required for growth at high temperatures. *J Bacteriol* **174**, 1240–1247.

Delaney, J. M., Wall, D. & Georgopoulos, C. (1993). Molecular characterization of the *Escherichia coli* *htrD* gene: cloning, sequence, regulation, and involvement with cytochrome *d* oxidase. *J Bacteriol* **175**, 166–175.

D'Mello, R., Hill, S. & Poole, R. K. (1996). The cytochrome *bd* quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity *in vivo* by oxygen inhibition. *Microbiology* **142**, 755–763.

Edwards, S. E., Loder, C. S., Wu, G., Corker, H., Bainbridge, B. W., Hill, S. & Poole, R. K. (2000). Mutation of cytochrome *bd* quinol oxidase results in reduced stationary phase survival, iron deprivation, metal toxicity and oxidative stress in *Azotobacter vinelandii*. *FEMS Microbiol Lett* **185**, 71–77.

Endley, S., McMurray, D. & Ficht, T. A. (2001). Interruption of the *cydB* locus in *Brucella abortus* attenuates intracellular survival and virulence in the mouse model of infection. *J Bacteriol* **183**, 2454–2462.

Esposti, D. E., De Vries, S., Crimi, M., Ghelli, A., Patarnello, T. & Meyer, A. (1993). Mitochondrial cytochrome *b*: evolution and structure of the protein. *Biochim Biophys Acta* **1143**, 243–271.

Ewart, G. D., Cannell, D., Cox, G. B. & Howells, A. J. (1994). Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in *Drosophila melanogaster*. Implications for structure-function relationships. *J Biol Chem* **269**, 10370–10377.

Gennis, R. B. & Stewart, V. (1996). Respiration. In *Escherichia coli and Salmonella. Cellular and Molecular Biology*, pp. 217–261. Washington, DC: American Society for Microbiology.

Georgiou, C. D., Fang, H. & 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*. *J Bacteriol* **169**, 2107–2112.

Giacomini, A., Corich, V., Ollero, F. J., Squartini, A. & Nuti, M. P. (1992). Experimental conditions may affect reproducibility of the beta-galactosidase assay. *FEMS Microbiol Lett* **79**, 87–90.

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

Goldman, B. S., Gabbert, K. K. & Kranz, R. G. (1996b). The temperature-sensitive growth and survival phenotypes of *Escherichia coli* *cydDC* and *cydAB* strains are due to deficiencies in cytochrome *bd* and are corrected by exogenous catalase and reducing agents. *J Bacteriol* **178**, 6348–6351.

Gort, A. S., Ferber, D. M. & Imlay, J. A. (1999). The regulation and role of the periplasmic copper, zinc superoxide dismutase of *Escherichia coli*. *Mol Microbiol* **32**, 179–191.

Holland, I. B. & Blight, M. A. (1999). ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules in organisms from bacteria to humans. *J Mol Biol* **293**, 381–399.

Itoh, T., Takemoto, K., Mori, H. & Gojobori, T. (1999). Evolutionary instability of operon structures disclosed by sequence comparisons of complete microbial genomes. *Mol Biol Evol* **16**, 332–346.

Jünemann, S. (1997). Cytochrome *bd* terminal oxidase. *Biochim Biophys Acta* **1321**, 107–127.

Juty, N. S., Moshiri, F., Merrick, M., Anthony, C. & Hill, S. (1997). The *Klebsiella pneumoniae* cytochrome *bd'* terminal oxidase complex and its role in microaerobic nitrogen fixation. *Microbiology* **143**, 2673–2683.

Kalnenieks, U., Galinina, N., Bringer-Meyer, S. & Poole, R. K. (1998). Membrane D-lactate oxidase in *Zymomonas mobilis*: evidence for a branched respiratory chain. *FEMS Microbiol Lett* **168**, 91–97.

Kelley, L. A., MacCallum, R. M. & Sternberg, M. J. E. (2000). Enhanced genome annotation using structural profiles in the program 3D-PSSM. *J Mol Biol* **299**, 499–520.

Kelly, M. J., Poole, R. K., Yates, M. G. & Kennedy, C. (1990). Cloning and mutagenesis of genes encoding the cytochrome *bd* terminal oxidase complex in *Azotobacter vinelandii*: mutants deficient in the cytochrome *d* complex are unable to fix nitrogen in air. *J Bacteriol* **172**, 6010–6019.

Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. L. (2001). Predicting transmembrane protein topology with a hidden Markov Model: application to complete genomes. *J Mol Biol* **305**, 567–580.

Lawrence, J. G. & Roth, J. R. (1996). Selfish operons: horizontal transfer may drive the evolution of gene clusters. *Genetics* **143**, 1843–1860.

Lee, C., Li, P., Inouye, H., Brickman, E. R. & Beckwith, J. (1989). Genetic studies on the inability of β -galactosidase to be translocated across the *Escherichia coli* cytoplasmic membrane. *J Bacteriol* **171**, 4609–4616.

- Lindqvist, A., Membrillo-Hernández, J., Poole, R. K. & Cook, G. M. (2000). Role of respiratory oxidases in protecting *Escherichia coli* K12 from oxidative stress. *Antonie van Leeuwenhoek* **78**, 23–31.
- Linton, K. J. & Higgins, C. F. (1998). The *Escherichia coli* ATP-binding cassette (ABC) proteins. *Mol Microbiol* **28**, 5–13.
- Mackenzie, S. M., Brooker, M. R., Gill, T. R., Cox, G. B., Howells, A. J. & Ewart, G. D. (1999). Mutations in the *white* gene of *Drosophila melanogaster* affecting ABC transporters that determine eye colouration. *Biochim Biophys Acta* **1419**, 173–185.
- Maloy, S. R., Stewart, V. J. & Taylor, R. K. (1996). *Genetic Analysis of Pathogenic Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Manoil, C. (1990). Analysis of protein localization by use of gene fusions with complementary properties. *J Bacteriol* **172**, 1035–1042.
- Manoil, C. & Bailey, J. (1997). A simple screen for permissive sites in proteins: analysis of *Escherichia coli* *lac* permease. *J Mol Biol* **267**, 250–263.
- Manoil, C. & Beckwith, J. (1985). *TnphoA*: a transposon probe for protein export signals. *Proc Natl Acad Sci U S A* **82**, 8129–8133.
- Markwell, M. A., Hass, S. M., Bieber, L. L. & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**, 206–210.
- Michaelis, S., Inouye, H., Oliver, D. & Beckwith, J. (1983). Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. *J Bacteriol* **154**, 366–374.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Moller, S., Croning, M. D. R. & Apweiler, R. (2001). Evaluation of methods for the prediction of membrane spanning regions. *Bioinformatics* **17**, 646–653.
- Nakashima, H. & Nishikawa, K. (1992). The amino acid composition is different between the cytoplasmic and extracellular sides in membrane proteins. *FEBS Lett* **303**, 141–146.
- Ninfa, E. G., Atkinson, M. R., Kamberov, E. S. & Ninfa, A. J. (1993). Mechanisms of autophosphorylation of *Escherichia coli* nitrogen regulator II (NRII or NtrB): trans-phosphorylation between subunits. *J Bacteriol* **175**, 7024–7032.
- Olsen, D. B., Sayers, J. R. & Eckstein, F. (1993). Site-directed mutagenesis of single-stranded and double-stranded DNA by phosphorothionate approach. *Methods Enzymol* **217**, 189–217.
- Osborne, J. P. & Gennis, R. B. (1999). Sequence analysis of cytochrome *bd* oxidase suggests a revised topology for subunit I. *Biochim Biophys Acta* **1410**, 32–50.
- Parsonage, D., Wilke-Mounts, S. & Senior, A. E. (1987). Directed mutagenesis of the β -subunit of F1-ATPase from *Escherichia coli*. *J Biol Chem* **262**, 8022–8026.
- Pi, J., Dogovski, C. & Pittard, A. J. (1998). Functional consequences of changing proline residues in the phenylalanine-specific permease of *Escherichia coli*. *J Bacteriol* **180**, 5515–5519.
- Pittman, M. S., Corker, H., Wu, G., Binet, M. B., Moir, A. J. G. & Poole, R. K. (2002). Cysteine is exported from the *Escherichia coli* cytoplasm by CydDC, an ABC-type transporter required for cytochrome assembly. *J Biol Chem* **277**, 49841–49849.
- Poole, R. K. & Cook, G. M. (2000). Redundancy of aerobic respiratory chains in bacteria? Routes, reasons and regulation. *Adv Microb Physiol* **43**, 165–224.
- Poole, R. K. & Haddock, B. A. (1974). Energy-linked reduction of nicotinamide-adenine dinucleotide in membranes derived from normal and various respiratory-deficient mutant strains of *Escherichia coli* K12. *Biochem J* **144**, 77–85.
- Poole, R. K., Williams, H. D., Downie, J. A. & Gibson, F. (1989). Mutations affecting the cytochrome *d*-containing oxidase complex of *Escherichia coli* K12: identification and mapping of a fourth locus, *cydD*. *J Gen Microbiol* **135**, 1865–1874.
- Poole, R. K., Hatch, L., Cleeter, M. W., Gibson, F., Cox, G. B. & Wu, G. (1993). Cytochrome *bd* biosynthesis in *Escherichia coli*: the sequences of the *cydC* and *cydD* genes suggest that they encode the components of an ABC membrane transporter. *Mol Microbiol* **10**, 421–430.
- Poole, R. K., Gibson, F. & 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 Microbiol Lett* **117**, 217–223.
- Roussel, A. & Cambillau, C. (1991). 'TurboFrodo', *Silicon Graphics Geometry Partners Directory*, p. 86. Mountain View, CA: Silicon Graphics.
- Sato, M. & Mueckler, M. (1999). A conserved amino acid motif (R-X-G-R-R) in the Glut1 glucose transporter is an important determinant of membrane topology. *J Biol Chem* **274**, 24721–24725.
- Schmitt, L. & Tampe, R. (2002). Structure and mechanism of ABC transporters. *Curr Opin Struct Biol* **12**, 754–760.
- Siegele, D. A., Imlay, K. R. & Imlay, J. A. (1996). The stationary-phase-exit defect of *cydC* (*surB*) mutants is due to the lack of a functional terminal cytochrome oxidase. *J Bacteriol* **178**, 6091–6096.
- Simons, R. W., Houman, F. & Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene* **53**, 85–96.
- Sonnhammer, E. L. L., von Heijne, G. & Krogh, A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. In *Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology*, vol. 5, pp. 175–182. Edited by J. Glasgow, T. Littlejohn, F. Major, R. Lathrop, D. Sankoff & C. Sensen. Menlo Park, CA: AAAI/MIT Press.
- Tamames, J. (2001). Evolution of gene order conservation in prokaryotes. *Genome Biol* **2**, 1–11.
- Tearle, R. G., Belote, J. M., McKeown, M., Baker, B. S. & Howells, A. J. (1989). Cloning and characterisation of the *scarlet* gene of *Drosophila melanogaster*. *Genetics* **122**, 595–606.
- Urbatsch, I. L., Sankaran, B., Weber, J. & Senior, A. E. (1995). P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. *J Biol Chem* **270**, 19383–19390.
- Vieira, J. & Messing, J. (1987). Production of single-stranded plasmid DNA. *Methods Enzymol* **153**, 3–11.
- von Heijne, G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J Mol Biol* **225**, 487–494.
- Way, S. S., Sallustio, S., Magliozzo, R. S. & Goldberg, M. B. (1999). Impact of either elevated or decreased levels of cytochrome *bd* expression on *Shigella flexneri* virulence. *J Bacteriol* **181**, 1229–1237.
- Winstedt, L., Yoshida, K., Fujita, Y. & von Wachenfeldt, C. (1998). Cytochrome *bd* biosynthesis in *Bacillus subtilis*: characterization of the *cydABCD* operon. *J Bacteriol* **180**, 6571–6580.