Roles for the *Rhodobacter sphaeroides* CcmA and CcmG Proteins

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***Rhodobacter sphaeroides* cells containing an in-frame deletion within *ccmA* lack detectable soluble and membrane-bound *c*-type cytochromes and are unable to grow under conditions where these proteins are required. Only strains merodiploid for *ccmABCDG* were found after attempting to generate cells containing either a *ccmG* null mutation or a *ccmA* allele that should be polar on to expression of *ccmBCDG*, suggesting that CcmG has another important role in *R. sphaeroides*.**

Because of their vital role in energy generation, there is considerable interest in determining how cytochromes bind their essential heme cofactor. Among cytochromes, *c*-type cytochromes are unique because heme is covalently attached to two cysteine thiolates of the polypeptide (22). From analyzing a series of genes (*ccm*) that are required for *c*-type cytochrome maturation (13, 31), it appears that a *c*-type cytochrome precursor protein and heme are translocated across the cytoplasmic membrane by the general export pathway and some combination of CcmABCD, respectively. Once exported, the cysteine thiolates at the *c*-type cytochrome heme attachment site are believed to form an intramolecular disulfide bond that must be reduced prior to covalent heme attachment (13, 31). CcmG is proposed to act in a pathway which facilitates the reduction of this disulfide bond because it contains a pair of redox active thiols (26) and has amino acid sequence similarity to disulfide oxidoreductases in the thioredoxin family (13, 31). The fact that some Ccm mutants have defects in assembly of heme *a*-containing proteins (19) or iron chelator production (9) underscores the importance of defining whether these proteins participate in other processes.

To assess the role of *Rhodobacter sphaeroides* Ccm proteins, we made mutations within the *ccmABCDG* locus. This facultative phototroph uses *c*-type cytochromes to generate energy when growing by aerobic respiration, by photosynthesis, or by anaerobic respiration (4, 16, 17). *R. sphaeroides* CcmA is involved in *c*-type cytochrome maturation since a nonpolar mutation in *ccmA* caused the loss of this class of electron carrier and an inability to grow under conditions when these proteins are required to generate energy. However, CcmG could have other roles in *R. sphaeroides* since strains containing a polar insertion in *ccmG* or a mutation in *ccmA* that should be polar on to expression of *ccmBCDG* were not isolated.

Growth and genetic procedures. *R. sphaeroides* was grown at 30°C in Sistrom's medium A (3, 28) containing spectinomycin (25 µg/ml), kanamycin (25 µg/ml), or tetracycline (1 µg/ml) when necessary. Sistrom's medium A containing 7.5% sucrose was used to screen for sucrose sensitivity. *Escherichia coli* was

grown at 37°C in Luria-Bertani medium (24) with appropriate combinations of ampicillin (50 µg/ml), spectinomycin (25 µg/ml), kanamycin (25 µg/ml), tetracycline (10 µg/ml), or chloramphenicol (50 µg/ml). *E. coli* DH5α (Table 1) was used as a plasmid host while S17-1 was used for plasmid transfer (2). Mating pairs were plated aerobically on Sistrom's minimal medium A containing appropriate antibiotics to obtain exconjugants.

Construction of probes and plasmids. Plasmid p2-9 was used to generate *Rhodobacter capsulatus ccmABC* and *ccmCD* probes (12). *R. sphaeroides ccmABCDG* was cloned as an ~9.5-kb *Pst*I restriction endonuclease fragment from pUI8298 (5) into pUC18 (pCSP4) or pRK415 (pRK4S). The ~3.5-kb and ~1.1-kb *ccm*-containing *Bam*HI restriction endonuclease fragments from pCSP4 were separately cloned into pUC18 (pCSP20 and pCSP110-1, respectively). For DNA sequencing of the *ccm* locus (Fig. 1) (GenBank accession number U83136), either *lac*- or *R. sphaeroides*-specific primers were used.

A spectinomycin resistance gene (*spc*) (7) was inserted as a *Bam*HI restriction endonuclease fragment into a unique *Bgl*II site of pCSP20 to create the *ccmAI::spc* allele (pCSP20Ω1). A *Bam*HI restriction fragment containing *ccmAI::spc* from pCSP20Ω1 was purified, made blunt with T4 DNA polymerase I, and ligated into a blunt *Eco*RI site of the mobilizable suicide plasmid, pSUP202 (pCSP91). To generate *ccmGI::spc*, *spc* (7) was inserted as a *Sma*I restriction fragment into a unique *Bsp*EI site of pCSP4 (pTP1). A *Pst*I restriction fragment from pTP1 containing *ccmGI::spc* was cloned into the *Pst*I site of pSup202 (pTP2).

Mutant isolation. Suicide plasmids were mated into *R. sphaeroides* (5) and Sp^r cells were screened for Tc^s under aerobic conditions to identify strains where *ccmAI::spc* or *ccmGI::spc* was incorporated by an even number of crossover events (resulting in loss of Tc^r).

Strains containing both a wild-type *ccmABCDG* locus and the *ccmAI::spc* or *ccmGI::spc* alleles (Sp^r, Tc^r strains CCMA2 and CCMG2, respectively) were grown aerobically in antibiotic-free liquid medium prior to plating onto antibiotic-free agar in the presence of O₂. Colonies were screened for Sp or Tc resistance to determine whether the *ccmAI::spc* or *ccmGI::spc* alleles (Sp^r) or the suicide plasmid (Tc^r) was present.

An in-frame deletion of *ccmA* codons 10 to 173 (*ccmA2*) was constructed by a two-step PCR process (17). Primers 5'-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
Strains		
<i>R. sphaeroides</i>		
2.4.1	Wild type	Laboratory strain
CCMA1	<i>ccmA1::Ωspc</i>	This work
CCMA2	<i>ccmA1::Ωspc</i> -pSUP202 (Tc ^r ; merodiploid)	This work
CCMA3	<i>ccmA2::pLO1</i> (Kn ^r sucrose ^s ; merodiploid)	This work
CCMA4	Δ <i>ccmA</i> because it contains the <i>ccmA2</i> allele	This work
CCMG2	<i>ccmG1::Ωspc</i> -pSUP202 (Tc ^r ; merodiploid)	This work
<i>E. coli</i>		
DH5 α	Plasmid maintenance	1
S17-1	Donor for plasmid transfer to <i>R. sphaeroides</i>	27
Plasmids		
p2-9	<i>R. capsulatus ccmABC</i>	12
pCSP4	Ap ^r ; ~9.5-kb <i>Pst</i> I <i>ccmABCDG</i> fragment cloned in pUC18 in opposite orientation as P _{lac}	This work
pCSP20	Ap ^r ; ~3.5-kb <i>Bam</i> HI <i>ccm</i> fragment cloned in pUC18 in opposite orientation as P _{lac}	This work
pCSP20 Ω 1	Ap ^r ; pCSP20 containing <i>spc</i> cloned into <i>ccmA</i> (source of <i>ccmA1::spc</i> allele)	This work
pCSP91	Ap ^r Sp ^r ; ~5.5-kb <i>Bam</i> HI fragment containing <i>ccmA1::spc</i> from pCSP20 Ω 1 cloned in pSup202	This work
pHP45 Ω	Source of <i>spc</i> gene	7
pLO1	Kn ^r ; <i>sacB</i> ; RP4 <i>oriT</i> ; ColE1 <i>ori</i>	14
pRC10	Kn ^r ; ~1.5-kb fragment containing <i>ccmA2</i> cloned into the <i>Pme</i> I site of pLO1	This work
pRK4S	Tc ^r ; ~9.5-kb <i>Pst</i> I fragment containing <i>ccmABCDG</i> cloned into pRK415	This work
pRK415	Tc ^r ; broad-host-range plasmid	11
pSUP202	Ap ^r Tc ^r Cm ^r Mob ⁺ ; pBR325 derivative	27
pTP1	Ap ^r ; ~11.5-kb <i>Pst</i> I fragment containing <i>ccmG1::spc</i> in pUC18	This work
pTP2	Tc ^r ; Sp ^r ; <i>ccmG1::spc</i> from pTP1 cloned into <i>Pst</i> I site of pSUP202	This work
pUI8298	Tc ^r ; ~29-kb fragment with <i>ccmABCDG</i> on pLA2917	This work
pUC18	Ap ^r ; cloning vector	32

CGGCAGTCCGAGATCGATGTGGGCGAGGTTGGTGAC GGT (CcmA-5' tail) and 5'-CGGTCAACGAGACGCTGA3' (CcmA-*Hinc*II 5') were used to generate a PCR product containing ~0.8 kb of upstream DNA and *ccmA* codons 1 to 9 fused in-frame to codons 174 to 179. To generate a PCR product that contains *ccmA* codons 4 to 9 fused in-frame to codons 174 to 210 plus ~0.75 kb of downstream DNA, primers 5'-ACCGTCAACCAACCTCGCCACATCGATCTCGGACT GGCC (CcmA-3' tailed) and 5'-GAATGGGGTCGCGAC ATC (CcmA-*Nru*I 3') were used. These two PCR products were purified, mixed with CcmA-*Hinc*II 5' and CcmA-*Nru*I 3', and used in a second round of PCR to generate a product that contained *ccmA2* flanked by ~0.80 kb and ~0.75 kb of upstream and downstream DNA. This product was purified, ligated into the *Pme*I site of the mobilizable suicide vector, pLO1 (pRC10), sequenced to verify the in-frame deletion of *ccmA* codons 10 to 173, and mated into *R. sphaeroides*. A resultant Kn^r, sucrose-sensitive exconjugant (CCMA3) was grown aerobically in the absence of antibiotics prior to plating ~10⁶ cells aerobically on medium supplemented with 7.5% sucrose. After 5 to 7 days of incubation, sucrose-resistant cells were screened for loss of Kn^r (from pLO1). DNA from sucrose-resistant, Kn^s cells was amplified and sequenced to verify that cells contained the *ccmA2* allele and no other mutations within this gene.

Cytochrome analysis. Soluble and membrane fractions were prepared from cultures grown under an atmosphere of 30% O₂, 69% N₂, 1% CO₂. Protein concentrations were determined by the Bio-Rad Protein Assay (Hercules, Calif.). Samples (~400 μ g of protein) were heated (70°C) for 10 min in the presence of 5% β -mercaptoethanol and analyzed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. After

electrophoresis, *c*-type cytochromes were visualized by peroxidase activity (30).

The *R. sphaeroides ccmABCDG* locus. Hybridization of *R. sphaeroides* DNA with *R. capsulatus ccmABCDG*-specific probes identified a related ~9.5-kb *Pst*I restriction fragment in genomic DNA and a wild-type gene library (pUI8298). When *R. sphaeroides* DNA was analyzed by pulse-field electrophoresis (29) and probed with *ccmABC*, this locus mapped to coordinate ~1500 on chromosome I (data not shown).

The DNA sequence of this region predicts the existence of seven complete open reading frames with codon usage typical of *R. sphaeroides* genes (Fig. 1). *R. sphaeroides* CcmA, CcmB, and CcmC each have significant amino acid similarity to other presumed family members (13, 31). Immediately downstream of *ccmABC* are two other genes (*ccmDG*) that could also encode Ccm homologues. The function of CcmD is unknown since the deduced polypeptide bears no significant relationship to proteins with known activity. CcmG has significant amino acid similarity to other family members, including a conserved sequence that is related to the active site of thioredoxin-type disulfide oxidoreductases. *R. sphaeroides ccmABCDG* could be cotranscribed since the initiation and termination codons for *ccmAB*, *ccmCD*, and *ccmDG* overlap (Fig. 1).

It appears that there are no other *ccm* genes in this region (Fig. 1). Upstream of *yheA*, which is not required for *c*-type cytochrome assembly in *R. capsulatus* (12), is a gene that encodes a predicted homologue of the general secretory protein, SecF (Fig. 1). Immediately downstream of *ccmABCDG*, and transcribed in the opposite direction (Fig. 1), is a gene that encodes a homologue of the tricarboxylic acid cycle enzyme aconitase.

Analysis of *R. sphaeroides* cells lacking CcmA. To test if these presumed *ccm* gene products functioned in *c*-type cyto-

FIG. 1. The *R. sphaeroides* *ccmABCDG* locus. Shown is the genetic organization of this locus (not drawn to scale; see below), the predicted direction of transcription (arrows), and the function of presumed gene products (bottom). Shaded boxes indicate where the predicted termination codon of one gene (*ccmA*, *ccmC*, and *ccmD*) overlaps the presumed initiation codon of the next gene (*ccmB*, *ccmD*, and *ccmG*, respectively).

chrome maturation, a strain containing an in-frame deletion within *ccmA* was generated (CCMA4). DNA sequence showed that this strain contains the *ccmA2* allele and that the coding overlap between *ccmA* and *ccmB* was preserved, so this mutation should not have a negative effect on expression of any downstream genes. Cells lacking CcmA grew under aerobic but not photosynthetic or anaerobic respiratory conditions, the phenotypes predicted for *R. sphaeroides* cells that lack *c*-type cytochromes. Also, growth under these conditions was restored when this mutant contained *ccmABCDG* on a stable, low-copy-number plasmid.

To test if the *ccmA2* allele caused the lack of *c*-type cytochromes, the accumulation of these proteins was measured in extracts from aerobically grown cells, a condition where *R. sphaeroides* contains several *c*-type cytochromes (4, 8, 17). The soluble and membrane proteins of wild-type cells that retain peroxidase activity under denaturing conditions (i.e., *c*-type cytochromes) were not detected in samples prepared from cells lacking CcmA (Fig. 2). In addition, reduced minus oxidized spectra of soluble and membrane extracts from aerobically grown cells which lack CcmA lacked the absorption feature that is present at ~550 nm in wild-type cells and that is diagnostic of the *c*-type cytochromes (data not shown; 4, 8, 17).

Could CcmG have additional roles in *R. sphaeroides*? To test the role of other products of this locus, we attempted to place the *ccmA1::spc* allele (which should be polar on to expression of *ccmBCDG*) in the genome. Of 1,700 Sp^r cells screened for Tc^s (Tc^r encoded by the suicide plasmid) under aerobic conditions, only one strain (CcmA1) was identified that had lost the suicide plasmid. Control experiments with a *cycA::kan* allele on a similar suicide plasmid (7) found that ~5% of the Kn^r cells (25/500) lost the suicide plasmid (i.e., became Tc^s), so the frequency at which CcmA1 was obtained is below what is typically observed when generating null mutations by marker exchange in *R. sphaeroides* (2). When CcmA1 was analyzed further, it was found to contain a duplication of the *ccmABCDG* locus, grow via photosynthesis, and contain a normal complement of *c*-type cytochromes (data not shown). Thus, the only strain from which the suicide plasmid was lost contained a second intact copy of the *ccmABCDG* locus. We also attempted to place a *ccmG1::spc* allele in wild-type cells. In this case, when Sp^r cells were screened for Tc^s , none were found among ~3,100 independent Sp^r isolates.

R. sphaeroides CcmG contains a motif found at the active site of disulfide oxidoreductases in the thioredoxin family. This, plus the ability of the *R. capsulatus* CcmG homologue to form an intramolecular disulfide bond (26), has been taken as provisional evidence that this protein has disulfide oxidoreduc-

tase activity. Thus, it is not surprising that supplementing growth media with low-molecular-weight thiols (cysteine, cystine, 2-mercaptoethanesulfonic acid, or dithiothreitol) can partially rescue CcmG mutants in some species (6, 15, 20), presumably because these thiols substitute for the proposed disulfide oxidoreductase activity of this protein. Adding cysteine, cystine, or dithiothreitol to media did not enable us to generate a strain lacking CcmG using either *ccmG1::spc* or the

FIG. 2. Heme peroxidase staining of soluble and membrane samples from aerobically grown cells. For each sample, ~400 μg of protein was heated at 70°C in the presence of 5% 2-mercaptoethanol and separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. Lanes 1 and 3 contain soluble and membrane samples from wild-type cells (2.4.1), respectively; lanes 2 and 4 contain soluble and membrane samples from cells lacking CcmA (CCMA4), respectively. Western blot analysis with antiserum against individual *c*-type cytochromes (data not shown) indicates that the soluble heme-staining protein in wild-type cells is mostly a combination of cytochrome c_2 and cytochrome c_{554} . Numbers at left are molecular weight markers (in thousands).

TABLE 2. Use of a merodiploid to generate null mutations at *ccmABCDG* or *cycA*

Strain/gene	No. of colonies found with genotype:		
	Loss of function (Tc ^r)	Merodiploid (Sp ^r Tc ^r)	Wild type (Sp ^r Tc ^r)
CYCA2/ <i>cycA</i>	24,913	42	45
CCMG2/ <i>ccmG</i>	0	39,985	15
CCMA2/ <i>ccmA</i>	0	19,956	44

ccmA1::spc allele that should block expression of *ccmBCDG* (data not shown).

The inability to obtain cells lacking CcmG could also reflect a low frequency of recombination in this region. To increase the probability of finding cells that arise from possibly rare recombination events, Tc^r cells containing either the *ccmA1::spc* or *ccmG1::spc* allele and a wild-type *ccmABCDG* locus (CCMA2 and CCMG2, respectively) were grown aerobically for ~20 generations in antibiotic-free media prior to screening for markers linked to the *ccm* mutation (Sp^r) or the suicide plasmid (Tc^r). After screening ~20,000 independent CCMA2 (*ccmA1::spc*) or ~40,000 CCMG2 (*ccmG1::spc*) colonies, no cells were found that had lost the suicide plasmid while retaining the mutant *ccm* allele (Tc^r Sp^r; Table 2). This procedure resulted in the generation of numerous cytochrome *c*₂ mutants using a strain that contains both a *cycA1::kan* allele and a wild-type *cycA* gene (Table 2). When the failure to isolate cells lacking CcmG at a reasonable frequency in controlled experiments is considered, it leads us to consider the possibility that this protein plays a role in some other important cellular process.

What additional functions might CcmG provide *R. sphaeroides*? If CcmG is a disulfide oxidoreductase, there are several reasons why *R. sphaeroides* might need such a periplasmic activity for purposes in addition to *c*-type cytochrome maturation. *R. sphaeroides* is believed to require a functional electron transport chain since it cannot generate sufficient energy to grow solely by substrate-level phosphorylation (25). Thus, one possibility is that CcmG also aids assembly of another respiratory enzyme. Indeed, a *Paracoccus denitrificans* ΔCcmG mutant contains reduced levels of the cytochrome *aa*₃ complex (19). Of the five terminal oxidases believed to be encoded in the *R. sphaeroides* genome, four of these enzymes are known or presumed to contain *c*- or *a*-type heme (<http://genome.ornl.gov/microbial/rsph/>). Thus, loss of CcmG could prevent *R. sphaeroides* from producing sufficient quantities of one or more terminal oxidases that are required to support respiration under our laboratory conditions. It is also possible that CcmG acts in a general pathway for the assembly of other periplasmic enzymes, in a manner reminiscent of the Dsb-dependent folding of extracytoplasmic proteins (23). In this regard, there is evidence that the CcmG homologue of *E. coli* interacts with DsbD (10). The cytoplasmic disulfide oxidoreductase, thioredoxin, is essential in *R. sphaeroides* (21), so this α-proteobacterium may also have roles for a periplasmic disulfide oxidoreductase that are more far-reaching than those in well-studied enteric bacteria (23).

In summary, the *R. sphaeroides ccmABCDG* gene cluster is generally similar to those found in other α-proteobacteria.

CcmA is required for *c*-type cytochrome maturation, but it appears that CcmG has a function outside its commonly accepted role in the assembly of these electron carriers. In the future, mutants lacking CcmA or other Ccm proteins will facilitate determining how they function in *c*-type cytochrome maturation. These strains will also help identify components of this bacterium's aerobic respiratory chain and dissect the pathway that couples aerobic electron transport to global changes in gene expression (18).

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