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Genetics

Cytochromes *c* biogenesis in a photosynthetic bacterium requires a periplasmic thioredoxin-like protein

(oxidoreductase/heme/secretion/assembly)

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ABSTRACT ***Rhodobacter capsulatus* is a Gram-negative photosynthetic bacterium that requires *c*-type cytochromes for photosynthetic electron transport. Our studies demonstrate that the gene *helX* is required for the biogenesis of *c*-type cytochromes in *R. capsulatus*. A *helX* chromosomal deletion mutant cannot grow photosynthetically, due to a deficiency of all *c*-type cytochromes. The predicted amino acid sequence of the *helX* gene product (176 residues) is related to that of thioredoxin and shares active-site homology with protein disulfide isomerase. Cytochrome c_2–alkaline phosphatase gene fusions are used to show that HelX is not required for the transcription, translation, or secretion of apocytochrome c_2. HelX–alkaline phosphatase and HelX–β-galactosidase gene fusions are used to demonstrate that HelX is a periplasmic protein, which is consistent with the presence of a typical signal sequence in HelX. Based on these results, we propose HelX functions as a periplasmic disulfide oxidoreductase that is essential for cytochromes *c* biogenesis. This role is in accordance with the observation that both heme and the cysteines of apocytochromes *c* (Cys-Xaa-Yaa-Cys-His) must be in the reduced state for covalent linkage between the two moieties to occur.**

The *c*-type cytochromes are electron transfer proteins that are essential to the mitochondrial electron transport system as well as to some electron transport systems of prokaryotes. These cytochromes are differentiated from other cytochromes by their heme, which is covalently attached by thioether bonds to the conserved cysteines (Cys-Xaa-Yaa-Cys-His) of the apoproteins. In prokaryotes, *c*-type cytochromes are further differentiated by their subcellular location: they are either soluble in the periplasm (e.g., cytochrome c_2) or oriented toward the periplasm when inserted in the cytoplasmic membrane (e.g., cytochrome c_1 of the bc_1 complex). The photosynthetic electron transport system in photosynthetic bacteria such as *Rhodobacter capsulatus* requires *c*-type cytochromes (e.g., cytochrome c_1) (1). In this system, electrons are cyclically transferred from the reaction-center complex via ubiquinone to cytochrome bc_1, then back to the reaction-center complex via cytochrome c_2.

Studies in eukaryotes have shown that apocytochromes *c* and c_1 are targeted to the mitochondrial intermembrane space (see ref. 2 for review) and that at least three components—apocytochromes *c*, heme, and heme lyase—are required in the intermembrane space for assembly to occur.

Some components required for cytochromes *c* biogenesis in prokaryotes have only recently been described. We identified six genes at two loci that are required for this process in *R. capsulatus* (3, 4). One of these loci contains four genes called *helA*, *helB*, *helC*, and *orf52* (hereafter called *helD*), which are proposed to encode an ABC-type heme transporter specific to cytochromes *c* biogenesis (4). Hel-like gene products have also been identified in one other prokaryote, the symbiotic Gram-negative bacterium *Bradyrhizobium japonicum* (5). HelA-, HelB-, HelC-, and HelD-like gene products called CycV, CycW, Orf263, and CycX, respectively, are 65% similar to their *R. capsulatus* counterparts. As is the case with *hel* mutants, mutants in *cycV*, *cycW*, or *cycX* are specifically missing *c*-type cytochromes (5). Analyses of the second locus required for cytochromes *c* biogenesis in *R. capsulatus* (4, 6) indicate that two genes, *ccl1* and *ccl2*, are required for this process (4). *ccl1* encodes a periplasmically oriented protein that is homologous to several chloroplast and mitochondrial open reading frames (ORFs) of unknown function (4). Ccl2 is predicted to be a periplasmic protein since it has a consensus signal sequence (4).

We report here that a *hel*-linked gene, *helX*, is essential for cytochromes *c* biogenesis in *R. capsulatus*. A *helX* chromosomal deletion mutant cannot grow photosynthetically, because it is specifically missing *c*-type cytochromes. We also show that *helX* encodes a periplasmic protein that is not required for the synthesis or secretion of apocytochrome c_2 to the periplasm.† HelX is significantly homologous to various bacterial, algal, and plant thioredoxins and shares active-site homology with protein disulfide isomerase, as well as *Escherichia coli* DsbA (7, 8) and *Vibrio cholerae* TcpG (9). Both DsbA and TcpG are members of a class of periplasmic proteins required for disulfide bond formation during the maturation of some secreted and membrane proteins. Our results suggest that HelX may function as a periplasmic protein disulfide oxidoreductase that is specific to cytochromes *c* biogenesis. Results with DsbA, TcpG, and HelX underscore the idea that specific proteins outside of the bacterial cytoplasmic membrane are required to mediate distinct oxidation or reduction reactions.

MATERIALS AND METHODS

Strains and Plasmids. *R. capsulatus* SB1003 (10), *E. coli* CC118 (11), and the cytochrome c_2–alkaline phosphatase fusion plasmids (4) have been described. Plasmids phelD and phelDX contain the indicated DNA restriction fragments (Fig. 1) cloned into pUC118 and into pUCA10, a derivative of the broad-host-range vector pUCA6 (12). Plasmid pA1Z118 is a 6.8-kb *Xba* I–*Sal* I subclone of pDFH100Bcl (12) in pUC118 and contains a *nifA–lacZ* gene fusion.

Plasmid pΔhelX contains the *Eco*RV–*Stu* I fragment of the *hel* locus (Fig. 1) cloned into pUCA10. This construct contains the *Sma* I-excised kanamycin-resistance cassette from pUC4 (Pharmacia) in the blunted *Rsr* II sites of the *R. capsulatus* DNA. To construct *R. capsulatus* ΔhelX, a chromosomal deletion mutant of *helX*, pΔhelX was conjugated

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; ORF, open reading frame; TMPD, tetramethylphenylenediamine.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M96013).

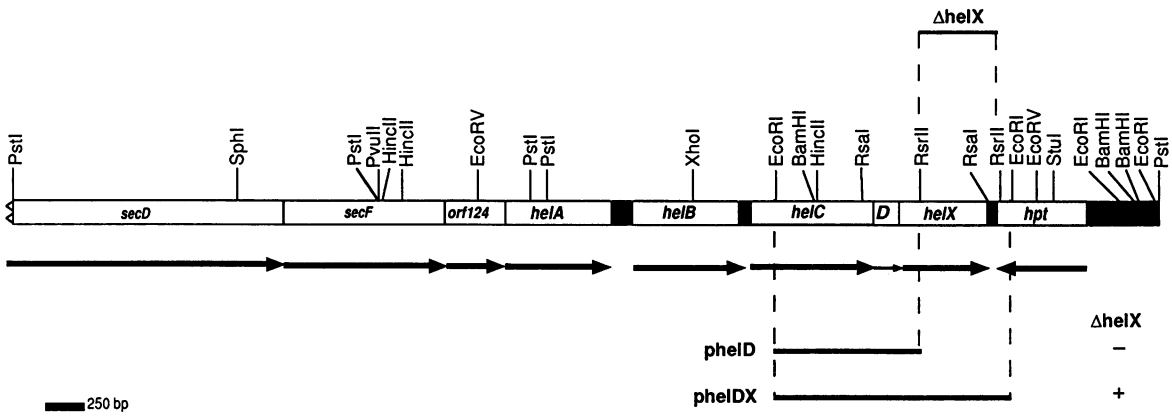

FIG. 1. Restriction map of the *helX* locus, including upstream and downstream regions, and the DNA complementation analysis of *R. capsulatus* ΔhelX. The *helX* chromosomal deletion strain ΔhelX contains a kanamycin-resistance cassette between the *Rsr* II sites of *helX* and *hpt*. Plasmids containing the indicated DNA restriction fragments were conjugated into the ΔhelX strain. Complementation was defined as the ability to oxidize the cytochromes *c*-specific electron donor tetramethylphenylenediamine (TMPD) and to grow photosynthetically.

into SB1003 and tetracycline-resistant colonies were selected. The incompatible plasmid pPH1J1 (13) was then conjugated into SB1003(pΔhelX). Kanamycin- and gentamicin-resistant colonies that were tetracycline-sensitive were selected and they exhibited a Hel$^-$ phenotype (i.e., could not oxidize TMPD and could not grow photosynthetically).

DNA Sequencing and Sequence Analyses. The Sanger dideoxy method was used to sequence *helX*. Both strands were sequenced according to methods previously described (4). Searches for related proteins were made in GenBank and EMBL databases by using TFASTA (14). In these analyses, optimal alignment scores > 100 were obtained for comparisons of HelX to various thioredoxins and to *B. japonicum* ORF132. LFASTA and RDF2 analyses (14) were used to determine the significance of each of the homologies. In all cases, scores > 10 were obtained, with RDF2 scores > 10 being significant (14).

Difference Spectroscopy. The *R. capsulatus* strains were grown in 500 ml of RCV medium (12) at 32°C for ≈40 hr. The strains were grown aerobically in 2-liter flasks, shaking at 300 rpm. The cells were harvested and suspended in 40 ml of 0.01 M Tris (pH 7.5). This suspension was sonicated for 15 min at 40% power (Branson sonifier 250). Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was ultracentrifuged for 1 hr at 200,000 × *g*. The supernatant of this centrifugation is called the soluble fraction and the resulting membrane pellet was resuspended in 2 ml of 0.1 M Tris (pH 7.5). Membrane proteins were solubilized by addition of 2 ml of 2% (vol/vol) Triton X-100 and incubation on ice for 30 min, followed by centrifugation at 12,000 × *g* for 5 min. Reduced (sodium hydrosulfite) minus oxidized (potassium ferricyanide) spectra were obtained on a Beckman model DU50 spectrophotometer.

Construction of *helX–phoA* and *helX–lacZ* Fusions. Plasmids phelX-pho and phelX-lacZ were constructed according to the strategy used to create the *cytA–phoA* gene fusions (4); an oligonucleotide was used to engineer a *Sal* I site into HelX (Fig. 2). A *phoA* gene and *lacZ* gene were cloned in-frame into this *Sal* I site. The *phoA* fusion vector pPH07 (15) was kindly provided by Claude Gutierrez (Centre de Biochemie et de Genetique Cellulaire). The *lacZ* fusion vector pLKC480 (16) was kindly provided by John Smith (Seattle Biomedical Research Institute). The junctions of the resulting fusion plasmids were confirmed by restriction digests and by sequence analysis.

Other Methods. Alkaline phosphatase activity was detected in bacterial colonies by the chromogenic indicator 5-bromo-4-chloro-3-indolyl phosphate (Sigma) (11). Bacterial cells were assayed for alkaline phosphatase (17) and β-galactosidase (18) activity as described. Protein was determined by a modified Lowry procedure (19).

To isolate periplasmic and cytoplasmic fractions, cells were grown at 37°C in 500 ml of LB medium (and antibiotic when necessary) to an OD_{550} of 0.6. At this point, 2 ml of 0.1 M isopropyl β-D-thiogalactopyranoside (IPTG) was added and the cells were cultured to an OD_{550} of 1.2–2. Cells were harvested and fractionated as described (20, 21).

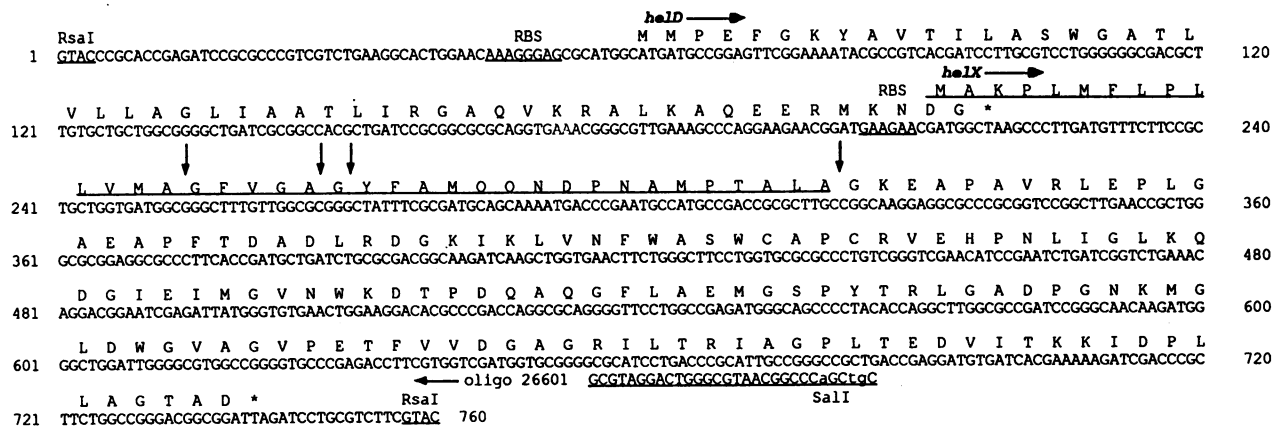

FIG. 2. DNA sequence (noncoding strand) of *helX*. The predicted amino acid sequence of HelX is shown above the nucleotide sequence. Putative ribosome binding sites are underlined and labeled RBS. The maximum putative 37-aa signal sequence is underlined, with potential cleavage sites indicated by arrows (see text). The sequence of oligonucleotide 26601, which was used to engineer a *Sal* I site into *helX*, is shown.

RESULTS

Sequence Analysis of *helX*. We previously reported (3, 4) that four linked genes called *helA*, *helB*, *helC*, and *helD* are specifically required for cytochromes *c* biogenesis in *R. capsulatus* and demonstrated that *orf124*, the gene upstream of *helA*, is not required for this process (see Fig. 1 for detailed map). Our continued sequence analysis of the DNA flanking this locus indicates that four additional ORFs with codon usage typical of *R. capsulatus* genes (data not shown) are present (Fig. 1). The predicted gene products of three of these ORFs exhibit significant homology to known proteins and were thus named accordingly. One of these ORFs, *hpt*, is transcribed opposite to the *hel* genes; its predicted gene product is homologous to various hypoxanthine (guanine) phosphoribosyltransferases (22). We designated the two ORFs upstream of *orf124* as *secD* and *secF* because their predicted gene products are homologous to *E. coli* SecD and SecF (ref. 23 and unpublished work). *E. coli* SecD and SecF are reported to be cytoplasmic membrane proteins that contain a large periplasmic domain and are essential for protein export (23). Chromosomal inserts in the *R. capsulatus secDF* locus could not be constructed (3). This inability suggests that, like *secDF* of *E. coli*, these are essential genes in *R. capsulatus*.

Overlapping the translational stop of *helD* is an ORF we designated *helX*. HelX comprises 176 aa (Fig. 2). Five of the first 32 aa of this predicted gene product are methionines. However, since only the methionine codon at nt 213 is preceded by a putative ribosome binding site and since this region contains good codon usage for *R. capsulatus*, this methionine codon is presented as the translational start codon of HelX. A typical bacterial signal sequence is present at the amino terminus of this ORF. Bacterial signal sequences are typically 15–30 aa long and contain a net positively charged region 5–8 aa in length, followed by an 8- to 12-aa central hydrophobic core, a predicted "turn," and a polar region of 5–7 aa that terminates with a site for signal cleavage (24). According to the "−3, −1 rule" (25), only residues that have small neutral side chains (e.g., Ala, Gly, Ser, and Thr) can occupy positions −3 and −1 preceding the cleavage site (26). While prokaryotic signal sequences often contain a Pro or turn-promoting residue at the junction between the hydrophobic core and cleavage regions (26), this residue has been shown not to be essential for transport (27). According to these criteria, four potential cleavage sites are present within the first 37 aa residues of HelX, with the minimal predicted signal sequence comprising the first 14 aa, and the maximum 37 aa (Fig. 2).

Inactivation of *helX*. To determine whether *helX* is required for cytochromes *c* biogenesis, a *helX* chromosomal deletion mutant (ΔhelX) was constructed as described in *Materials and Methods*. Southern blots of ΔhelX genomic DNA confirmed that *helX* was replaced with the kanamycin-resistance cassette (data not shown). The *R. capsulatus* ΔhelX strain was unable to grow photosynthetically but grew under aerobic conditions. Additionally, the ΔhelX strain was unable to oxidize the cytochrome *c*-specific electron donor TMPD. The ΔhelX strain was complemented to a wild-type phenotype by a plasmid containing *helX* and *helD* (plasmid phelDX), but not by a plasmid containing only *helD* (phelD, Fig. 1). As expected, these complementation results also indicate that *hpt* is not involved in the mutant phenotype.

Hydrophilicity Analysis and Homology Comparisons. HelX is a predominantly hydrophilic protein with the only hydrophobic region contained within the first 20 residues of the predicted signal sequence (Fig. 3).

The TFASTA (14) program was used to search the GenBank (Version 73) and EMBL (October 1992) databases for HelX-related proteins. HelX showed significant homology to bacterial, algal, and plant thioredoxins (see refs. 30 and 31 for reviews on thioredoxin). Of the first nine significant matches, eight were to thioredoxins, with the ninth match being to *B. japonicum* Orf132 (see below). Interestingly, although prokaryotic thioredoxins are ≈50% identical to each other (31), HelX is only 25% identical and 53% similar to the thioredoxin of the closely related *Rhodobacter sphaeroides* and only 23% identical and 49% similar to the thioredoxin of *E. coli* (Fig. 4 *A* and *B*). The greatest degree of homology (39% identical, 63% similar; data not shown) was obtained with the thioredoxin-like *orf132*-encoded protein of *B. japonicum* (5). Although *orf132* is adjacent to *hel*-like genes in *B. japonicum*, a kanamycin-resistance cassette could not be inserted into the chromosomal *orf132* (5). Thus, a specific role in cytochromes *c* biogenesis could not be confirmed and further analogies to the *B. japonicum* system are not possible. HelX does not have significant overall homology to either *E. coli* DsbA or *V. cholerae* TcpG (see Introduction), but it does have the purported consensus active site of DsbA (ref. 7; Fig. 4 *B* and *C*).

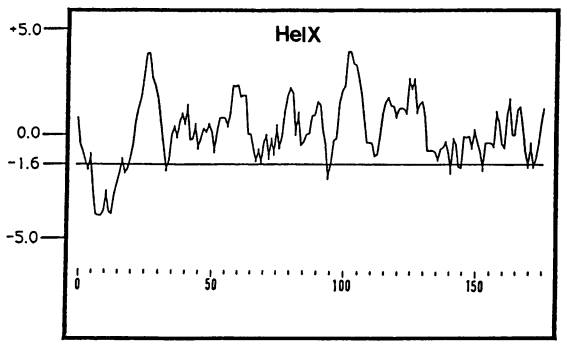

FIG. 3. Hydrophilicity analysis of HelX. The analysis was performed using a window length of 19 residues (28) with programs from the Wisconsin Genetics Computer Group (29). Regions with values of >0 are hydrophilic; regions of <0 are hydrophobic, with regions below −1.6 being predicted transmembrane regions (28).

Spectral Analysis of ΔhelX. We used difference spectroscopy to show that *R. capsulatus* ΔhelX was specifically missing all *c*-type cytochromes. Reduced-minus-oxidized spectra of cytochromes extracted from the ΔhelX strain show that this mutant is lacking soluble and membrane-bound *c*-type cytochromes (≈552 nm) but has *b*-type cytochromes (560 nm) (Fig. 5). Similar results were obtained for ΔhelAB and Δccl12 mutant strains (unpublished work). SDS/PAGE was used to separate proteins from wild-type and ΔhelX cells; heme-stained proteins of membrane and crude sonicated extracts confirmed that all *c*-type cytochromes are absent in ΔhelX (data not shown). These results are identical to those previously reported for *hel* mutants (3).

Apocytochrome Secretion Is Independent of HelX Function. To determine whether the mutant phenotype of ΔhelX was due to a defect in the transcription, translation, or secretion of apocytochrome c_2 to the periplasm, we assayed the alkaline phosphatase activity of the ΔhelX strain containing a cytochrome c_2–alkaline phosphatase gene fusion plasmid. Alkaline phosphatase gene (*phoA*) fusions are useful in secretion studies because alkaline phosphatase is active only if it is secreted into the periplasm (see ref. 35 for review). *R. capsulatus* ΔhelX synthesized and secreted the cytochrome c_2–alkaline phosphatase fusion protein at a level similar to that produced by the wild-type strain (Table 1). These results prove that HelX is not required for the transcription, translation, or secretion of apocytochrome c_2.

HelX Is a Periplasmic Protein. We constructed *helX–phoA* and *helX–lacZ* gene fusions to confirm the use of the consensus signal sequence of HelX. Although *R. capsulatus* strains containing the *helX–phoA* fusion showed alkaline phosphatase activity (data not shown), the levels of activity

A

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HelX          39 KEAPAVRLEPLGAEAPFTDADLRDGKIKLVNFWASWCAPCRVEHPNLIGLKQDGIEIMGVNWKDTPDQAQGFLAEMGSPY 118
E.coli         1 MSDKIIHL....TDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTV..................... 56
R.sphaeroides  2 ...STVPV....TDATFDTEVRKSDVPVVVDFWAEWCGPCRQIGPALEELSKEYAGKVKI..................... 54

HelX         119 TRLGADPGNKMGLDWGVAGVPETFVVDGAGRILTRIAGPLTEDVITKKIDPLLAG 173
E.coli        57 AKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVASATKVGALSKGQLKEFLDANLA* 110
R.sphaeroides 55 VKVNVDENPESPAMLGVRGIPALFLFKNGQVVS.NKVGAAPKAALATWIASAL* 106
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B

	HelX	E.coli	R.sphaeroides	PDI	TcpG	DsbA
HelX	100%	49% (23%)	53% (25%)	N.S.	N.S.	N.S.
E.coli		100%	72% (50%)	59% (33%)	N.S.	N.S.
R.sphaeroides			100%	72% (50%)	N.S.	N.S.
PDI				100%	N.S.	N.S.
TcpG					100%	63% (40%)
DsbA						100%

C

HelX	D	G	K	I	K	L	V	N	F	W	A	S	W	C	A	P	C	R	V	E	H	P	N	
R.sphaeroides Trx	S	D	V	P	V	V	V	D	F	W	A	E	W	C	G	P	C	R	Q	I	G	P	A	
E.coli Trx	A	D	G	A	I	L	V	D	F	W	A	E	W	C	G	P	C	K	M	I	A	P	I	
PDI C-terminus	E	K	K	N	V	F	V	E	F	Y	A	P	W	C	G	H	C	K	Q	L	A	P	I	
PDI N-terminus	A	H	N	Y	L	L	V	E	F	Y	A	P	W	C	G	H	C	K	A	L	A	P	E	
TcpG	S	S	S	P	V	V	S	E	F	F	S	F	Y	C	P	H	C	N	T	F	E	P	I	
DsbA	A	G	A	P	Q	V	L	E	F	F	S	F	F	C	P	H	C	Y	Q	F	E	E	V	

FIG. 4. (*A*) Pairwise alignment of HelX and *R. sphaeroides* thioredoxin (32) with *E. coli* thioredoxin (33). The program BESTFIT (29) was used to create this alignment. (*B*) Degree of homology between HelX and *E. coli* thioredoxin (33), *R. sphaeroides* thioredoxin (32), rat hepatic protein disulfide isomerase (PDI) (34), *V. cholerae* TcpG (9), and *E. coli* DsbA (7). Percent similarities are shown, with percent identity in the parentheses; nonsignificant homologies are indicated (N.S.). These values were obtained from the program BESTFIT (29). (*C*) Homology at the active site. HelX residues 62–84 are aligned with the region surrounding the catalytic site of *R. sphaeroides* thioredoxin (Trx) (32), *E. coli* thioredoxin (33), rat hepatic PDI (34), *V. cholerae* TcpG (9), and *E. coli* DsbA (7). Arrowheads mark residues conserved between the thioredoxins and HelX that are not conserved in TcpG or DsbA.

were much higher when expressed from the *lacZ* promoter in *E. coli*. Periplasmic and cytoplasmic fractions of *E. coli* cells containing the HelX–PhoA or the NifA–LacZ fusion protein were assayed, respectively, for alkaline phosphatase or β-galactosidase activity (Table 2). The NifA–LacZ fusion protein (12) was used as a cytoplasmic reporter. This fusion construct, which is also directed from the *lacZ* promoter, contains the amino terminus of NifA, a DNA-binding regulatory protein. Periplasmic fractions of *E. coli* cells containing the *helX–phoA* gene fusion were 50-fold enriched for alkaline phosphatase activity when compared with the β-galactosidase activity of the cytoplasmic fractions (Table 2).

When the *helX–lacZ* fusion was expressed from the *lacZ* promoter in *E. coli*, IPTG-induced lethality occurred (data not shown). This lethality is consistent with the concept of hybrid jamming, which occurs when the β-galactosidase part of the fusion protein interferes with the export machinery, resulting in the jamming of the export process (36, 37). This IPTG-induced lethality suggests that HelX is secreted into the periplasm via a general secretory route.

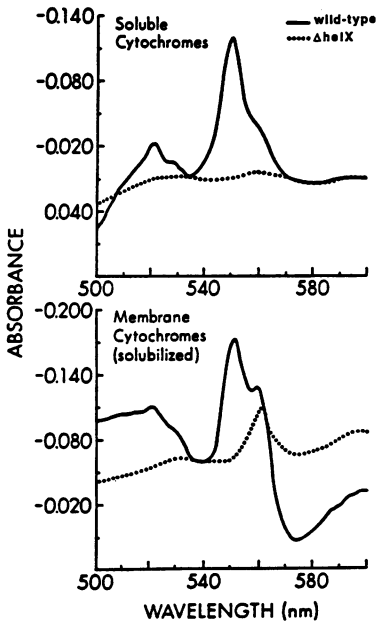

FIG. 5. Reduced-minus-oxidized spectra of Triton X-100-solubilized membranes (*Lower*) and soluble cytochromes (*Upper*) from ΔhelX (·····) and the wild-type strain SB1003 (——). The *c*-type cytochromes show peaks at 552 nm and the *b*-type cytochromes at 560 nm. The extracts in each spectra are at equal protein concentrations.

DISCUSSION

We report here a gene, *helX*, whose product is specifically required for the biogenesis of *c*-type cytochromes in the

Table 1. Alkaline phosphatase activities of bacteria with *cytA–phoA* gene fusions

Strain	Alkaline phosphatase activity
SB1003	140*
SB1003(pCytphoA)	12,000
ΔhelX	200*
ΔhelX(pCytphoA)	13,100

Activities with whole cells are measured in OD units/min per mg of protein. See Table 2 for assay conditions. Averages of three experiments are shown. The variability between ΔhelX(pCytphoA) and SB1003(pCytphoA) was always less than the variability between SB1003(pCytphoA) experimental values. Results were confirmed by plate assays using the indicator 5-bromo-4-chloro-3-indolyl phosphate (data not shown). Cells were grown in RCV basal medium with incubation at 34°C. Aerobic growth conditions were used and antibiotics were added to the medium with strains containing plasmids. **R. capsulatus* backgrounds differ, depending on amounts of photopigments (which vary with strains).

Table 2. Specific activities of fusion proteins in fractions

Fraction	HelX–PhoA activity	NifA–LacZ activity
Whole cells	3,500	10,700
Periplasmic fraction	26,200	4,300
Cytoplasmic fraction	2,100	18,000

Values are averages of two separate trials with five assays per trial. Fractionations were performed as described in *Materials and Methods*. Activities are measured in OD units/min per mg of protein. Alkaline phosphatase (PhoA) OD units are defined as $OD_{420} \times 1000$ after incubation in 2.2-ml assay volume at 37°C with *o*-nitrophenyl phosphate. β-Galactosidase (LacZ) OD units are defined as $(OD_{420}/0.0045) \times$ volume in 3.4 ml after incubation at 37°C with *o*-nitrophenyl phosphate. Cells were grown in LB medium with incubation at 37°C. Aerobic growth conditions were used and antibiotics were added to the medium with strains containing plasmids. All fractions reported here were sonicated.

photosynthetic bacterium *R. capsulatus*. The chromosomal deletion mutant ΔhelX is specifically missing *c*-type cytochromes. Cytochromes *c* deficiencies were indicated by the inability of ΔhelX to oxidize the cytochromes *c*-specific electron donor TMPD, the inability to grow photosynthetically, and the absence of *c*-type cytochromes as detected either spectrally or with denaturing protein gels stained for heme. HelX is homologous to various thioredoxins and is not required for the transcription, translation, or transport of apocytochrome c_2 to the periplasm. We created and used *helX–phoA* and *helX–lacZ* gene fusions to confirm that HelX is a periplasmic protein as suggested by its signal sequence.

These results indicate that a periplasmic thioredoxin-like protein is required specifically for cytochromes *c* biogenesis. Recently, an *E. coli* periplasmic disulfide oxidoreductase has been identified that is essential for the *in vivo* formation of disulfide bonds of some secreted and membrane proteins (7, 8). This protein, DsbA, appears to represent a novel class of periplasmic disulfide oxidoreductases (7). Another member of this class has been reported: TcpG is a *V. cholerae* periplasmic protein that is 40% identical to DsbA and has been shown to have disulfide oxidoreductase activity (9). Neither DsbA nor TcpG has overall significant homology to bacterial thioredoxins, although their putative active sites have a Cys-Pro-His-Cys domain (vs. Cys-Xaa-Pro-Cys for thioredoxin and Cys-Gly-His-Cys for protein disulfide isomerase). In contrast, HelX has significant overall homology to bacterial thioredoxins, albeit at a lower level than that observed when individual bacterial thioredoxins are compared with each other. HelX also has a cysteine domain, Cys-Ala-Pro-Cys, which is more like that of thioredoxin (see Fig. 4*C*). Based on these observations, we propose that HelX represents a different class of periplasmic disulfide oxidoreductase.

It is clear that the function of HelX is not the same as that of DsbA. Alkaline phosphatase itself requires disulfide bonds (in *E. coli* via oxidation by DsbA), presumably for proper folding, yet high levels of alkaline phosphatase activity were observed in both the *R. capsulatus* wild-type and ΔhelX strains containing the cytochrome c_2–alkaline phosphatase gene fusion.

The simplest function envisioned for HelX is to retain cysteine residues of apocytochromes *c* (Cys-Xaa-Yaa-Cys-His) in a reduced state prior to heme ligation. *In vitro* studies suggest that these cysteine residues must be reduced for this reaction to occur (38). Another possible function of HelX would be to keep heme or some other biosynthetic component reduced. *In vitro* experiments using intact mitochondria have demonstrated that heme must be in a reduced state to be ligated to apocytochromes *c* (39) or c_1 (40). In these experiments, heme reduction was achieved by addition of NADH and a flavin nucleotide. It remains to be determined whether specific thioredoxin-like components are required in mitochondria (or chloroplasts) for cytochromes *c* biogenesis. We have already noted that Ccl- and Hel-like components are encoded by chloroplast genomes (4) and, very recently, we have discovered liverwort mitochondrial (41) homologs to *R. capsulatus* Ccl1, HelB, and HelC (unpublished work).

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1. Prince, R. C. & Daldal, F. (1987) *Biochim. Biophys. Acta* **894**, 370–378.
2. Gonzales, D. H. & Neupert, W. (1990) *J. Bioenerg. Biomembr.* **22**, 753–768.
3. Kranz, R. G. (1989) *J. Bacteriol.* **171**, 456–464.
4. Beckman, D. L., Trawick, D. R. & Kranz, R. G. (1992) *Genes Dev.* **6**, 268–283.
5. Ramseier, T. M., Winteler, H. V. & Hennecke, H. (1991) *J. Biol. Chem.* **266**, 7793–7803.
6. Biel, S. W. & Biel, A. J. (1990) *J. Bacteriol.* **172**, 1321–1326.
7. Bardwell, J. C. A., McGovern, K. & Beckwith, J. (1991) *Cell* **67**, 581–589.
8. Kamitani, S., Akiyama, Y. & Ito, K. (1992) *EMBO J.* **11**, 57–62.
9. Peek, J. A. & Taylor, R. K. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6210–6214.
10. Yen, H.-C. & Marrs, B. (1976) *J. Bacteriol.* **126**, 619–629.
11. Manoil, C. & Beckwith, J. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8129–8133.
12. Foster-Hartnett, D. & Kranz, R. G. (1992) *Mol. Microbiol.* **6**, 1049–1060.
13. Hirsch, P. R. & Beringer, J. E. (1984) *Plasmid* **12**, 139–141.
14. Pearson, W. R. & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2444–2448.
15. Gutierrez, C. & Devedjian, J. C. (1989) *Nucleic Acids Res.* **17**, 3999.
16. Tiedeman, A. A. & Smith, J. M. (1988) *Nucleic Acids Res.* **16**, 3587.
17. Brickman, E. & Beckwith, J. (1975) *J. Mol. Biol.* **96**, 307–316.
18. Kranz, R. G. & Haselkorn, R. (1985) *Gene* **40**, 203–215.
19. Markwell, M. K., Haas, S. M., Bieber, L. L. & Tolbert, N. E. (1978) *Anal. Biochem.* **87**, 206–210.
20. Weiss, R. L. (1976) *J. Bacteriol.* **128**, 668–670.
21. Tai, S.-P. & Kaplan, S. (1985) *J. Bacteriol.* **164**, 181–186.
22. Beckman, D. L. & Kranz, R. G. (1991) *Biochim. Biophys. Acta* **1129**, 112–114.
23. Gardel, C., Johnson, K., Jacq, A. & Beckwith, J. (1990) *EMBO J.* **9**, 3209–3216.
24. von Heijne, G. (1985) *J. Mol. Biol.* **184**, 99–105.
25. von Heijne, G. (1984) *J. Mol. Biol.* **173**, 243–251.
26. von Heijne, G. (1986) *Nucleic Acids Res.* **14**, 4683–4690.
27. Laforet, G. A. & Kendall, D. A. (1991) *J. Biol. Chem.* **266**, 1326–1334.
28. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
29. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.
30. Holmgren, A. (1989) *J. Biol. Chem.* **264**, 13963–13966.
31. Ecklund, H., Gleason, F. K. & Holmgren, A. (1991) *Proteins Struct. Funct. Genet.* **11**, 13–28.
32. Pille, S., Chuat, J.-C., Breton, A. M., Clement-Metral, J. D. & Galibert, F. (1990) *J. Bacteriol.* **172**, 1556–1561.
33. Lim, C.-J., Geraghty, D. & Fuchs, J. A. (1985) *J. Bacteriol.* **163**, 311–316.
34. Edam, J. C., Ellis, L., Blacher, R. W., Roth, R. A. & Rutter, W. J. (1985) *Nature (London)* **317**, 267–270.
35. Manoil, C., Mekalanos, J. J. & Beckwith, J. (1990) *J. Bacteriol.* **172**, 515–518.
36. Bieker, K. L., Phillips, G. J. & Silhavy, T. J. (1990) *J. Bioenerg. Biomembr.* **22**, 291–310.
37. Lee, C., Li, P., Inouye, H., Brickman, E. R. & Beckwith, J. (1989) *J. Bacteriol.* **171**, 4609–4616.
38. Enosawa, S. & Ohashi, A. (1987) *Anal. Biochem.* **160**, 211–216.
39. Nicholson, D. W. & Neupert, W. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4340–4344.
40. Nicholson, D. W., Stuart, R. A. & Neupert, W. (1989) *J. Biol. Chem.* **264**, 10156–10168.
41. Oda, K., Yamato, K., Ohta, E., Nakamura, Y., Takemura, M., Nozato, N., Akashi, K., Kanegae, T., Ogura, Y., Kohchi, T. & Ohyama, K. (1992) *J. Mol. Biol.* **223**, 1–7.