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 c2–alkaline phosphatase gene fusions were used to show that apocytochrome c2 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 CcU-alkaline phosphatase gene fusion. The CcU–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 c1 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 c1 are transported from the cytosol to the mitochondrial intermembrane space. Apocytochrome c1 transport takes place by two sequential translocations [Nicholson et al. 1989]. Initially, preapocytochrome c1 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 c1 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 c1 requires the ligation of heme to the apoproteins in the intermembrane space. This ligation occurs at two distinct cysteine residues [CXCH] within the apocytochromes c and c1 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 c1 (called CCjHL), appear to be required. S. cerevisiae mutants deficient in CCjHL 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, HL 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 Kurltzes 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 Hedderstedt 1990) and Gram-negative bacteria have demonstrated the presence of covalently bound heme at CX-YCH 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 endosymbiont (Woese 1987). We 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 gene (cytA) and to ccl are used to investigate targeting processes. Sequence analyses of the hel and ccl loci and the cytA-phoA and ccl-phoA analyses of hel and ccl mutants provide evidence for the hypothesis that hel genes encode a heme transporter and that apocytochromes c, Cc1, 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 PvuII-RsrII fragment is shown with the locations of orf124, 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 Tn5 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 Tn5 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 PvuII and EcoRI 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 Tn5 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 EcoRV–EcoRI 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 Cct1 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 adenylyl 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 locus: cytA–phoA gene fusion analyses.

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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* gene as a probe, we cloned the *R. capsulatus* cytochrome *c* gene ([cytA]). A cytA–phoA gene fusion was constructed with 88 of 116 amino acid residues of the apocytochrome *c* 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 phosphatase 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
need for a heme transporter. We therefore decided to analyze candidate lyase genes (called \( ccl \) genes).

Two other mutants that are pleiotropically missing \( c \)-type cytochromes in \( R. \) \( \text{capsulatus} \) have been reported: MT113 (Davidson et al. 1987) and AJB530 (Biel and Biel 1990). Biel and Biel (1990) have shown recently that the Tn5 transposon is inserted in AJB530, and this gene is probably in the same gene that the \( ccl2 \) 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 \( P. \) \( \text{aurelia} \) mitochondrial genome (Pritchard et al. 1990). Of the 111 amino acid residues shown in Figure 9, \( ccl1 \) is \( \approx 52\% \) 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 \( E. \) \( \text{coli} \) and \( R. \) \( \text{capsulatus} \) 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
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 cytochromo 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 aerobic 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 bc1 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 bc1 complex. Yet, except for cytochrome b of the bc1 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,
were shown to be present in \( \text{B. japonicum} \). The production of b-type cytochromes is not impaired in Hel" yields a strain with a Hel~ phenotype. Genetic analysis of the hel~ phenotype must take these specific deficiencies into account. The helC gene, and ORF263, respectively, show ~65% similarity to the \( \text{R. capsulatus} \) counterparts described here. Ramseier et al. (1991) demonstrated that a small ORF called cycX, equivalent to \( \text{R. capsulatus} \) orf52, is required for cytochromes c biogenesis. They also suggest that the \( \text{B. japonicum} \) 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 \( \text{R. capsulatus} \) 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 \( \text{R. capsulatus} \) [Kranz 1989]. Although an explanation for the different results with helC requires further experimentation, the results in \( \text{R. capsulatus} \) and \( \text{B. japonicum} \) 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 \( \text{E. coli} \) requires HlyB, which contains the nucleotide-binding domain, and HlyD. Some ABC exporters are predicted to transport small molecules; these include a \( \beta-1,2 \) glucan transporter in \( \text{Rhizobium meliloti} \) (called ndv) [Stanfield et al. 1988] and in \( \text{Agrobacterium tumefaciens} \) (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 \( \text{R. sphaeroides} \), 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 \( \text{R. capsulatus} \) hel mutants are as sensitive as wild type to the iron chelator dipyrindyl, and multiple sources of iron do not correct the hel defect [R. Kranz, unpubl.]. Finally, iron importers analyzed in \( \text{E. coli} \) (e.g., Zimmermann et al. 1984; Burkhardt and Braun 1987) and \( \text{Serratia marcescens} \) [Angerer et al. 1990 and references therein] are composed of ABC-type transporters 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 \( \text{B. japonicum} \) [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 \( \text{R. capsulatus} \) counterparts described here. Ramseier et al. (1991) demonstrated that a small ORF called cycX, equivalent to \( \text{R. capsulatus} \) orf52, is required for cytochromes c biogenesis. They also suggest that the \( \text{B. japonicum} \) 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 \( \text{R. capsulatus} \) 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 \( \text{R. capsulatus} \) [Kranz 1989]. Although an explanation for the different results with helC requires further experimentation, the results in \( \text{R. capsulatus} \) and \( \text{B. japonicum} \) do indicate that hel genes are specifically involved in cytochromes c biogenesis in both photosynthetic and nonphotosynthetic Gram-negative bacteria.

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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,
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 c2-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 c2-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 c2 export takes place in the absence of heme ligation in R. capsulatus. We suggest that apocytochromes c2 and c1 are transported to the periplasm by a signal sequence-dependent mechanism, since both cytochrome c1 [Daldal et al. 1987] and cytochrome c2 [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, Cell, does not require hel genes for transport to the periplasm, as a Cell–PhoA fusion protein is transported to the periplasm in the hel mutants [Table 2]. These results exclude Cell 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 Cell 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.

### Figure 8. Hydrophilicity analyses of Ccl1 and Ccl2

Hydropathicity 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].

### Figure 9. Cell and HelC homology to other proteins

Genes to myc. 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 ORF328 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.
whether homologs could be found in eukaryotes. We car­
cell has indicated that are required for cy­
and whose mutation mapped to a different locus. Sequence
analyzing other genes involved in cytochromes c biogen­
eel2, were present in an operon. Deletion mutagenesis
analysis of this locus showed that two genes,
and previously to have a similar phenotype to the
hel
AJB530 (Biel and Biel 1990) that had been shown previ­
sional, the lyase, and any other assembly components
must also be present in the periplasm. Our reason for
the heme, the lyase, and any other assembly components
If cytochrome c heme ligation occurs in the periplasm,
sequence-dependent general secretory mechanism.
transport of Cell and possibly Ccl2 occurs by a signal
fusion studies, at least

cell and Ccl2 and the phenotype of the
mutants, a
roles that Cell and Cell play in cytochromes c biogen­
a question of considerable importance concerns the
roles that Cell1 and Cell2 play in cytochromes c biogen­
Considering the probable periplasmic orientation of
Cell1 and Cell2 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 deter­
mined that reduced heme is a direct substrate for the
CCHL- and CcHL- catalyzed ligation to apocy­
mitochondrial [Minami-Ishii et al. 1989] enoyl-CoA hy­
Ccl proteins may be required for each lyase. A yeast
[1987]. Mutants in cyc3 are missing both isoforms of
cycloporin c [Matner and Sherman 1982], and an in
CCHL (Dumont et al.

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

| Strain         | Strain description | Growth condition | Alkaline phosphatase activity* |
|----------------|--------------------|------------------|-------------------------------|
| E. coli CC118  | PhoA [Manoil and Beckwith 1985] | LB + 0.2% glucose | <20                           |
| E. coli CC118  | PhoA [Manoil and Beckwith 1985] | LB + IPTGb       | <20                           |
| E. coli CC118  | PhoA [Manoil and Beckwith 1985] | LB + 0.2% glucose | <20                           |
| E. coli CC118  | PhoA [Manoil and Beckwith 1985] | LB + IPTG        | 340                           |
| R. capsulatus SB1003 | wild type (Yen and Marrs 1976) | RCVc            | <20                           |
| R. capsulatus SB1003 | wild type (Yen and Marrs 1976) | RCVc            | 430                           |
| R. capsulatus KR7.8 | HeLA- [Kranz 1989] | RCVc            | 750                           |

*CActivities are measured in OD units/min per milligram of protein. OD units are defined as OD420 × 1000 after incubation at 25°C
with 6 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].
*IPPTG] Isopropyl-thiogalactoside was added 4 hr before cells were harvested.
[RVC] R. capsulatus basal media [Kranz 1989].

Cell1 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 biogen­
essis 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 car­
cel, where deletion mutagenesis has indicated that
cell1 and cell2 are required for cy­
tochromes c biogenesis [D.L. Beckman and R.G. Kranz,
in prep.]. The cell2 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 mito­
chondrial [Minami-Ishii et al. 1989] enoyl-CoA hy­
derases [Beckman and Kranz 1991]. Proximal to cell1,
but transcribed in the opposite direction, is a gene that
encodes a protein homologous to acetylornithine ami­
o transferase [Fig. 6]. Thus, cell1 and cell2 appear to be
the only genes at this locus involved in cytochromes c
biogenesis.

Analysis of the primary sequence of Cell1 and Cell2
indicates that both possess amino-terminal consensus
signal sequences. We have confirmed the periplasmic
orientation of Cell1 by cell1-phoA fusion studies, at least
at the position of insertion of the phoA gene. Both E.
coli and R. capsulatus are able to transport Cell1 to the
periplasm. On the basis of these data, we suggest that
transport of Cell1 and possibly Cell2 occurs by a signal
sequence-dependent general secretory mechanism.
Moreover, the periplasmic orientation of other compo­
nents necessary for cytochromes c biogenesis supports
the hypothesis that heme is required in the periplasm.

Cell1 and Ccl2: roles in cytochromes c biogenesis

A question of considerable importance concerns the
roles that Cell1 and Cell2 play in cytochromes c biogen­
essis. Considering the probable periplasmic orientation of
Cell1 and Cell2 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
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1. Early reports proposed that horse apocytochrome c
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Ccl proteins may be required for each lyase. A yeast
[1987]. Mutants in cyc3 are missing both isoforms of
cycloporin c [Matner and Sherman 1982], and an in
CCHL (Dumont et al.

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. capsula­
tus [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.
These hypotheses, and the exact roles of Cell and the homologous mitochondrial and chloroplast ORFs, await 3. The significant homologies that are observed between Cc1 and the plastid 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 c1. Cytochrome f in chloroplasts is part of the cytochrome b6f complex involved in photosynthetic electron transport. If it is assumed that the mitochondrial ORF238 and chloroplast ORF320 are not CChL or CClHL, 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, CClHL, and/or the apocytochromes c. The amino terminus of Ccl2 has the amino acid sequence CPVMQGDN (Dumont et al. 1987). In either of these hypotheses, heme ligation could be catalyzed by individual lyases or Cc1/Ccl2.

These hypotheses, and the exact roles of Cc1 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 loci are involved in the membrane-bound Cell and heme ligation. Alternatively, RCPVCQGEN in Ccl2 may represent a heme-binding domain, the HAP1 [hem-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 CPVMQCDN (Dumont et al. 1987). In either of these hypotheses, heme ligation could be catalyzed by individual lyases or Cc1/Ccl2.

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, pHinclI, 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 dyeideox 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 [MI101 [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. aerulias 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 P. aerulis ORF228. A RDF2 analysis of the comparison of HelC and 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 [Kryc and Doolittie 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 c2 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
library with an \textit{R. sphaeroides} cytA gene probe. The \textit{R. sphaeroides} cytA gene [Donohue et al. 1986] was kindly provided by Dr. Tim Donohue (University of Wisconsin, Madison, WI). Construction of the \textit{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 ccll-phoA fusions

The 2.2-kbp BamHI fragment that contains most of \textit{ccll} and upstream DNA (see Fig. 6) was ligated into pUC118 such that \textit{ccll} was in the direction of the lacZ promoter. This plasmid is designated pllccll-phoA. To construct a \textit{ccll-phoA} plasmid that was mobilizable and replicates in \textit{R. capsulatus}, a 4.2-kbp BamHI-HindIII fragment from pllccll-phoA, containing the \textit{ccll-phoA} gene fusion, was ligated into pUC6, that was also digested with BamHI and HindIII. The resulting \textit{ccll-phoA} fusion plasmid is designated pl1-6-1.

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](image-url)

Construction of cytochrome \textit{c}_2-alkaline phosphatase gene fusions. A cosmid containing the cytochrome \textit{c}_2 gene, cytA, was isolated from a \textit{R. capsulatus} genomic library as described in Materials and methods. A 2-kbp BamHI-Sall 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 BamHI 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 BamHI and Sall and cloned into pUC119 to yield pC2. Using the alkaline phosphatase gene fusion vector pPH07 [Gutierrez and Devedjian 1989], a 2-kbp phoA fragment was excised with BamHI and ligated to BamHI-linearized pC2. The BamHI oligonucleotide used in the PCR step above was designed such that it would ligate to BamHI-cut phoA fragment and form a cytA-phoA in-frame fusion. For mobilization and replication in \textit{R. capsulatus}, the entire pC2 was linearized with HindIII and ligated to HindIII linearized pUC6. 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 \textit{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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