

Differential Levels of Specific Cytochrome *c* Biogenesis Proteins in Response to Oxygen: Analysis of the *ccl* Operon in *Rhodobacter capsulatus*

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The photosynthetic bacterium *Rhodobacter capsulatus* synthesizes *c*-type cytochromes under a variety of growth conditions. For example, under aerobic growth, *c*-type cytochromes are synthesized as part of an electron transport pathway, using oxygen as the terminal electron acceptor. Anaerobically in the light, *R. capsulatus* requires cytochrome *bc*₁ and other *c*-type cytochromes for the photosynthetic electron transport pathway. It is shown here that the *ccl1* and *ccl2* genes of *R. capsulatus* are required for the synthesis of all *c*-type cytochromes, including the cytochrome *c'* protein of unknown function but of structural similarity to cytochrome *b*₅₆₂. Polar and nonpolar mutations constructed in each gene demonstrated that the *ccl12* genes form an operon. Expression of the *ccl12* genes was examined by using *lacZ* and *phoA* fusions as translational reporters. Primer extension analysis was used to determine transcriptional control and the start site of the *ccl12* promoter. Finally, antiserum to the Ccl2 protein was used to quantitate levels of Ccl2 under six different growth conditions. The Ccl2 protein is present at 20-fold-higher levels under conditions where oxygen is present. In contrast, other cytochromes *c* biogenesis proteins, HelA and HelX, previously shown to be part of an *helABCDX* operon, are at relatively similar levels under these six growth conditions. This discovery is discussed in terms of the physiology and evolution of cytochromes *c* biogenesis, with particular attention to oxidative environments.

Cytochrome *c* biogenesis in the gram-negative bacterium *Rhodobacter capsulatus* requires at least eight specific genes at three loci: *ccl1-ccl2* (3, 4), *helABCDX* (2, 3), and *cycH* (31). The *helABCDX* genes constitute an operon, and by using epitope tagging and immunological analysis of the Hel proteins, it was demonstrated that HelABCD polypeptides form a four-subunit ABC exporter complex in the cytoplasmic membrane (19). It is proposed that this complex transports heme to the Ccl1 protein at the periplasmic surface of the cytoplasmic membrane (3). It is at this surface that the ligation of the vinyl groups of heme to the highly conserved cysteine residues of cytochromes *c* is predicted to occur. This thioether ligation of heme to CysXxxYyyCysHis of the polypeptides is the hallmark of *c*-type cytochromes. The CysXxxYyyCys motif of Ccl2 is proposed to react with cysteines of the apocytochrome *c* polypeptides, forming mixed disulfides (33). Subsequently, this mixed disulfide product is brought into proximity of the heme, which is presented by the Ccl1 protein, for complete apocytochrome *c* reduction and spontaneous ligation. Acting as a more general reductant, the thioredoxin-like HelX protein then rereduces the Ccl2 cysteines for another round of synthesis (2, 33). Some of the genetics of cytochrome *c* biogenesis in *R. capsulatus* and other bacteria have been previously reviewed (29, 41).

In some organisms, such as *Escherichia coli* and *Haemophilus influenzae*, the eight genes described above are present in a single operon and called *ccm* (16, 20, 23, 40). For *E. coli*, these genes are expressed primarily when *c*-type cytochromes are used (i.e., anaerobically in the presence of an alternative electron acceptor [8, 11, 23]). *Bradyrhizobium japonicum* contains cytochrome *c* biogenesis genes at two loci: *cycHJKL* (*ccl1/cycK*,

ccl2/cycL) (35, 36) and *cycVWorf263cycXtlpB* (*helABCDX*) (32, 34). The present study was undertaken to determine if the *R. capsulatus ccl12* genes form an operon and if so to construct defined nonpolar deletion strains. The phenotype of these strains and the expression of the *ccl12* genes were characterized further. Using an antiserum to the Ccl2 protein, we demonstrate that a remarkable increase in concentration is observed in cells grown under aerobic conditions. In contrast, Hel proteins are present at similar levels in cells grown under aerobic or anaerobic environments. The results are discussed in the context of the evolution and physiological requirements of cytochrome *c* biogenesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. Figure 1 shows the map of the *ccl* locus and restriction sites used to create *R. capsulatus* mutants. The *R. capsulatus* chromosomal deletion mutants RGK292 ($\Delta ccl12$), RGK290 ($\Delta ccl1$), RGK293 ($\Delta ccl1$), and RGK291 ($\Delta ccl2$) contain an integrated kanamycin resistance (Km^r) cassette in the indicated direction from pUC4-KIXX (Pharmacia, Piscataway, N.J.). Plasmids pRGK206, pRGK208, pRGK279, and pRGK210 contain the Km^r cassette *Sma*I fragment and were used to create the $\Delta ccl12$, $\Delta ccl1$ polar, $\Delta ccl1$ nonpolar, and $\Delta ccl2$ mutants, respectively. pRGK206 has the Km^r cassette ligated between the *Bgl*II sites (filled in with Klenow enzyme) of the *ccl1* and *fadB1* genes. pRGK208 contains the Km^r cassette ligated into the *Not*I sites of the *ccl1* gene. pRGK279 contains the Km^r cassette ligated into the *Bsa*BI sites of the *ccl1* gene. pRGK210 contains the Km^r cassette ligated between the *Msc*I and second *Ear*I sites (filled in with Klenow enzyme) of the *ccl2* gene. pRGK208, pRGK279, and pRGK210 have DNA that includes the 3.46-kb *Bam*HI-*Xcm*I fragment (Fig. 1). The deletion mutants RGK290, RGK291, and RGK292 were constructed by using the incompatible plasmid pPH1J1, which carries a gentamicin resistance gene, as previously described (1, 25). Kanamycin- and gentamicin-resistant colonies that were tetracycline sensitive were selected, and these exhibited a cytochrome *c*-deficient phenotype. To generate mutant RGK293, the *Bam*HI-*Sa*I fragment of pRGK279 was subcloned into the suicide vector pSUP202 to yield pRGK280 and then conjugated into SB1003. Double recombinants that had lost pSUP202 (Tet^r) were detected by selecting for Km^r (indicating that the Km^r cassette was recombined into the chromosome), tetracycline-sensitive colonies. These exhibited a cytochrome *c*-deficient phenotype.

For the *ccl12* expression reporters, an in-frame translational *lacZ* fusion to the cytoplasmic C terminus of Ccl2 (in pRGK243) and an in-frame translational

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid(s)	Description	Reference or source
<i>R. capsulatus</i>		
SB1003	Wild type	43
RGK277	$\Delta helAB$ (Km ^r)	19
RGK290	$\Delta ccl1$ (Km ^r in <i>NotI-NotI</i> , opposite orientation)	This study
RGK291	$\Delta ccl2$ (Km ^r in <i>MscI-EarI</i>)	This study
RGK292	$\Delta ccl12$ (Km ^r in <i>BglII-BglII</i>)	This study
RGK293	$\Delta ccl1$ (Km ^r in <i>BsaBI-BsaBI</i> , forward orientation)	This study
Plasmids		
pUC118 and -119	Ap ^r , M13 intergenic region	42
pUC4-KIXX	Km ^r cassette	Pharmacia
pSUP202	Ap ^r , Cm ^r , Tc ^r , suicide vector	38
pRK2013	Km ^r , <i>tra</i> ⁺	13
pUCA5	Tc ^r , pUCA6 derivative	17
pUCA6	Km ^r , Tc ^r , 9.2 kb; mobilizable by pRK2013	W. Buikema and J. Meeks
pUCA9	Tc ^r , pUCA6 derivative	28
pUCA10	Tc ^r , 7.7 kb; mobilizable by pRK2013	18
pPH1J1	Gen ^r (incompatible with pLAFR1-derived plasmids)	25
pRGK204	Ap ^r , Tc ^r , 3.46-kb <i>BamHI-XcmI</i> fragment containing <i>ccl12</i> in pUCA10	This study
pRGK206	Tc ^r , Km ^r , $\Delta ccl12$ fragment in pUCA6	This study
pRGK208	Km ^r , Tc ^r , $\Delta ccl1$ fragment in pUCA5	This study
pRGK210	Km ^r , Tc ^r , $\Delta ccl12$ fragment in pUCA10	This study
pRGK211	Tc ^r , 1.52-kb <i>BamHI-XcmI</i> fragment containing <i>ccl2</i> in pUCA9	This study
pRGK212	Tc ^r , 1.56-kb <i>SmaI ccl</i> fragment in pUCA6	This study
pRGK213	Tc ^r , 2.92-kb <i>PstI-AccI</i> fragment containing <i>ccl2</i> in pUCA10	This study
pRGK243	Km ^r , Tc ^r , Ccl1-Ccl2-LacZ (carboxy terminus) in pUCA10	33
pRGK246	Ap ^r , Tc ^r , Ccl1-PhoA in pUCA10	Unpublished
pRGK279	Km ^r cassette in <i>BsaBI</i> sites of <i>ccl1</i> gene in pUC118	This study
pRGK280	Km ^r , Tc ^r , pRGK279 <i>BamHI-SalI</i> in pSUP202	This study

phoA fusion to a periplasmic domain of Ccl1 (in pRGK245) were constructed. These plasmids will be described elsewhere. Both exhibit high enzymatic activity of their respective fusion proteins in *R. capsulatus*, and both contain the entire upstream region of *ccl12* to the *BamHI* site shown in Fig. 1.

Media and growth conditions. Peptone-yeast extract medium (PYE), *R. capsulatus* basal medium (RCV), and drug concentrations have been described

previously (1). *R. capsulatus* cells were grown in 2-liter flasks with 500 ml of culture aerobically at 300 rpm to an optical density at 550 nm (OD₅₅₀) of 0.5, microaerobically at 300 rpm to an OD₅₅₀ of 1.7, and anaerobically photosynthetically to an OD₅₅₀ of 2.3 in screw-cap bottles.

Difference spectroscopy. The preparation of *R. capsulatus* extracts for reduced-minus-oxidized spectra was described previously (2). The reduced (sodium hy-

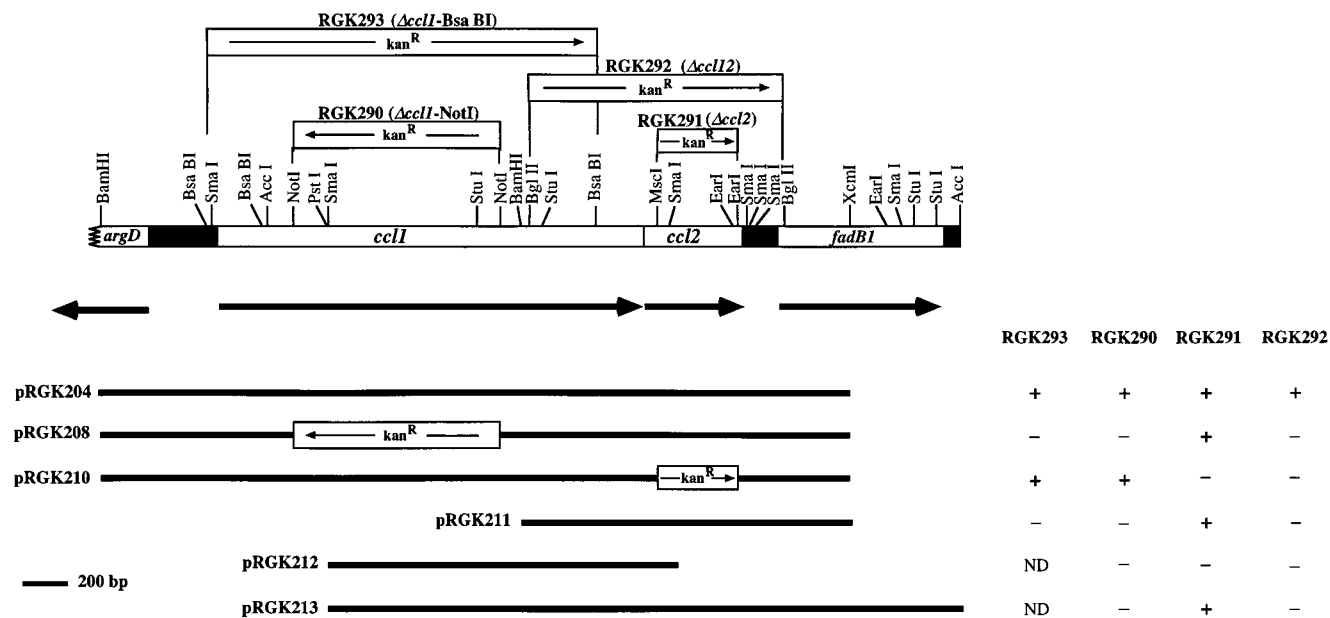


FIG. 1. Restriction map of the *ccl* locus and DNA complementation analysis of *R. capsulatus* mutant strains. Each mutant strain contains a chromosomal Km^r gene between the indicated restriction sites. Plasmids containing the indicated restriction fragments were conjugated into the *ccl* mutant strains. Complementation was defined as the ability to grow photosynthetically and oxidize the cytochrome *c*-specific electron donor *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (The Km^r gene is not drawn to scale.) ND, not determined.

drosulfite)-minus-oxidized (ammonium persulfate) spectra were obtained on either a Beckman model DU50 or a Shimadzu UV-2101-PC spectrophotometer. For membrane fractions, extracts were solubilized in 1% Triton X-100 for 1 h prior to spectral analysis. The cytochrome *c'* reduced (sodium hydrosulfite)-plus-CO-minus-reduced (ammonium persulfate) spectra were obtained on a Shimadzu UV-2101-PC spectrophotometer.

Cell fractionations. Extracts for Western analysis were prepared as follows. Cell cultures were harvested by centrifugation at $15,000 \times g$ for 10 min, washed with 10 mM Tris (pH 8), and frozen at -80°C overnight. Frozen cells were resuspended in 4 to 8 ml of 10 mM Tris (pH 8) and sonicated three times for 3 min each time (Branson 200 sonicator with a microtip at a power setting of 40%) at 4°C . Unbroken cells were removed by centrifugation at $12,000 \times g$ for 10 min at 4°C . The sonicates were separated into membrane and soluble protein fractions by ultracentrifugation at $150,000 \times g$ for 1 h. The resulting pellet is the membrane fraction; it was resuspended in 10 mM Tris (pH 8).

Western blotting. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electroblotted onto Hybond-C nitrocellulose (Amersham, Arlington Heights, Ill.). Polyclonal antibodies against the purified *R. capsulatus* Ccl2 protein are described elsewhere (33). A polyclonal antiserum was obtained in New Zealand White rabbits against *R. capsulatus* cytochrome *c*₂, which was kindly provided by Michael Cusanovich (University of Arizona, Tucson). Western analyses were performed by using SuperSignal chemiluminescent substrate (Pierce Chemical Co., Rockford, Ill.), with protein A peroxidase (Sigma, St. Louis, Mo.) as the secondary label.

Other methods. Primer extension analysis was performed and RNA was prepared as described previously (18) except that a 100 mM hexadecyltrimethyl ammonium bromide extraction was performed on the RNA preparations; 50 μg of RNA was used in each reaction. The primer 3'-GTAAAGCGGGACTAGG ACCGGGACACGCAC-5' is complementary to bases +19 to +48 relative to the predicted translational start site of *ccl1*. DNA sequencing was done by the dideoxy method of Sanger et al. (37), using [³⁵S]ATP, a Sequenase 2.0 kit (Amersham), and double-stranded denatured template. β -Galactosidase activities of cell extracts were determined as described previously (30). Alkaline phosphatase assays were performed with whole cells at 23°C as described previously (6). Protein was determined by the bicinchoninic acid assay (Pierce), using bovine serum albumin as the standard. Conjugations were carried out with triparental matings as described previously (1).

RESULTS

Construction of defined *ccl12* deletion strains and analysis of the *ccl1-ccl2* operon. The *ccl1* and *ccl2* genes were each deleted and replaced with a *Km*^r cassette by using homologous recombination (Fig. 1). A mutant containing a deletion of both *ccl* genes was also generated. For the *ccl1* gene, the *Km*^r cassette was engineered in both orientations to examine possible polarity on *ccl2*. Complementation studies using defined *ccl1* and/or *ccl2* plasmids for each strain were performed by testing transconjugates for photosynthetic growth. This growth mode requires cytochrome *c*₁ of the cytochrome *bc*₁ complex in addition to either cytochrome *c*₂ or *c*_V (10, 27). A plasmid with both *ccl1* and *ccl2* complemented all four strains. A plasmid containing only the *ccl2* gene, pRGK211, complemented the *ccl2* deletion strain, while a plasmid containing only the *ccl1* gene, pRGK210, complemented either *ccl1* deletion strain. Results of the complementation analysis described above are not due to reversion, since we have been unable to isolate revertants of the *ccl1* or *ccl2* deletion mutant.

These results indicate that both *ccl1* and *ccl2* are required for cytochrome *c* biogenesis and that from complementation analysis, *ccl1* mutations are not polar on *ccl2*. However, in quantitating the levels of *c*-type cytochromes produced in these strains, we observed that strain RGK290 containing pRGK210 produced only 10% of the spectrally detectable amount in RGK293 containing pRGK210. This result suggested that when the *Km*^r gene is transcribed in the opposite orientation as *ccl2*, polarity on *ccl2* results. To further analyze the effects of polarity on *ccl2* expression, we used antibodies to the Ccl2 protein to detect its levels in various genetic backgrounds (Fig. 2). Strains were grown under microaerobic conditions. As expected, the *ccl2* deletion strain does not contain the Ccl2 polypeptide in Western blot analysis (lane 1). When *ccl2* is supplied in *trans* on a plasmid in either the *ccl2* (lane 2) or *ccl1* (lane 5)

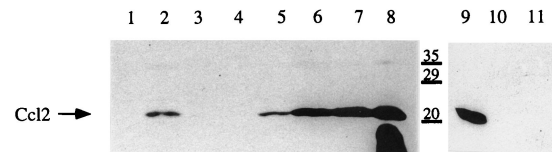


FIG. 2. Mutations in the *ccl1* gene are polar on *ccl2* expression. Western analysis of membrane extracts, using an antiserum to Ccl2. Cells were grown microaerobically to an approximate OD_{600} of 2.0 in 40 ml of medium in 125-ml flasks. Lane 1, RGK291 ($\Delta ccl2$); lane 2, RGK291 containing pRGK211 (*ccl2*); lane 3, RGK290 ($\Delta ccl1$ polar); lane 4, RGK290 containing pRGK210 (*ccl1*); lane 5, RGK290 containing pRGK211; lane 6, RGK277 ($\Delta helAB$); lane 7, SB1003 (wild type); lane 8, RGK290 containing pRGK204 (*ccl12*); lane 9, RGK293 ($\Delta ccl1$ nonpolar); lane 10, RGK290 ($\Delta ccl1$ polar); lane 11, RGK291 ($\Delta ccl2$). Protein amount in lanes 1 to 8 is 25 μg ; protein amount in lanes 9 to 11 is 50 μg . The reactive material running at the dye front in lane 8 could be degraded Ccl2 since a plasmid-borne *ccl12* produces more protein; however, this has not been investigated further.

strain, the Ccl2 protein is produced. These levels are lower than those in the wild type (lane 7) or an *helAB* deletion strain (lane 6). It is likely that a vector promoter and plasmid copy number are responsible for the synthesis of this level of Ccl2 protein, although a promoter located immediately upstream from *ccl2* cannot be ruled out from these experiments. Strains RGK290 and RGK290(pRGK210) produced Ccl2 polypeptide at undetectable levels (lanes 3 and 4, respectively). Considering the sensitivity of these Western blots, we estimate that less than 5% of wild-type levels of the Ccl2 antigen could be detected. Thus, when grown microaerobically, these strains have at most 5% of the wild-type levels of Ccl2. Nevertheless, anaerobically there is enough Ccl2 protein to yield the photosynthetic growth noted above for RGK290(pRGK210). Strain RGK293, a *ccl1* deletion strain which has the *Km*^r gene promoter in the *ccl2* orientation, produces levels of Ccl2 polypeptide that are close to the wild-type level (lane 9). Since the orientation of the insert in *ccl1* determines the levels of Ccl2 protein when grown under identical conditions, these results demonstrate that a *ccl12* operon is present.

***R. capsulatus ccl1* and *ccl2* mutants are deficient in all *c*-type cytochromes, including cytochrome *c'*.** To confirm the loss of *c*-type cytochromes in *ccl1* and *ccl2* mutants, reduced-minus-oxidized absorption spectra were taken on the soluble and membrane fractions of mutant and wild-type cells grown under aerobic conditions (Fig. 3). *c*-type cytochromes typically show absorption maxima at approximately 550 nm, while *b*-type cytochromes absorb at 560 nm. The soluble fraction of the wild type had high levels of *c*-type cytochromes, while the *ccl12* and *helAB* double mutants showed no detectable cytochromes *c*. The membrane fraction of each strain exhibited significant absorption at 560 nm, indicating that all strains synthesize *b*-type cytochromes as expected. The approximate 50% reduction in the *ccl* and *hel* mutants is probably due to the loss of the cytochrome *bc*₁ complex. Using cytochrome *bc*₁ antibodies, we previously noted that the entire complex is degraded in *hel* mutants (28), and an identical result was obtained for the *ccl* mutants constructed in this study (data not shown). The membrane fractions of the *ccl* and *hel* mutants are clearly missing the absorption peak at 550 nm that is observed in the wild type, and thus these membranes are deficient in the holo forms of cytochromes *c*. Identical results were obtained with the single *ccl1* and *ccl2* strains (data not shown).

To determine whether the soluble cytochrome *c*₂ apoprotein is also degraded in *ccl* and *hel* mutants, an antiserum to cytochrome *c*₂ was produced. This antiserum reacted intensely with purified cytochrome *c*₂ and the cytochrome *c*₂ in extracts of the wild-type strain (Fig. 4). The *R. capsulatus* cytochrome *c*₂ de-

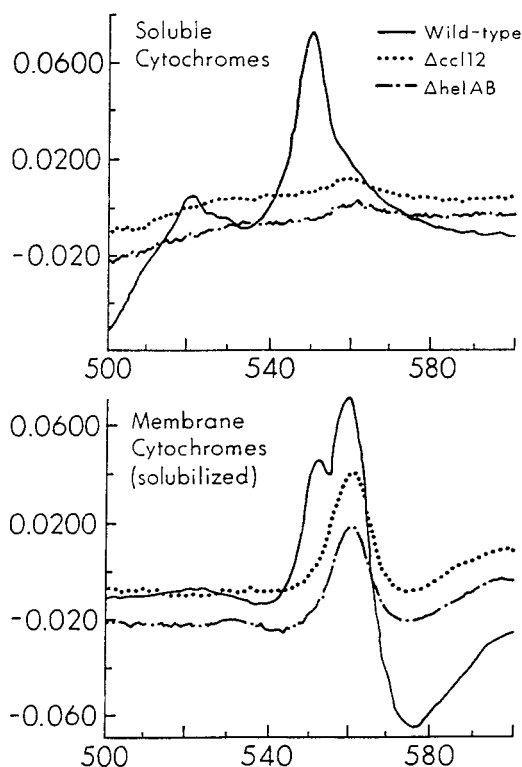


FIG. 3. *c*-type cytochromes are absent in spectra of *ccl* and *hel* mutants. Reduced-minus-oxidized spectra of soluble cytochromes (upper) and Triton X-100-solubilized membranes (lower) from mutant strains RGK292 ($\Delta ccl12$) (· · ·) and RGK277 ($\Delta helAB$) (— —) and wild-type strain SB1003 (—). Cells were grown aerobically to late log phase in minimal (RCV) medium. Cytochromes *c* absorb at approximately 550 nm; *b*-type cytochromes absorb at approximately 560 nm. The extracts in each spectrum contain approximately 5 mg of protein per ml.

letion strain ($\Delta cytA$, also called $\Delta cycA$) does not contain this antigen. Since the proteins for these Western blots are denatured in SDS, it is likely that the antiserum reacts with the unfolded forms of cytochrome *c*₂, including determinants that are not located at the heme ligation motif. None of the mutants in the cytochrome *c* biogenesis pathway have detectable cytochrome *c*₂ polypeptide, suggesting that neither the holo nor the apo form is present. We estimate that at least 100-fold less cytochrome *c*₂ than in the wild type could have been detected in these experiments. Since a cytochrome *c*₂-alkaline phosphatase fusion protein is secreted to wild-type levels in *hel* (3) and *ccl* mutants (specific activities are within 10% of the wild-type level), we conclude that the apocytochrome *c*₂ is rapidly degraded in the absence of assembly in the periplasm.

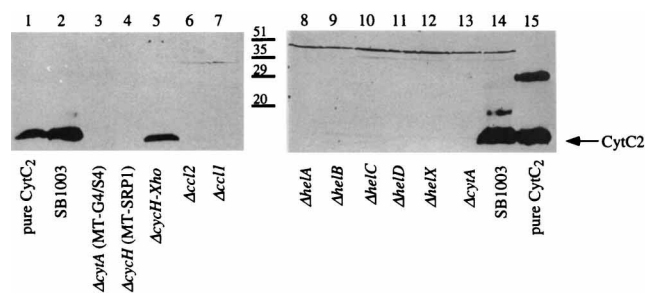


FIG. 4. Western analysis of *R. capsulatus* extracts, using an antiserum to cytochrome *c*₂. Lanes contain 26 μ g of protein of the soluble fraction of the indicated *R. capsulatus* strains. Ten nanograms of pure *R. capsulatus* cytochrome *c*₂ was loaded as a control in lanes 1 and 15. For construction of the *hel* strains, see reference 19); for construction of the *cycH* strains, see reference 31; for construction of $\Delta cytA$, see reference 9. The blot on the right was exposed for a longer period of time to increase sensitivity.

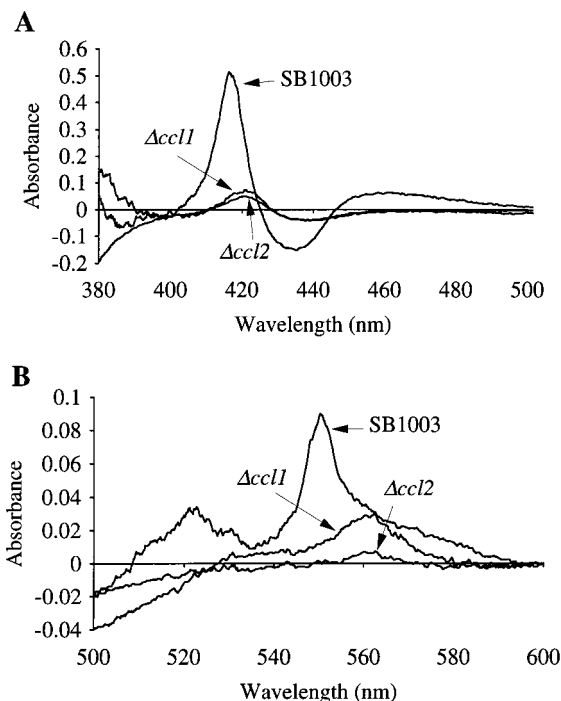


FIG. 5. Cytochrome *c*' is not made in Δccl strains. (A) Reduced-plus-carbon monoxide-minus-reduced spectra of the soluble fractions of wild-type, $\Delta ccl1$, and $\Delta ccl2$ strains. The minor absorption at approximately 420 nm in the $\Delta ccl2$ and $\Delta ccl1$ strains is due to contaminating *b*-type cytochromes that can also be observed in the lower spectra. (B) Reduced-minus-oxidized spectra of the extracts used for panel A. The extracts for each spectra contain approximately 5 mg of protein per ml.

tase fusion protein is secreted to wild-type levels in *hel* (3) and *ccl* mutants (specific activities are within 10% of the wild-type level), we conclude that the apocytochrome *c*₂ is rapidly degraded in the absence of assembly in the periplasm.

Cytochrome *c*' is a protein of unknown function in *R. capsulatus* that is structurally related to the periplasmic cytochrome *b*₅₆₂ of *E. coli* (7). While the function of cytochrome *b*₅₆₂ is also unknown, a major distinction is that in cytochrome *c*', the heme vinyl groups are covalently linked to the CysXxxYyyCys His motif present in the C terminus of the cytochrome. We have recently shown that synthesis of cytochrome *b*₅₆₂ of *E. coli* does not use the Hel/Ccl biogenesis pathway whereas endogenous *c*-type cytochromes require the *hel* and *ccl* genes (19). We wanted to determine if the *ccl* genes are required for the synthesis of cytochrome *c*' in *R. capsulatus*. The *ccl* mutants and wild-type strain were grown under identical conditions, and soluble fractions were tested for the presence of cytochrome *c*' by staining for heme after SDS-polyacrylamide gel electrophoresis. No *c*-type cytochromes were observed in the *ccl* mutants, including those that would migrate at the predicted size for cytochrome *c*', 14 kDa (data not shown). Cytochrome *c*' can also be detected by reduced-plus-carbon monoxide-minus-reduced spectroscopy (12). This cytochrome binds to CO and yields a difference spectrum at a maximum of approximately 418 nm. Wild-type *R. capsulatus* contains this cytochrome *c*', but the *ccl1* and *ccl2* mutants do not (Fig. 5A). The minor absorption at approximately 420 nm in the $\Delta ccl2$ and $\Delta ccl1$ strains is due to contaminating *b*-type cytochromes that are also observed (at 560 nm) in the reduced-minus-oxidized spectra of the same extracts (Fig. 5B).

Regulation of Ccl and Hel proteins in response to oxygen. We have generated antisera to some of the Hel and Ccl pro-

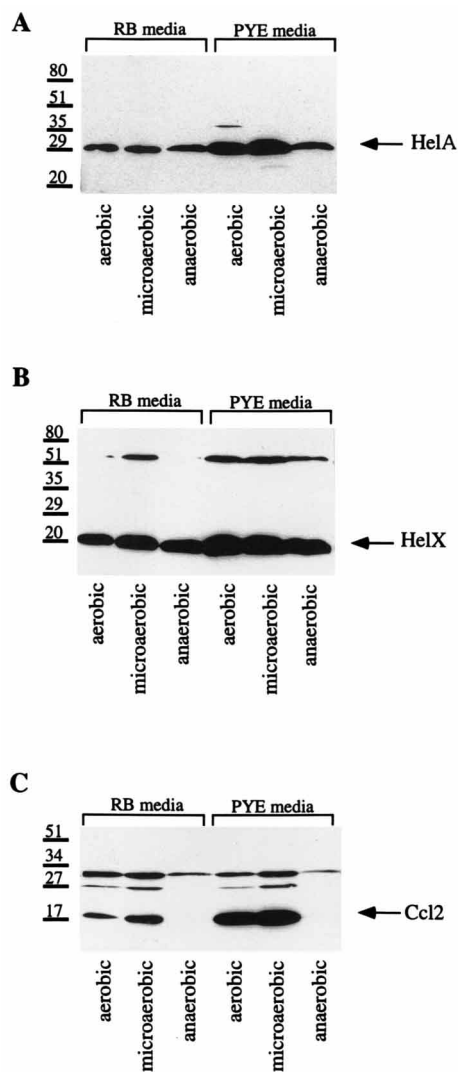


FIG. 6. Levels of the HelA, HelX, and Ccl2 proteins when wild-type *R. capsulatus* is grown aerobically, microaerobically, and anaerobically in RCV (RB) and PYE. Shown is Western analysis of extracts with 30 μ g of protein from wild-type *R. capsulatus* membrane fractions. Extracts were run on an SDS-17% polyacrylamide gel and transferred to nitrocellulose. (A) The HelA protein is produced constitutively under the various growth conditions. (B) The HelX protein is produced constitutively under the various growth conditions. A contaminating antigen at approximately 51 kDa is observed which is still present in *helX* deletion strains (unpublished data). (C) The Ccl2 protein is produced at 20-fold-higher levels under aerobic growth than under anaerobic growth. The polypeptides observed above the Ccl2 protein are actually *c*-type cytochromes that naturally stain with this peroxidase-based immunoblot during the time used for detection with Ccl2 antibodies. The polypeptide observed at approximately 27 kDa is associated with the aerobically induced cytochrome *cb*-type cytochrome *c* oxidase (22).

teins. Using these antisera, we have begun to explore whether expression differences may explain why some organisms contain the complete set of cytochrome *c* biogenesis genes in a single operon whereas others, such as *R. capsulatus*, have separate loci. Extracts of wild-type *R. capsulatus* cells grown under different conditions were assayed with antisera to Ccl2, HelX, and HelA to determine the levels of these proteins (Fig. 6). Six different modes of growth were analyzed: aerobic, microaerobic, and anaerobic (photosynthetic) in minimal and rich media. The minimal medium contained malate as the carbon source and ammonia as the source of fixed nitrogen, while the rich

medium contained peptone and yeast extract. A dramatic increase in Ccl2 levels is observed under aerobic or microaerobic environments (Fig. 6C). As discussed above, the sensitivity of the Ccl2 Western blots indicates that there is at least a 20-fold increase in Ccl2 levels when oxygen is present. In contrast, the HelA (Fig. 6A) and HelX (Fig. 6B) proteins are present at similar levels, aerobically or anaerobically, when grown in minimal medium. It is worth noting that all three proteins are made at higher levels in the rich (PYE) medium than in minimal medium under the same aeration levels. Although these differences are less than that observed for aeration differences, it suggests that more biogenesis components may be necessary in rich media.

To determine whether this effect is transcriptional, translational, or posttranslational, we initially used *ccl1-phoA* (pRGK246) and *ccl12-lacZ* (pRGK243) translational fusions as reporters. Alkaline phosphatase (for *ccl1-phoA*) and β -galactosidase (for *ccl1-ccl2-lacZ*) assays on *R. capsulatus* carrying these plasmid-borne fusions indicated that activities were approximately twofold higher in aerobic cells than in anaerobic cells (β -galactosidase activities of 464 ± 14 and 220 ± 40 nmol of *o*-nitrophenol formed per min per mg of protein [averages of three independent cultures \pm standard deviations], respectively; alkaline phosphatase activities of 50 ± 1 and 36 ± 1 U, respectively; in all cases, activities were <1 for plasmidless SB1003). Thus, transcription or translation does not appear to mediate the major aerobic effect. To look for major differences in transcript levels and to locate the promoter of the *ccl12* operon, primer extension analysis was carried out. Results of the primer extension study confirmed that the transcriptional effect does not explain the 20-fold difference (Fig. 7). A single transcript at similar levels is present in both the aerobic and anaerobic cells, although a twofold difference may not have been detected. The start site of this transcript is located approximately five nucleotides upstream of the *ccl1* ribosome binding site (Fig. 7).

DISCUSSION

The results presented above clearly demonstrate that the *ccl1* and *ccl2* genes in *R. capsulatus* form an operon and that they are required for the biogenesis of all *c*-type cytochromes, including cytochrome *c'*. Like the cytochrome *bc*₁ polypeptides, the cytochrome *c*₂ polypeptide is rapidly degraded in *ccl* and *hel* mutants. This result is similar to that observed for other systems. For example, a yeast isocytochrome *c* is degraded when heme is not attached (15). Thus, unlike some *b*-type cytochromes and hemoglobins (20), the bacterial *c*-type cytochromes must enter the heme ligation pathway, mediated by Hel, Ccl, and CycH proteins, before folding occurs.

The levels of the Ccl2 protein are dramatically increased (at least 20-fold) in the presence of oxygen. This effect is due to growth in the presence of oxygen and not the phase of growth. We have immunoblotted the Ccl2 protein in extracts of cells grown in aerobic and photosynthetic (anaerobic) cultures at different ODs (not shown). These results reveal no correlation to cell density. Moreover, the immunoblots shown in Fig. 6C clearly demonstrate that a correlation exists between the presence of the aerobic *bc*-type cytochrome *c* oxidase and the levels of Ccl2 protein (see the legend to Fig. 6). These results indicate that oxygen is a key determinant of Ccl2 levels. Although an antiserum to the Ccl1 protein is presently not available, the *ccl1-phoA* and *ccl1-ccl2-lacZ* reporter results indicate that only a minor component (twofold) of the oxygen effect is transcriptional or translational. Thus, if such plasmid-borne *pho* and *lac* expression can be used as a measure of transcription and trans-

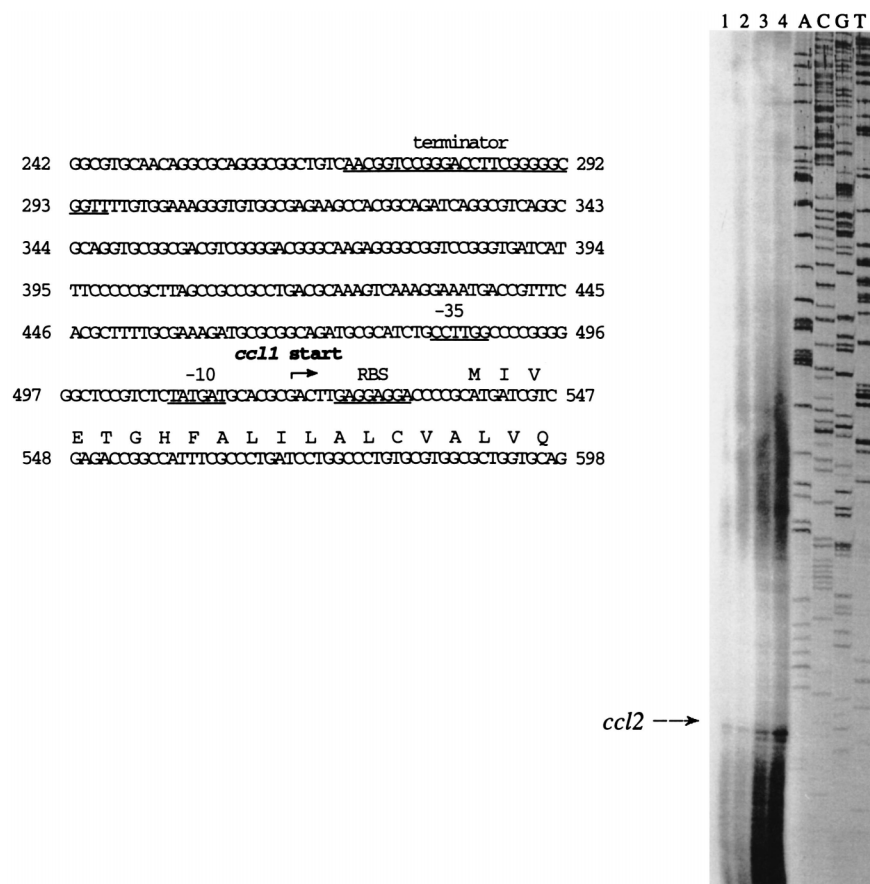


FIG. 7. Primer extension analysis of the *ccl2* operon in *R. capsulatus*. The start site was mapped to 5 bp upstream of the *ccl1* ribosome binding site. Each lane contains 50 μ g of RNA. Lane 1, SB1003 grown aerobically; lane 2, SB1003 grown anaerobically, lanes 3 and 4, the same as lanes 1 and 2 but with twice the amount of primer.

lation that is directed from the chromosome, we favor the hypothesis that the major mechanism explaining the high levels of Ccl2 under aerobic conditions is posttranslational. Studies to address this mechanism(s) are currently in progress. From a physiological viewpoint, it is worth considering that increased levels of Ccl2 (and possibly Ccl1) in an aerobic environment are required for increased protection of the heme and apocytochrome *c* cysteine residues from oxidation. We have shown recently that the Ccl2 protein is a thioredox protein that contains two essential cysteine residues (33). The Ccl2 protein is oxidized by an apocytochrome *c* peptide containing the CysXxxYyyCysHis motif. It is envisioned that the Ccl2 protein directly reduces the apocytochrome cysteine residues prior to ligation to heme. The Ccl1 protein is presumed to present heme to this complex via a conserved heme-binding domain that is also present in HelC (3). Thus, these two key proteins, Ccl1 and Ccl2, are positioned within the pathway to specifically interact with and protect the substrates, heme and apocytochromes *c*. We suggest that modulation of their levels is consistent with these key roles in biogenesis. Another result that suggests a fundamental role for these proteins is the inability to find suppressor strains (to photosynthetic growth) in any of the *ccl* deletions described here (unpublished data). This is in contrast to *cycH* and *helX* deletion mutants, for which suppressors are easily isolated.

It is probable that bacteria (and eukaryotes) that use and synthesize *c*-type cytochromes in aerobic environments have

evolved mechanisms to protect the substrates from oxidation during biogenesis. The results presented here provide the first clue to how a bacterium may adjust the biogenesis pathway such that it can synthesize these heme proteins under drastically different and rapidly changing environments. Interestingly, none of the *hel* or *ccl* genes have homologs in the yeast genome (discussed in reference 19). Rather, yeasts, vertebrates, and invertebrates have the cytochrome *c* heme lyase gene (*CYT3*) first discovered in *Saccharomyces cerevisiae* (14). These organisms may maintain a relatively reduced milieu at the site of cytochrome *c* synthesis, the mitochondrial intermembrane space. The lyase may recognize reduced heme (39) and apocytochrome *c* and act as a scaffold for assembly. On the other hand, the Ccl1, HelB, and HelC proteins are present in the mitochondria of plants (5, 21, 24, 26). This complex biogenesis system may have been retained in plants because of the ability of the components to protect the assembly process during times of oxygen evolution within the cell.

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