Cysteine Is Exported from the *Escherichia coli* Cytoplasm by CydDC, an ATP-binding Cassette-type Transporter Required for Cytochrome Assembly*

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Assembly of *Escherichia coli* cytochrome *bd* and periplasmic cytochromes requires the ATP-binding cassette transporter CydDC, whose substrate is unknown. Two-dimensional SDS-PAGE comparison of periplasm from wild-type and cydD mutant strains revealed that the latter was deficient in several periplasmic transport binding proteins, but no single major protein was missing in the cydD periplasm. Instead, CydDC exports from cytoplasm to periplasm the amino acid cysteine, demonstrated using everted membrane vesicles that transported radiolabeled cysteine inward in an ATP-dependent, uncoupler-independent manner. New pleiotropic cydD phenotypes are reported, including sensitivity to benzylpenicillin and dithiothreitol, and loss of motility, consistent with periplasmic defects in disulfide bond formation. Exogenous cysteine reversed these phenotypes and affected levels of periplasmic c-type cytochromes in cydD and wild-type strains but did not restore cytochrome *d*. Consistent with CydDC being a cytochrome exporter, cydD mutant growth was hypersensitive to high cytochrome *d* concentrations and accumulated higher cytoplasmic cysteine levels, as did a mutant defective in *orf299*, encoding a transporter of the major facilitator superfamily. A cydD orf299 double mutant was extremely cysteine-sensitive and had higher cytoplasmic cysteine levels, whereas CydDC overexpression conferred resistance to high extracellular cysteine concentrations. We propose that CydDC exports cysteine, crucial for redox homeostasis in the periplasm.

*Escherichia coli* possesses two major membrane-bound terminal respiratory oxidases, namely cytochromes *bo*’ (“bo3” encoded by *cyoABCDE*) and *bd*. The latter comprises two polypeptide subunits (encoded by *cydA* and *cydB*) and hemes b$_{153}$, b$_{161}$, and d (1–3). Both oxidases catalyze ubiquinol oxidation and oxygen reduction but differ in the efficiency with which electron transfer is coupled to proton translocation (2, 4), and the pattern of expression in response to environment (1, 2, 4). Significantly, cytochrome *bd* is required for resistance to a number of environmental stresses and its loss attenuates virulence in certain bacteria (5, 6).

Assembly of cytochrome *bd* is dependent not only on the structural genes *cydAB*, but also on the unlinked *cydDC* operon (7–9). The latter genes are predicted to encode a heterodimeric ABC*1*-type transporter (traffic ATPase) (9) with an unknown export function (10, 11). Unlike traffic ATPases involved in uptake, CydDC is thought not to interact with a cognate periplasmic-binding protein. Strains defective in either cydD or cydC display complex phenotypes in addition to loss of cytochrome *bd*. These include loss of periplasmic b-type and c-type cytochromes (10, 12); increased sensitivity to high temperature, H$_2$O$_2$, azide, and Zn$^{2+}$ ions (8, 12, 13); and inability to exit stationary phase at 37 °C under aerobic conditions (14). We hypothesized that the substrate of CydDC might be heme (9, 10) that would be assembled into apocytochromes following export to the periplasm. However, the assembly of heme into heterologous apoproteins (e.g. *Ascaris* hemoglobin) exported to the periplasm of *E. coli* does not require cydC (12), suggesting that outward transport of heme is not absolutely dependent on CydDC. Furthermore, transport studies using inside-out vesicles derived from wild-type and cydD mutant strains revealed no discernible differences between the two strains in association of radiolabeled heme with, or transport by, vesicle membranes (15).

An important clue to the function of CydDC was the finding (12) that the periplasm of a cydD mutant is more oxidizing, as assayed using 5,5’-dithiobis(2-nitrobenzoic acid), than that of a wild-type strain. This suggests that CydDC exports a reducing molecule to the periplasm and therefore contributes to the maintenance of the balanced redox conditions required for cytochrome *c* biogenesis in the periplasm. CcmH, containing a conserved CXXC motif, is required in *E. coli* for keeping the heme-binding site of apocytochrome *c* in a reduced form for subsequent heme ligation (16). Several other protein thiols: disulfide oxidoreductases are required for cytochrome *c* maturation; loss of DsbA, DsbB, or DsbD (DipZ) each results in a loss of c-type cytochromes (17–19). DsbA and DsbB are involved in the formation of disulfide bonds in various periplasmic proteins (20, 21), whereas DsbD translocates electrons from the cytoplasm to the periplasm (22), thereby providing a source of reducing power to an otherwise oxidized environment.

The aim of this work was to identify the substrate exported by CydDC. We failed to find an obvious protein candidate, but show instead that CydDC exports cysteine to the periplasm,

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1 The abbreviations used are: ABC, ATP-binding cassette; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; IPG, immobilized pH gradient; LB, Luria-Bertani; EDDHA, ethylenediamine dithiorectetamin:o, hydroxypolyacetic acid; CCCP, carbonyl cyanide p-chlorophenylhydrazone; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
the first demonstration of ATP-driven L-cysteine export. Support for this conclusion comes from: (a) direct demonstration that everted membrane vesicles take up cysteine in an ATP- and CydDC-dependent manner, corresponding to export in vivo; (b) correction by exogenous L-cysteine of newly reported cydD phenotypes, specifically loss of motility and increased sensitivity to benzylpenicillin; (c) detection of higher cytoplasmic ATP in cydD mutant cells; (d) susceptibility of cydD mutants to growth inhibition by external cysteine; and (e) increased resistance to cytotoxic levels of cysteine by strains that overexpress CydDC.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli strain AN2343 carrying the mutant cydD1 allele and its isogenic wild-type parent strain AN2342 have been described before (8). Strains RKP4611 and RKP4612 were constructed by P1vir transduction (23) of the orf299::Km allele from strain MC4100(orf299) (24) into strains AN2342 and AN2343, respectively. Strains RKP2634 and RKP2005 were obtained by transformation of the wild-type and cydD mutant strain, respectively, with plasmid pRFP33 (9) that has the cydDC operon cloned into vector pBR328.

Media and Culture Conditions—Cells were grown in Luria-Bertani (LB) broth (pH 7.0) (25), or in MOPS-buffered minimal medium (pH 7.4) (26). Anaerobically grown cultures were used to obtain a 400 ml culture was used and the spheroplasts were constructed by P1vir transduction (23) of the mid pRP33 (9) that has the orf299::Km allele and its isogenic wild-type parent strain AN2342. Strains RKP2634 and RKP2005 were obtained by transforma-

Preparation of Membrane Vesicles—Membrane vesicles were prepared by the method of Markwell et al. (36). An equal volume of 0.1% Triton X-100 (w/v) solution containing 53 mM Tris-HCl (pH 7.5) and 2 mM EDTA, and incubated at 4 °C overnight was used to prepare a 1:1 ratio of membrane vesicles. Everted vesicles were thawed slowly on ice and diluted to 1.0 mg protein/mL. Everted vesicles were then disrupted by a single 2-fold dilution in a second buffer (5 vol/g of wet cells). Everted vesicles were then disrupted by a single passage through a French pressure cell at 2800 psi, and the colonies were counted.

Preparation of Subcellular Fractions—Periplasmic fractions were isolated using a modified procedure of Willis et al. (27). In brief, 200 ml of culture was conditioned for osmotic shock by the addition of 6 ml of 1 M NaCl and 6 ml of 1 M Tris-HCl buffer (pH 7.3). An equal volume of the conditioned culture was incubated with 40 ml lactose plus 10% (w/v) LB, Kanamycin and benzylpenicillin (penicillin G) were added to give final concentrations of 30 and 20 μg/mL, respectively. L-Cysteine was added as a filter-sterilized 100 mM stock solution to media, giving the final concentrations of 0.2 mg of protein/mL. The samples were incubated overnight at 37 °C for 14 h.

Motility Assays—Cells were grown to stationary phase in LB broth at 30 °C, and 2 μl drops were spotted onto semi-solid LB medium (0.3% Difco agar). The motility assays were performed by the method of Willis et al. (27). In brief, 200 μl of culture was conditioned for osmotic shock by the addition of 6 ml of 1 x NaCl and 6 ml of 1 x Tris-HCl buffer (pH 7.3). An equal volume of the conditioned culture was incubated with 40 ml lactose plus 10% (w/v) LB, Kanamycin and benzylpenicillin (penicillin G) were added to give final concentrations of 30 and 20 μg/mL, respectively. L-Cysteine was added as a filter-sterilized 100 mM stock solution to media, giving the final concentrations of 0.2 mg of protein/mL. The samples were incubated overnight at 37 °C for 14 h.

Preparation of Everted Membrane Vesicles—E. coli strain AN2343 carrying the mutant cydD1 allele and its isogenic wild-type parent strain AN2342 have been described before (8). Strains RKP4611 and RKP4612 were constructed by P1vir transduction (23) of the orf299::Km allele from strain MC4100(orf299) (24) into strains AN2342 and AN2343, respectively. Strains RKP2634 and RKP2005 were obtained by transformation of the wild-type and cydD mutant strain, respectively, with plasmid pRFP33 (9) that has the cydDC operon cloned into vector pBR328.

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Two-dimensional Gel Electrophoresis—Periplasmic samples were concentrated 2-fold with a Centricon YM-3 centrifugal filter device (Amicon Bioseparations-Millipore Corp.) with a maximum volume of 2 ml and a molecular mass cut-off of 3,000 Da. A portion (2 ml) of each sample was spun (10,000 × g for 10 min) at 4 °C and the supernatant was concentrated to one-tenth of the original volume. An additional 2 ml of sample was centrifuged exactly as above, and samples were pooled. Concentrated periplasm (~0.2 mg of protein) was included in 125 μl (total volume) of rehydration solution (8 M urea, 2% (v/v) CHAPS, 0.5% (v/v) I PG buffer pH 3–10 (non-linear) (Amersham Biosciences), 0.28% dithiothreitol, and a few grains of bromphenol blue) and applied to a 7 cm IPG strip. After rehydration (18–24 h), two-dimensional gel electrophoresis was carried out using a Multiphor II horizontal unit with immobilized pH gradients (pre-cast IPG strip, pH 3–10, non-linear) in the first dimension and a sodium dodecyl sulfate (SDS)-polyacrylamide gel (6–18% polyacrylamide) in the second dimen-

**Determination of N-terminal Sequence—** Proteins were electroblotted onto ProBlott (Applied Biosystems) membranes at 400–500 mA for 1.5–2 h before staining with Coomassie Blue. The N-terminal sequences of the protein spots were determined by sequential Edman degradation (30). Sequence identity was computed using the Colibri web site (geno
dbase.biologie.uni-frankfurt.de/Colibri/) FASTA function (31). Further information on sequenced proteins was found on the SWISS-PROT web site (www.expasy.ch).

**Cysteine Assay—** This was carried out using the method of Gaitonde (32). A standard curve (0–0.5 μmol of cysteine-HCl) was prepared, and used to quantify cysteine levels in cytoplasmic fractions, which had been treated with acetic acid and acid ninhydrin “Reagent 2” (250 mg ninhydrin dissolved in a mixture of 6 ml of acetic acid and 4 ml of HCl). Samples were heated in a boiling water bath for 10 min, then cooled rapidly in water before dilution to 5 or 10 ml using 95% ethanol. After 30 min at room temperature, the reaction products were measured at 561 nm. To correct for interference by other ninhydrin-reactive compounds, a standard curve was prepared as described above to minimize interference by other cytochromes with overlapping spectral features. Reduced minus oxidized difference spectra at room temperature were recorded as in Ref. 10 but in the SDB4 dual wavelength scanning spectrophotometer (33) was used. For cytochrome d, an absorption coefficient ε (622 minus 644 nm) of 12.6 μM·cm⁻¹ was used in CO difference spectra. For c-type cytochromes, periplasmic samples were used to measure CO difference spectra. Reduced minus oxidized difference spectra at room temperature were recorded as in Ref. 10 but in the SDB4 dual wavelength scanning spectrophotometer. Correction for baseline drift in the Soret region was accomplished by dropping a vertical from the absorption peak at ~425 nm (NHaF has a maximum in absolute spectra at 420.5 nm; Ref 35) to a base line drawn between 404 and 450 nm. The absorption coefficient ε was used as 146 μM·cm⁻¹·nm⁻¹ (10), determined by using the absorption coefficient ε₅₅₀–₅₄₀ for the aₕ-band (10) and a ½ ε₅₅₀ of 7.5 measured in spectra of concentrated periplasmic fractions. Protein contents of cell suspensions and periplasmic fractions were assayed using the method of Markwell et al. (36).

Preparation of Everted Membrane Vesicles—Up to 6 liters of culture was grown aerobically at 37 °C to the mid-exponential phase of growth (A₅₄₆₀ = 0.6) in MOPS minimal medium supplemented with lactose and LB. Cells were harvested by centrifugation and the cell pellet washed with pre-cooled 10 mM Tris-HCl (pH 7.5), containing 140 mM choline chloride, 0.5 mM dihydrothreitol, and 10% glycerol (v/v) followed by re-

The first demonstration of ATP-driven L-cysteine export. Support for this conclusion comes from: (a) direct demonstration that everted membrane vesicles take up cysteine in an ATP- and CydDC-dependent manner, corresponding to export in vivo; (b) correction by exogenous L-cysteine of newly reported cydD phenotypes, specifically loss of motility and increased sensitivity to benzylpenicillin; (c) detection of higher cytoplasmic ATP in cydD mutant cells; (d) susceptibility of cydD mutants to growth inhibition by external cysteine; and (e) increased resistance to cytotoxic levels of cysteine by strains that overexpress CydDC.
transport, vesicles were energized for 15 min prior to lactose addition with 20 mM L-lactate. [35S]Cysteine transport was initiated by the addition of cysteine for 5 min prior to the addition of 10 mM ATP. Vesicles were de-energized with either CCCP (2 μM) to dissipate the proton gradient (15), or sodium orthovanadate (50 μM), an analogue of inorganic phosphate that mimics the γ-phosphate of ATP in the transition state for ATP hydrolysis (38). Transport was terminated by rapidly pouring the contents onto cellulose-nitrate filters (0.45-μm pore size), which were washed twice with 4 ml of 100 mM LiCl, and dried. Radioactivity was measured by liquid scintillation counting. To minimize nonspecific binding of substrate to filters, the filters were pre-soaked in 100 mM LiCl.

RESULTS

Periplasmic Fractions of Wild-type and cydD Mutant Strains Have Different Levels of Periplasmic Transport Proteins—The periplasm of a cydC mutant is more oxidized than that of a wild-type strain (12). It seems plausible, therefore, that candidate substrates for the CydDC transporter are any reducing or oxygen-scavenging agents. Interestingly, the cydC operon is adjacent to the trxB (thioredoxin reductase) gene (9, 39) on the E. coli chromosome but trxB mutants do synthesize cytochromes c and bd (10), ruling out TrxB as a candidate substrate. Mutants defective in trxA (encoding thioredoxin) and grx (glutaredoxin) also synthesize cytochrome bd (15). Although trxA mutants are unable to assemble c-type cytochromes unless complemented with 2-mercaptoethanesulfonic acid (40), this demonstrates that TrxA is not essential for cytochrome bd assembly either. However, a redox protein other than TrxB, thioredoxin, or glutaredoxin remains an intriguing candidate, as this would explain the plethora of redox-associated phenotypes of cydDC mutants.

We therefore sought a protein that might be transported by CydDC by using two-dimensional SDS-PAGE and N-terminal sequencing to analyze periplasmic fractions of wild-type and cydD strains. Marker enzyme assays on both periplasmic and cytoplasmic fractions revealed <5% contamination by cytoplasmic and periplasmic enzymes, respectively (results not shown). Comparison of two-dimensional gels (Fig. 1 and Table I) revealed several major differences, and, of the spots chosen for excision and subsequent Edman degradation, all were found to be periplasmic proteins, the determined sequences of which began after a signal sequence. This strongly suggests that all proteins identified were exported from the cytoplasm to the periplasm by a Sec-dependent mechanism (41). The proteins represented by spots 1 and 9 were identified as OppA (42) and AnsB (43), respectively, and were expressed at significantly higher levels in the periplasm of the wild type than that of the mutant (Fig. 1). A minor spot (number 8) was also OppA and may result from post-translational alteration or modification of lysine residues during electrophoresis (44). Proteins OsmY (45) and HisJ (46) (spots 5 and 6, respectively) were expressed at slightly more elevated levels in the cydD mutant periplasm compared with that of the wild type (Fig. 1). The remaining five sequenced proteins (MalE, GlnH, ProX, HisJ, and DppA) were expressed at slightly higher levels in the wild type compared with the cydD mutant periplasm and are the periplasmic binding–proteins of secondary type transport systems in E. coli (see Ref. 47 and references therein). Transport mechanisms for all of these proteins are already established, so it seems unlikely that they are substrates of the CydDC transporter.

A cydD Mutant Displays a Cysteine-reversible Defect in Motility—Many of the well documented phenotypes associated with loss of CydDC are actually attributable to the consequent loss of cytochrome bd (48). These include sensitivity to several agents including cysteine and inability to grow or exit stationary phase at 42 °C (8, 13, 14, 49). Loss of periplasmic c-type cytochromes, however, appears directly attributable to the loss of the CydDC transporter (10). We report here an additional phenotype of a cydD mutant, namely a defect in motility. When inoculated onto semi-solid agar and incubated overnight at 30 °C, a temperature that enhances expression of flagellar genes (50), the mutant was non-motile (Fig. 2), spreading only slightly beyond the inoculation site. In comparison, the wild-type strain displayed a normal swimming phenotype, producing a halo approximately 50 mm in diameter (Fig. 2), punctuated with concentric rings, a characteristic associated with swarmer cell morphology (51).

Addition to growth media of compounds containing thiol groups, such as cysteine and 2-mercaptopethanesulfonic acid, complements a dsbD mutation (18) with respect to cytochrome c assembly, suggesting the requirement for a reductant in the periplasm. Furthermore, cysteine corrects the temperature-sensitive phenotypes of Cyd− mutants (48). We therefore investigated the possibility that cysteine might also reverse the motility defect of cydD mutants. The cydD mutant was inoculated onto LB (0.3% agar) containing 2 mM L-cysteine and incubated at 30 °C for 8 h. This strain was non-motile (see above), but, in the presence of cysteine, a zone of swarming, typically 18 mm (Fig. 2), was observed. In contrast, the ability of the wild-type strain to swarm decreased in the presence of cysteine (Fig. 2), suggesting that a finely poised periplasmic

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**Fig. 1.** Two-dimensional polyacrylamide gel electrophoresis of polypeptides from periplasmic fractions of wild-type strain AN2342 (A) and cydD mutant AN2343 (B). Arrows identify polypeptides for which levels were found either to decrease (1–4 and 7–10 in panel A) or to increase (5 and 6 in panel B) in the cydD mutant.
mean values and standard deviations in three experiments. 30 °C (ior of E. coli culture) the presence or absence of 2 mM cysteine. In the absence of both culture) redox potential is required for optimal motility. AN2342 (wild type) and AN2343 (cydD mutant) were grown in liquid broth to stationary phase at 30 °C, then inoculated with sterile toothpicks onto LB (0.3% Difco agar) with or without cysteine and incubated at 30 °C for 8 h. Bars show mean values and standard deviations in three experiments. redox potential is required for optimal motility.

Effects of Cysteine on Penicillin Sensitivity—Sensitivity to benzylpenicillin is observed when there are defects in disulfide bond formation in the periplasm (52). Cultures of strains AN2342 (wild type) and AN2343 (cydD mutant), at similar culture densities, were challenged with benzylpenicillin (20 µg ml−1) in the presence or absence of 2 mM cysteine. In the absence of both penicillin and cysteine, the number of viable cells was slightly lower for the cydD mutant (corresponding to 1.3 × 10^6 cells (ml culture)−1) than for the wild-type parent (2.1 × 10^6 cells (ml culture)−1), consistent with the compromised viability of cydDC mutants (14). Extracellular cysteine alone slightly increased the viable counts of both strains. Although both strains were incapable of growth on plates containing penicillin alone (Fig. 3), cysteine suppressed the antibacterial effect more strongly, the colony morphologies of the two strains when plated (52). Cultures of strains AN2342 (white bars) and AN2343 (black bars) were grown in liquid broth to stationary phase at 30 °C, then inoculated with sterile toothpicks onto LB (0.3% Difco agar) with or without cysteine and incubated at 30 °C for 8 h. Bars show mean values and standard deviations in three experiments. finding (48) that a cydD mutant is hypersensitive to DTT, a powerful reductant (mid-point redox potential of approximately −330 mV (Ref. 53)) that is used for determining sensitivity to disulfide bond formation (52). We confirmed that a cydD mutant is also DTT-sensitive; zones of growth inhibition for wild-type and cydD strains were recorded around sterile filter discs soaked in 7 or 15 mM filter-sterilized DTT. This reductant exerted a dose-dependent inhibition of the wild-type strain but, in the cydD mutant, the diameter of the zone of inhibition was 3-fold higher than for the wild-type strain at both concentrations. Thus, in the absence of other environmental stresses, the cydD mutant is DTT-sensitive, as well as cysteine-sensitive (see below).

Exogenous Cysteine Modulates Levels of c-type Cytochromes—Poole et al. (10) reported that periplasmic c-type cytochromes were barely detectable in a cydD mutant; the Soret absorbance at 418.5 nm (77 K) was less than 10% of wild-type levels and the distinctive α-band at 550 nm (77 K, expected at 552 nm at room temperature) was undetectable. Assembly of c-type cytochromes takes place in the oxidizing environment of the periplasm; thus, cysteine residues of the apocytochrome must be reduced before ligation of heme to the apocytochrome (54–56). DsbD, an integral membrane protein, provides this reducing power by translocating electrons from the cytoplasm to the periplasm (22, 57). Loss of DsbD results in a loss of c-type cytochromes, which can be corrected by the addition of specific thiol-containing compounds (18, 40). Because cysteine also corrects defects in motility of a cydD

| Protein | Sequence (with amino acid residue nos.) | Enzyme function | pI | M r | Accession nos. |
|---------|----------------------------------------|-----------------|----|-----|----------------|
| Spot 1 (OppA) | 27ADVPGVTLAEK38 Periplasmic oligopeptide-binding protein precursor | 5.85 | 58.36 | P23843 |
| Spot 2 (OppA) | 29KTLVXSEGDEP40 Periplasmic dipeptide transport protein precursor (dipeptide-binding protein) | 5.75 | 57.41 | P23847 |
| Spot 3 (MalE) | 27KIEEKLIVWIN38 Maltose-binding periplasmic protein precursor | 5.22 | 58.36 | P23843 |
| Spot 4 (ProK) | 22ATPGKGTYNVPQV38 High affinity glycine betaine-binding protein | 5.65 | 33.73 | P4177 |
| Spot 5 (OsmY) | 32TTNESAGQKND42 Periplasmic, sigma S-dependent protein | 5.42 | 18.16 | P27291 |
| Spot 6 (HisJ) | 23AIPQNIRI30 histidine-binding protein of high affinity | 5.17 | 26.23 | P23843 |
| Spot 7 | Not available | Near 5.8 | — | 50 |
| Spot 8 (OppA) | 27ADVPGA32 Same as spot 1 | 5.85 | 58.36 | P23843 |
| Spot 9 (AnsB) | 23LPNITILA30 L-Asparaginase II (precursor) | 5.66 | 34.59 | P00805 |
| Spot 10 (GlnH) | 23ADKLVYAT31 Glutamine-binding periplasmic protein | 5.87 | 24.96 | P10344 |

Table I
N-terminal sequences of proteins extracted from selected spots in two-dimensional PAGE gels of periplasmic fractions

FIG. 3. Exogenous cysteine confers resistance to benzylpenicillin (penicillin G) differentially in wild-type and cydD mutant strains. Strains AN2342 (cydD+) (white bars) and AN2343 (cydD) (black bars) were grown at 37 °C to stationary phase. Portions (5 µl) of serially diluted cultures were drop-plated onto LB containing benzylpenicillin (20 µg ml−1) and/or cysteine (2 mM). Bars show the mean of 10 replicates with standard deviations. Total colony counts are shown; note that, in the presence of both penicillin and cysteine, the cydD mutant produced full-sized colonies in 16–18 h, whereas the wild-type strain produced tiny "pin-prick" colonies.
mutant and modulates sensitivity to benzylpenicillin in a cydD mutant (see above), the effects of cysteine on levels of c-type cytochromes were investigated. Wild-type and cydD strains were grown anaerobically in LB plus 20 mM KNO₃ to elevate cytochrome c levels, without or with cysteine (0.2 and 2 mM) supplements. Reduced minus oxidized difference spectra of the periplasm from the wild-type strain showed a Soret band at 423 nm, a β-band at 525 nm, and an α-band at 552.5 nm (data not shown). These signals are attributable to the NrfA/NrfB cytochrome c nitrite reductase, maxima for reduced NfrA being, for example, 420.5, 523.5, and 552 nm (35), whereas NfrB has a sharp absorbance maximum at 551 nm, all at room temperature.² Cysteine (0.2 and 2 mM) in the growth media was without effect on the band positions but progressively decreased cytochrome c concentration in the periplasm of the wild-type strain (Fig. 4). Qualitatively similar spectra were also recorded for the cydD mutant but, in the absence of cysteine, the cytochrome c level was 30% of the wild-type level. In previous work (10), we could find no periplasmic cytochrome c in the cydD mutant; the difference may be attributable to the use of fumarate as an electron acceptor (10) or the poorer sensitivity of the earlier spectrophotometer. In the cydD mutant, cytochrome c levels were increased ~1.7-fold when cells were grown with 0.2 mM cysteine compared with cells grown without cysteine, but decreased at 2 mM cysteine (Fig. 4). The opposing effects of 0.2 mM cysteine on the wild-type (decreased cytochrome c) and the cydD mutant strains (increased cytochrome c) again suggest the significance of an appropriate cysteine-modulated redox poise in the periplasm.

**Exogenous Cysteine Does Not Restore Cytochrome bd in a cydD Mutant**—Because cysteine increased cytochrome c levels in a cydD mutant, we hypothesized that cysteine might also restore cytochrome bd. CO difference spectra of the wild-type strain showed a band at 644 nm, corresponding to the cytochrome d form of cytochrome d, as described previously (8, 9). Spectra of the cydD mutant revealed no cytochrome d signal at 644 nm, and cells grown in the presence of 0.2 and 2 mM cysteine also lacked cytochrome d.

**cydD and orf299 Mutants Are Sensitive to Cysteine**—Delaney et al. (13) reported that htrD (cydD) mutants show growth defects in the presence of cysteine, and that there is increased cysteine uptake compared with a wild-type strain. We therefore considered the possibility that the cysteine sensitivity of cydDC mutants and the ability of exogenous cysteine to modulate physiology (Figs. 2 and 3) and cytochrome assembly (Fig. 4) are linked, and result from the failure of cydDC mutants to export cysteine. The effect of cysteine on the growth of a cydD mutant was therefore compared with the effect on the orf299 mutant recently described by Dassler et al. (24). This gene product is a putative member of the major facilitator superfamily of transport proteins, and its expression promotes cysteine and O-acetyl-l-serine excretion (24).

To clarify the role(s) of each gene in cysteine metabolism, isogenic strains were first constructed by transducing the orf299 allele into wild-type and cydD mutant strains. The resulting strain RKP4611 (orf299) was insensitive to EDDHA as anticipated for Cyd⁺ strains (58), and CO difference spectra revealed the presence of cytochrome d. However, RKP4612 (orf299 cydD) was sensitive to EDDHA and cytochrome d was absent. The growth phenotypes of the orf299 and cydD orf299 strains were compared with those of wild-type and cydD mutant strains in the presence of 0, 2, and 5 mM cysteine (Fig. 5). Growth of the wild type was not substantially affected by cysteine. At 5 mM cysteine, there was a slight lag in reaching the stationary phase but optical density was not significantly different after 8 h (Fig. 5A). Growth of the cydD mutant was slightly inhibited at 2 mM cysteine, as characterized by a slower growth rate and a reduction in optical density after 8 h. However, 5 mM cysteine was severely inhibitory and growth was arrested after 4 h (Fig. 5B). The orf299 mutant displayed an extended lag phase and did not reach mid-exponential growth until 3–4 h after inoculation compared with 2 h for the wild-type and cydD strains. Cysteine (5 mM) extended the lag phase by ~1 h. The double mutant (orf299 cydD) displayed growth that was highly sensitive to cysteine. At 2 mM, growth was arrested after 4 h, and at 5 mM, A₆₀₀ was further reduced (Fig. 5D). Therefore, a cydD mutant strain displays greater cysteine sensitivity than an orf299 mutant, but defects in both genes result in extreme sensitivity to cysteine. The data suggest that both gene products are involved in cysteine resistance.

**Cysteine Is Transported by CydDC in an ATP-dependent Manner**—In view of the ability of cysteine to reverse some of the pleiotropic defects of a cydD mutant, particularly those associated with periplasm physiology, it was considered a candidate substrate of CydDC. To demonstrate this directly, we measured uptake of [³⁵S]cysteine by everted membrane vesicles of the orf299 and cydD orf299 strains. Extensive studies (37, 59–61) have already demonstrated the efficacy of French pressure cell treatment or ultrasonication of Gram-negative bacteria in producing predominantly everted (inside-out) vesicles that actively take up solutes, particularly toxic metal or metalloid ions, that would be exported in vivo. To demonstrate that the everted vesicles support active transport under the present conditions, we measured the ability of the vesicles to accumulate [¹⁴C]lactose in response to an energized membrane. The lactose transporter, LacY, can support lactose transport in either right-sided or everted vesicles, provided a proton motive force ( Δp) of the appropriate polarity is applied. Everted vesicles prepared from cells grown in MOPS medium supplemented with glucose have a low rate of lactose transport (15). Therefore, everted vesicles were prepared from the orf299 strain grown with lactose. Significant accumulation of [¹⁴C]lactose occurred only if β-lactate was added as an energy source; typical rates of [¹⁴C]lactose transport were 0.18 nmol min⁻¹ (mg protein)⁻¹ (data not shown). The addition of the protonophore, CCCP, abolished transport, demonstrating that accumulation of [¹⁴C]lactose was dependent upon Δp (data not shown).

² D. J. Richardson, personal communication.
The role of CydDC in cysteine transport was investigated using an orf299 genetic background, because this gene is also proposed to play a role in exporting cysteine from cells (and thus into everted vesicles). \[^{35}\text{S}]\text{cysteine uptake assays were done using everted membrane vesicles from the orf299 mutant and the cydD orf299 double mutant. Because ATP does not permeate the lipid membrane and is hydrolyzed only on the inner aspect of the membrane \((61)\), the ability of ATP to drive transport can be taken as evidence of vesicle inversion. In the 5-min period prior to ATP addition, no uptake of \[^{35}\text{S}]\text{cysteine was observed in the orf299 vesicles (Fig. 6A). Upon addition of ATP, however, \[^{35}\text{S}]\text{cysteine uptake was rapid with maximal uptake occurring over a 3-min period following ATP addition (Fig. 6A). The maximum rate of \[^{35}\text{S}]\text{cysteine uptake observed under the conditions tested was 0.31 nmol min}^{-1} \text{mg protein}^{-1}.\) The uptake of \[^{35}\text{S}]\text{cysteine reached a maximal level 4 min after the addition of ATP, and apparent saturation was observed (Fig. 6A). In contrast, no \[^{35}\text{S}]\text{cysteine uptake was observed in vesicles of RKP4612 (cydD orf299) before or after the addition of ATP (Fig. 6A).\)

Transport inhibitors were used to test whether the CydDC transporter derives energy for transport directly from ATP. CCCP (2 \(\mu\text{M}\)) had no discernible effect on the uptake of \[^{35}\text{S}]\text{cysteine by vesicles of the orf299 mutant (Fig. 6B), and the rate of \[^{35}\text{S}]\text{cysteine uptake was \(-0.21\) nmol (mg protein)}^{-1}.\) There was no \[^{35}\text{S}]\text{cysteine uptake into everted vesicles of RKP4612 (cydD orf299) in the presence of CCCP (Fig. 6B). Incubation of orf299 vesicles with sodium orthovanadate abolished transport such that the rate was indistinguishable from that observed with everted vesicles of RKP4612 (cydD orf299) (Fig. 6C).\) These studies confirm that the CydDC transporter does not derive energy from the \(\Delta\text{p}\) but directly from ATP hydrolysis.

Overexpression of CydDC Confers Resistance to Exogenous Cysteine Toxicity—The finding that, \textit{in vivo}, CydDC exports cysteine and that a cydD mutant is hypersensitive to exogenous cysteine (Fig. 5) predicts that overexpression of the transporter might confer additional resistance at very high concentrations of extracellular cysteine. We have already demonstrated that expression of the entire cydDC operon under the control of its own promoter on a multicopy plasmid results in increased levels of the cydDC transcript and elevated levels of the CydD protein detectable by a polyclonal antibody \((62)\). We therefore compared the growth of various strains in liquid medium containing a cysteine concentration that barely permitted growth of the wild-type strain. At 20 mm cysteine, poor growth of the wild-type and cydD mutant strains was observed 3 h after inoculation (Table II), but, after 9 h, culture turbidity declined markedly. Control cultures in the absence of cysteine showed little difference between these two strains at 3 or 9 h. Strain RKP2634, a wild-type strain harboring plasmid pRP33 \((\text{cydDC}^{a})\), showed slightly better growth than the corresponding plasmid-free strain at 3 h and continued to grow over the 9-h course of the experiment. Similar results were obtained when this plasmid was present in a cydD mutant background (strain RKP2005), showing that the plasmid not only complemented the chromosomal mutation but also conferred enhanced cysteine resistance.

Mutation of cydD or orf299 Increases Cytoplasmic Pool Sizes of Cysteine—A further prediction of the hypothesis that both CydDC and the orf299 gene product contribute to cysteine export from the cytoplasm is that the cytoplasmic pool size of cysteine should be elevated in these mutants. Periplasmic fractions from various isogenic strains were removed by osmotic shock, and a cytoplasmic fraction was prepared by disruption of the resulting spheroplasts and removal of membrane material by high speed centrifugation. In the wild-type strain, the cysteine concentration of the cytoplasm was 0.38 nmol of cysteine (mg protein)\(^{-1}\). This level was elevated 1.6-fold in the cydD mutant, 1.3-fold in the orf299 mutant (RKP4611), and 2.2-fold in the double mutant (RKP4612). In a replicate experiment, similar elevations of cysteine content relative to the wild type were observed. In an attempt to prevent cysteine oxidation to cystine during extraction, the use of DTT was explored, but this reagent interfered with the colorimetric cysteine assay. Despite potential complications from loss of cysteine during cytoplasm preparation or cysteine pool turnover, these data strongly suggest that both CydDC and the orf299 gene product contribute to cysteine export to the periplasm.

**DISCUSSION**

Mutation of the CydDC transporter causes a loss of cytochrome \(bd\) and consequently other phenotypes including sensitivities to \(\text{Zn}^{2+}\), azide, and the iron-chelating agent EDDHA; the inability to grow at 42 °C; and stationary-phase exit defects \((13, 48, 58)\). Thus, CydDC plays a critical role in bacterial physiology, stress responses, and pathogenicity \((5, 6)\). Previous
studies have implicated heme as a possible substrate for CydDC in part because (a) the oxidase subunits are detected as apoproteins in the membranes of cydDC mutants (63) and (b) all periplasmic cytochromes, as well as cytochrome bd, are affected (9, 10, 14). However, the transport of heme was shown not to be energy-dependent, and uptake was observed at similar rates in both a cydD mutant and an isogenic wild type, suggesting that heme may be delivered to the periplasm by binding and passive crossing of the cytoplasmic membrane (14) or by an unknown heme transporter (64). In the present work, protein profiles of the periplasmic fractions of cydD mutant and wild-type strains revealed no evidence that the substrate of CydDC is a major protein. Interestingly, OsmY and HisJ were present at elevated levels in the periplasm of the cydD mutant compared with that of the wild-type strain and this might be interpreted as a stress response employed to counteract the periplasmic defects associated with loss of CydD. None of the proteins identified in this study (Table I) contain disulfide bonds.

Goldman et al. (12) first postulated that the substrate of the CydDC transporter is involved in redox homeostasis. Consistent with this, we present direct evidence here that cysteine is exported by CydDC and also report new cydD phenotypes, including loss of motility. The flagellum required for motility of Gram-negative bacteria is arguably the most complex organelle found in bacteria (50). The basal body or rotor contains two rings (L and P) in a position equivalent to the outer membrane, these proteins having the classical Sec export signal sequence. A defect in the assembly of these rings in dsbB mutants appears to result from an inability to form a disulfide bond in the P-ring protein (65); indeed, cystine, but not cysteine, added to media suppresses this defect (65). In our work, a non-motile phenotype (lack of swarming on soft agar) is also observed in a cydD mutant but cysteine suppresses this phenotype (Fig. 2). The apparently opposite requirements for cystine and cysteine in these reports may result from these amino acids correcting opposing extremes of periplasmic redox status, or the amino acids may undergo oxidation/reduction transformations in the perturbed periplasm to suppress the phenotypes. The penicillin sensitivity assays (Fig. 3) also suggest a difference in periplasmic redox poise between wild-type and cydD mutant strains. Hypersensitivity to benzylpenicillin is a phenotype commonly associated with mutants unable to cope with changes in the redox environment and results when penicillin-binding protein 4 (which contains two disulfide bonds) (66) is not correctly folded. This may lead to an increase in drug binding.

Fig. 6. CydDC is an ATP-driven cysteine transporter in everted membrane vesicles. The cells used were strain RKP4611 (cydD+; orf299; white circles in A–C) and RKP4612 (cydD; orf299; black circles in A–C). Panels A and B both show ATP-driven uptake for the wild-type strain only in the absence of inhibitors (A) or in the presence of 2 mM CCCP (B). Panel C shows data for strain RKP4611 (white circles, no vanadate; white triangles, + vanadate) and RKP4612 (black circles) in the presence of 50 mM sodium orthovanadate. In each case, the arrow represents the time point at which 10 mM ATP was added. In A, the bars show standard deviations of three experiments. In B and C, a result typical of three replicates is illustrated.

Table II
Effects of CydDC overexpression on growth of E. coli at cytotoxic cysteine concentrations
Growth was measured in LB broth; values are corrected for the turbidity of the inoculum.

| Strain no. | Strain | Relevant genotype | Culture growth (Klett units, red filter) |
|-----------|--------|-------------------|----------------------------------------|
|           |        |                   | No added cysteine | Plus 20 mM cysteine |
|           |        |                   | 3 h     | 9 h     | 3 h     | 9 h     |
| AN2342    | Wild-type | 127 | 377 | 5 | 1 |
| AN2343    | cydD    | 118 | 377 | 3 | 1 |
| RKP2634   | Wild-type/pRP33 (cydDC+) | 102 | 422 | 7 | 9 |
| RKP2005   | cydD/pRP33 (cydDC+) | 92 | 392 | 5 | 9 |
Further evidence for redox perturbation of the periplasm of cydDC mutants is provided by the dramatically lowered level of periplasmic cytochromes (10, 12). In this study, the observed 423-nm Soret absorbance used for quantitation in difference spectra is very close to the band position reported for absolute spectra of the reduced form of NrFA cytochrome c (35) and distinct from the Soret band of reduced cytochrome \( b_{562} \) (427 nm) in absolute spectra (67). Furthermore, the \( e \)-band was at 550–552 nm as expected for \( e \)-type cytochromes, but not cytochrome \( b_{562} \) (67). The spectral characteristics and the use of anaerobic growth conditions strongly suggest that the measured signals arise largely from cytochromes \( c \), but a small contribution from \( b_{562} \) cannot be ruled out. Indeed, this cytochrome is made at only 10% of wild-type levels in a cydC mutant (12). Collectively, the data show a dramatic decrease in the levels of periplasmic cytochrome(s).

Endoglycanase (0.2 mg) increased the level of cytochrome \( c \) in a cydD mutant (although not to wild-type levels) and is reminiscent of the effects of cysteine in restoring cytochrome \( c \) in dbfD and trxA mutants (18, 40). The present data suggest that the requirement for redox homeostasis in cytochrome \( c \) assembly is met in part by efflux of cysteine from the cytoplasm via the CydDC ABC-type transporter or, in cydD mutants, by externally added cysteine.

That the ability of CydDC to transport cysteine is of physiological significance is evident from comparing growth of wild-type, orf299, and cydD strains. The single mutants are each cysteine-sensitive, but the double mutant is hypersensitive.

L-Cysteine is toxic to enteric bacteria (68), as it inhibits enzymes involved in serine and methionine metabolism (69). As a result, Salmonella can utilize it only in a limited capacity as a sole nitrogen and carbon source (70), whereas \( E. coli \) cannot utilize cysteine thus (71). The various degrees of sensitivity displayed by the strains examined here are probably a reflection of their ability to remove cysteine from their cellular compartments. Indeed, cytoplasmic cysteine concentrations reflect export of larger substrates also. The substrate(s) of CydDC may be DsbB and involved in maintaining disulfide bonds between CXXC motifs in target proteins such as the P- and L-rings of the flagellar basal body and penicillin-binding protein 4. Interestingly, Cuzzo and Kaiser (72) have demonstrated a similar model in the endoplasmic reticulum of eukaryotes, a cellular compartment with parallels to the oxidized bacterial periplasm. Glutathione enters the endoplasmic reticulum by an as-yet unidentified transporter and is the target for oxidation by Ero1, an analogue of DsbB (73).

Although rapid progress has been made in identifying two pathways involved in disulfide bond formation in the periplasm (74), many questions remain, including the matter of how reducing equivalents cross the membrane. Our demonstration that the cydSC mutants are transported by CydDC, and that CydDC mutants have pleiotropic phenotypes with striking similarities to those of \( dsbA \) and \( dsbB \) mutants, strongly suggests that CydDC is involved in the same oxidation-reduction pathways. Further work will be required to determine whether the exported cysteine plays a direct role in reduction reactions, such as the re-reduction of DsbA or of “incorrect” disulfides in periplasmic proteins.

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