Roles for the *Rhodobacter sphaeroides* CcmA and CcmG Proteins

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Received 6 March 2001/Accepted 16 May 2001

*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 where 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 (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 *PstI* restriction endonuclease fragment from pU8298 (5) into pUC18 (pCSP4) or pRK415 (pRK4S). The ~3.5-kb and ~1.1-kb *ccm*-containing *BamHI* 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 *BamHI* restriction endonuclease fragment into a unique *BglII* site of *R. sphaeroides* ccmABCDG to create the *ccmAI*::*spc* allele (pCSP20). A *BamHI* restriction fragment containing *ccmAI*::*spc* from pCSP2001 was purified, made blunt with T4 DNA polymerase I, and ligated into a blunt *EcoRI* site of the mobilizable suicide plasmid, pSUP202 (pCSP91). To generate *ccmG1*::*spc*, *spc* (7) was inserted as a *SmaI* restriction fragment into a unique *BspEI* site of pCSP4 (pTP1). A *PstI* restriction fragment from pPT1 containing *ccmG1*::*spc* was cloned into the *PstI* site of pSup202 (pTP2).

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

Strains containing both a wild-type *ccmABCDG* locus and the *ccmAI::spc* or *ccmG1::spc* alleles (Sp', Tc') 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 *ccmG1::spc* alleles (Sp') or the suicide plasmid (Tc') was present.

An in-frame deletion of *ccmA* codons 10 to 173 (*ccmA2*) was constructed by a two-step PCR process (17). Primers 5'-
CGGAGTCCGAGATGATGGCCGAGTTGTTGAC
GGT (Cma-A5' tail) and 5'-CCGTTAAGGAGACCGTTCGAG3' (CcmA-HincII 5') were used to generate a PCR product containing ~0.8 kb of upstream DNA and cmaA codons 1 to 9 fused in-frame to codons 174 to 179. To generate a PCR product that contains cmaA codons 4 to 9 fused in-frame to codons 174 to 210 plus ~0.75 kb of downstream DNA, primers 5'-AACGTCACCCACCTGCCCACATCGATCTGGCCTGAC
GGCC (Cma-A3' tail) and 5'-GAATGGGGTCGCGACT
were used. These two PCR products were purified, mixed with CmaA-HincII 5' and CmaA-HincII 3', and used in a second round of PCR to generate a product that contained cmaA2 flanked by ~0.80 kb and ~0.75 kb of upstream and downstream DNA. This product was purified, ligated into the pM1 site of the mobilizable suicide vector, pL01 (pRC10), sequenced to verify the in-frame deletion of cmaA. A resultant Kn'-sensitive exconjugant (CCMA1) was isolated and used in a second round of PCR to generate a product that contained cmaA2 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 μg 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 PstI 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 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 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 *ccmA2* 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 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 a *ccmA1::spc* allele (which should be polar on to expression of *ccmBCDG*) in the genome. Of 1,700 Sp^r^ cells screened for Tc^r^ (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^r^), 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. We also attempted to place a *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
cmmA1::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, TcR cells containing either the ccmA1::spc or ccmG1::spc allele and a wild-type ccmABCDG locus (CCM2A and CCM2G, respectively) were grown aerobically for ~20 generations in antibiotic-free media prior to screening for markers linked to the ccm mutation (SpR) or the suicide plasmid (TcR). After screening ~20,000 independent CCM2A (ccmA1::spc) or ~40,000 CCM2G (ccmG1::spc) colonies, no cells were found that had lost the suicide plasmid while retaining the mutant ccm allele (TcR SpR; Table 2). This procedure resulted in the generation of numerous cytochrome c2 mutants using a strain that contains both a cyaA1::kan allele and a wild-type cyaA 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 aa3 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/rspb/). 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. 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).

This work was supported by NIH grant GM37509 to T.J.D. We thank R. Kranz for supplying R. capsulatus ccm probes and T. Pastijn for help in constructing mutants.

REFERENCES


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

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