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Use of Heme Reporters for Studies of Cytochrome Biosynthesis and Heme Transport

BARRY S. GOLDMAN, KAREN K. GABBERT, AND ROBERT G. KRANZ*

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Strains of *Escherichia coli* containing mutations in the *cydDC* genes are defective for synthesis of the heme proteins cytochrome *bd* and *c*-type cytochromes. The *cydDC* genes encode a putative heterodimeric ATP-binding cassette transporter that has been proposed to act as an exporter of heme to the periplasm. To more fully understand the role of this transporter (and other factors) in heme protein biosynthesis, we developed plasmids that produce various heme proteins (e.g., cytochrome b_5, cytochrome b_{562}, and hemoglobin) in the periplasm of *E. coli*. By using these reporters, it was shown that the steady-state levels of polypeptides of heme proteins known to be stable without heme (e.g., cytochrome b_5 and hemoglobin apoprotein) are significantly reduced in a *cydC* mutant. Exogenous addition of hemin to the *cydC* mutant still resulted in <10% of wild-type steady-state levels of apohemoglobin in the periplasm. Since the results of heme reporter studies are not consistent with lower heme availability (i.e., heme export) in a *cydC* mutant, we analyzed other properties of the periplasm in *cydC* mutants and compared them with those of the periplasm in *cydAB* (encoding cytochrome *bd*) mutants and wild-type cells. Our results led us to favor a hypothesis whereby *cydDC* mutants are defective in the reduction environment within the periplasmic space. Such an imbalance could lead to defects in the synthesis of heme-liganded proteins. The heme reporters were also used to analyze strains of *E. coli* with a defect in genes encoding homologs of a different ABC transporter (*helABC*). The *helABC* genes have previously been shown to be required for the assembly of *c*-type cytochromes in *Rhodobacter capsulatus* (R. G. Kranz, J. Bacteriol. 171:456–464, 1989; D. L. Beckman, D. R. Trawick, and R. G. Kranz, Genes Dev. 6:268–283, 1992). This locus was shown to be essential in *E. coli* for endogenous cytochrome *c* biogenesis but not cytochrome b_{562} synthesis. Consistent with these and previous results, it is proposed that the HelABC transporter is specifically involved in heme export for ligation (*hel*). This class of periplasmic cytochromes is proposed to require heme liganding before undergoing correct folding.

A number of reports have recently elucidated extragenic factors that are important in the assembly of extracellular cytochromes in bacteria. For example, genetic analysis has led to the isolation of eight genes required for the assembly of *c*-type cytochromes in the photosynthetic bacterium *Rhodobacter capsulatus*. These genes are present at three loci, the *hel*, *ccl* (3, 4, 8, 24), and *cyc* (26) loci. Previously, we proposed that a transporter of the ATP-binding cassette (ABC) family, encoded by the *helABC* genes (24), is required for the specific export of heme to the periplasm (4). The *helA* gene encodes the predicted ATP-binding protein, while the *helBC* genes encode integral membrane proteins (4). The recent discoveries of *helBC* homologs in plant mitochondrial genomes suggest that this putative heme transporter also operates in eukaryotic organisms (see, e.g., references 20 and 38).

In *Escherichia coli*, the *cydDC* genes are necessary for the synthesis of *c*-type cytochromes and the membrane-bound cytochrome *bd* encoded by the *cydAB* genes (18, 30–32). Recently, the *cydDC* genes were proposed to encode an exporter specific for the heme of *c*-type cytochromes and cytochrome *bd* of *E. coli* (30). Genes homologous to the *helABC* genes have recently been discovered in genomic sequencing projects in a number of prokaryotic organisms including *E. coli* (34). Therefore, two different ABC-type transporters previously proposed as heme exporters are present in the same organism, the HelABC proteins (4, 24) and the CydDC transporter (30, 31). However, comparison of the sequences of the CydDC and HelABC proteins showed no similarities outside the ABC region.

The present study analyzes various aspects of heme protein synthesis in *E. coli*. Plasmids and specific methods were developed to study the biosynthesis of several periplasmic (cytochromes *c*, b_{562}, and b_5 and *Ascaris* periplasmic hemoglobin domain 1) and cytoplasmic (cytochrome b_5 and *Ascaris* cytoplasmic hemoglobin domain 1) heme proteins. The properties of these heme reporters in *cydC* mutants were studied in hopes of further defining the role of the CydDC proteins in heme export. We have analyzed the ability of *E. coli* to make these heme proteins in *cydC* mutants compared with *cydAB* mutants. The results show that the *cydC* mutants are partially but not completely defective for the synthesis of the periplasmic heme reporters. We also show that the locus in *E. coli* containing homologs to the *R. capsulatus helABC* genes is required for cytochrome *c* synthesis but not cytochrome b_{562} synthesis. However, since (i) even a *cydAB* mutant is reduced in the production of some periplasmic cytochromes and (ii) steady-state levels of apoforms of some heme proteins (e.g., cytochrome b_5 and hemoglobin apoprotein) are decreased in *cydDC* cells, the conclusion that the CydDC proteins make up a heme exporter may be oversimplified.

We tested other properties of the periplasm in *cydDC* and *cydAB* strains that might be important for protein, particularly cytochrome, synthesis. In addition to dithionitrobenzoic acid (DTNB) assays, we analyzed the reducing environment of the periplasm by overexpressing the soluble cysteine-containing domain of the periplasmic Ccl2 protein of *R. capsulatus* and then probed the state of sulfhydryl reduction of the Ccl2 protein in various *E. coli* strains. We demonstrate that the reducing environment of a *cydC* mutant is defective compared with

* Corresponding author. Mailing address: Department of Biology, Washington University, One Brookings Dr., St. Louis, MO 63130. Phone: (314) 935-4278. Fax: (314) 935-4432. Electronic mail address: Kranz@wustlb.wustl.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
E. coli K-12		
GO103	Δ*cydAB*::Kn^r	29
GN04	*cydC*::1MudKan	18
TB1	*hsdR ara* Δ(*lac-proAB*) *rpsL* [ϕ80d *lac* (Δ*lacZ*)*M15*]	1
RK5278	*narL*::Tn*10*	40
VJS4745	*aeg-46.5*::Kn^r	13a
RGK263	*aeg-46.5*::Kn^r in TB1	This work
RGK264	*cydC*::1MudKan in TB1	This work
RGK265	Δ*cydAB*::Kn^r in TB1	This work
RGK266	*narL*::Tn*10* in TB1	This work
RGK267	*narL*::Tn*10 aeg-46.5*::Kn^r in RGK263	This work
RGK268	*narL*::Tn*10 cydC*::λMudKan in RGK264	This work
RGK269	*narL*::Tn*10* Δ*cydAB*::Kn^r in RGK265	This work
E. coli B		
BL21	λDE3 T7 gene *l* (RNA polymerase) *ompT hsdS*	Novagen
RGK270	*aeg-46.5*::Kn^r in BL21	This work
RGK271	*cydC*::lMudKan in BL21	This work
RGK272	Δ*cydAB*::Kn^r in BL21	This work
Plasmids		
pUC18	Ap^r	46
pET-8c	Ap^r	Novagen
pET-21b	Ap^r; T7 promoter	Novagen
pET-22b	Ap^r; T7 promoter *pelB* leader	Novagen
pB5	Ap^r; rat cytochrome b_5 in *Pst*I and *Eco*RI site of pUC13	5
pD1	Ap^r; *A. suum* hemoglobin domain 1 in *Nco*I site of pET-8c	23
pNS207	Ap^r; *E. coli* cytochrome b_{562} in *Sal*I and *Sma*I site of pUC18	28
pRGK260	Ap^r; T7 promoter rat cytochrome b_5 in *Nco*I and *Eco*RI site of pET-22b	This work
pRGK261	Ap^r; T7 promoter *Ascaris* hemoglobin domain 1 in *Nco*I site of pET-22b	This work
pRGK262	Ap^r; T7 promoter *R. capsulatus ccl2* in *Nde*I and *Xho*I site of pET-21b	This work

a *cydAB* mutant and suggest that the CydDC transporter could be involved in periplasmic sulfhydryl homeostasis in *E. coli*. Such a homeostasis may be necessary for optimal synthesis of periplasmic heme-liganded proteins, which are particularly sensitive to oxidation reactions.

MATERIALS AND METHODS

Bacterial strains and plasmids and tests for reversion. The strains and plasmids used in this study are listed in Table 1. The parental strains in these studies were the *E. coli* K-12 strain TB1 and B strain BL21(DE3). Because *cyd* mutations demonstrate a very high degree of reversion (43) to wild-type growth, it was necessary to ensure that these strains had not reverted in each experiment. To ensure this, at the time of harvest, all *cydC* and *cydAB* cells were tested for temperature-sensitive growth at 42°C or for sensitivity to zinc, both of which are indicative of defects in cytochrome *bd* (16, 32). For assaying zinc sensitivity, 3 μl of cells was dispensed into 0.6% agar containing Luria broth (LB) medium at 42°C and spread evenly onto LB plates. A sterile disk of filter paper (3 MM; Whatman, Fairfield, N.J.) was placed in the middle of the plate, 5 μl of a 0.4 M $ZnCl_2$ solution was placed on the disk, and the cells were grown overnight at 30°C.

Media and culture conditions. Cells were grown at 30°C in LB medium unless otherwise indicated. To induce the proteins encoded by genes cloned into pET vectors, overnight cultures of strains containing the pET vector were used to inoculate 100 ml of LB medium containing ampicillin (200 μg/ml), and cells were grown to mid-exponential phase (roughly 0.5 optical density at 600 nm unit), induced with isopropyl-β-D-thiogalactopyranoside (IPTG) added to 1 mM, typically grown for 4 h, and then harvested. For the experiments in Fig. 2B and 8B, cells were induced for only 2 h before harvest. Overnight cultures of strains containing cytochrome b_{562} were used to inoculate 100 ml of LB medium containing ampicillin, and the cells were grown to late exponential phase (~1.5 to 2.0 optical density at 600 nm units) and harvested. Strains containing *Ascaris* hemoglobin were grown, induced, and harvested as described by Kloek et al. (23). Hemin was added from a stock solution of 10 mg/ml in dimethyl sulfoxide to a final concentration of 40 μg/ml. For determining *c*-type cytochromes, 500 ml of LB medium containing 40 mM potassium nitrate (KNO_3) was inoculated with 1 ml of cells from an overnight culture. These cultures were grown with shaking for 3 h and then incubated without shaking overnight at 30°C.

Cell fractionations. Cell cultures were harvested by centrifugation at 15,000 × *g* for 10 min, washed with 10 mM Tris (pH 8), and frozen at −80°C overnight. Frozen cells were resuspended in 4 to 8 ml of 10 mM Tris (pH 8) and sonicated three times for 3 min each (Branson 200 sonicator with a microtip at a power setting of 40%) at 4°C. Unbroken cells were removed by centrifugation at 12,000 × *g* for 10 min at 4°C. Soluble fractions were prepared by ultracentrifugation of crude sonicate fractions at 100,000 × *g* for 1 h. Protein concentrations were determined by using the Pierce protein assay kit with bovine serum albumin as standard. Periplasmic fractions for cytochrome c_{552} were prepared by the method of Weiss (44). Periplasmic fractions for all other cytochromes were prepared by osmotic shock (27).

Cytochrome *c* analysis. We found that a defect in the *narL* gene in *E. coli* TB1 increased the levels of *c*-type cytochromes significantly. We therefore transduced a *narL*::Tn*10* insertion (provided by V. Stewart) into TB1 and the *aeg-46.5*, *cydAB*, and *cydC* derivatives of TB1 to increase the resolution of *c*-type cytochromes. While analyzing the above strains, we discovered that the *narL* mutation corrected both *cydAB* and *cydC* mutants for growth at 42°C. However, these mutants were still defective for synthesis of the cytochrome *bd* (data not shown).

DTNB assays. Cells (0.1 ml) grown from single colonies in LB medium were used to inoculate 10 ml of LB medium. These cells were grown to stationary phase and harvested by centrifugation (10,000 × *g*) for 10 min, and the periplasmic fraction was isolated. DTNB was added to periplasmic fractions to 0.8 mM, and the A_{412} was measured. Three separate tubes were tested for each culture. The results of the DTNB assays of exponential-phase cultures of *cydC* mutants were inconsistent from trial to trial for unknown reasons.

Spectral analysis. Reduced (sodium hydrosulfite)-minus-oxidized (ammonium persulfate or potassium ferricyanide) spectroscopy was performed on a Shimadzu UV-2101-PC dual-beam spectrophotometer. All experiments involving *cydC* mutants were repeated at least twice with separate cultures. The spectra in Fig. 3 to 8 and 10 were obtained by using computer software to subtract the absorbance of extracts of the strains without plasmids from the absorbance of the extracts from strains with the plasmid over the entire spectral wavelength. Therefore, any signal present in the extracts should be due to the plasmid-encoded heme reporter. Levels of active heme protein were determined by absorption at the following wavelengths; cytochrome b_5, 424 and 555 nm; cytochrome b_{562}, 428 and 562 nm; hemoglobin, 433 to 434 nm.

Analysis of the redox state of the Ccl2 protein. Strains containing the *ccl2* plasmid (pRGK262) were grown in LB medium to exponential or stationary phase and induced with 1 mM IPTG for 1 h. Iodoacetic acid was added to 100 mM and cells were incubated for 15 min. Cells were harvested and subjected to periplasmic shock in the presence of 10 mM iodoacetic acid as described previously (2). The shock fractions were separated by nondenaturing polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose, and immunoblotting was performed with anti-Ccl2 polyclonal antisera. The production and purification of the Ccl2 protein and antibodies will be described elsewhere. For controls, purified Ccl2 protein was either left untreated, treated with 5 mM dithiotrietol (DTT) for 20 min at room temperature, or treated with 5 mM DTT for 20 min and 100 mM iodoacetic acid for 20 min at room temperature.

Western blotting. Proteins were separated by sodium dodecyl sulfate (SDS)-PAGE and electroblotted onto Hybond-C nitrocellulose filters (Amersham). Western blot (immunoblot) analyses were performed with the enhanced chemiluminescence detection system (Amersham) with protein A peroxidase (Sigma) as the secondary label.

DTT sensitivity. Cells were streaked onto plates containing LB medium to which DTT had been added to a final concentration of either 1, 3, 10, or 30 mM DTT. The cells were grown at 30°C overnight and were considered positive for growth if single colonies were formed.

Heme stain. The covalently bound heme of *c*-type cytochromes from soluble fractions was visualized as described by Thomas et al. (41) after separation by SDS-PAGE (15% polyacrylamide).

RESULTS

Use of heme reporters to study *cydDC* and *cydAB* defects. Because the *cydDC* genes are required for the synthesis of *c*-type cytochromes and cytochrome *bd*, it has been proposed that the CydDC transporter exports heme for all periplasmic cytochromes (30). Since many defects associated with *cydDC* mutations are in fact due to a lack of cytochrome *bd*, it is critical to compare *cydDC* mutants with *cydAB* mutants in such studies, as in the study reported in the accompanying paper

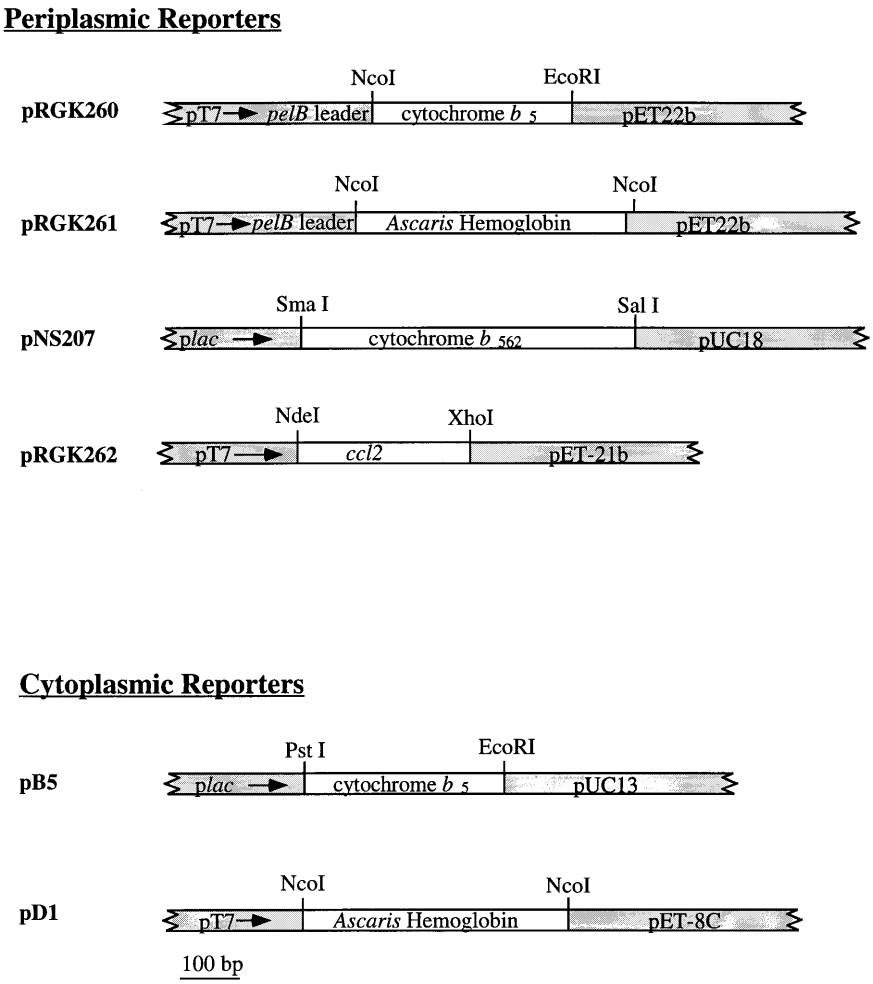

FIG. 1. Schematic representation of the plasmids encoding the periplasmic and cytoplasmic heme reporter molecules. The plasmids pRGK260 and pRGK261 contain genes that form in-frame fusions with the *pelB* leader sequence of pET22b and are recognized by the *sec*-dependent export machinery of *E. coli*. Cytochrome b_{562} and the Ccl2 protein have their own signal sequences. The periplasmic reporter plasmid pNS207 (26) and the cytoplasmic reporter plasmids (5, 23) have been described previously.

(19). Moreover, the *cydDC* heme exporter hypothesis predicts that for heme proteins that can be made as stable apoforms (e.g., hemoglobin and cytochrome b_5), no differences should be apparent in *cydDC* and *cydAB* strains with respect to polypeptide synthesis in the periplasm. We have obtained and constructed plasmids with genes encoding homologous and heterologous heme-binding proteins to serve as reporters for heme export (Fig. 1). These plasmids were used to determine the levels of heme available in the periplasm and cytoplasm. The periplasmic heme reporters pRGK260 (cytochrome b_5 containing a signal sequence), pNS207 (cytochrome b_{562}, a native periplasmic protein), and pRGK261 (*Ascaris* hemoglobin domain 1 containing a signal sequence) were used to test the ability of the cell to export both heme and the apoprotein to the periplasm. The cytoplasmic heme reporters pB5 (cytochrome b_5) and pD1 (*Ascaris* hemoglobin domain 1) were used to test the ability of the cell to synthesize heme and the apoproteins. These reporters were transformed into the *cydAB*, *cydC*, and wild-type strains in the BL21 background. Synthesis of the heme proteins was assayed by differential (reduced-minus-oxidized) spectroscopy and SDS-PAGE. The results are discussed below and summarized in Table 2.

Synthesis of periplasmic reporters. (i) Cytochrome b_5. Differential spectroscopy of wild-type and *cyd* mutant strains containing pRGK260 was performed. Because nearly 90% of the total protein in the wild-type periplasmic shock fractions represents cytochrome b_5, comparisons based on total protein are biased (Fig. 2C, lanes 2 and 6). Thus, the estimates in Table 2 are presented as a percentage of the wild-type levels of reporter protein and normalized for the total protein present in extracts from cells that did not contain the plasmid. Both the *cydC* and *cydAB* mutants made spectrally detectable periplasmic cytochrome b_5 (Fig. 2A and B). The protein analysis showed that the cytochrome b_5 polypeptide was present at approximately the same level in the *cydAB* mutant (Fig. 2C, lane 3) as the *cydC* mutant (lane 5) but at only 20% of the wild-type level. The amino terminus of the cytochrome b_5 protein from the periplasmic fraction of the wild-type strain was sequenced. The signal sequence is cleaved in more than 95% of the molecules, confirming that this protein is secreted into the periplasm as the fractionation experiments indicated. The identical molecular weights of cytochrome b_5 in the *cydC* and *cydAB* strains, as determined by SDS-PAGE (Fig. 2C), show that these were also processed.

(ii) Cytochrome b_{562}. Spectrally detectable periplasmic cytochrome b_{562} was made at only 10% of wild-type levels in the *cydC* mutant and at 100% of wild-type levels in the *cydAB* mutant (Fig. 3A). SDS-PAGE analysis of these fractions (Fig.

TABLE 2. Spectral and polypeptide analyses of cytoplasmic and periplasmic heme proteins

Analysis type and relevant genotype	% of reporter molecules made in mutants compared with that in wild type							
	Periplasmic					Cytoplasmic		
	b_5	b_{562}	*Ascaris* hemoglobin (no hemin)	*Ascaris* hemoglobin (plus hemin)	c_{552}	b_5	*Ascaris* hemoglobin (no hemin)	*Ascaris* hemoglobin (plus hemin)
Spectral analysis[a]								
cydC	50	10	25	75	0	100	75	>400[b]
cydAB	20	100	50–100	100	100	ND[c]	ND	100
Wild type	100	100	100	200	100	100	100	200
Polypeptide analysis[d]								
cydC	20	10	5	5			50–60	50–60
cydAB	10–20	100	50	20			ND	20–50
Wild type	100	100	100	100			100	100

[a] Results determined by reduced-minus-oxidized spectra (see Materials and Methods for details).
[b] Values vary, but at least twice wild-type levels were seen.
[c] ND, not determined.
[d] Results determined by densitometric analysis of SDS-PAGE of apopolypeptides.

3B) showed that cytochrome b_{562} polypeptide levels were similar to the holocytochrome levels in each strain. Thus, the *cydC* mutant is partially defective for periplasmic cytochrome b_{562} biosynthesis, but the *cydAB* mutant is proficient for its synthesis.

(iii) Periplasmic hemoglobin domain 1. Domain 1 from the hemoglobin of the parasitic nematode *Ascaris suum* has previously been overproduced as the holoform in the cytoplasm of *E. coli* with the addition of exogenous hemin (23, 39). We have found that the holoform of this 17.1-kDa protein can be made either with or without exogenous hemin (see below). The *Ascaris* hemoglobin protein was directed to the periplasm by using the *pelB* signal sequence of pRGK261, and the amounts of protein that are synthesized were measured. The estimates in Table 2 are normalized to the amount of hemoglobin made in the wild-type strain containing pRGK261 without added hemin. After normalization, it was determined that the *cydC* mutant made spectrally detectable periplasmic *Ascaris* hemoglobin at 25% of the wild-type level and the *cydAB* mutant made at least 50% of the wild-type level (Fig. 4A). When exogenous hemin was added, the amount of the holoform in the wild-type strain doubled (Fig. 5A). When exogenous hemin was added to the *cydC* mutant, the holoform remained at much lower than wild-type levels (Fig. 5A). Importantly, even the hemoglobin polypeptides were synthesized at very low levels in the *cydC* mutant whether hemin was added (Fig. 5B, lane 5) or not (Fig. 4B, lane 5).

Synthesis of cytoplasmic reporters. (i) Cytochrome b_5. To determine if the lack of cytochrome synthesis was due to defects in transcriptional or translational expression or a defect in the general capacity of the cells for heme synthesis, we analyzed the synthesis of cytoplasmic heme proteins of *Ascaris* hemoglobin domain 1 and cytochrome b_5 in these backgrounds. Cytochrome b_5 was made to wild-type levels in the *cydC* strain, as determined spectrally (Fig. 6).

(ii) Cytoplasmic hemoglobin domain 1. The protein and spectral levels of *Ascaris* hemoglobin domain 1 were detected at 50 to 75% of wild-type levels in the *cydC* mutant when hemin was omitted from the culture (Fig. 7). However, for unknown reasons, levels of *Ascaris* hemoglobin domain 1 greater than wild-type levels were detected spectrally when hemin was added to the *cydC* cultures. By SDS-PAGE analysis, the polypeptide was still made at 50% of wild-type levels (Fig. 8C, compare lanes 1 and 2). The *cydAB* mutant made spectrally

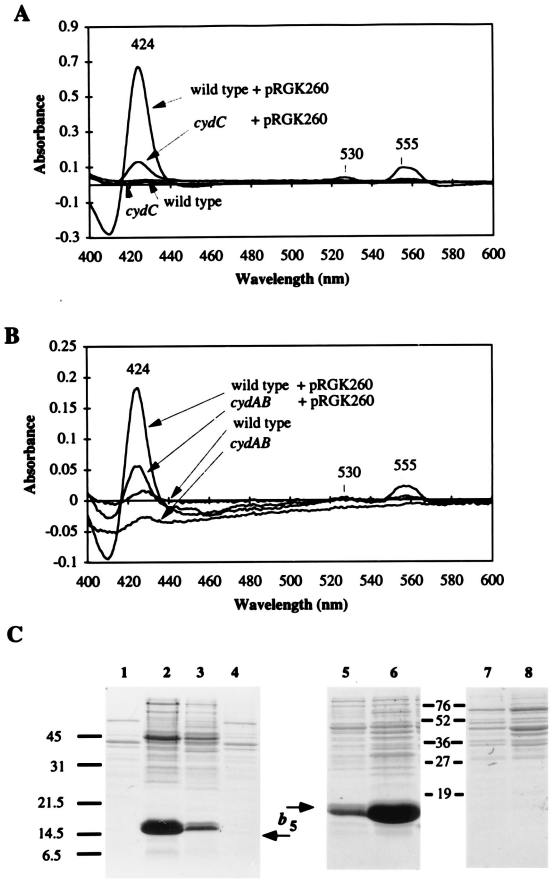

FIG. 2. Difference spectroscopy and SDS-PAGE of periplasmic fractions with and without periplasmic cytochrome b_5. (A) Reduced-minus-oxidized spectra of the periplasmic fraction of *cydC* and wild-type (BL21) strains with and without plasmid pRGK260. Protein concentrations: wild type, 0.6 mg/ml; *cydC*, 0.3 mg/ml; wild type plus pRGK260, 1.6 mg/ml; *cydC* plus pRGK260, 0.5 mg/ml. (B) Reduced-minus-oxidized spectra of the periplasmic fraction of *cydAB* and wild-type (BL21) strains with and without plasmid pRGK260. Protein concentrations: wild type and *cydAB*, 0.3 mg/ml; wild type plus pRGK260, 1.4 mg/ml; *cydAB* plus pRGK260, 0.8 mg/ml. Note that protein concentrations are biased by the large percentage of sig-b_5 in the periplasm. See the text for a detailed explanation. (C) SDS-PAGE showing the amount of cytochrome b_5 polypeptide in the different genetic backgrounds. Lanes: 1, wild type, 6 μg; 2, wild type plus pRGK260, 28 μg; 3, *cydAB* plus pRGK260, 16 μg; 4, *cydAB*, 5 μg; 5, *cydC* plus pRGK260, 11 μg; 6, wild type plus pRGK260, 32 μg; 7, *cydC*, 6 μg; 8, wild type, 10 μg.

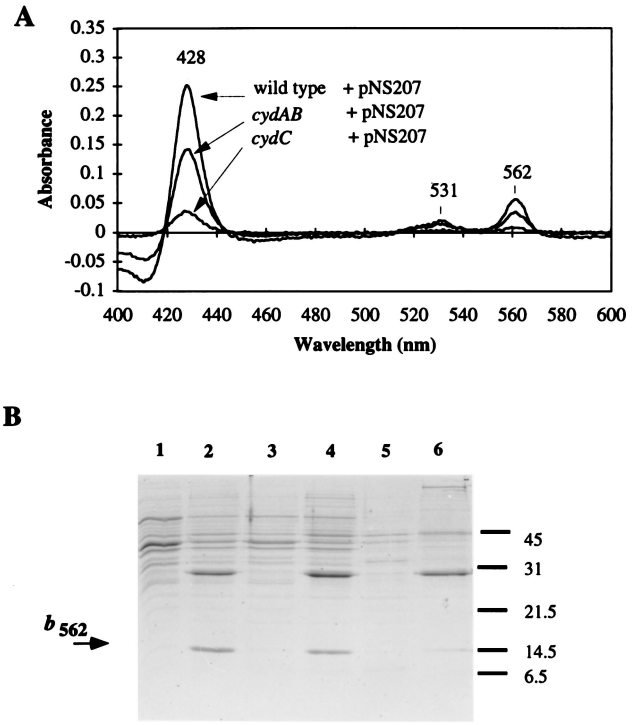

FIG. 3. Difference spectroscopy and SDS-PAGE of periplasmic fractions with cytochrome b_{562}. (A) Reduced-minus-oxidized spectra of the periplasmic fraction of *cydAB*, *cydC*, and wild-type (BL21) strains with plasmid pNS207. Protein concentrations were approximately 0.4 mg/ml in all strains. (B) SDS-PAGE showing the amount of cytochrome b_{562} polypeptide in the different genetic backgrounds. Lanes: 1, wild type; 2, wild type plus pNS207; 3, *cydAB*; 4, *cydAB* plus pNS207; 5, *cydC*; 6, *cydC* plus pNS207. The amount of protein loaded was approximately 8 μg in all lanes.

detectable hemoglobin at 100% of wild-type levels and made up to 50% of the wild-type polypeptide levels.

Cytochrome c_{552} is not made in either *cydC* or *aeg-46.5* mutants. The recent sequencing of the 47-min region of *E. coli* uncovered eight genes with sequence homology to the genes necessary for *c*-type cytochrome synthesis, including the *helABC* genes (3, 4, 34). These genes are located at (and proposed to be in an operon with) the previously described *aeg-46.5* locus (7, 9, 10, 33). Sequence analysis of the open reading frames at the *aeg-46.5* locus predicts the presence of a periplasmic nitrate reductase system as well as the eight proteins associated with cytochrome *c* biogenesis (6, 13, 34). This locus is under the negative control of the NarL protein and the positive control of the NarP protein (9, 10, 13, 34). Mutations in the promoter region of *aeg-46.5* reduce the expression of this operon to less than 1% of wild-type levels (13). To test whether the *aeg-46.5* genes were necessary for *c*-type cytochrome biosynthesis, we transduced an *aeg-46.5*::Kan promoter insertion (constructed by Darwin and Stewart [13a]) into *narL*::Tn*10* derivatives of *E. coli* TB1. We determined that either soluble (Fig. 9A) or periplasmic (data not shown) extracts of the *narL* strain (RGK266) generated a peak at 552 nm in reduced-minus-oxidized spectra and produced a 50-kDa protein that stained for heme on SDS-PAGE (data not shown). The 50-kDa protein is the cytochrome c_{552} protein associated with the formate-dependent nitrite reductase encoded by the *nrfA* gene (12, 21). Neither the *aeg narL* (Fig. 9A) nor the *cydC narL* (Fig. 9B) mutant makes this *c*-type cytochrome. The *cydAB* mutant makes this *c*-type cytochrome in the *narL* background (data not shown). To demonstrate that the *aeg-46.5* mutant is specifically defective for *c*-type cytochrome biosynthesis, we transformed the plasmid encoding cytochrome b_{562} into the *narL* and *aeg-46.5* mutants. Figure 10 demonstrates that both these strains make this cytochrome to near wild-type levels.

In the accompanying paper (19) we present data that the temperature-sensitive growth defects associated with *cydDC* mutants are due to defects of the cytochrome *bd* and specifically to an increase in oxygen radical-mediated growth inhibition. The data above demonstrate that defects in the CydDC transporter affect several periplasmic processes that are distinguished from specific defects in the cytochrome *bd*. The following sections describe experiments that are intended to help further elucidate the role of the CydDC transporter.

Strains containing *cyd* mutations are hypersensitive to DTT. In the accompanying paper (19), we demonstrate that at certain concentrations, reducing agents can suppress the temperature-sensitive phenotypes of both *cydAB* and *cydC* mutants. However, in that study, we also noted that the *cydAB* and *cydC* mutants were more sensitive than the wild type to growth inhibition by certain concentrations of DTT. To analyze and quantitate this effect, we screened the ability of *cydC*, *cydAB*, and wild-type cells to grow on LB plates containing either 1, 3, 10, or 30 mM DTT. The cells were grown at 30°C for 24 to 48 h and were considered positive for growth if single colonies were formed. We found that both *cydC* and *cydAB* mutants were sensitive to 3 and 10 mM DTT and resistant to 1 mM DTT while the wild-type strains were resistant to DTT at these

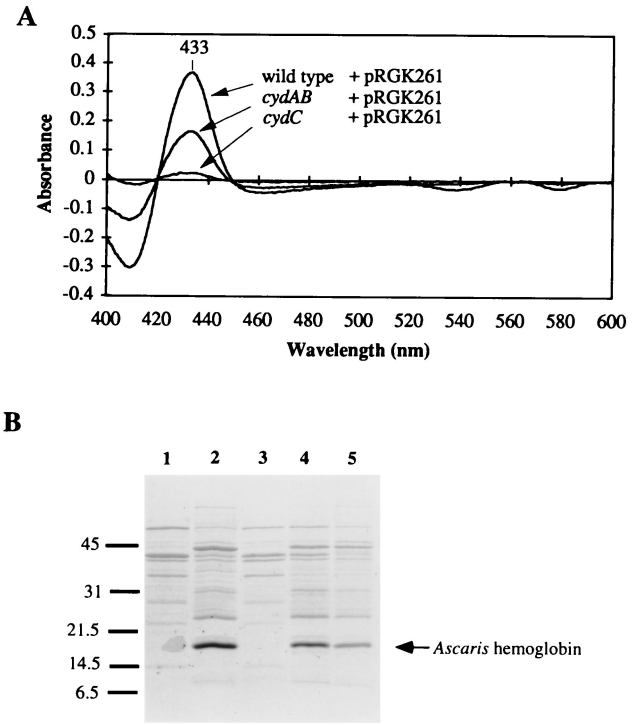

FIG. 4. Difference spectroscopy and SDS-PAGE of periplasmic fractions of cells grown with signal-containing *A. suum* hemoglobin domain 1 without exogenously added hemin. (A) Reduced-minus-oxidized spectra of the periplasmic fraction of *cydAB*, *cydC*, and wild-type (BL21) strains with plasmid pRGK261. Protein concentrations: wild type plus pRGK261, 0.7 mg/ml; *cydAB* plus pRGK261, 0.6 mg/ml; *cydC* plus pRGK261, 0.3 mg/ml. (B) SDS-PAGE showing the amount of *Ascaris* hemoglobin polypeptide in the different genetic backgrounds. Lanes: 1, wild type; 2, wild type plus pRGK261; 3, *cydAB*; 4, *cydAB* plus pRGK261; 5, *cydC* plus pRGK261. The amount of protein loaded was approximately 6 μg in all lanes.

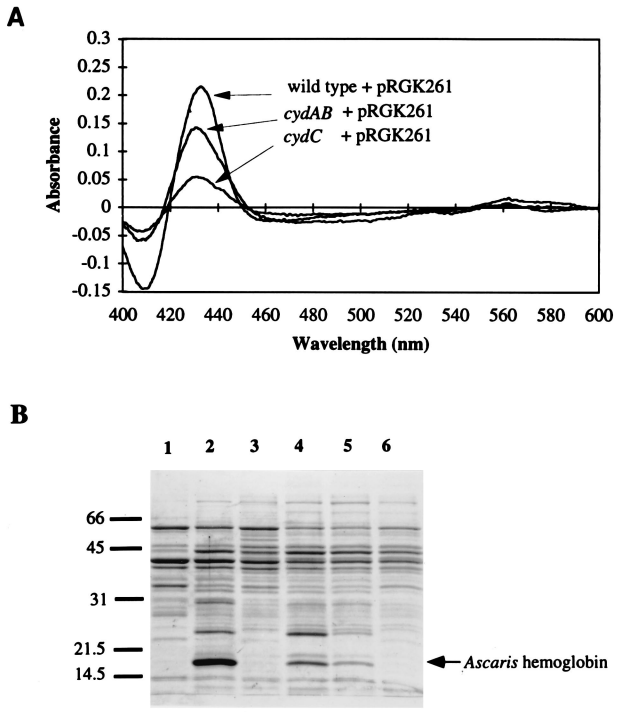

FIG. 5. Difference spectroscopy and SDS-PAGE of periplasmic fractions of cells with signal-containing *A. suum* hemoglobin domain 1 grown with exogenously added hemin. (A) Reduced-minus-oxidized spectra of the periplasmic fraction of *cydAB*, *cydDC*, and wild-type (BL21) strains with plasmid pRGK261. Protein concentrations were approximately 0.3 mg/ml in all strains. (B) SDS-PAGE showing the amount of *Ascaris* hemoglobin polypeptide in the different genetic backgrounds. Lanes: 1, wild type; 2, wild type plus pRGK261; 3, *cydAB*; 4, *cydAB* plus pRGK261, 5, *cydC* plus pRGK261; 6, *cydC*. The amount of protein loaded was approximately 6 μg in all lanes.

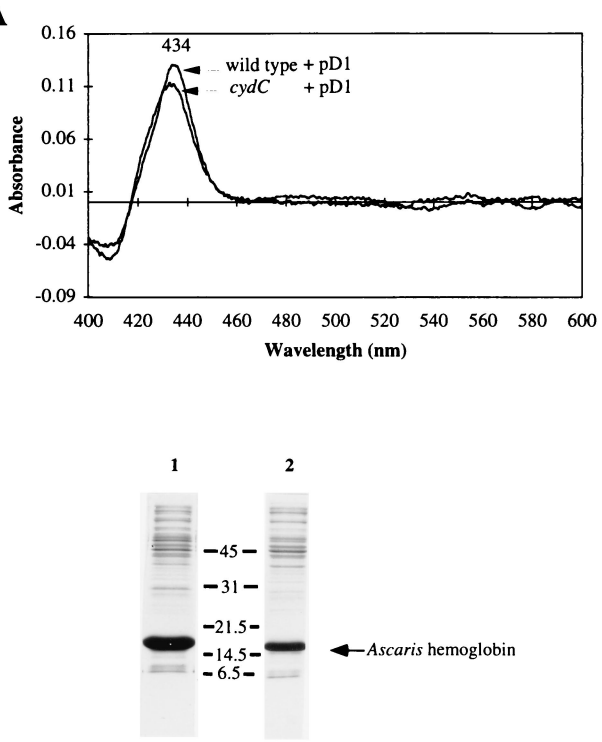

FIG. 7. Difference spectroscopy and SDS-PAGE of soluble fractions of cells with *A. suum* hemoglobin domain 1 grown without exogenously added hemin. (A) Reduced-minus-oxidized spectra of the soluble fraction of *cydC* and wild-type (BL21) strains with plasmid pD1. Protein concentrations were approximately 6 mg/ml in both strains. (B) SDS-PAGE showing the amount of *Ascaris* hemoglobin polypeptide in the different genetic backgrounds. Lanes: 1, wild type plus pD1; 2, *cydC* plus pD1. The amount of protein loaded was approximately 20 μg in both lanes.

concentrations. All strains were sensitive for growth at 30 mM DTT.

DTNB assays to determine the redox state of the periplasm. Since both *cyd* mutants were hypersensitive to DTT and H_2O_2 (19, 43) and since the synthesis of some of the periplasmic reporter proteins was affected, it seemed possible that *cyd* mutations reflected some general periplasmic defect, such as an altered redox state. We therefore measured the overall redox state of the periplasm by using the sulfhydryl reporter DTNB (35). DTNB, a colorless compound in solution, is reduced nonenzymatically by sulfhydryls to thionitrobenzoic acid, which is yellow in solution and whose absorbance can be measured spectrophotometrically at 412 nm. Periplasmic shock fractions of stationary-phase cultures of the mutants and the wild type were reacted with DTNB. On a relative scale of wild type as 1.0 U/mg of protein released, *cydAB* shock fractions possessed close to wild-type levels of reducing equivalents (~0.8 U/mg) and the *cydC* mutant had 0.4 U/mg. Thus, the sulfhydryl periplasmic environment of the *cydC* mutants in the stationary phase is oxidized in comparison with the wild-type and *cydAB* strains.

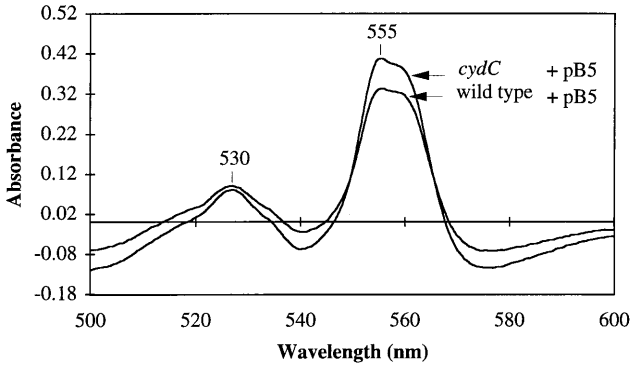

FIG. 6. Difference spectroscopy of soluble fractions with rat cytochrome b_5 showing reduced-minus-oxidized spectra of the soluble fraction of *cydC* and wild-type (BL21) strains with plasmid pB5. Protein concentrations: wild type plus pB5, 23 mg/ml; *cydC* plus pB5, 14 mg/ml.

The Ccl2 protein is in the oxidized state in *cydC* mutants and the reduced state in *cydAB* mutants. To specifically demonstrate the altered redox state of the periplasm, the Ccl2 protein of *R. capsulatus* was used as a reporter. The Ccl2 protein is a component of a redox pathway and contains a CysXxxYyyCys motif that is necessary and redox active in the periplasmic space for cytochrome *c* biogenesis (26a). Additionally, we have developed assays to determine the in vivo reduction state of the Ccl2 CysXxxYyyCys motifs (24a). An open reading frame encoding the soluble portion of the Ccl2 protein was cloned into the pET21b vector (pRGK262). The soluble Ccl2 protein contains its own signal sequence and is directed upon synthesis to the periplasm with subsequent processing of its signal sequence (26a). The plasmid was transformed into the *cydAB* and *cydC* mutants and the wild-type strain BL21. Cells were grown, treated with iodoacetic acid and osmotically shocked as

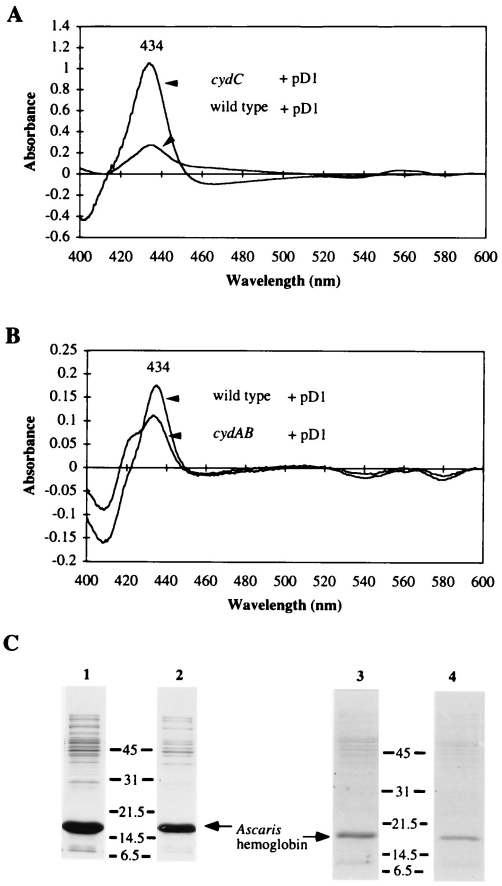

FIG. 8. Difference spectroscopy and SDS-PAGE of soluble fractions of cells with *A. suum* hemoglobin domain 1 grown with exogenously added hemin. (A) Reduced-minus-oxidized spectra of the soluble fraction of *cydC* and wild-type (BL21) strains with plasmid pD1. Protein concentrations were approximately 8 mg/ml in both strains. (B) Reduced-minus-oxidized spectra of the soluble fraction of *cydAB* and wild-type (BL21) strains with plasmid pD1. Protein concentrations were approximately 10 mg/ml in both strains. (C) SDS-PAGE showing the amount of *Ascaris* hemoglobin polypeptide in the different genetic backgrounds. Lanes: 1, wild type plus pD1, 20 μg; 2, *cydC* plus pD1, 20 μg; 3, wild type plus pD1, 10 μg; 4, *cydAB* plus pD1, 10 μg.

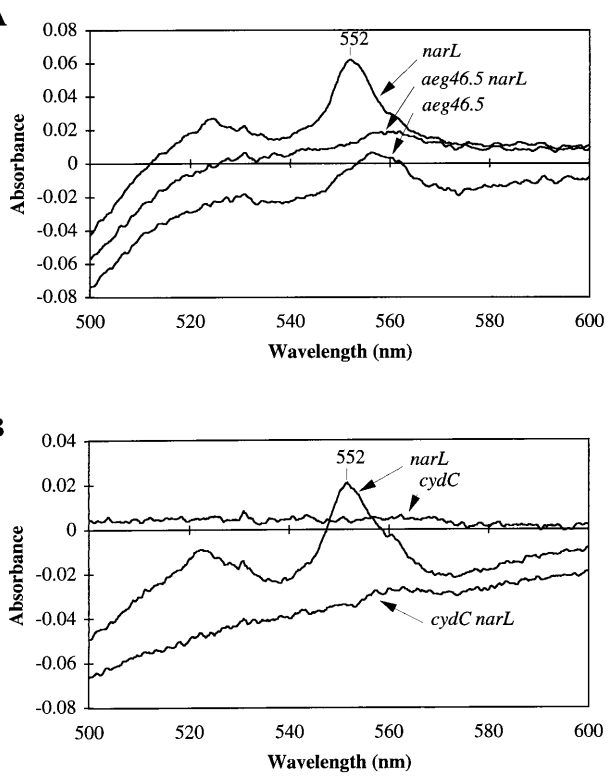

FIG. 9. Difference spectroscopy to detect *c*-type cytochromes in various strains. (A) Reduced-minus-oxidized spectra of the soluble fraction of *narL*, *aeg-46.5 narL*, and *aeg-46.5* strains. Protein concentrations were approximately 5 mg/ml in all strains. (B) Reduced-minus-oxidized spectra of the soluble fraction of *narL*, *cydC narL*, and *cydC* strains in the TB1 background. Protein concentrations were approximately 4 mg/ml in all strains.

described in Materials and Methods. When cells were grown to either exponential or stationary phase, more than 90% of the Ccl2 protein was in the reduced state in the *cydAB* periplasm (Fig. 11, lane 3). However, in the wild type (lane 1) and the *cydC* mutant (lane 2), more than 90% of the Ccl2 protein was in the oxidized state.

DISCUSSION

Most *cydDC* phenotypes, including temperature sensitivity for growth and stationary-phase arrest, are attributable to a cytochrome *bd* defect (19). These temperature sensitivity and survival defects can be corrected by exogenous reductants, including catalase, and thus are due to oxidation of an unknown factor required for high-temperature growth. Key to understanding the role of the CydDC transporter in cell physiology is distinguishing differences between *cydDC* and *cydAB* mutants. Four basic differences between *cydDC* and *cydAB* mutants have been discerned from the present study: (i) synthesis of the cytochrome b_{562} apo- and holocytochromes; (ii) synthesis of *c*-type cytochromes; (iii) less synthesis of the periplasmic hemoglobin holoform and the polypeptide in the *cydC* mutant; (iv) a different periplasmic sulfhydryl redox environment between the two. Each of these differences is addressed below.

Differences between *cydDC* and *cydAB* mutants with respect to heme protein synthesis. On the basis of the inability of *cydDC* mutants to make either cytochrome *c* or *d*, Poole et al. (30) suggested that the *cydDC* genes encode a heme transporter. The possibility that CydDC proteins transport heme to the periplasm was analyzed by using heterologous heme re-

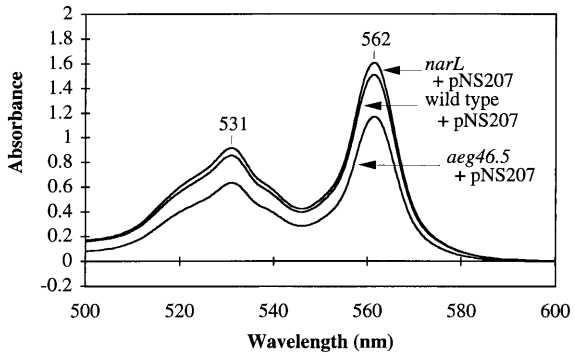

FIG. 10. Difference spectroscopy of soluble fractions with cytochrome b_{562}. Reduced-minus-oxidized spectra of the soluble fractions of *narL*, *aeg-46.5*, and wild-type strains (TB1) containing the plasmid pNS207 are shown. Cells were grown aerobically. Protein concentrations: wild type and *narL*, 24 mg/ml; *aeg-46.5*, 14 mg/ml.

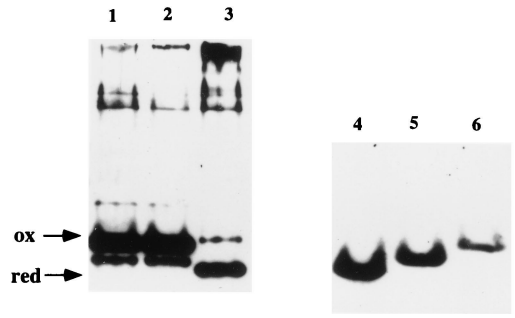

FIG. 11. Western blot with Ccl2 antibodies demonstrating the redox state of the soluble Ccl2 protein in periplasmic fractions of *cydAB*, *cydC*, and wild-type (BL21) strains. A Western blot of the periplasmic fractions of wild-type (lane 1), *cydC* (lane 2), and *cydAB* (lane 3) strains containing pRGK262 is shown. Cells were treated with iodoacetic acid, and extracts were prepared and immunoblotted as described in Materials and Methods. As controls, purified Ccl2 protein was treated with DTT and iodoacetic acid (lane 4) or DTT alone (lane 5) or was left untreated (lane 6). ox, oxidized Ccl2; red, reduced Ccl2.

porters in the present study. In general, the results of our study indicate that periplasmic heme proteins are produced at lower levels in the *cydC* mutant. However, these *cydDC*-specific defects were distinguished from those due to increased oxidative stress brought on by a cytochrome *bd* deficiency (i.e., in a *cydAB* mutant). Additionally, the question whether such deficiencies are due to heme availability or a general periplasmic defect (e.g., the reducing environment) still needed to be addressed.

A clue to the deficiency in a *cydC* mutant may come from the poor production of even the apo form of many of the heme proteins. Both cytochrome b_5 (5) and hemoglobin (23, 39) are produced and stable without heme (or with oxidized rather than reduced heme). Why are the steady-state levels of these reporters, even the apo form, so low in *cydDC* mutants? In fact, even *cydAB* mutants have dramatically lower levels of periplasmic apo- and holo-cytochrome b_5 (Table 2). A recent report on signal-containing cytochrome b_5, when produced in *E. coli*, suggests that the holoform is produced after a stable apoform is folded in the periplasm (22). Although the levels of apo- and holo-cytochrome b_5 produced in the periplasm of the wild-type strain in the present study are 10-fold higher than in the study in reference 22, there is no reason to believe that a stable apoform cannot form in our wild-type strain. It should also be noted that even exogenous hemin did not increase the levels of hemoglobin polypeptide produced in the periplasm of the *cydC* mutant (5% [Table 2]). Taken together, our results suggest that *cydDC* mutants are defective in a step not specifically associated with heme availability. It is possible that a general periplasmic defect is present in *cydDC* mutants that affects a subset of secreted proteins and extracellular domains of proteins. This defect may involve a redox or scavenging function; for example, tryptophan- or histidine-containing proteins, typical for heme proteins, are particularly sensitive to the hydroxyl radical concentration in vitro (14). In fact, a *cydDC* defect in the reducing environment and/or scavenger function is consistent with all the data presented here. The reason that apo- or holo-cytochrome b_5 is produced at low levels in *cydAB* mutants (20% of wild type) may be the increase in the number of oxygen radicals such that the sensitivity of cytochrome b_5 to these radicals is similar to the proposed sensitivity of the molecule(s) necessary for high-temperature growth (19).

The periplasmic reducing environment of the *cydAB* and *cydC* mutants is different. We analyzed the sulfhydryl redox state of the periplasm of the wild-type and mutant strains by using the sulfhydryl indicator DTNB. We found that the periplasm was more oxidized in the *cydC* mutant strain than in either the *cydAB* mutant or the wild type when cells were grown to stationary phase. Finally, we analyzed the in vivo redox state of the periplasm by using the periplasmically located Ccl2 protein as a sensor. Previously, we showed that the Ccl2 protein is required for cytochrome *c* biogenesis (4), and recently we have shown that the CysProValCys motif is redox active and can oxidize the HelX protein in vitro (24a), a periplasmic thioredoxin-like protein (3). We have discovered that the vicinal cysteines of the Ccl2 protein are in the reduced state in the *cydAB* mutant and in the oxidized state in the *cydC* mutant and the wild type (Fig. 11). The effect on the redox state appears to be protein specific, since the HelX protein of *R. capsulatus* was found in the oxidized state in all three strains and alkaline phosphatase activities of strains containing a *phoA* protein fusion were similar in all three (data not shown). The latter result indicates that the traditional *dsb* oxidation pathway is probably not directly affected by the CydDC and CydAB gene products. Hence, we believe that only specific periplasmic redox pathways involve the CydDC transporter and cytochrome *bd.*

Because a *cydDC* mutant is in effect a *cydDC cydAB* double mutant (i.e., it lacks cytochrome *bd*), we can conclude that for this particular Ccl2 pathway, the *cydDC* transporter defect results in a dramatically limited ability to reduce sulfhydryls (compared with a *cydAB* mutant). Although other explanations are possible, we postulate that compared with the wild type, a *cydAB* mutant is defective in reoxidation of the Ccl2 protein because an electron transport pathway for this (i.e., cytochrome *bd*) is absent. This hypothesis suggests that cytochrome *bd* may be a sink for some sulfhydryl redox pathways in the periplasm and is consistent with the increased sensitivity to DTT as shown here. It is interesting that cytochrome *bd* may accept electrons from both ubiquinone and structurally unrelated donors (e.g., tetramethylphenylenediamine) at different sites within the oxidase complex (25). This phenomenon may be physiologically significant. If the cytochrome *bd* and the CydDC transporter are both involved in periplasmic reduction/oxidation homeostasis, separating and defining the exact functions will be a particular challenge for the future. In this respect, it may be that oxidation or reduction of heme to the chlorin (i.e., cytochrome *d* heme) also requires the molecule secreted by the CydDC ABC transporter either directly or indirectly. Candidate substrates for the CydDC transporter include any reducing or oxygen-scavenging agent, not necessarily a specific cysteine-reducing molecule. It is worth mentioning that in both *E. coli* and *Haemophilus influenzae*, *cydDC* is adjacent to the *trxB* gene, which encodes the thioredoxin reductase (15, 17). Poole et al. have shown that a *trxB* mutant still synthesizes cytochrome *bd* (30). We cannot completely rule out the possibility, however unlikely, that the substrate for the CydDC transporter is thioredoxin reductase, that this enzyme could be responsible for reduction reactions in both the cytoplasm and periplasm, and that asymmetric distribution might result in a cytochrome *bd* synthesis defect.

Mutants with *cydC* or *aeg-46.5* defects are unable to synthesize *c*-type cytochromes, but *aeg-46.5* mutants are proficient for cytochrome b_{562} synthesis. Eight open reading frames found at the *aeg-46.5* locus of *E. coli* are highly homologous to genes necessary for *c*-type cytochrome biosynthesis in *R. capsulatus* (34). We have used an *E. coli* strain containing a Kan^r insertion in the promoter region of the *aeg-46.5* operon to demonstrate that no *c*-type cytochromes are made spectrally or by heme stain analysis (of SDS-PAG) in this mutant. This

defect is specific for *c*-type cytochromes, since the periplasmically located cytochrome b_{562} is made to wild-type levels. While the present work was in progress, Thöny-Meyer et al. (42) demonstrated that a single deletion of all eight genes at the *aeg-46.5* locus renders the cells unable to make *c*-type cytochromes, consistent with our results.

We have previously proposed that the *helABC* genes encode a heme exporter in *R. capsulatus* (4). Since defects in either the *cydC* or *aeg-46.5* locus prevent cytochrome *c* synthesis, it is unlikely that both encode heme transporters, particularly since they contain no obvious regions of homology. We believe that it is likely that the general periplasmic defects observed in the *cydDC* mutants are responsible for the cytochrome *c* deficiency. For example, if the redox environment of the periplasm is unbalanced, a number of pathways required for cytochrome *c* biogenesis will be impaired. These include the DipZ (11, 36, 37), HelX (3), and Ccl2 (4) proteins, all of which are required for cytochrome *c* assembly.

Conclusions. Four major points have been addressed by the present study.

(i) The CydDC ABC transporter is probably not a specific heme exporter but may affect a post-secretory step in the assembly pathway of select proteins.

(ii) The reducing environment of the periplasm of *cydC* mutants is different from that of either the *cydAB* mutant or the wild-type strain. This result suggests that the CydDC transporter may be involved in periplasmic reduction pathways and homeostasis.

(iii) A locus with another ABC transporter, the *aeg-46.5* locus (*helABC*), is required for cytochrome *c* but not cytochrome b_{562} biogenesis. We suggest that a specific heme transporter may be necessary for *c*-type cytochrome synthesis but that a different (or nonspecific or diffusional) mechanism for heme export to periplasmic cytochromes b_{562}, cytochrome b_5, and hemoglobin is present in *E. coli.*

(iv) The reporters used in this study will be useful in understanding the biosynthesis of heme proteins and the transport and biosynthesis of heme itself. Concerning the latter, we note that hemin added exogenously increased the levels of hemoglobin (holoform) independent of the strain or final location of the hemoglobin. Thus, heme synthesis is limiting, but we note that the use of heme sinks such as periplasmic or cytoplasmic cytochrome b_5 can increase the total heme synthesis in the cell. This is in agreement with a recent report on heme synthesis in the *E. coli* cytoplasm (45).

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