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Bacterial cytochromes *c* biogenesis

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We report the primary sequence analyses of two loci, *hel* and *ccl*, whose gene products are required specifically for the biogenesis of *c*-type cytochromes in the Gram-negative photosynthetic bacterium *Rhodobacter capsulatus*. Genetic and molecular analyses show that the *hel* locus contains at least four genes, *helA*, *helB*, *helC*, and *orf52*, and the *ccl* locus contains two genes, *ccl1* and *ccl2*, that are essential for cytochromes *c* biogenesis. HelA is homologous to a class of proteins called ABC transporters and *helA*, *helB*, and *helC* are proposed to encode an export complex. Cytochrome c_2–alkaline phosphatase gene fusions were used to show that apocytochrome c_2 synthesis and secretion are not affected by the *hel* and *ccl* defects. Ccl1 and Ccl2 possess typical signal sequences to direct them to the periplasm. The periplasmic orientation of Ccl1 was confirmed using a Ccl1–alkaline phosphatase gene fusion. The Ccl1–alkaline phosphatase gene fusion analysis also demonstrated that Ccl1 does not require *hel* genes for its synthesis and secretion. Ccl1 is homologous to proteins encoded by chloroplast and mitochondrial genes, suggesting analogous functions in these organelles. Taken together, these results support the hypothesis that the *hel*-encoded proteins are required for the export of heme to the periplasm where it is subsequently ligated to the *c*-type apocytochromes.

[*Key Words*: Biogenesis; cytochrome; heme; secretion; transporter]

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All eukaryotes and many prokaryotes have *c*-type cytochromes. These cytochromes function as electron transfer proteins and differ from other cytochromes in that their heme is attached covalently (Pettigrew and Moore 1987). Eukaryotes have a soluble cytochrome *c* and a membrane-bound cytochrome c_1 positioned in the mitochondrial intermembrane space, whereas bacterial *c*-type cytochromes are topologically oriented outside the cytoplasmic membrane in the periplasmic space (Pettigrew and Moore 1987; Page and Ferguson 1989). These biosynthetic distinctions of *c*-type cytochromes raise a number of important questions in biology. What enzymes are required for the heme ligation process? Where do the ligation and assembly processes take place? How are components necessary for biogenesis transported to the assembly sites? To begin to answer these questions we have characterized bacterial genes involved specifically in cytochromes *c* biogenesis and studied the targeting of components of this process.

Most studies on the process of cytochromes *c* biogenesis in eukaryotes have been carried out with *Saccharomyces cerevisiae* or *Neurospora crassa*. In these organisms, both apocytochromes *c* and c_1 are transported from the cytosol to the mitochondrial intermembrane space. Apocytochrome c_1 transport takes place by two sequential translocations (Nicholson et al. 1989). Initially, preapocytochrome c_1 is translocated through contact sites between the mitochondrial outer and inner membranes to the matrix. Here, the first part of the bipartite signal sequence is removed. The preapocytochrome c_1 is then translocated to the outer surface of the inner membrane with subsequent ligation of heme and removal of the entire signal sequence. Final assembly of functional cytochromes *c* and c_1 requires the ligation of heme to the apoproteins in the intermembrane space. This ligation occurs at two distinct cysteine residues (CXYCH) within the apocytochromes *c* and c_1 to two vinyl moieties in the heme (Taniuchi et al. 1983). Two separate cytochrome *c* heme lyases, one for cytochrome *c* (called CCHL) and one for cytochrome c_1 (called CC_1HL), appear to be required. *S. cerevisiae* mutants deficient in CC_1HL have been reported (Tzagoloff and Dieckmann 1990), but their characterization and the genes that correct the defects have not been published. A *N. crassa* mutant, *cyt-2-1* (Nargang et al. 1988), and a *S. cerevisiae* mutant, *cyc3* (Matner and Sherman 1982), are unable to ligate heme to apocytochrome *c* in vivo and in vitro. The *cyc3* gene has been cloned, sequenced, and characterized, and its gene product is predicted to function as a CCHL within the intermembrane space (Dumont et al. 1987). Moreover, the *cyt-2-1* gene (Nargang et al. 1988) and the *cyc3* gene (Dumont et al. 1988) are essential for the transport of nuclear encoded apocytochrome *c* through the mitochondrial outer membrane into the intermembrane space. The assembly of a *c*-type cytochrome in a mitochondrion, therefore, requires at least three compo-

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nents—heme, a lyase, and the apocytochrome *c*—to be targeted to the intermembrane space. For eukaryotes, major questions remain concerning the nature of CC_1HL and the transport of heme and individual lyases. It is also unknown whether other components are required for the in vivo assembly process. This report suggests that other components may be involved.

Little is known about cytochrome *c* biogenesis in prokaryotes. Many bacteria, including *Escherichia coli* and related species, do not require *c*-type cytochromes for aerobic or anaerobic growth. These organisms use cytochromes in which heme is bound noncovalently (e.g., *b*-type cytochromes) for electron transport systems (Lin and Kuritzbes 1987; Poole and Ingledew 1987). Nevertheless, cytochromes *c* are present in a wide variety of prokaryotes. Biochemical analyses of cytochromes *c* from both Gram-positive (e.g., Von Wachenfeldt and Hederstedt 1990) and Gram-negative bacteria have demonstrated the presence of covalently bound heme at CXYCH by thioether linkages (Pettigrew and Moore 1987). A recent report suggests that the heme ligation process occurs in the periplasm in *Paracoccus denitrificans* (Page and Ferguson 1990). Along with *P. denitrificans*, photosynthetic bacteria such as *Rhodobacter capsulatus* and symbiotic bacteria such as *Bradyrhizobium japonicum* are prokaryotic paradigms for studies on mitochondrial-like electron transport systems (e.g., cytochromes *c* and cytochrome bc_1). These Gram-negative eubacteria are considered to be phylogenetically related to the ancestral mitochondrial endosymbiont (Woese 1987). We have undertaken investigations into the process of cytochromes *c* biogenesis in *R. capsulatus* and have previously isolated mutants and genes called *helA*, *helB*, and *helC* that are involved in this process (Kranz 1989). Very recently, three *hel*-like genes in *B. japonicum* were reported to be involved in cytochromes *c* biosynthesis (Ramseier et al. 1991). In this report, we describe genetic and molecular analyses of the *hel* locus from *R. capsulatus*, and we show that four genes, *helA*, *helB*, *helC*, and *orf52*, are required for cytochromes *c* biogenesis. In addition, we characterize a second locus from *R. capsulatus* that encodes two additional genes, *ccl1* and *ccl2*, that are also essential for cytochromes *c* biogenesis. Alkaline phosphatase gene (*phoA*) fusions to the cytochrome c_2 gene (*cytA*) and to *ccl1* are used to investigate targeting processes. Sequence analyses of the *hel* and *ccl* loci and the *cytA–phoA* and *ccl1–phoA* analyses of *hel* and *ccl* mutants provide evidence for the hypothesis that *hel* genes encode a heme transporter and that apocytochromes *c*, Ccl1, and Ccl2 are targeted to the bacterial periplasm by a general secretory mechanism.

Results

Sequence analysis of the hel *locus*

We reported previously that three linked genes called *helA*, *helB*, and *helC* are required for the specific biogenesis of *c*-type cytochromes in *R. capsulatus* (for detailed map, see Fig. 1; Kranz 1989) . These genes were defined genetically and physically by mini-Mu and Tn5 transposon mutagenesis and by DNA restriction fragment complementation analyses of spontaneous *hel* mutants (Kranz 1989). The DNA sequence of the 3200 nucleotides encompassing *helA*, *helB*, and *helC* indicates that five complete open reading frames (ORFs) are present within this region (Fig. 2). All five ORFs have typical codon usage for *R. capsulatus* genes (not shown). Putative ribosome-binding sites are located in front of the deduced translational start codons of *helB*, *helC*, and *orf52*. A consensus ribosome-binding site is not observed 5–10 nucleotides proximal to the putative *helA* start codon, but this observation has been noted for other *R. capsulatus* genes (e.g., Masepohl et al. 1988). The genetic

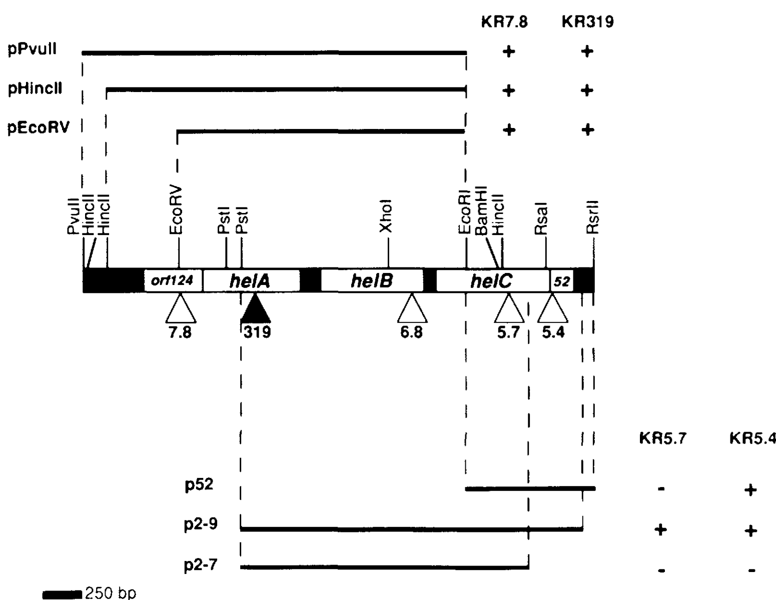

Figure 1. Restriction map of the *hel* locus and DNA complementation studies. The 3.1-kbp *Pvu*II–*Rsr*II fragment is shown with the locations of *orf 124*, *helA*, *helB*, *helC*, and *orf52*. Plasmids containing the indicated DNA fragments were conjugated into the mini-Mu insertion mutant strains KR7.8, KR5.7, and KR5.4 and into the Tn*5* insertion mutant strain KR319. Complementation was defined as the ability to oxidize the cytochrome *c*-specific electron donor tetramethylphenylenediamine.

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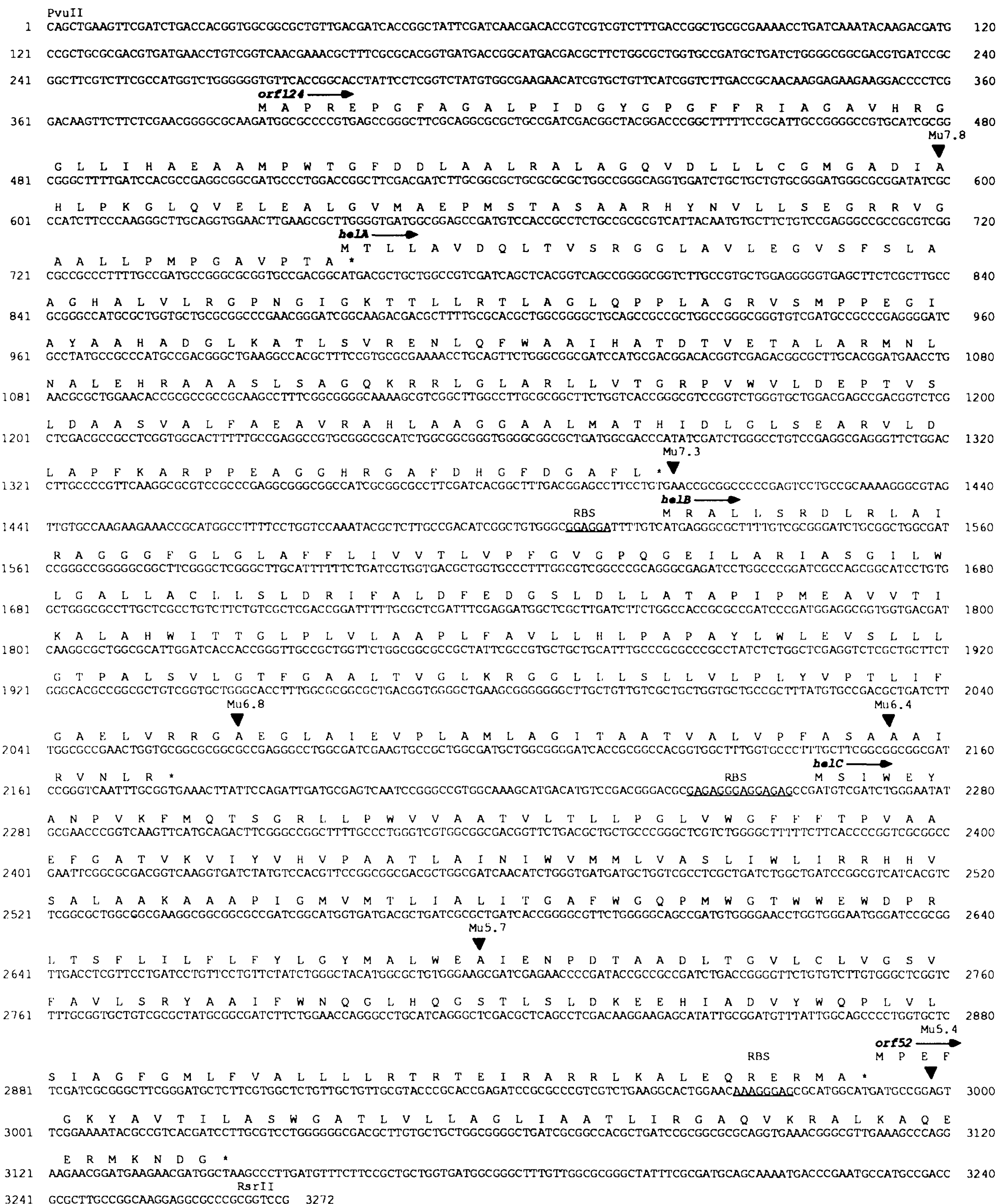

Figure 2. The DNA sequence (noncoding strand) of the *hel* locus. The predicted amino acid sequences of HelA, HelB, HelC, ORF124, and ORF52 are shown above the nucleotide sequence. Putative ribosome-binding sites (RBS) are underlined. The positions of mini-Mu insertions that define the *hel* genes are also indicated.

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analysis (see below) and the typical *R. capsulatus* codon usage indicates position 761 as a likely methionine start codon for HelA.

Genetic analysis of the hel *locus:* helA, helB, helC, *and* orf52 *but not* orf124 *are required for cytochromes* c *biogenesis*

Because five potential ORFs were present at the *hel* locus, we needed to determine which ORFs are required for cytochromes *c* biogenesis. To determine whether *orf124* and *orf52* are required and to further define *helA*, *helB*, and *helC*, the position of each mini-Mu insertion was identified by DNA sequence analysis (Fig. 2). When recombined into the chromosome, each of the mini-Mu insertions and a Tn*5* insertion (shown in Fig. 1) yielded a strain with a Hel$^-$ phenotype (Kranz 1989). Our previous genetic studies (Kranz 1989) indicated that at least two genes required for cytochromes *c* biogenesis were located between the *Pvu*II and *Eco*RI sites (Fig. 1). One of these genes, *helB*, was clearly defined by those studies, and its designation is confirmed here by the mini-Mu sequencing. In addition, the Hel$^-$ strain KR319 (319 in Fig. 1), containing the Tn*5* insertion in *helA*, confirmed that *helA* is required. To determine whether ORF124 is required for cytochromes *c* biogenesis, complementation studies were carried out with the Hel$^-$ strain KR7.8 containing the mini-Mu 7.8 insertion in *orf124*. This strain was complemented to a Hel$^+$ phenotype by a plasmid containing only *helAB* (plasmid pEcoRV; Fig. 1). Because this complementation occurred only when *helAB* on the *Eco*RV–*Eco*RI insert was in the direction of the *lac* promoter of the vector, we conclude that the mini-Mu 7.8 insertion shows polarity on *helA* and that *orf124* is not required for cytochrome *c* biogenesis.

Although our previous genetic studies defined *helC* as containing mini-Mu 5.7 and 5.4 (Kranz 1989), the sequence analysis indicated that mini-Mu 5.4 is located in *orf52* and mini-Mu 5.7 is in *helC* (Fig. 2). Therefore, strain KR5.4 may be Hel$^-$ owing either to the destabilization of the *helC* transcript or to the requirement of *orf52*, whereas strain KR5.7 may be Hel$^-$ owing either to polarity on *orf52* or to inactivation of *helC*. To determine which possibility was the correct one for each mutant strain, complementation studies were done (Fig. 1). Strain KR5.4 was complemented to Hel$^+$ only by plasmids containing the complete ORF52 gene, indicating that *orf52* is required. Thus, p52 complements KR5.4 and supplies only a functional *orf52* gene. These studies also show that the mini-Mu 5.7 insertion is not polar on *orf52* but, rather, inactivates *helC*; this is shown by the complementation of KR5.7 by p2-9 but not by p52. Moreover, mini-Mu 5.7 cannot be affecting the upstream *helB* as KR5.7 is not complemented by the plasmid p2-7.

HelA, HelB, and HelC comparisons to sequence data bases and hydrophilicity analyses

Using TFASTA and FASTA programs, respectively, GenBank and NBRF data bases were searched for ORF124-, ORF52-, HelA-, HelB-, and HelC-related proteins. Significant homologies to HelB, ORF124, and ORF52 were not obtained. HelC showed significant homology to a chloroplast-encoded ORF and to a *Paramecium aurelia* mitochondrial-encoded ORF. These homologies and their homology to the Ccl1 protein are discussed below.

HelA showed significant homology to a superfamily of transport proteins, recently called ABC transporters (Fig. 3; Hyde et al. 1990). HelA, like ABC transporters, has an adenylate kinase-like ATP-binding cassette (hence ABC) in addition to more extensive regions of homology (termed loops 2, 3, and 4 in Fig. 3) in the carboxy-terminal region of the protein. ABC transporters that have these regions of homology include the cystic fibrosis protein (Riordan et al. 1989), the *hisP* component of the histidine importer in *E. coli* (Kraft and Leinwand 1987), and the *hlyB* gene product involved in the secretion of hemolysin out of *E. coli* (Felmlee et al. 1985). Pairwise comparisons of HelA to the individual ABC component of these transporters show ~40% similarity. This value is comparable to the degree of similarity observed between any two ABC components of family members. A hydrophilicity analysis demonstrates that most of the HelA protein is hydrophilic (Fig. 4), much like HisP and other similarly sized ABC components of the bacterial transporters. HelB and HelC show highly periodic hydrophobic profiles, typical of integral membrane proteins with transmembrane helices (Fig. 4).

Analysis of the transport defect in hel *mutants:* cytA–phoA *gene fusion analyses*

The significant homology observed between HelA and the ABC transporters strongly implicated the *hel* mutations to be transport defects associated specifically with cytochromes *c* biogenesis. Moreover, all of the bacterial

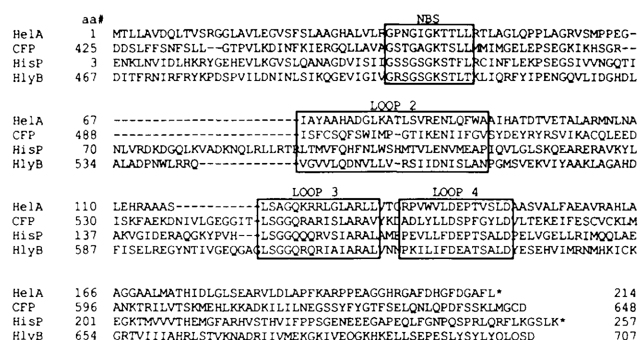

Figure 3. Amino acid homology between HelA and ABC transporters. Amino acid sequence homologies of HelA to the cystic fibrosis protein (CFP) and to the *hisP* and *hlyB* gene products of *E. coli* were determined by TFASTA analysis. The predicted nucleotide-binding site (NBS) of HelA contains the necessary G–G-GK sequence and is boxed. The conserved structural motifs, loops 2, 3, and 4, are also boxed (Hyde et al. 1990). The HisP sequence is from Kraft and Leinwand (1987); HlyB is from Felmlee et al. (1985); and CFP is from Riordan et al. (1989). For homology significance scores, see Materials and methods.

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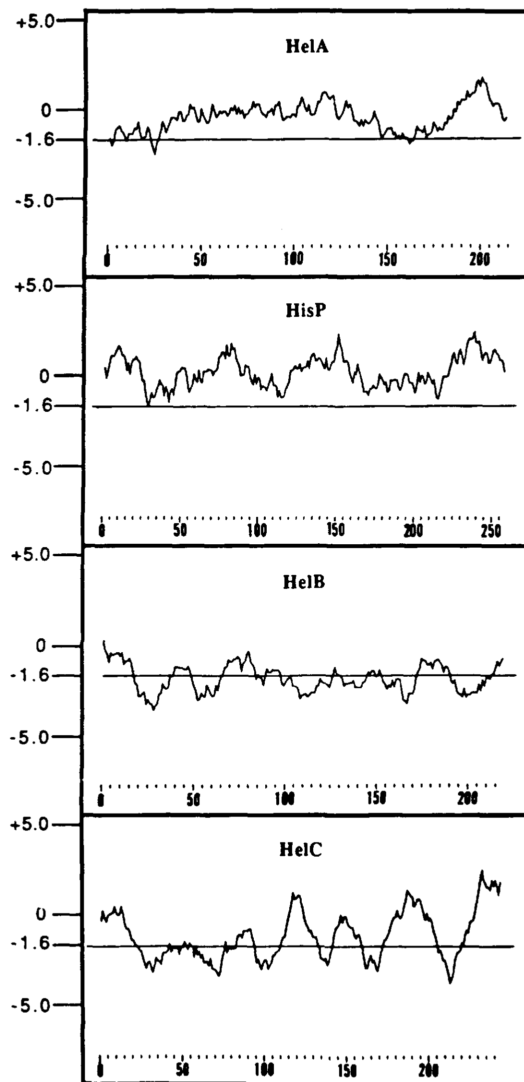

Figure 4. Hydrophilicity analyses of HelA, HelB, and HelC. Hydrophilicity analyses were performed using a window length of 19 residues (Kyte and Doolittle 1982) with programs from Wisconsin GCG. Regions above zero are hydrophilic; regions below zero are hydrophobic with regions less than −1.6 being predicted transmembrane regions (Kyte and Doolittle 1982).

ABC transporters have adjacent genes that encode hydrophobic proteins required for the transport process (for review, see Ames 1986; Hyde et al. 1990). Accordingly, the hydrophobic proteins HelB and HelC are likely membrane components for the *hel* transport system.

To test the possibility of a cytochrome *c*-specific transport defect in *hel* mutants, we constructed cytochrome c_2–alkaline phosphatase gene fusions. Because alkaline phosphatase is only active if it is secreted into the bacterial periplasm, alkaline phosphatase gene (*phoA*) fusions are useful in secretion studies in bacteria (e.g., Hoffman and Wright 1985; Manoil and Beckwith 1985). The *phoA*-coding region, minus its own signal sequence, is engineered distal to a potential signal sequence such that the coding frame is retained. The fusion protein produced from this construct exhibits alkaline phosphatase activity only if it is secreted to the periplasm. Using an *Rhodobacter sphaeroides* cytochrome c_2 gene as a probe, we cloned the *R. capsulatus* cytochrome c_2 gene (*cytA*). A *cytA–phoA* gene fusion was constructed with 88 of 116 amino acid residues of the apocytochrome c_2 protein retained, including the CXYCH region near the amino terminus of cytochrome c_2. This gene fusion was cloned behind the *E. coli lacZ* promoter such that when pC42pho is present in *E. coli*, alkaline phosphatase activity is inducible by IPTG (not shown). The *cytA–phoA* gene was cloned into a plasmid that replicates in *R. capsulatus*, and this plasmid was conjugated into various *R. capsulatus* and *E. coli* strains. Wild-type *R. capsulatus* and *E. coli* were able to synthesize and secrete the fusion protein (Fig. 5; Table 1). We confirmed that alkaline phosphatase is a secretion reporter in *R. capsulatus* by isolating periplasmic fractions and then assaying for the cytochrome c_2–alkaline phosphatase fusion protein. In these experiments, the periplasmic fraction was enriched for alkaline phosphatase activity (Table 1). Similar levels of activity and periplasmic enrichment results have been reported previously with *R. sphaeroides cytA–phoA* fusions (Varga and Kaplan 1989). These and subsequent studies (Moore and Kaplan 1989; Yun et al. 1991) have confirmed the use of *phoA* fusions as periplasmic reporters in photosynthetic bacteria such as *Rhodobacter*.

Hel^- and Ccl^- *R. capsulatus* strains containing the *cytA–phoA* plasmid synthesized and secreted levels of alkaline phophatase fusion protein similar to that secreted by the wild-type strain (Fig. 5; Table 1). Moreover, both *helAB* and *ccl12* deletion strains secreted the fusion protein (not shown). These results prove that the phenotypes of the Hel^- and Ccl^- strains are not the result of a defect in the transcription, translation, or secretion of the apocytochrome c_2. Other possible *hel* transport defects that would result in specific deficiencies of *c*-type cytochromes but not *b*-type cytochromes are discussed below.

Sequence analysis of the ccl *locus*

The results with apocytochrome c_2–alkaline phosphatase gene fusions demonstrated that the transport defect in Hel^- strains and the defect in the Ccl^- strains were not the result of an apocytochrome c_2 transport defect; both the Hel^- and Ccl^- strains could secrete the apocytochrome c_2 even though heme was not ligated to the apoprotein. These results, along with those reported for *P. denitrificans* (Page and Ferguson 1990), support the hypothesis that heme ligation occurs independently of apocytochromes *c* secretion. If heme ligation occurs subsequent to apocytochromes *c* secretion, then heme must be transported to the periplasm for the ligation reaction. In addition, the lyase and any other components necessary for this ligation must be transported to the periplasm. Identifying periplasmic components required for cytochromes *c* biogenesis would support further the

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

Strain	Strain description (reference)	Alkaline phosphatase activities[a]	
		sonicates	periplasmic fraction
E. coli TB1	Baldwin (1984)	20[b]	20
E. coli TB1 (pCyt:pho9)	Baldwin (1984)	170	2200
R. capsulatus SB1003	wild type (Yen and Marrs 1976)	70[c]	70
R. capsulatus SB1003 (pCytpho9)	wild type (Yen and Marrs 1976)	640	1900
R. capsulatus SB1003 (pCytpho12)	wild type (Yen and Marrs 1976)	840	3100
R. capsulatus KR7.8	HelA⁻ (Kranz 1989)	80	ND[d]
R. capsulatus KR7.8 (pCytpho9)	HelA⁻ (Kranz 1989)	1500	ND
R. capsulatus KR7.8 (pCytpho12)	HelA⁻ (Kranz 1989)	1100	ND
R. capsulatus AJB530	Ccl1⁻ (Biel and Biel 1990)	10	ND
R. capsulatus AJB530 (pCytpho9)	Ccl1⁻ (Biel and Biel 1990)	750	ND

[a]Activities are measured in OD units/min per milligram of protein. OD units are defined as $OD_{420} \times 1000$ after incubation at 25°C with ϕ nitrophenyl phosphate. Either sonicated whole cells or a periplasmic fraction were assayed. Except for AJB530 constructs, fractionations and assays were performed a minimum of three times with each showing similar results (averages are shown). The assay results with AJB530 were confirmed by plate assays using the indicator XP (not shown). Cells were grown in LB broth (for *E. coli*) or *R. capsulatus* RCV basal media with incubation at 34°C for *R. capsulatus* and 37°C for *E. coli*. Aerobic growth conditions were used, and antibiotics were added to the media with strains containing plasmids.
[b]*E. coli* TB1 was used. This strain is PhoA⁺, and 20 units represents the background activity in this media.
[c]*R. capsulatus* backgrounds differ, depending on amount of photopigments (which vary with strains).
[d](ND) Not determined.

need for a heme transporter. We therefore decided to analyze candidate lyase genes (called *ccl* genes).

Two other mutants that are pleiotrophically missing *c*-type cytochromes in *R. capsulatus* have been reported: MT113 (Davidson et al. 1987) and AJB530 (Biel and Biel 1990). Biel and Biel (1990) have shown recently that the defect in MT113 is probably in the same gene that the Tn5 transposon is inserted in AJB530, and this gene is not in the *hel* locus. We cloned the DNA region from *R. capsulatus* AJB530 that contains the Tn5 and the same region from the wild-type *R. capsulatus* strain SB1003 (Fig. 6). This DNA was then sequenced to determine the primary structure of potential lyase genes. The Tn5 is present within the 0.9-kbp *Pst*I–*Bam*HI fragment (Biel and Biel 1990) that is internal to *ccl1*. The initial sequence analysis indicated that the gene required to complement AJB530 is not present to the right of the *Pst*I site as was originally reported (Biel and Biel 1990). The amino terminus of Ccl1 is encoded by DNA to the left of the *Pst*I site (Fig. 6). Genetic complementation analyses have shown that only *ccl1* is required to correct the AJB530 defect, and complementation analyses of a *ccl12* deletion mutant have proved that both *ccl1* and *ccl2* are required for cytochromes *c* biogenesis (D.L. Beckman and R.G. Kranz, in prep.).

The sequence analysis of the *ccl* locus (Fig. 6) is shown in Figure 7. Ccl1 and Ccl2 comprise 653 amino acid residues and 149 amino acid residues, respectively. Both genes have putative ribosome-binding sites upstream of their predicted start codons. In addition, both Ccl1 and Ccl2 have amino-terminal consensus signal sequences (e.g., von Heijne 1986). These putative signal sequences contain a charged amino acid residue followed by a stretch of hydrophobic amino acids, a turn (e.g., Proline), and VXA (Fig. 7). Ccl1 shows extensive regions of hydrophobicity with a number of predicted transmembrane helices, whereas Ccl2 is a predominantly hydrophilic protein (Fig. 8).

Ccl1 and Ccl2 comparisons to sequence data bases and hydrophilicity analyses

A Ccl2 comparison to proteins in the GenBank and NBRF data bases gave no significant homologies. Ccl1 showed significant homology to four ORFs (Fig. 9). Three of these ORFs are from chloroplast genomes (Shinozaki et al. 1986; Ohyama et al. 1988; Hiratsuka et al. 1989) and are similar in size, but their function is unknown. The fourth match is to an undesignated ORF from the *P. aurelia* mitochondrial genome (Pritchard et al. 1990). Of the 111 amino acid residues shown in Figure 9, Ccl1 is ~52% similar and 28% identical to the organelle ORFs. Interestingly, these same four ORFs (and Ccl1) show limited but significant homology to HelC (Fig. 9). The possible importance of this homology is discussed below.

ccl1–phoA *gene fusions*

To test whether Ccl1 is a periplasmic protein and to determine whether it is the substrate recognized by the Hel transporter, we constructed *ccl1–phoA* gene fusions. Both *E. coli* and *R. capsulatus* strains containing the *ccl1–phoA* gene fusions showed high levels of alkaline phosphatase activity, as determined by plate assays using the alkaline phosphatase indicator XP (not shown). Colonies of strains containing *ccl1–phoA* gene fusion

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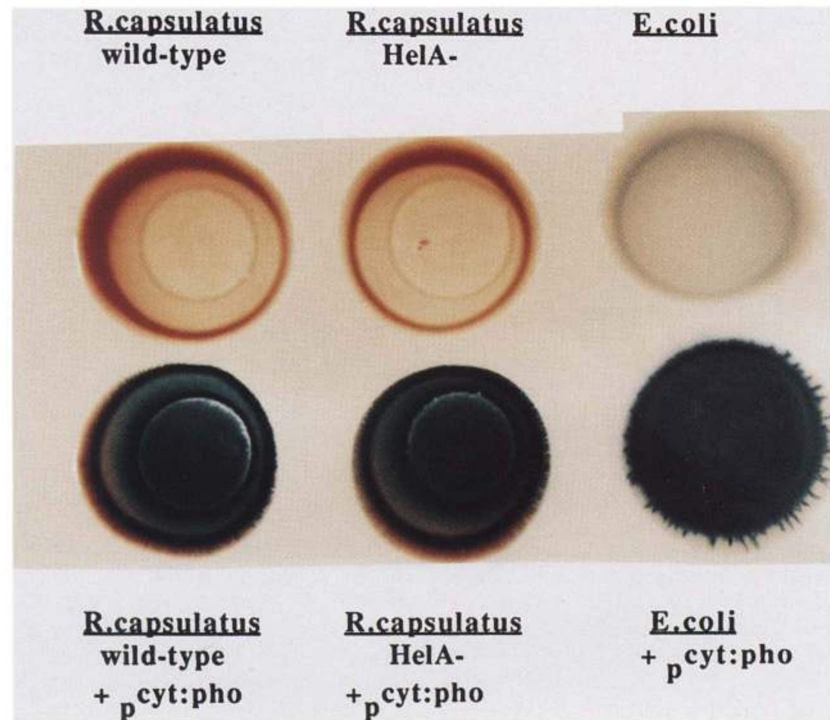

Figure 5. Cytochrome c_2–alkaline phosphatase analysis. Detection of secretion of cytochrome c_2–alkaline phosphatase in colonies of different bacterial strains (as indicated). The blue color is the result of cleavage of the alkaline phosphatase indicator XP. All colonies were grown aerobically on the same culture plate (with 0.3% peptone, 0.3% yeast extract, and 1.2% agar) at 28°C. The *R. capsulatus* wild-type strain is SB1003, and the HelA strain is KR7.8. *E. coli* TB1 is shown with and without pCyt–pho. pCyt–pho9 was used in these experiments.

plasmids looked similar to the same strains containing *cytA–phoA* gene fusion plasmids (see Fig. 5). When a *ccl1–phoA* gene fusion expressed from the *E. coli lacZ* promoter is present in *E. coli* CC118, high levels of alkaline phosphatase activity are observed (Table 2). This activity is not observed when cells are grown under conditions of *lacZ* repression, that is, the *lac* inducer IPTG is omitted and when the cells are grown in 0.2% glucose.

R. capsulatus wild type and a HelA$^-$ strain, KR7.8, are able to express and secrete the *ccl1–phoA* fusion protein. High alkaline phosphatase activities are observed when the *ccl1–phoA* fusion plasmid p11-6-1 are present in either strain (Table 2).

Discussion

Genes at two loci are required for cytochromes c *biogenesis*

It is possible to isolate mutants specific to a cytochromes *c* biogenesis pathway due to the various electron transport pathways and, hence, different growth modes present in *R. capsulatus*. Previous studies have shown that aerobic growth can occur in *R. capsulatus* mutants lacking the cytochrome *c* oxidase electron transfer branch due to the presence of an alternative ubiquinol oxidase electron transfer branch that only contains *b*-type heme (La Monica and Marrs 1976; Hüdig and Drews 1982). The *c*-type cytochromes are required for photosynthetic growth in *R. capsulatus* (Daldal et al. 1987), and a *c*-type cytochrome is required for dark, anaerobic growth. The latter conclusion is based on the phenotype of the *hel* mutants (Kranz 1989) and the observation that a specific *c*-type cytochrome is induced under dark anaerobic growth conditions (Zsebo and Hearst 1984; Kranz 1989). Accordingly, *hel* and *ccl* mutants cannot grow in an anaerobic light or dark environment. This is in contrast to mutants in photosynthetic genes, such as reaction center polypeptide genes (e.g., Zsebo and Hearst 1984), or in cytochrome bc_1 genes (Daldal et al. 1987), which can grow under dark anaerobic conditions.

The Hel$^-$ and Ccl$^-$ strains are specifically missing *c*-type but not *b*-type cytochromes (Kranz 1989; Biel and Biel 1990). We analyzed these mutants and the genes that correct the defects to better understand bacterial cytochromes *c* biogenesis. Our analyses indicate that there are a minimum of six genes at two loci required for cytochromes *c* biogenesis in *R. capsulatus*. Recently, using pulsed field-gel and cosmid mapping strategies, it has been shown that the *ccl* locus is at least 300 kbp distant from the *hel* locus (M. Fonstein and R. Haselkorn, pers. comm.). We discuss the possible functions of *hel*- and *ccl*-encoded proteins separately.

Functions of genes at the hel *locus*

What is the defect in the Hel$^-$ strains? Hel$^-$ strains are specifically missing all *c*-type cytochromes including the cytochrome bc_1 complex. Yet, except for cytochrome *b* of the bc_1 complex, Hel$^-$ strains synthesize normal amounts of *b*-type cytochromes (Kranz 1989). The amounts of *b*-type cytochromes produced by a *helAB* deletion strain are equivalent to the amounts produced by a *ccl12* deletion strain (D.L. Beckman and R.G. Kranz,

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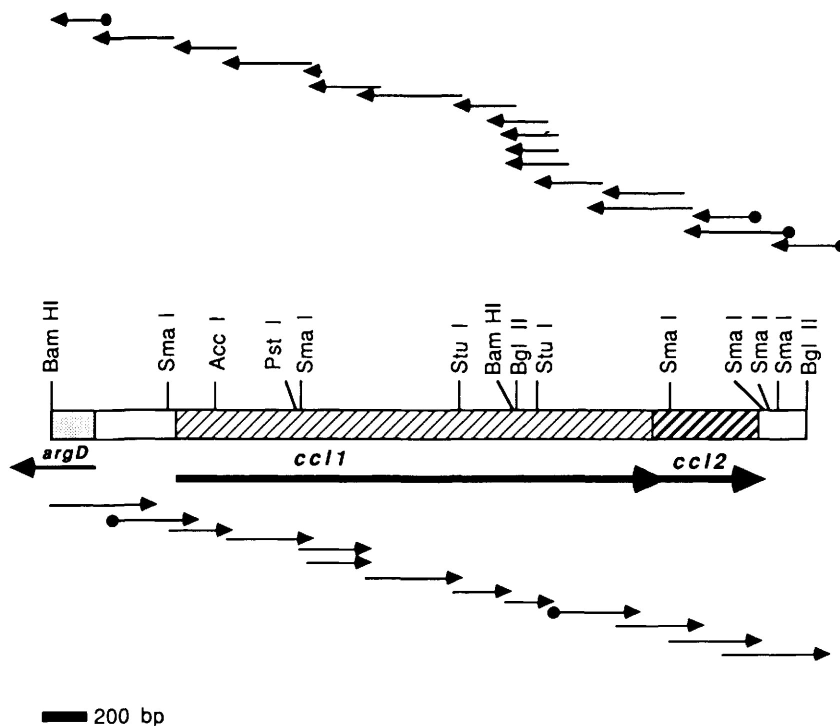

Figure 6. Restriction map of the *ccl* locus and DNA sequencing strategy. The 3.1-kbp *Bam*HI–*Bgl*II restriction map is shown with the locations of *ccl1* and *ccl2* (hatched boxes). Proximal to *ccl1* is a putative ORF showing significant homology to *argD* of *E. coli* (Heimberg et al. 1990). *argD* encodes an acetylornithine aminotransferase. The small arrows above and below the restriction map indicate the sequencing strategy. Arrows with a solid circle represent areas sequenced using synthetic oligonucleotide primers. Arrows without the circle represent areas sequenced using ordered deletions as described in the Materials and methods.

in prep.); therefore, the ability to synthesize heme and produce *b*-type cytochromes is not impaired in Hel$^-$ strains. Analysis of the *hel* defects must take these specific deficiencies into account.

During the preparation of this paper, *hel*-like genes were shown to be present in *B. japonicum* (Ramseier et al. 1991). HelA-, HelB-, and HelC-like gene products in that organism, called CycV, CycW, and ORF263, respectively, show ~65% similarity to the *R. capsulatus* counterparts described here. Ramseier et al. (1991) demonstrated that a small ORF called *cycX*, equivalent to *R. capsulatus orf52*, is required for cytochromes c biogenesis. They also suggest that the *B. japonicum helC*-like gene *orf263* is not required since a Tn5 insertion that mapped in *orf263* gave a wild-type phenotype. In contrast to that study, we have shown that a mini-Mu insertion (Mu 5.7 in Figs. 1 and 2) within *R. capsulatus helC* yields a strain with a Hel$^-$ phenotype. Genetic complementation analyses confirm that *helA*, *helB*, *helC*, and *orf52* are required for cytochromes *c* biogenesis in *R. capsulatus* (Fig. 1; Kranz 1989). Although an explanation for the different results with *helC* requires further experimentation, the results in *R. capsulatus* and *B. japonicum* do indicate that *hel* genes are specifically involved in cytochromes *c* biogenesis in both photosynthetic and nonphotosynthetic Gram-negative bacteria.

HelA is homologous to a superfamily of proteins called ABC transporters. These transporters have been shown (Bishop et al. 1989; Mimmack et al. 1989) or predicted to use ATP as an energy source for transporting specific molecules. Of the bacterial ABC transporters, the importers comprise approximately four gene products (e.g., histidine or maltose importers). One of these gene products is a periplasmic-binding protein that has a typical signal sequence to direct it to the periplasm (for review, see Ames 1986). The other components include the conserved HelA-like ATP-binding subunit and usually two predicted hydrophobic polypeptides, analogous to the topological profiles of HelB and HelC. In contrast, bacterial exporters do not appear to require periplasmic proteins (for review, see Blight and Holland 1990). For example, hemolysin export out of *E. coli* requires HlyB, which contains the nucleotide-binding domain, and HlyD. Some ABC exporters are predicted to transport small molecules; these include a β-1,2 glucan transporter in *Rhizobium meliloti* (called *ndv*) (Stanfield et al. 1988) and in *Agrobacterium tumefaciens* (called *chv*) (Cangelosi et al. 1989). The small ORF52 has no counterpart in bacterial ABC transporters; thus, its role in the translocation process and in cytochromes *c* biogenesis awaits further experimentation.

Although we cannot eliminate completely *hel* involvement in iron import, this role seems unlikely for several reasons. In the closely related species *R. sphaeroides*, it has been shown that a comparable decrease in *c*- and *b*-type cytochromes is observed when the cells are limited for iron (Moody and Dailey 1985). In addition, the *R. capsulatus hel* mutants are as sensitive as wild type to the iron chelator dipyridyl, and multiple sources of iron do not correct the *hel* defect (R. Kranz, unpubl.). Finally, iron importers analyzed in *E. coli* (e.g., Zimmermann et al. 1984; Burkhardt and Braun 1987) and *Serratia marsescens* (Angerer et al. 1990 and references therein) are composed of ABC-type transporters

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```
      BamHI
      I  R  Y  L  N  S  V  H  W  L  K  G  A  Q  E  T  L  T  A  V  L  D  P  A  A  H  G  L  A  N  V  A  I  G  A  G  L  D  L
   1  GGATCCGGTAAAGGTTCGAGACATGCCAGAGCTTGCCCGCCTGTTCGGTCAGCGTCGCCACAAGGTCGGGCGCGGCATGGCCCAGCGCATTGACCGCGATCCCGGCGCCCAGATCCAGAT   120
                                                                                         <---- argD
      Y  R  S  G  D  A  T  W  L  W  S  G  E  G  R  V  F  A  L  P  A  R  T  Y  T  P  L  V  S  A  I  M        RBS
 121  ATCGGCTGCCATCCGCGGTCCAAAGCCAGGAGCCCTCGCCGCGCACGAAGGCGAGGGGGGCACGGGTGTAGGTCGGCAGCACGGAAGCGATCATTTGCGGCTCCTGAAAAAGGAAGGCCA   240
                                   terminator
 241  AGGCGTGCAACAGGCGCAGGGCGGCTGTCAACGGTCCGGGACCTTCGGGGGCGGTTTTGTGGAAAGGGTGTGGCGAGAAGCCACGGCAGATCAGGCGTCAGGCGCAGGTGCGGCGACGTC   360

 361  GGGGACGGGCAAGAGGGGCGGTCCGGGTGATCATTTCCCCCGCTTAGCCGCCGCCTGACGCAAAGTCAAAGGAAATGACCGTTTCACGCTTTTGCGAAAGATGCGCGGCAGATGCGCATC   480
                                                                     ccl1 ---->
                                                      RBS            M  I  V  E  T  G  H  F  A  L  I  L  A  L  C  V  A  L  V  Q  A
 481  TGCCTTGGCCCCGGGGGGCTCCGTCTCTATGATGCACGCGACTTGAGGAGGACCCCGCATGATCGTCGAGACCGGCCATTTCGCCCTGATCCTGGCCCTGTGCGTGGCGCTGGTGCAGGC   600

      V  I  P  L  V  G  A  Q  K  G  W  S  G  W  M  A  V  A  T  P  A  A  L  A  Q  F  G  L  I  A  I  A  F  A  A  L  T  Y  A  F
 601  GGTCATTCCCCTGGTGGGCGCGCAAAAGGGCTGGTCGGGCTGGATGGCCGTTGCCACCCCCGCGGCGCTGGCGCAGTTCGGGCTGATCGCCATCGCCTTTGCCGCGCTGACCTATGCCTT   720

      V  T  S  D  F  S  L  K  L  V  Y  E  N  S  H  T  D  K  P  M  L  Y  K  V  T  G  V  W  G  N  H  E  G  S  M  L  L  W  V  L
 721  TGTGACGTCTGACTTTTCGCTGAAGCTCGTCTACGAAAACTCGCATACCGACAAGCCGATGCTCTACAAGGTCACCGGGGTCTGGGGCAACCATGAGGGCTCGATGCTGCTTTGGGTGCT   840

      I  L  A  M  F  G  A  A  A  A  A  F  G  G  A  L  P  E  R  L  R  A  R  V  L  A  V  Q  G  T  I  G  V  A  F  L  V  F  V  L
 841  GATCCTCGCCATGTTCGGCGCGGCGGCCGCGGCCTTTGGCGGTGCCTTGCCGGAGCGGCTGCGGGCGCGGGTGCTGGCGGTGCAGGGCACCATCGGGGTCGCGTTTCTGGTCTTTGTGCT   960

      F  T  S  N  P  F  L  R  L  E  E  A  P  F  N  G  R  D  M  N  P  L  L  Q  D  P  G  L  A  F  H  P  P  F  L  Y  L  G  Y  V
 961  TTTCACCTCGAACCCGTTCCTGCGGCTGGAAGAAGCGCCGTTCAACGGCCGCGACATGAACCCGCTGCTGCAGGACCCGGGTCTCGCCTTCCATCCGCCGTTCCTGTACCTTGGCTATGT  1080

      G  L  S  M  A  F  S  F  A  V  A  A  L  I  E  G  R  V  D  A  A  W  A  R  W  V  R  P  W  T  L  A  A  W  I  F  L  T  I  G
1081  CGGGCTTTCGATGGCCTTCAGCTTCGCCGTCGCCGCGCTGATCGAGGGGCGGGTCGATGCCGCCTGGGCGCGCTGGGTCCGGCCCTGGACCTTGGCCGCCTGGATCTTCCTGACCATCGG  1200

      I  A  L  G  S  W  W  A  Y  Y  E  L  G  W  G  G  F  W  F  W  D  P  V  E  N  A  S  L  M  P  W  L  L  A  A  A  L  L  H  S
1201  CATCGCGCTGGGATCGTGGTGGGCCTATTACGAGCTCGGCTGGGGCGGCTTCTGGTTCTGGGACCCGGTGGAAAACGCCTCGCTGATGCCCTGGCTGCTGGCGGCGGCGCTGTTGCATTC  1320

      A  I  V  V  E  K  R  E  A  L  K  S  W  T  I  L  L  A  I  M  A  F  G  F  S  L  I  G  T  F  L  V  R  S  G  V  I  S  S  V
1321  CGCCATCGTCGTCGAAAAGCGCGAGGCGCTGAAAAGCTGGACGATCCTGCTCGCCATCATGGCCTTCGGCTTTTCGCTGATCGGCACGTTCCTGGTGCGGTCCGGGGTGATTTCCTCGGT  1440

      H  S  F  A  N  D  P  E  R  G  V  F  I  L  F  I  L  A  F  F  T  G  G  A  L  T  L  Y  A  A  R  A  S  E  M  Q  A  K  G  L
1441  GCACAGTTTCGCCAATGACCCCGAGCGCGGGGTGTTCATCCTGTTCATCCTTGCCTTCTTCACCGGCGGGGCGCTGACGCTTTATGCCGCGCGGGCCTCGGAGATGCAGGCGAAGGGGCT  1560

      F  S  M  V  S  R  E  S  A  L  V  M  N  N  V  L  L  A  V  A  A  L  V  V  F  T  G  T  V  W  P  L  I  A  E  L  F  W  D  R
1561  GTTTTCCATGGTCAGCCGCGAATCGGCGCTGGTGATGAACAACGTGCTTTTGGCGGTGGCGGCGCTGGTGGTGTTCACCGGCACGGTCTGGCCGCTGATCGCGGAGCTGTTCTGGGACCG  1680

      K  L  S  V  G  A  P  F  F  E  K  A  F  T  P  F  M  V  G  L  A  L  L  L  P  L  G  S  M  M  P  W  K  R  A  S  L  G  K  L
1681  CAAGCTCTCGGTGGGGGCGCCGTTCTTCGAAAAGGCCTTTACCCCCTTCATGGTCGGGCTGGCGCTGCTGTTGCCGCTGGGATCGATGATGCCGTGGAAACGCGCGAGCCTCGGCAAGCT  1800

      V  R  P  L  L  P  A  L  V  L  T  L  A  V  L  A  L  V  W  V  M  A  T  G  R  P  M  L  A  L  G  A  A  G  L  G  A  W  I  L
1801  GGTGCGGCCGCTGTTGCCCGCGCTTGTGCTGACGCTGGCGGTGCTGGCGCTGGTCTGGGTGATGGCGACGGGGCGGCCGATGCTGGCGCTTGGCGCGGCGGGGCTGGGCGCCTGGATCCT  1920

      F  G  A  L  A  E  I  W  Q  R  A  G  R  T  P  G  R  I  L  R  L  P  R  A  D  W  G  K  A  F  A  H  G  G  L  G  I  V  F  A
1921  TTTTGGCGCCCTGGCCGAGATCTGGCAACGCGCCGGGCGGACGCCGGGACGCATCCTGCGGCTGCCGCGGGCGGATTGGGGCAAGGCCTTCGCGCATGGCGGGCTGGGCATCGTCTTTGC  2040

      G  V  G  L  L  M  A  G  Q  V  E  D  I  R  V  A  K  A  G  D  S  F  E  V  A  G  Y  T  I  T  L  V  S  V  E  D  V  P  G  P
2041  GGGCGTCGGCCTCTTGATGGCCGGGCAGGTCGAGGATATCCGCGTGGCGAAGGCGGGCGACAGTTTCGAGGTGGCAGGTTACACGATCACGCTGGTGTCGGTCGAGGATGTGCCGGGGCC  2160

      N  F  T  A  K  T  A  T  M  E  V  R  Q  G  G  K  L  V  A  T  L  H  P  E  K  R  I  Y  P  V  Q  A  M  P  T  T  E  A  D  I
2161  GAACTTCACCGCCAAGACCGCGACGATGGAGGTGCGGCAGGGCGGCAAGCTGGTGGCGACCCTGCATCCCGAAAAGCGCATCTATCCGGTGCAGGCGATGCCGACGACCGAGGCCGATAT  2280

      D  N  G  F  W  R  D  V  Y  L  V  I  G  D  P  Q  E  G  G  G  W  A  V  R  T  Y  V  K  P  F  A  N  W  I  W  A  G  C  L  L
2281  CGACAACGGCTTCTGGCGCGATGTCTATCTGGTGATCGGCGACCCGCAGGAGGGCGGCGGCTGGGCGGTGCGGACCTATGTCAAGCCTTTCGCGAACTGGATCTGGGCGGGCTGTCTGCT  2400
                                                                                                      RBS    ccl2 ---->
      M  A  F  G  G  G  L  S  L  T  D  R  R  Y  R  S  A  A  G  A  R  R  A  T  V  A  D  A  V  A  A  E  *        M  L  K  R  L  L
2401  GATGGCCTTTGGCGGCGGGCTGAGCCTGACCGACCGGCGTTATCGCAGCGCCGCCGGGGCGCGGCGGGCGACGGTGGCCGATGCTGTTGCGGCGGAGTGAGCGATGCTGAAACGACTTCT  2520

      L  L  L  V  L  A  T  P  V  H  A  V  Q  P  D  E  V  L  S  D  P  G  L  E  A  R  A  R  Q  I  S  Q  V  L  R  C  P  V  C  Q
2521  GCTTTTGCTGGTGCTGGCCACGCCCGTTCATGCGGTGCAGCCCGACGAGGTTCTGTCCGATCCGGGGCTTGAAGCCCGGGCGCGGCAGATTTCGCAGGTGCTGCGCTGCCCGGTCTGTCA  2640

      G  E  N  I  D  E  S  N  A  G  V  S  R  D  L  R  L  A  V  R  E  R  L  V  A  G  D  S  D  A  Q  V  I  D  Y  I  K  D  R  F
2641  GGGCGAGAATATCGACGAATCGAATGCCGGGGTGTCGCGCGATCTGCGGCTTGCGGTGCGCGAGCGGCTGGTGGCGGGCGACAGCGACGCGCAGGTGATCGACTATATCAAGGACCGTTT  2760

      G  E  Y  V  L  F  E  P  E  R  R  G  A  N  L  I  L  Y  W  I  G  P  A  V  L  V  V  A  L  G  G  I  F  L  W  L  R  G  R  R
2761  CGGGGAATATGTGCTGTTCGAGCCCGAGCGGCGCGGGGCGAACCTGATCCTGTACTGGATCGGGCCTGCGGTGCTGGTGGTGGCCCTGGGCGGCATCTTCCTGTGGCTGCGCGGGCGGCG  2880

      R  E  E  E  P  V  P  V  L  S  A  E  E  E  A  R  L  K  D  L  L  K  D  *
2881  GCGCGAGGAAGAGCCGGTGCCGGTGCTCTCGGCCGAGGAAGAGGCGCGGCTGAAAGACCTGCTGAAGGACTAGGGATTTCGCCCGGGGCTTGCGCCCCGGGCGACCCGCGGGCGGCTCCG  3000
         terminator
3001  CCCCCCGCACCCCCCGGGCGTATTTGCCAAGGAAAAGCGGGGGTCTGGTCGGTGCACCGTTTGGCTTTCGCTTTCGCTTTCGCGGCCCGCGCGGCCCAAGCTGGGGCAAAGAGGAGGCA  3120
                                  Bgl II
3121  GGCCATGAGCTATCACACGATCCGCTACGAGATCT  3155
```

Figure 7. The DNA sequence of the *ccl* locus. The noncoding strand (with respect to *ccl1* and *ccl2* genes) is shown. The predicted amino acid sequences of Ccl1 and Ccl2 are listed above the nucleotide sequence with the consensus signal sequence of each underlined. Predicted transcriptional terminators flanking *ccl1* and *ccl2* are underlined and labeled. Putative ribosome-binding sites (RBS) are underlined. Proximal to *ccl1* is an ORF showing significant homology to *argD* of *E. coli* (Heimberg et al. 1990). *argD* encodes an acetylornithine aminotransferase.

that include a periplasmic-binding protein. In all cases studied, the periplasmic-binding protein is a hydrophilic protein that has a typical signal sequence. None of the *hel* genes (or adjacent ORFs) have consensus signal sequences.

Therefore, similar to the rationale of Ramseier et al. (1991), we suggest that the *R. capsulatus hel* genes encode an ABC transporter specific to cytochromes *c* biogenesis. Because *c*-type cytochromes are periplasmically located and heme is covalently linked to the apoprotein,

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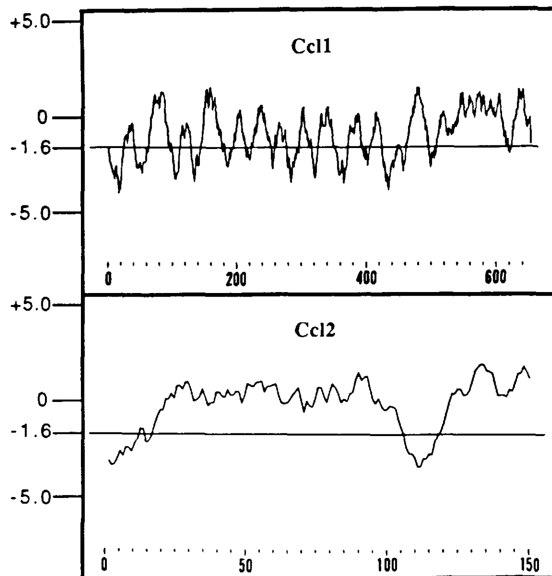

Figure 8. Hydrophilicity analyses of Ccl1 and Ccl2. Hydrophilicity analyses were performed using a window length of 19 residues (Kyte and Doolittle 1982) with Wisconsin GCG programs. Regions >0 are hydrophilic; regions <0 are hydrophobic, with regions less than − 1.6 being predicted as transmembrane regions (Kyte and Doolittle 1982).

four substrates are envisioned as candidate molecules recognized by the Hel transporter: If heme is ligated to the apoprotein in the cytoplasm, then (1) holocytochrome *c* must be secreted to the periplasm; if heme ligation occurs in the periplasm, then (2) apocytochromes *c*, (3) other ligation components (e.g., lyase), and (4) heme must be transported to the periplasm.

Of these four possibilities, the first one seems unlikely. If heme ligation occurs in the cytoplasm before transport, then transport-specific mutants should still have heme ligated to their apocytochromes. Holocytochromes *c* were not detected in the *R. capsulatus hel* mutants (Kranz 1989) or in the *B. japonicum* mutants (Ramseier et al. 1991). Moreover, a previous study with *P. denitrificans* demonstrated that apocytochrome *c* is still transported to the periplasm in the absence of heme ligation (Page and Ferguson 1990), suggesting that the ligation process occurs in the periplasm. These results, along with the cytochrome c_2–alkaline phosphatase transport studies presented here, indicate that holocytochrome *c* is an unlikely substrate for the *hel*-encoded transporter.

The second possibility, apocytochromes *c* export, cannot be the defect in *hel* mutants. We demonstrated that an apocytochrome c_2–alkaline phosphatase fusion protein is secreted as efficiently in *hel* mutants as in the wild-type *R. capsulatus* strain. These results also indicate that apocytochrome c_2 export takes place in the absence of heme ligation in *R. capsulatus*. We suggest that apocytochromes c_2 and c_1 are transported to the periplasm by a signal sequence-dependent mechanism, since both cytochrome c_1 (Daldal et al. 1987) and cytochrome c_2 (Daldal et al. 1986) possess consensus signal sequences. This suggestion is supported by the fact that *E. coli*, which does not require *c*-type cytochromes for aerobic growth, is able to secrete the *cytA–phoA* fusion protein (Fig. 5; Table 1).

In addition, we have shown that another component required for cytochromes *c* biogenesis, Ccl1, does not require *hel* genes for transport to the periplasm, as a Ccl1–PhoA fusion protein is transported to the periplasm in the *hel* mutants (Table 2). These results exclude Ccl1 as a substrate of the Hel transporter.

It should be noted that Ramseier et al. (1991) also favored the idea that the *B. japonicum hel*-like genes encode a transporter that exports (apo)cytochrome *c* or heme to the periplasm. We have eliminated apocytochrome *c* and two other possible substrates and are thus left with the hypothesis that the Hel transporter is necessary for the translocation of heme to the periplasmic space. It is tempting to speculate that the limited but significant homology observed between HelC and Ccl1 define heme-binding residues. Nevertheless, confirmation that the *hel* genes encode a specific heme transporter awaits the development of in vitro heme translocation assays to test *R. capsulatus* Hel⁺ and Hel⁻ strains.

```
                  aa#
Ccl1              206  WVRPWTLAAWIFLTIGIALGSWWAYYELGWGGFWFWDPVENASLMPWLLAAALLHS
Paramecium ORF238 127  WRLDIYKKFFLWTGGSIVLGAVWAQHELNWGGFWSWDQVEIISLFYFAVALFLLHF
Liverwort ORF320  224  WSYRVISLGFPLLTIGILSGAVWA--NEAWGSYWNWDPKETWALITWLIFAIYLH-
HelC              102              TLIALITGAFWGQ--PMWGTWWEWDPRLT-SFL--ILFLFYL
==============================================================================
Identities             W           ltigI lG  WA  el WGgfW WDpvE  sL  wl aa lLH


Ccl1              262  AIVVEKREALKSWTILLAIMAFG-FSLIGTFLVRSGVISSVHSFANDPERGVFILF.
Paramecium ORF238 183  KRPLFFFSAALAFSYFFFGLRFNYFTSVHSFVSKRAAAQSLNFFFFGPWPWPWGPS*
Liverwort ORF320  277  TRMIKGWQGKKP--AIIASLGF--FIVWICYLGVNLLGKGLHSYGWLI*
==============================================================================
Identities                     a k      a   F  F     fl       s hsf   p
```

Figure 9. Ccl1 and HelC homology to other proteins. Homology to an ORF from the mitochondrial genome of *P. aurelia* is shown. The start site of this ORF has not been defined (Pritchard et al. 1990), and the designation as ORF238 is tentative. Also shown is homology to ORF320 from the liverwort chloroplast genome. Not shown are homologies to the tobacco chloroplast ORF313 and the rice chloroplast ORF321, that are nearly identical to the liverwort ORF320 in this region. The letters beneath the line summarize the identities between Ccl1 and the other proteins. An uppercase letter represents an identity between Ccl and both the mitochondrial ORF and the chloroplast ORF; a lowercase letter represents an identity between Ccl1 and either the mitochondrial ORF or the chloroplast ORF. The *P. aurelia* ORF sequence is from Pritchard et al. (1990). The liverwort ORF320 is from Ohyama et al. (1988). For homology significance scores, see Materials and methods.

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Table 2. *Alkaline phosphatase activities of bacteria with* ccl1:phoA *gene fusions*

Strain	Strain description (reference)	Growth condition	Alkaline phosphatase activity[a]
E. coli CC118	PhoA⁻ (Manoil and Beckwith 1985)	LB + 0.2% glucose	<20
E. coli CC118	PhoA⁻ (Manoil and Beckwith 1985)	LB + IPTG[b]	<20
E. coli CC118 (p1ccl1:phoA)	PhoA⁻ (Manoil and Beckwith 1985)	LB + 0.2% glucose	<20
E. coli CC118 (p1ccl1:phoA)	PhoA⁻ (Manoil and Beckwith 1985)	LB + IPTG	340
R. capsulatus SB1003	wild type (Yen and Marrs 1976)	RCV[c]	<20
R. capsulatus SB1003 (p11-6-1)	wild type (Yen and Marrs 1976)	RCV[c]	430
R. capsulatus KR7.8 (p11-6-1)	HelA⁻ (Kranz 1989)	RCV[c]	750

[a]Activities are measured in OD units/min per milligram of protein. OD units are defined as $OD_{420} \times 1000$ after incubation at 25°C with ϕ nitrophenyl phosphate. Whole cells were used in the assays. Cells were induced and assayed a minimum of four times. Each experiment showed the same pattern with at least 10-fold increase of activity over background. Cells were grown in LB broth (for *E. coli*) or *R. capsulatus* RCV basal media, with incubation at 34°C for *R. capsulatus* and 37°C for *E. coli*. Aerobic growth conditions were used, and antibiotics were added to the media with strains containing plasmids (see Kranz 1990).
[b](IPTG) Isopropyl-thiogalactoside was added 4 hr before cells were harvested.
[c](RCV) *R. capsulatus* basal media (Kranz 1989).

Ccl1 and Ccl2: periplasmic topology

If cytochrome *c* heme ligation occurs in the periplasm, the heme, the lyase, and any other assembly components must also be present in the periplasm. Our reason for analyzing other genes involved in cytochromes *c* biogenesis was to determine whether their gene products have signal sequences to direct them to the periplasm and whether homologs could be found in eukaryotes. We carried out genetic and molecular analyses of the mutant AJB530 (Biel and Biel 1990) that had been shown previously to have a similar phenotype to the *hel* mutants but whose mutation mapped to a different locus. Sequence analysis of this locus showed that two genes, *ccl1* and *ccl2*, were present in an operon. Deletion mutagenesis has indicated that *ccl1* and *ccl2* are required for cytochromes *c* biogenesis (D.L. Beckman and R.G. Kranz, in prep.). The *ccl12* operon is followed by a predicted Rho-independent transcription terminator (Fig. 6). Distal to this terminator is a gene that encodes a protein homologous to peroxisomal (Osumi et al. 1985) and mitochondrial (Minami-Ishii et al. 1989) enoyl–CoA hydratases (Beckman and Kranz 1991). Proximal to *ccl1*, but transcribed in the opposite direction, is a gene that encodes a protein homologous to acetylornithine aminotransferase (Fig. 6). Thus, *ccl1* and *ccl2* appear to be the only genes at this locus involved in cytochromes *c* biogenesis.

Analysis of the primary sequence of Ccl1 and Ccl2 indicates that both possess amino-terminal consensus signal sequences. We have confirmed the periplasmic orientation of Ccl1 by *ccl1–phoA* fusion studies, at least at the position of insertion of the *phoA* gene. Both *E. coli* and *R. capsulatus* are able to transport Ccl1 to the periplasm. On the basis of these data, we suggest that transport of Ccl1 and possibly Ccl2 occurs by a signal sequence-dependent general secretory mechanism. Moreover, the periplasmic orientation of other components necessary for cytochromes *c* biogenesis supports the hypothesis that heme is required in the periplasm.

Ccl1 and Ccl2: roles in cytochromes c *biogenesis*

A question of considerable importance concerns the roles that Ccl1 and Ccl2 play in cytochromes *c* biogenesis. Considering the probable periplasmic orientation of Ccl1 and Ccl2 and the phenotype of the *ccl* mutants, a number of possible roles are envisioned. These include (1) enzymatic modifiers of heme and/or apocytochromes, (2) a role in the ligation process (i.e., a general lyase), and (3) periplasmic chaperone proteins that deliver heme and/or apocytochromes *c* to the lyases. The following is an analysis of each of these possibilities.

1. Early reports proposed that horse apocytochrome *c* might be linked to protoporphyrinogen followed by the insertion of reduced iron (Sano and Tanaka 1964). Subsequently, using *N. crassa* extracts, it was determined that reduced heme is a direct substrate for the CCHL- and Cc_1HL- catalyzed ligation to apocytochromes *c* (Schleyer and Neupert 1985; Nicholson and Neupert 1989). It is possible that the Ccl proteins are involved in the modification or reduction of protopophyrin, heme, or the apocytochromes before or after ligation.
2. Ccl mutants are deficient in all *c*-type cytochromes, suggesting that if Ccl1 and/or Ccl2 are involved in ligation, a single lyase may be responsible for ligation of heme to all cytochromes *c*. With the existence of at least five separate *c*-type cytochromes in *R. capsulatus* (e.g., Kranz 1989), it makes biological sense to evolve or retain a single lyase system. Alternatively, if individual lyases exist for each *c*-type cytochrome, Ccl proteins may be required for each lyase. A yeast nuclear gene called *cyc3* has been shown to encode the *S. cerevisiae* mitochondrial CCHL (Dumont et al. 1987). Mutants in *cyc3* are missing both isoforms of cytochrome *c* (Matner and Sherman 1982), and an in vitro assay for CCHL was used to show that Cyc3⁻ strains are deficient in CCHL activity (Dumont et al.

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1987). Neither Ccl1 nor Ccl2 show significant homology to Cyc3 (see below). The gene for *S. cerevisiae* CC_1HL has been cloned and sequenced recently (A. Haid, pers. comm.). The CC_1HL gene product is homologous to Cyc3 (A. Haid, pers. comm.) but not to Ccl1, Ccl2, or the Ccl1-related mitochondrial and chloroplast ORFs.

3. The significant homologies that are observed between Ccl1 and the chloroplast and mitochondrial ORFs (Fig. 9) suggest potential functional analogies between these proteins and may offer the best clue as to the possible functions of Ccl1. Mitochondria and chloroplasts have *c*-type cytochromes with thioether-linked hemes. Chloroplasts require cytochrome *f* (e.g., Willey et al. 1984), which is the structural and functional equivalent to the mitochondrial cytochrome c_1. Cytochrome *f* in chloroplasts is part of the cytochrome b_6f complex involved in photosynthetic electron transport. If it is assumed that the mitochondrial ORF238 and chloroplast ORF320 are not CCHL or CC_1HL, then these proteins may play novel roles in eukaryotic cytochromes *c* biogenesis. Perhaps the Ccl1 class of proteins function as specific heme chaperones that serve as in vivo-docking proteins for CCHL, CC_1HL, and/or the apocytochromes *c*. The amino terminus of Ccl2 has the amino acid sequence RCPVCQGEN. It is tempting to speculate that secreted Ccl2 may form disulfide bonds to CXYCH of apocytochromes *c*, with subsequent docking onto the membrane-bound Ccl1 and heme ligation. Alternatively, RCPVCQGEN in Ccl2 may represent a heme-binding domain; the HAP1 (heme-activating protein) from *S. cerevisiae* has been shown recently to possess the motif RCPVDH, possibly involved in heme binding (Creusot et al. 1988; Pfeifer et al. 1989). In addition, *S. cerevisiae* CCHL has an amino-terminal sequence CPVMQGDN (Dumont et al. 1987). In either of these hypotheses, heme ligation could be catalyzed by individual lyases or Ccl1/Ccl2.

These hypotheses, and the exact roles of Ccl1 and the homologous mitochondrial and chloroplast ORFs, await confirmation by biochemical analysis and reconstitution of the assembly process. Considering the close phylogenetic relationship between photosynthetic bacteria and the ancestral mitochondrial endosymbiont (Woese 1987), it would not be surprising that the *hel* and *ccl* components described here have functional equivalents in eukaryotes. Continued studies on these bacterial systems will undoubtedly lead to a better understanding of cytochromes *c* biogenesis and heme transport in general.

Materials and methods

Strains and plasmids

R. capsulatus SB1003 (Yen and Marrs 1976), KR7.8 ($HelA^-$) (Kranz 1989), and the plasmids containing *hel* genes (Kranz 1989) have been described previously. Plasmids pPvuII, pHincII, pEcoRV, and p52 contain the indicated DNA restriction fragments (see Fig. 1) cloned into derivatives of the broad host range vector pUCA6. Plasmids p2-9 and p2-7 contain the indicated exonuclease III ordered deletion fragments (see Fig. 1) cloned into a pUCA6 derivative. The cosmid vector pUCA6 was constructed by Dr. William Buikema (University of Chicago, Chicago, IL) and Dr. Jack Meeks (University of California, Davis, CA). It is ~10 kbp in size and has kanamycin and tetracycline resistance. It also has transfer functions and an origin of replication used by *R. capsulatus*. It is mobilized into *R. capsulatus* using the helper plasmid pRK2013 by methods described previously (Kranz 1989). Cloning of cosmids and plasmids containing *ccl1* and *ccl2* will be described elsewhere (D.L. Beckman and R.G. Kranz, in prep.).

DNA sequencing

The Sanger dideoxy method was used to sequence the *hel* and *ccl* loci. The 65% GC content of *R. capsulatus* DNA made it necessary to use *Taq* polymerase (Promega, Madison, WI) in most GC-rich regions; otherwise, Sequenase (U.S. Biochemical, San Diego, CA) was used. Single-stranded DNA templates were used in this sequencing. The M13 K07 helper phage was infected into JM101 [containing pUC118 and pUC119 derivatives (Vieira and Messing 1987)]. The positions of the mini-Mu insertions that defined the *hel* genes were determined by sequencing the double-stranded, denatured mini-Mu plasmids (Kranz 1989) using an oligonucleotide specific to the *lacZ* gene present in the mini-Mu.

The sequencing strategy for the *hel* locus was to sequence overlapping ordered deletions, using specific oligonucleotides whenever necessary. The same strategy was used for the *ccl* locus, and it is illustrated in Figure 6. Ordered deletions were made by exonuclease III nuclease digestion or by methods described previously (Kranz et al. 1990). The *ccl* (X63461) and *hel* (X63462) DNA sequences presented in this paper can be accessed in the EMBL Data Library.

Data base searches, homology significance, and hydrophilicity analyses

Searches for related proteins were made to GenBank and NBRF using TFASTA and FASTA, respectively (Pearson and Lipman 1988). In these analyses, optimal alignment scores for HelA to ABC transporters were >100 . Likewise, optimal alignment scores for Ccl1 and HelC to *P. aurelia* ORF228 and to the chloroplast ORFs were >100. To determine the significance of the similarity between the sequences shown in Figure 3 (HelA) and Figure 8 (Ccl1 and HelC), LFASTA and RDF2 programs were used. In these analyses, for example, HelA gave a score of 14 when compared with HisP, and Ccl1 gave a score of 13 when compared with the *P. aurelia* ORF228. A RDF2 analysis of the comparison of HelC with the liverwort ORF320 (shown in Fig. 8) yielded a score of 8. RDF2 scores >10 are significant, >6 are probably significant, and >3 are possibly significant (Pearson and Lipman 1988).

Hydrophilicity analyses were carried out as described previously (Kyte and Doolittle 1982) using the recommended parameters (described in the legends to Figs. 4 and 9). The Wisconsin GCG programs were used to implement these algorithms (Devereux et al. 1984).

Cytochrome c_2 gene cloning and construction of a cytA–phoA *fusion*

The *R. capsulatus cytA* gene was cloned from an *R. capsulatus* genomic library, which has been described previously (Kranz 1989). A cosmid containing *cytA* was obtained by screening the

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library with an *R. sphaeroides cytA* gene probe. The *R. sphaeroides cytA* gene (Donohue et al. 1986) was kindly provided by Dr. Tim Donohue (University of Wisconsin, Madison, WI). Construction of the *R. capsulatus cytA–phoA* fusion plasmids was carried out as shown in Figure 10. The *phoA* fusion vector pPH07 (Gutierrez and Devedjian 1989) was kindly provided by Dr. Claude Gutierrez (Centre de Biochemie et de Genetique Cellulaire du CWRS, Toulouse, France).

Construction of ccl1–phoA *fusions*

The 2.2-kbp *Bam*HI fragment that contains most of *ccl1* and upstream DNA (see Fig. 6) was ligated into pUC118 such that *ccl1* was in the direction of the *lacZ* promoter. This plasmid was digested with *Stu*I and *Sal*I, and a 2-kbp *Sma*I–*Sal*I *phoA* fragment from pPHO7 was inserted. From the *ccl1* sequence analysis, this *Sma*I–*Stu*I ligation was predicted to form an in-frame *ccl1–phoA* fusion. The products from this ligation were transformed into *E. coli* CC118, and cells were plated on LB ampicillin media containing XP and IPTG (see Table 2). Plasmids from blue colonies had a *ccl1–phoA* fusion as determined by restriction enzyme mapping. This plasmid is designated p11ccl1–phoA.

To construct a *ccl1–phoA* plasmid that was mobilizable and replicates in *R. capsulatus*, a 4.2-kbp *Bam*HI–*Hin*dIII fragment from p11ccl1–phoA, containing the *ccl1–phoA* gene fusion, was ligated into pUCA6, that was also digested with *Bam*HI and *Hin*dIII. The resulting *ccl1–phoA* fusion plasmid is designated p11-6-1.

Alkaline phosphatase assays and periplasmic fractionations

Alkaline phosphatase activity was detected in bacterial colonies using the chromogenic indicator 5-bromo-4-chloro-3-indole phosphate (XP, Sigma Chemical Co.), as described previously (Manoil and Beckwith 1985). Bacterial cells were broken by sonication (Kranz and Haselkorn 1985) and assayed for alkaline phosphatase activity as described previously (Brickman and Beckwith 1975). Periplasmic fractions were isolated by a procedure similar to one used by Tai and Kaplan (1985). Pelleted cells were resuspended to one-third original culture volume in 0.1 M Tris (pH 8.0), 20% sucrose (wt/wt). After 10 min incubation at

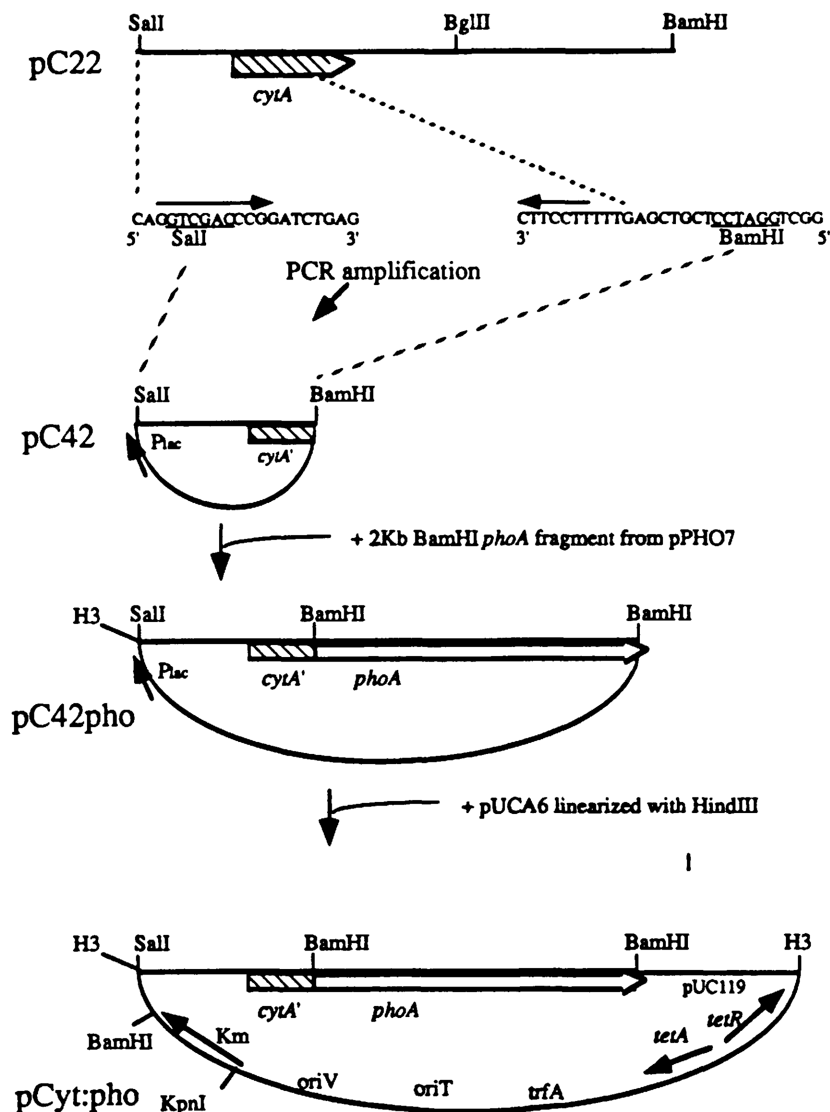

Figure 10. Construction of cytochrome c_2–alkaline phosphatase gene fusions. A cosmid containing the cytochrome c_2 gene, *cytA*, was isolated from a *R. capsulatus* genomic library as described in Materials and methods. A 2-kbp *Bam*HI–*Sal*I fragment that contains the entire *cytA* gene (Daldal et al. 1986) was then subcloned into pUC118 to make pC22. Only the DNA insert in pC22 is shown. A *Bam*HI restriction enzyme site was engineered into *cytA* by using the two oligonucleotides shown and amplifying the indicated fragment with pC22 as template by the polymerase chain reaction (PCR) technique (Saiki et al. 1985). The resulting 0.7-kbp was digested with *Bam*HI and *Sal*I and cloned into pUC119 to yield pC42. Using the alkaline phosphatase gene fusion vector pPH07 (Gutierrez and Devedjian 1989), a 2-kbp *phoA* fragment was excised with *Bam*HI and ligated to *Bam*HI-linearized pC42. The *Bam*HI oligonucleotide used in the PCR step above was designed such that it would ligate to *Bam*HI-cut *phoA* fragment and form a *cytA–phoA* in-frame fusion. For mobilization and replication in *R. capsulatus*, the entire pC42 was linearized with *Hin*dIII and ligated to *Hin*dIII linearized pUCA6. The resulting pCyt–pho was obtained with the insert in both orientations and is shown (pCyt:pho). pCyt–pho12 is the opposite orientation. pCyt–pho constructs are kanamycin, ampicillin, and tetracycline resistant. All others shown are ampicillin resistant. The *cytA–phoA* gene fusion plasmids contain 88 of 116 amino acids of the apocytochrome c_2 protein.

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37°C, lysozyme was added to a final concentration of 100 μg/ml while stirring. After incubation for 12 min at 37°C, the preparation was brought to a final concentration of 10 mM ethylenediaminetetraacetic acid (pH 8.0) using a 0.25 M stock solution. After incubation for ~3 min, spheroplasts were pelleted by centrifugation at 10,000*g* for 10 min. The supernatant, consisting of outer membrane and periplasmic components, was centrifuged at 150,000*g* for 2 hr to pellet outer membrane components. The resulting supernatant is the periplasmic fraction.

Protein determinations were carried out using a modified Lowry procedure (Markwell et al. 1978).

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