Roles for the *Rhodobacter sphaeroides* CcmA and CcmG Proteins

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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).

R. *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*.

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), 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 pU18298 (5) into pUC18 (pCSP4) or pRK415 (pRK485). 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 *ccmA::spc* allele (pCSP201). A *Bam*HI restriction fragment containing ccmG1::spc from pCSP201 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 ccmG1::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 ccmG1::spc was cloned into the *Pst*I site of pSup202 (pTP2).

**Mutant isolation.** Suicide plasmids were mated into *R. sphaeroides* (5) and Sp⁺ cells were screened for Te⁺ under aerobic conditions to identify strains where ccmA::spc or ccmG1::spc was incorporated by an even number of crossover events (resulting in loss of Te⁺).

Strains containing both a wild-type *ccmABCDG* locus and the ccmA::spc or ccmG1::spc alleles (Sp⁺, Te⁺ 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 Te resistance to determine whether the ccmA::spc or ccmG1::spc alleles (Sp⁺) or the suicide plasmid (Te⁺) was present.

An in-frame deletion of ccmA codons 10 to 173 (ccmA2) was constructed by a two-step PCR process (17). Primers 5′-
CGCGAGTCGAGATCGTGGGCGAGGTTGGTAGAC
GGT (CcmA-5' tail) and 5'-CCGTTCAACGAGCAGGGTAGA3' (CcmA-HincII 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'-ACCGTCGAACACCTGCCACATCGATCTCGGACT
GGCC (CcmA-3' tail) and 5'-GAATGGGGTCGCGAC
TGTAATCCACGTGATGAGAAGA3' were used. These two PCR products were purified, mixed with CcmA-HincII 5' and CcmA-NruI 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 PmeI site of the mobilizable suicide vector, pLO1 (pRC10), sequenced to verify the in-frame deletion of ccmA and used in a second round of PCR to generate a product that contains ccmA2 cloned into the PmeI site of pLO1. The resultant Kn' cells, sucrose-sensitive exconjugant (CCMA3) was grown aerobically in the absence of antibiotics prior to plating on September 8, 2017 by guest http://jb.asm.org/ Downloaded from
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 Psrl restriction fragment in genomic DNA and a wild-type gene library (pU18298). 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 sheA, 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 aconitate.

Analysis of R. sphaeroides cells lacking CcmA. To test if these presumed ccm gene products functioned in c-type cyto-
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 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 lacking 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 ccmABCDG) in the genome. Of 1,700 Sp r cells screened for ccmA1::spc allele in wild-type cells. In this case, when Sp r cells were screened for Tc r, 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 oxidoreductase 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. 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).](http://jb.asm.org/)

![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, 11); 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 c2 and cytochrome c554. Numbers at left are molecular weight markers (in thousands).](http://jb.asm.org/)
generally similar to those found in other a- proteobacteria. CcmG 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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REFERENCES