

Published in final edited form as:

Mol Microbiol. 2008 November ; 70(3): 652–666. doi:10.1111/j.1365-2958.2008.06441.x.

Compensatory Thio-redox Interactions Between DsbA, CcdA and CcmG Unveil the Apocytochrome *c* Holdase Role of CcmG During Cytochrome *c* Maturation

Serdar Turkarslan^{*}, Carsten Sanders^{*}, Seda Ekici, and Fevzi Daldal[†]

Department of Biology, Plant Science Institute, University of Pennsylvania, PA, 19104, USA

Summary

During cytochrome *c* maturation (Ccm), the DsbA-dependent thio-oxidative protein-folding pathway is thought to introduce a disulfide bond into the heme-binding motif of apocytochromes *c*. This disulfide bond is believed to be reduced through a thio-reductive pathway involving the Ccm components CcdA (DsbD), CcmG and CcmH. Here, we show in *Rhodobacter capsulatus* that in the absence of DsbA cytochrome *c* levels were decreased and CcdA or CcmG or the putative glutathione transporter CydDC were not needed for Ccm. This decrease was not due to overproduction of the periplasmic protease DegP as a secondary effect of DsbA absence. In contrast, CcmH was absolutely necessary regardless of DsbA, indicating that compensatory thio-redox interactions excluded it. Remarkably, the double (DsbA-CcmG) and triple (DsbA-CcmG-CcdA) mutants produced cytochromes *c* at lower levels than the DsbA-null mutants, unless they contained a CcmG derivative (CcmG^{*}) lacking its thio-reductive activity. Purified CcmG^{*} can bind apocytochrome *c* *in vitro*, revealing for the first time a thiol-independent, direct interaction between apocytochrome *c* and CcmG. Furthermore, elimination of the thio-redox components does not abolish cytochrome *c* production, restricting the number of Ccm components essential for heme-apocyt *c* ligation *per se* during Ccm.

Keywords

cytochrome *c* maturation; thio-oxidation; thio-reduction; *Rhodobacter capsulatus*; photosynthetic and respiratory electron transfer

Introduction

The cytochromes (cyts) *c* are membrane-attached or soluble ubiquitous electron carrier proteins essential for energy metabolisms of almost all organisms. They contain Fe-protoporphyrin IX (heme), which is stereo-specifically and covalently attached through two thioether bonds between the heme vinyl groups and the cysteine sulfhydryls of the apocyt *c* C₁XXC₂H motif. The covalent heme-apocyt *c* ligation is carried out by a post-translation and post-export process called cyt *c* maturation (Ccm), which involves in most gram-negative bacteria ten essential components (CcmABCDEFGHI and CcdA (or DsbD) (Barker and Ferguson, 1999; Thony-Meyer, 2000). During Ccm, it is thought that the cysteine thiols of apocyt *c* C₁XXC₂H motif are first oxidized by the DsbA-dependent extracytoplasmic thio-oxidative protein-folding pathway (Fig. 1) (Allen *et al.*, 2003b; Kranz *et al.*, 1998; Thony-Meyer, 2002). Subsequent reduction of this disulfide bond then becomes a

[†]Corresponding author: Phone: 215-898-4394, Fax: 215-898-8780, Email: fdaldal@sas.upenn.edu.

^{*}Both authors contributed equally to this work.

prerequisite for heme ligation (Daltrop *et al.*, 2002). The reduction is accomplished by a thio-reductive pathway consisting of CcdA, CcmG and CcmH (Monika *et al.*, 1997). In *Rhodobacter capsulatus*, CcdA transfers electrons from cytoplasmic thioredoxins to CcmG (Katzen and Beckwith, 2000; Katzen *et al.*, 2002). CcmG is believed to be re-oxidized by shuttling its electrons to CcmH, which then transfers them to apocyts *c* (Fig. 1) (Fabianek *et al.*, 1999; Monika *et al.*, 1997).

Formation of a disulfide bond in the apocyt *c* C₁XXC₂H motif occurs *in vitro*, and is considered to be an intermediate of Ccm (Daltrop *et al.*, 2002). However, whether this is always the case *in vivo* is unclear. Initially, DsbA and DsbB mutants in *E. coli* were identified as unable to produce cyts *c* (Metheringham *et al.*, 1996). Recent works in *E. coli* (Allen *et al.*, 2003a; Kojima *et al.*, 2005) and other bacteria (Erlendsson and Hederstedt, 2002; Deshmukh *et al.*, 2003) indicated that DsbA- and DsbB-null mutants are Ccm proficient. Furthermore, the lack of DsbA or DsbB functions suppresses the cyt *c* deficiency of CcdA-null mutants in *B. subtilis* or *R. capsulatus* (Deshmukh *et al.*, 2003; Erlendsson and Hederstedt, 2002), demonstrating that the thio-reductive branch becomes dispensable in the absence of the thio-oxidative pathway. Therefore, why a thio-redox loop (*i. e.*, thio-oxidation followed by thio-reduction) occurs at the apocyt *c* heme binding motifs during Ccm remains unclear (Fig. 1). Here, we examined cyt *c* production without such a thio-redox loop in *R. capsulatus*. We found that mutants lacking DsbA produced less cyts *c*, but this decrease was not due to the overproduction of periplasmic protease DegP that occurs in the absence of DsbA in *R. capsulatus* (Onder *et al.*, 2008). Moreover, when the DsbA-dependent thio-oxidative pathway was compromised, only CcmH, but neither CcdA nor CcmG, was required for heme-apocyt *c* ligation. The compensatory thio-redox interactions were also independent of the glutathione transporter CydDC (Pittman *et al.*, 2005), unlike in *E. coli* (Poole *et al.*, 1994). Remarkably though, the DsbA-CcmG double and DsbA-CcmG-CcdA triple mutants produced consistently lower levels of cyt *c* than the DsbA-null mutants, unless they contained a CcmG derivative devoid of its thio-reductive activity. This finding revealed a second role of CcmG acting as an apocyt *c* holdase during Ccm in addition to its thio-reductive function. Our findings show that the thio-redox pathway is important, but not essential, for cyt *c* production, and reduce the complex Ccm apparatus to those that are absolutely required for heme-apocyt *c* ligation *per se* in *R. capsulatus*.

Results

R. capsulatus* mutants lacking DsbA produce lower amounts of cyts *c

R. capsulatus mutants lacking DsbA are proficient for Ccm, unlike those lacking CcdA (Deshmukh *et al.*, 2000). To further assess the role of DsbA during Ccm, we compared the total amounts of periplasmic soluble and membrane-associated cyts *c* produced in a wild type strain of *R. capsulatus* with an isogenic DsbA-null mutant (Fig. 1). Qualitative SDS-PAGE/TMBZ analyses showed quasi-wild type like cyts *c* profiles in DsbA-null mutants (Fig. 2A) (Deshmukh *et al.*, 2003). Although this staining technique is very useful to monitor the overall cyts *c* profiles, the band intensities are time-dependent and may not be directly proportional to cyts *c* amounts. Thus, we used optical redox difference spectroscopy to obtain more quantitative estimations. Ascorbate-reduced *minus* ferricyanide-oxidized spectra recorded between 500 and 600 nm using membranes and membrane supernatants revealed that supernatant fractions of a DsbA-null mutant contained twice less (~ 40 % per 0.5 mg /ml of total proteins) 550 nm absorbing materials relative to a wild type strain (Fig. 2B). In *R. capsulatus*, this absorbance reflects almost exclusively the characteristic α -band of cyt *c*₂ (Daldal *et al.*, 1986). Using membrane fractions representing the pool of membrane-associated cyts *c* that comprises of the cyts *c*₁, *c*_y, *c*_o and *c*_p (Davidson and Daldal, 1987; Gray *et al.*, 1994; Koch *et al.*, 1998; Zannoni and Daldal, 1993) similar spectral data were also obtained (not shown). Furthermore, optical spectra acquired with a

Ccm proficient mutant lacking both CcdA and DsbA revealed that the amounts of cyts *c* produced were similar to those seen in the absence of DsbA (Fig. 2B, top and bottom two spectra). We therefore concluded that, although the absence of DsbA did not abolish covalent heme-apocyt *c* ligation *per se*, it decreased cyt *c* production.

Neither the overproduction of DegP in the absence of DsbA nor the lack of CydDC affects cyt *c* production in *R. capsulatus*

Recently, it was reported that an *E. coli* mutant lacking the periplasmic protease DegP accumulates the heterologously expressed *Bradyrhizobium japonicum* apocyt *c*₅₅₀ when the Ccm system is defective (Gao and O'Brian, 2007). In an earlier work, we found that *R. capsulatus* mutants lacking DsbA overproduce DegP, and that DsbA derivatives containing low amounts of DegP are viable (Onder *et al.*, 2008). We therefore investigated whether the decreased amounts of cyts *c* in mutants lacking DsbA or DsbA CcdA were due to the overproduction of DegP. Comparison of a DsbA mutant (overproducing DegP) with its derivative harboring very low amounts of DegP (*degPR3*) showed that both strains produced similar amounts of cyts *c* (Fig. 2B, middle spectrum). Similar data were also obtained with DegP derivatives of other Ccm mutants (Table 1). Thus, the observed decrease of cyt *c* amounts in the absence of DsbA or the entire thio-redox pathway was unrelated to DegP overproduction in this mutant background.

Earlier work indicated that the *E. coli* putative ABC-type glutathione transporter CydDC (Pittman *et al.*, 2005) is a component required for the *bd*-type hydroquinone oxidase assembly and for cyt *c* production (Georgiou *et al.*, 1987; Poole *et al.*, 1994). We found that *R. capsulatus* mutants lacking CydC or CydD, or their derivatives lacking DsbA or DsbA CcdA (Table 2) also did not contain any active hydroquinone oxidase (Zhang & Daldal, unpublished data). However, all CydDC derivatives still produced cyts *c*, unlike their *E. coli* counterparts (Fig. 3A). Thus, in *R. capsulatus*, the putative ABC-type glutathione transporter CydDC was not involved in cyt *c* production, and the compensatory thio-redox interactions seen between DsbA and CcdA did not rely on the periplasmic reductive power that might be due the CydDC transporter.

Compensatory thio-redox interactions during Ccm is not restricted to CcdA and DsbA

CcmG and CcmH are also thought to be parts of the Ccm thio-reductive branch (Deshmukh *et al.*, 2003). Therefore, we probed whether the compensatory thio-redox interactions seen between DsbA and CcdA also extended to CcmG and CcmH. Like CcdA-null mutants, CcmG-null or CcmH-null mutants cannot grow under photosynthetic conditions (Ps⁻) due to the absence of cyts *c* (Beckman *et al.*, 1992). However, unlike the CcdA-null mutants that revert readily to Ps⁺ by DsbA inactivation (Deshmukh *et al.*, 2003), CcmG-null mutants required supplementation of the growth medium with thiol-reactive reagents (0.33 mM L-cysteine and 0.165 mM L-cystine) in the case of MD11, and without supplement in the case of an SB1003 derivative on enriched medium, to obtain Ps⁺ revertants. In contrast, CcmH-null mutants never yielded any Ps⁺ revertants under the conditions tested. Analyses of several Ps⁺ revertants of CcmG-null mutants indicated that they all produced cyts *c* (*e. g.*, *ccmG-Rev2* and *-Rev3* in Fig. 3B), and that their Ps⁺ growth was suppressed upon introduction of a wild type allele of *dsbA* in *trans*. DNA sequence analyses revealed that the revertants carried chromosomal mutations inactivating *dsbA*. These mutations included a deletion encompassing the base pairs 55 to 68 in MD11R2, 5 and 7, a G insertion at position 135 in MD11R1, 3, 4, 6, 8, and Δ*helXR5*, 2, an A insertion at position 363 in Δ*helXR6* and 7, and a T to G substitution at position 629 in Δ*helXR1*, 3, 4 and 8 (Fig. 4 and Table 2).

Indeed, construction of CcmG-null DsbA-null and CcmH-null DsbA-null double mutants confirmed that the loss of DsbA suppressed the Ccm deficiency of CcmG-null, but not that

of CcmH-null, mutants (Table 2). The DsbA-null CcmG-null double mutants became Ps⁺ in minimal medium supplemented with thiol-reactive reagents and produced cyts *c* (Fig. 3B and C). In contrast, the DsbA-null CcmH-null double mutants remained Ps⁻, independent of thiol-reactive reagents addition to the growth medium, and produced no cyt *c* (Fig. 5A and B). Thus, the compensatory thio-redox interactions seen between DsbA and CcdA also occurred between DsbA and CcmG, but not between DsbA and CcmH. In agreement with these findings, the triple mutant lacking DsbA CcdA CcmG (but not DsbA CcdA CcmH) (Table 2) was Ps⁺ in thiol-reactive reagents supplemented minimal medium and exhibited cyts *c* profiles similar to those seen with the CcmG-null DsbA-null double mutant (Fig. 3B and C). We concluded that heme-apocyt *c* ligation occurred in the absence of the Ccm thio-redox loop encompassing DsbA, DsbB, CcdA and CcmG (Fig. 1).

An additional role for CcmG as an apocyt *c* holdase during Ccm

The observation that the CcmG-null DsbA-null double mutants required thiol-reactive supplements to grow under Ps conditions, unlike the CcdA-null DsbA-null mutants, suggested that absence of CcmG hampered Ccm efficiency more severely than CcdA. Examination of the optical redox difference spectra indicated that the CcmG-null DsbA-null double or CcdA-null CcmG-null DsbA-null triple mutants indeed contained much less (~ 10-15 % of wild type) (Fig. 3C) 550 nm absorbing materials than the CcdA-null DsbA-null double mutants (~ 40-50 % of wild type) (Fig. 2B). Since the only difference between these thio-redox loop mutants was the absence of CcmG, we probed whether the decrease of cyt *c* production was linked to the absence of the CcmG protein *per se*. CcmG has two active site cysteines (C75 and C78 in *R. capsulatus*) (Monika *et al.*, 1997), which face the periplasm and accept electrons from CcdA (Katzen and Beckwith, 2000; Katzen *et al.*, 2002). Substitution of these active site cysteines with serine yielded the CcmG^{C75S}, CcmG^{C78S} and CcmG^{C75S/C78S} (the latter dubbed here as CcmG*) mutants. As expected, these mutants were unable to complement a CcmG-null strain for Ps growth in any growth medium, regardless of thiol-reactive supplement. They also did not produce any cyt *c* that can be detected by SDS-PAGE/TMBZ (Fig. 6A and B, top panels) or optical redox difference spectra analyses (Fig. 7A) (Table 1). Immunoblot data indicated that all strains (except CcmG^{C78S} in enriched medium) produced mutant forms of CcmG at wild type or higher amounts (Fig. 6A and B, lower panels). We also noted that the amounts of CcmG were variable in various mutants grown under different conditions, and this point deserves future studies.

Interestingly, the cysteine-less derivative of CcmG complemented even in the absence of thiol-reactive supplements the DsbA-null CcmG-null mutant for Ps growth in minimal medium. Moreover, it also produced more cyts *c* as shown both by SDS-PAGE/TMBZ (Fig. 6A and B) and optical redox difference spectra analyses (Fig. 7B) (Table 1). Furthermore, the DsbA-null CcmG-null mutant carrying the CcmG* derivative was, like the DsbA-null or the DsbA-null CcdA-null mutants, Ps⁺ in minimal medium regardless of any thiol-reactive supplement, and contained the highest amounts of cyts *c* among the CcmG mutants (Fig. 6). Optical spectra again showed that the amounts of the 550 nm absorbing materials found in the DsbA-null CcmG-null mutants harboring CcmG* were comparable (~ 40-50 % of wild type) to those found in the DsbA-null or DsbA-null CcdA-null mutants (Fig. 7B). Thus, the presence of CcmG* devoid of its thio-reductive activity increased drastically cyt *c* production in the absence of DsbA, indicating that CcmG had an additional, thio-reduction-independent function referred to as apocyt *c* holdase.

Similar studies were also conducted with CcmH and its cysteine-less derivatives. *R. capsulatus* CcmH has two functionally essential cysteine residues at positions 42 and 45 facing the periplasm (Monika *et al.*, 1997). CcmH mutants lacking them (CcmH^{C42S}, CcmH^{C45S} and CcmH^{C42S/C45S}, the latter dubbed CcmH*) did not complement for Ps

growth the CcmH-null or DsbA-null CcmH-null mutants, and produced no *cyt c* (Fig. 5A and B) (Table 1) (unlike in *E. coli*, see (Robertson *et al.*, 2008)) although they had similar amounts of wild type or mutant CcmH proteins, as indicated by immunoblot analyses (Fig. 5A, lower panel). Overall comparison of the data obtained with CcmH and CcmG indicated that the two thio-reductive components behaved very differently in respect to the DsbA- and CcdA-dependent thio-redox loop, suggesting that their reductive roles during Ccm might be different.

R. capsulatus* apocyt *c*₂ physically interacts with CcmG* *in vitro

The additional, thioredoxin-independent apocyt *c* holdase function of CcmG during Ccm was investigated *in vitro* by probing the direct physical interactions between purified apocyt *c* and partially purified CcmG*. A C-terminally His epitope-tagged mature form of *R. capsulatus* CcmG* was expressed in *E. coli*, and partially purified by affinity chromatography. Similarly, a double (N-terminal His and C-terminal Strep) epitope-tagged derivative of *R. capsulatus* apocyt *c*₂ devoid of its signal sequence was expressed in *E. coli* cytoplasm, and tandem affinity purified using His-Bind and then Strep-Tactin affinity columns, as described in Experimental Procedures. As the apocyt *c*₂ was highly unstable, use of tandem affinity tags facilitated the isolation of intact apocyt *c*₂. Purified apocyt *c*₂ and CcmG* containing extracts (50 and 40 µg total proteins, respectively) were incubated together for two hours at 4 °C, and apocyt *c*₂ was re-isolated using Strep-Tactin affinity chromatography. Column fractions were probed for the presence of apocyt *c*₂ using anti-Strep and anti-His (not shown) antibodies, as well as for CcmG using anti-CcmG antibodies (Fig. 8). A similar mock experiment using partially purified CcmG* but no apocyt *c*₂ was also conducted to show that CcmG* itself was not retained by the Strep-Tactin affinity column (Fig. 8A). However, when an incubation mixture containing both CcmG* and apocyt *c*₂ were loaded onto the same column, and eluted with desthiobiotin (DTB), apparently CcmG* was retained by the column as both apocyt *c*₂ and CcmG* were co-eluted in the same fractions (mainly E3) (Fig. 8B and C). We therefore concluded that a CcmG* lacking its active site cysteines can recognize and bind apocyt *c*₂ *in vitro* as an apocyt *c* holdase.

Discussion

This work was initiated in order to assess whether the compensatory thio-redox interactions previously seen between DsbA and CcdA mutants (Deshmukh *et al.*, 2003) also extended to other thio-reductive Ccm components. By doing so we hoped to dissect apart the Ccm components involved in preparing apocyt *c* as an adequate substrate from those catalyzing the subsequent heme-apocyt *c* ligation *per se*. Searches for spontaneous Ps⁺ revertants of CcmG-null and CcmH-null strains, and construction of appropriate double and triple knock-out mutants, demonstrated for the first time that the DsbA-dependent compensatory thio-redox interactions involved solely CcdA and CcmG, and not CcmH (or CcmI, data not shown). Earlier, it was observed that Ps⁺ revertants of CcmG-null mutants, unlike CcdA-null mutants, could only be obtained if the growth medium was supplemented with thiol-reactive chemicals (Monika *et al.*, 1997). Similarly, we noted that CcmG DsbA and CcdA CcmG DsbA knock-out mutants were Ps⁺ only upon supplementation with thiol-reactive reagents. The basis for this redox constraint is unclear. Thus, in the absence of CcmG (in contrast to that of CcdA), *R. capsulatus* cells might be metabolically more limited to reduce their growth environment to initiate anoxygenic photosynthesis.

Although the cysteine-less derivatives (Fabianek *et al.*, 1999; Monika *et al.*, 1997) of the unusual thioredoxin-like CcmH (Di Matteo *et al.*, 2007) were indeed Ccm deficient, the thio-reductive function of CcmH could not be compensated by a thio-oxidative defect. These findings limited the thio-redox loop to DsbA, DsbB, CcdA and CcmG (Fig. 1) establishing

that these components were not essential for cyt *c* production *in vivo*. Moreover, they simplified the number of Ccm components required for heme-apocyct *c* ligation *per se* (Fig. 9). Importantly, the data also suggested that both CcmG and CcmH might have additional function(s) distinct from their known thio-reductive roles (Monika *et al.*, 1997). In respect to CcmH, a possibility is that its oxidized form might interact with CcmG-reduced apocyct *c* during its transfer to heme ligation complex containing CcmF. Indeed, CcmH is known to co-immunoprecipitate with CcmF (Ren *et al.*, 2002), and its overproduction together with CcmF bypasses the Ccm deficiency of CcmI-null mutants (Deshmukh *et al.*, 2002; Sanders *et al.*, 2005a). Furthermore, our recent data indicate that CcmH is a component of a complex also containing CcmF and CcmI in *R. capsulatus* membranes (Sanders *et al.*, submitted).

Unlike other thioredoxins, CcmG is highly specific for Ccm (Edeling *et al.*, 2002; Fabianek *et al.*, 1998; Monika *et al.*, 1997) with an active site unusually acidic, proposed to provide its substrate specificity (Edeling *et al.*, 2004). Our finding that the cysteine-less CcmG derivative CcmG* produced high amounts of cyts *c* and exhibited Ps growth regardless of the thio-reactive supplements, indicated that CcmG protein *per se* also had a thioredoxin-independent role(s) referred to as apocyct *c* holdase during Ccm (Fig. 9). Earlier, the active site cysteines of *E. coli* CcmG was found to be important, but not essential, for Ccm in the presence of reducing agents (Fabianek *et al.*, 1998). In the light of the compensatory thio-redox interactions seen between DsbA and CcmG, this finding could also be interpreted as counteracting the DsbA oxidative power, and not necessarily as an additional activity of CcmG. The significant increase of cyts *c* production in the presence of CcmG* in the absence of DsbA, as seen here, established unambiguously a thioredoxin-independent role for the CcmG protein *per se* (Fig. 9). This additional function of CcmG was further supported by the fact that purified apocyct *c* and CcmG* devoid of its cysteines directly interact with each other *in vitro*. Growing evidence suggests that CcmG acts as a holdase, conveying apocyct *c* to the heme ligation core complex. The crystal structure of *B. subtilis* CcmG homologue ResA revealed a cavity, close to its active site cysteines, speculated to form a binding surface for apocyct *c* (Colbert *et al.*, 2006; Crow *et al.*, 2004). Consistent with this proposal, we observed that overproduction of CcmG or apocyct *c* (Sanders *et al.*, 2007) in addition to CcmF and CcmH also increased cyt *c* production in *R. capsulatus* mutants lacking CcmI (Deshmukh *et al.*, 2002; Sanders *et al.*, 2005a).

It should be emphasized that DsbA is not essential for Ccm, but its absence decreases cyt *c* production irrespective of the thio-reductive pathway (*i. e.*, CcdA and CcmG). Although this observation could rationalize why the Ccm thio-redox loop might be conserved evolutionarily, why it occurs remains unknown. That the thio-redox loop reactions might protect some apocyts *c* intermediates (Sanders *et al.*, 2005a), or improve the fidelity of stereo-specific heme ligation (Sanders *et al.*, 2005b) has been suggested. Unfolded apocyts *c* might be more vulnerable to degradation unless protected by chaperones such as CcmG* (depicted as dashed outline in Fig. 9). Formation of a disulfide bond at the apocyct *c* C₁XXC₂H motif, although requires subsequent reduction by the thio-reductive pathway, might be beneficial by promoting partial folding and decreasing degradation of apocyct *c*. Consequently, in the absence of the thio-redox loop and CcmG*, cyt *c* production would be much lower than native amounts. Absence of the Ccm thio-redox loop might also compromise stereo-specific heme ligation (Sanders *et al.*, 2005b), yielding non-native cyts *c* that are prone to degradation. Indeed the DsbA, CcdA DsbA, CcmG* DsbA or CcdA CcmG* DsbA mutants produced lower amounts of cyts *c* than a wild type strain of *R. capsulatus*. In summary, this study established that the Ccm thio-redox loop components DsbA, CcdA and CcmG are not essential for cyt *c* production, hence decreasing the number of Ccm components absolutely required for heme-apocyct *c* ligation. Furthermore, it revealed a second, thio-reduction independent role of CcmG, which is critical for cyt *c* production, and raised the possibility that the oxidized form of CcmH might be required for transferring

CcmG-reduced apocyt *c* to a heme ligation complex containing CcmF and CcmI. Future studies would hopefully better define the interactions between apocyt *c* and the essential Ccm components during cyt *c* production in bacteria.

Experimental Procedures

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are described in Table 2. *R. capsulatus* strains were grown at 35 °C on enriched (Daldal *et al.*, 1986) or minimal (Sistrom, 1960) media, supplemented with appropriate antibiotics (2.5, 10, 10, and 1 µg/ml tetracycline, kanamycin, spectinomycin and gentamycin, respectively), either chemoheterotrophically (Res growth) or photoheterotrophically (Ps growth) in anaerobic jars with H₂ + CO₂ generating gas packs from BBL Microbiology Systems (Cockeysville, MD). All growth media were supplemented with 0.33 mM L-cysteine and 0.165 mM L-cystine when appropriate. *E. coli* strains were grown on Luria Bertani (LB) broth supplemented with appropriate antibiotics (12.5, 50, 10, 100 and 12 µg/ml tetracycline, kanamycin, spectinomycin, ampicillin and gentamycin, respectively) as needed.

Molecular Genetics Techniques

Standard molecular genetic techniques were performed as described (Sambrook, 2001). Plasmid pBS-DsbA^{wt} (Deshmukh *et al.*, 2003) carrying the *R. capsulatus* wild type *dsbA* was digested with BglII and SfiI to replace its 0.5 kb internal fragment with a 2.0 kb DNA polymerase blunted BamHI spectinomycin resistance cassette from pHP45Ωspec to yield pBS-STA3 harboring $\Delta(dsbA::spe)$ (Table 2). The XbaI-KpnI fragment of pBS-STA3 was then cloned into the respective sites of pRK415 to yield pSTA1. A 1.2 kb gentamycin resistance cassette under the control of the cyt *c*₂ gene promoter (*PcycA-gen*) was first amplified from pCHB::Gm (Table 2) with M13-forward and M13-reverse primers, phosphorylated using T4 polynucleotide kinase and ligated into the EcoRV-digested pBluescript to yield pST1 (Table 2). To obtain a $\Delta(ccdA::PcycA-gen)$ allele pST1 was restricted with BamHI and the 1.2 kb fragment harboring *PcycA-gen* ligated into BglII restricted pK6, eliminating a 0.3 kb internal fragment of *ccdA*. The 2.7 kb insert from the resulting plasmid pST3 was then transferred into the BamHI site of pRK415 to yield pST4. Plasmids pCS1540 and pCS1545 containing wild type *ccmG* and the *ccmG** variant with both cysteines (C75 and C78) to serine substitutions, respectively, were constructed as described (Sanders *et al.*, 2005a) (Table 2). To obtain pCS1543 and pCS1544, the C75 or C78 of *ccmG* in pCS1540 were substituted with serine using the QuikChange® XL Site-Directed Mutagenesis Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA) and the mutagenic primers HelX-C75Ss (5'-CTT CTG GGC TTC CTG GAG CGC GCC CTG TCG-3') and HelX-C75Sas (5'-ACC CGA CAG GGC GCG CTC CAG GAA GCC CAG-3') for pCS1543, and HelX-C78Ss (5'-TCC TGG TGC GCG CCC AGT CGG GTC GAA CA-3') and HelX-C78Sas (5'-GGA TGT TCG ACC CGA CTG GGC GCG CAC CAG-3') for pCS1544 (Table 2). PCR-amplification of *ccmH* using pYZ1 as a template and CcmH/XbaI-Fwd (5'- CTC TCT AGA GTT GCG GCG GAG TGA GCG ATG CTG AAA CG-3') and CcmH/KpnI-Rev (5'-GAG GGT ACC CTA GTC CTT CAG CAG GTC TTT CAG CCG CGC CTC-3') as primers generated a 0.48 kb DNA fragment, which was digested with XbaI and KpnI and ligated into the respective sites of pBluescript II KS(+) (pBSK) to yield pST7 (Table 2). Plasmid pST8 was constructed by engineering a Strep-tag sequence at the 3'-end of *ccmH* via PCR amplification using pST7 as a template and the primers CcmH-StrepC/XbaI-Fwd (5'-CTC TCT AGA GTT GCG GCG GAG TGA GCG ATG CTG AAA CG-3') and CcmH-StrepC/KpnI-Rev (5'- GAG GGT ACC CTA TTT TTC GAA CTG CGG GTG GCT CCA AGC GCT GTC CTT CAG CAG GTC TTT CAG CCG CGC CTC-3'). The 0.51 kb DNA fragment thus generated was cut with XbaI and KpnI and

ligated into the same sites of pBSK. Plasmid pST8 was digested with XbaI and KpnI and the fragment carrying *ccmH-Strep* was ligated into the respective sites of pCHB500 to yield pST6. The cysteine-less derivative of CcmH was obtained by substituting C42 and C45 of *ccmH* (with an in-frame Strep-tag sequence at its 3'-end) with serine as described above by using pST8 as a template and CcmH-C42S/C45Ss (5'-TCG CAG GTG CTG CGC AGC CCG GTC AGT CAG GGC GAG A-3') and CcmH-C42S/C45Sas (5'-GAT ATT CTC GCC CTG ACT GAC CGG GCT GCG CAG CAC CTG C-3') as mutagenic primers to yield pST11 (Table 2). Plasmid pST11 was digested with XbaI and KpnI, and the fragment carrying mutant *ccmH* was ligated into the respective sites of pCHB500 to yield pST14. A 4.8 kb PCR fragment encompassing *cydDC* genes was amplified with primers CydDCF (5'-GCA CTT GCT CGT CGA ACA GGA TCC AG-3') and CydDCR (5'-CCG GAA TTC GAA ACA GAC GCG CGA CA-3'), digested with BamHI and EcoRI (introduced sites in the primers are underlined) and cloned into respective sites of pBluescript to yield pKZ66. In order to create deletion-insertion allele of *cydC*, a 1.2 kb PstI fragment of pKZ66 was replaced with 1.2 kb Gentamycin resistance cartridge from pCHB::Gm yielding pKZ68. XbaI-KpnI fragment from pKZ68 carrying *cydD⁺ΔC::Gm* allele was inserted into the corresponding sites of pRK415 to yield pKZ69. Plasmid pKZ66 was digested with HindIII and ligated back, eliminating *BbsI* site immediately downstream of *cydC* to yield pKZ7. To create deletion-insertion allele of *cydD*, a 320 bp *BbsI* fragment from pKZ7 was excised, the remaining vector was made blunt and ligated to Gm cartridge from pCHB::Gm to yield pKZ71. XbaI-KpnI fragment of pKZ71 was then cloned into XbaI-KpnI site of pRK415 to create pKZ72 (Table 2). Plasmid pQE60-helX was obtained by cloning into pQE60 (Qiagen, Valencia, CA) *R. capsulatus ccmG* lacking its N-terminal signal peptide/transmembrane helix by using the NcoI and BamHI sites. Plasmid pCS1554 expressing the C-terminally His tagged mature form of CcmG* was obtained by mutating to serine the cysteines 75 and 78 of *ccmG* in pQE60-helX using the primers HelX-C75S/C78Ss (5'-CTT CTG GGC TTC CTG GAG CGC GCC CAG TCG GGT CGA ACA-3') and HelX-C75S/C78Sas (5'-GGA TGT TCG ACC CGA CTG GGC GCG CTC CAG GAA GCC CAG-3') according to the instruction of the QuikChange® XL Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA). The mature form of *R. capsulatus* *cyt c₂* gene (*cycA^{mat}*) was PCR-amplified using the primers CS24 (5'-GTT GAC AAT TAA TCA TCG GC-3') and RcccACtHis6BamHIr (5'-GCC GGA TCC TAG TGA TGG TGA TGG TGA TGT TTC ACG ACC GAG GCC AGA TAG GC-3') and pCS1726 (Sanders *et al.*, submitted) as a template. The 0.5 kb fragment thus generated was digested with NdeI and BamHI, and cloned into the same sites of pCS1302 (Sanders *et al.*, submitted) to yield pCS1757 that carries *cycA^{mat}* with an in frame Strep-tag sequence at its 5'-end and a 6xHis-tag at its 3'-end. The expression of the double-tagged *cycA^{mat}* derivative is controlled by a *Ptac-lac* promoter-operator system in *E. coli*.

Automated DNA sequencing with the Big-Dye™ terminator cycle sequencing kit (AmpliTag FS; Applied Biosystems) was performed according to the manufacturer by using the M13-forward and M13-reverse primers to verify all plasmids constructed in this study. DNA sequence analyses, homology searches and genome sequence comparisons were done using MacVector (IBI; Kodak) and BLAST software packages (Altschul *et al.*, 1990).

Conjugal transfers of plasmids from *E. coli* to *R. capsulatus* and chromosomal allele replacement via interposon mutagenesis using the gene transfer agent (Yen *et al.*, 1979) were carried out as described earlier (Daldal *et al.*, 1986). Briefly, the mutant strain ST40 (*ccmG::kan dsbA::spe degPR3*) mutant was obtained using pHX-1 (*ccmG::kan*) as a donor and ST38 (*dsbA::spe, degPR3*), which is a Spec^R derivative of MD20R3 (Onder *et al.*, 2008) as a recipient strain. The CcmH-null DsbA-null double mutant ST27, and CcdA-null CcmH-null DsbA-null triple mutant ST36, were constructed using pTC4-1K (*dsbA::kan*) and pST4

(*ccdA::gen*) as donors and MD14 (*ccmH::spe*) and ST27 (*ccmH::spe dsbA::kan*) as recipient strains, respectively.

Biochemical techniques

Intracytoplasmic membrane vesicles (chromatophore membranes) were prepared in 50 mM MOPS (pH 7.0) containing 100 mM KCl for spectral, or 1 mM KCl for SDS-PAGE, analyses as described earlier (Gray *et al.*, 1994). All preparations contained 10 mM EDTA, 1 mM PMFS and 0.1 mM ϵ -amino-caproic acid as protease inhibitors. Protein concentrations were determined according to (Lowry *et al.*, 1951) and SDS-PAGE was used as in (Schagger and von Jagow, 1987) using 16.5 % polyacrylamide gels. Cyts *c* were revealed based on the endogenous peroxidase activity of covalently attached heme by using 3,3',5,5'-tetramethylbenzidine (TMBZ) (Thomas *et al.*, 1976). For CcmG and CcmH immunodetection, protein samples were separated using 15% SDS-PAGE (Laemmli, 1970). Following electrophoresis, gels were electroblotted onto Immobilon-P PVDF membranes (Millipore, Billerica, MA). CcmG and CcmH were detected using anti-CcmG and anti-CcmH polyclonal antibodies (Monika *et al.*, 1997), ECL anti-rabbit IgG/horseradish peroxidase-conjugated secondary antibodies from Amersham-Pharmacia (Piscataway, NJ) and SuperSignal West Pico Chemiluminescent substrate from Pierce (Rockford, IL).

Expression and purification of apocytochrome c_2 and CcmG*

E. coli strains containing pCS1757 (for apocyt c_2) or pCS1554 (for CcmG*) were grown in Luria Broth (LB) liquid medium (four liters each) containing 100 μ g/ml ampicillin at 37 °C with shaking (200 rpm) until an OD₆₀₀ of approximately 0.8. Cultures were then induced under the same growth conditions for two hours by adding isopropyl-thio- β -D-galactoside (IPTG) to a final concentration of 10 mM. Cells were harvested by centrifugation for 10 min at 10,000 g and resuspended in Tris Buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl and 1mM PMSF), and disrupted on ice by using a French Pressure cell as described in (Daldal *et al.*, 1986). Cell lysates were centrifuged at 25,571 g for 1 hour, followed by at 117,734 g for 1 hour. Cleared supernatants were filtered through a 0.45 μ m filter and applied to a His-bind purification chromatography column (His-Bind Kit from Novagen, Inc.) following the manufacturer's instructions. Final concentrations of 25 and 100 mM imidazole in the washing and elution buffers, respectively, were used, and column fractions monitored by SDS-PAGE and Western Blot analyses. His-bind chromatography fraction containing apocyt c_2 were pooled, concentrated by ultrafiltration (3,000 MWCO, Amicon Ultra, Millipore) to eliminate imidazole, diluted ten fold in Strep Wash buffer before loading onto the StrepTactin® Sepharose affinity column, and purified according to manufacturer's instruction (IBA, Inc.). Direct interactions between purified apocyt c_2 and CcmG* were probed by mixing 50 μ g of His and Strep purified apocyt c_2 with 40 μ g of CcmG* enriched extracts and incubating for two hours at 4 °C, in the presence of 20 mM Tris-HCl pH = 7.9, 50 mM NaCl, 50 μ g DTB and 10 mM imidazole (final concentrations). After incubation, the reaction mixture was applied to the Strep-Tactin affinity chromatography column and purification was performed according to the manufacturer's instructions (IBA, Inc). Column fractions were subjected to SDS-PAGE and Western Blot analyses, and apocyt c_2 was detected with anti-His and anti-Strep antibodies whereas CcmG was identified with CcmG antisera.

Spectroscopic analysis

Optical absorbance redox difference spectra were recorded using a Hitachi U-3210 UV/vis spectrophotometer between 500-600 nm. Chromatophore membrane supernatants were centrifuged at 120000 \times g for 2 hours to eliminate membrane debris. The supernatants thus obtained were adjusted with 50 mM MOPS/100 mM KCl to a protein concentration of 0.5 mg/ml and oxidized by adding a crystal of potassium ferricyanide, and subsequently reduced

by either solid sodium ascorbate or sodium dithionite to measure the total amounts of reduced cyts *c*.

Chemicals

All chemicals were of reagent grade and obtained from commercial sources.

Acknowledgments

This work was supported by grants from DOE 91ER20052 and NIH GM 38237 to F. D. The authors thank to Drs. R. G. Kranz for providing anti-CcmG and anti-CcmH antibodies, J. F. Collet for plasmid pQE60-*helX*, and K. Zhang and M. Deshmukh for construction of various pKZ plasmids and MD strains, respectively.

References

- Allen JW, Barker PD, Ferguson SJ. A cytochrome b562 variant with a c-type cytochrome CXXCH heme-binding motif as a probe of the *Escherichia coli* cytochrome *c* maturation system. *J Biol Chem*. 2003a; 278:52075–52083. [PubMed: 14534316]
- Allen JW, Daltrop O, Stevens JM, Ferguson SJ. C-type cytochromes: diverse structures and biogenesis systems pose evolutionary problems. *Philos Trans R Soc Lond B Biol Sci*. 2003b; 358:255–266. [PubMed: 12594933]
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990; 215:403–410. [PubMed: 2231712]
- Barker PD, Ferguson SJ. Still a puzzle: why is haem covalently attached in c-type cytochromes? *Structure*. 1999; 7:281–290.
- Beckman DL, Trawick DR, Kranz RG. Bacterial cytochromes *c* biogenesis. *Genes Dev*. 1992; 6:268–283. [PubMed: 1310666]
- Beckman DL, Kranz RG. Cytochromes *c* biogenesis in a photosynthetic bacterium requires a periplasmic thioredoxin-like protein. *Proc Natl Acad Sci U S A*. 1993; 90:2179–2183. [PubMed: 8384715]
- Benning C, Somerville CR. Isolation and genetic complementation of a sulfolipid-deficient mutant of *Rhodobacter sphaeroides*. *J Bacteriol*. 1992; 174:2352–2360. [PubMed: 1551852]
- Colbert CL, Wu Q, Erbel PJ, Gardner KH, Deisenhofer J. Mechanism of substrate specificity in *Bacillus subtilis* ResA, a thioredoxin-like protein involved in cytochrome *c* maturation. *Proc Natl Acad Sci U S A*. 2006; 103:4410–4415. [PubMed: 16537372]
- Crow A, Acheson RM, Le Brun NE, Oubrie A. Structural basis of Redox-coupled protein substrate selection by the cytochrome *c* biosynthesis protein ResA. *J Biol Chem*. 2004; 279:23654–23660. [PubMed: 15047692]
- Daldal F, Cheng S, Applebaum J, Davidson E, Prince RC. Cytochrome *c*(2) is not essential for photosynthetic growth of *Rhodospseudomonas capsulata*. *Proc Natl Acad Sci U S A*. 1986; 83:2012–2016. [PubMed: 16593675]
- Daltrop O, Allen JW, Willis AC, Ferguson SJ. In vitro formation of a c-type cytochrome. *Proc Natl Acad Sci U S A*. 2002; 99:7872–7876. [PubMed: 12060734]
- Davidson E, Daldal F. Primary structure of the bc1 complex of *Rhodospseudomonas capsulata*. Nucleotide sequence of the *pet* operon encoding the Rieske cytochrome *b*, and cytochrome *c*1 apoproteins. *J Mol Biol*. 1987; 195:13–24. [PubMed: 2821268]
- Deshmukh M, Bresseur G, Daldal F. Novel *Rhodobacter capsulatus* genes required for the biogenesis of various c-type cytochromes. *Mol Microbiol*. 2000; 35:123–138. [PubMed: 10632883]
- Deshmukh M, May M, Zhang Y, Gabbert KK, Karberg KA, Kranz RG, Daldal F. Overexpression of *ccl1-2* can bypass the need for the putative apocytochrome chaperone Cych during the biogenesis of c-type cytochromes. *Mol Microbiol*. 2002; 46:1069–1080. [PubMed: 12421312]
- Deshmukh M, Turkarslan S, Astor D, Valkova-Valchanova M, Daldal F. The dithiol:disulfide oxidoreductases DsbA and DsbB of *Rhodobacter capsulatus* are not directly involved in cytochrome *c* biogenesis, but their inactivation restores the cytochrome *c* biogenesis defect of CcdA-null mutants. *J Bacteriol*. 2003; 185:3361–3372. [PubMed: 12754234]

- Di Matteo A, Gianni S, Schinina ME, Giorgi A, Altieri F, Calosci N, Brunori M, Travaglini-Allocatelli C. A strategic protein in cytochrome c maturation: three-dimensional structure of CcmH and binding to apocytochrome c. *J Biol Chem.* 2007; 282:27012–27019. [PubMed: 17623665]
- Ditta G, Schmidhauser T, Yakobson E, Lu P, Liang XW, Finlay DR, Guiney D, Helinski DR. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid.* 1985; 13:149–153. [PubMed: 2987994]
- Edeling MA, Guddat LW, Fabianek RA, Thony-Meyer L, Martin JL. Structure of CcmG/DsbE at 1.14 Å resolution: high-fidelity reducing activity in an indiscriminately oxidizing environment. *Structure.* 2002; 10:973–979. [PubMed: 12121652]
- Edeling MA, Ahuja U, Heras B, Thony-Meyer L, Martin JL. The acidic nature of the CcmG redox-active center is important for cytochrome c maturation in *Escherichia coli*. *J Bacteriol.* 2004; 186:4030–4033. [PubMed: 15175318]
- Erlendsson LS, Hederstedt L. Mutations in the thiol-disulfide oxidoreductases BdbC and BdbD can suppress cytochrome c deficiency of CcdA-defective *Bacillus subtilis* cells. *J Bacteriol.* 2002; 184:1423–1429. [PubMed: 11844773]
- Fabianek RA, Hennecke H, Thony-Meyer L. The active-site cysteines of the periplasmic thioredoxin-like protein CcmG of *Escherichia coli* are important but not essential for cytochrome c maturation in vivo. *J Bacteriol.* 1998; 180:1947–1950. [PubMed: 9537397]
- Fabianek RA, Hofer T, Thony-Meyer L. Characterization of the *Escherichia coli* CcmH protein reveals new insights into the redox pathway required for cytochrome c maturation. *Arch Microbiol.* 1999; 171:92–100. [PubMed: 9914305]
- Gao T, O'Brian MR. Control of DegP-dependent degradation of c-type cytochromes by heme and the cytochrome c maturation system in *Escherichia coli*. *J Bacteriol.* 2007; 189:6253–6259. [PubMed: 17616605]
- Georgiou CD, Fang H, Gennis RB. Identification of the *cydC* locus required for expression of the functional form of the cytochrome d terminal oxidase complex in *Escherichia coli*. *J Bacteriol.* 1987; 169:2107–2112. [PubMed: 3032907]
- Gray KA, Grooms M, Myllykallio H, Moomaw C, Slaughter C, Daldal F. *Rhodobacter capsulatus* contains a novel cb-type cytochrome c oxidase without a CuA center. *Biochemistry.* 1994; 33:3120–3127. [PubMed: 8130227]
- Katzen F, Beckwith J. Transmembrane electron transfer by the membrane protein DsbD occurs via a disulfide bond cascade. *Cell.* 2000; 103:769–779. [PubMed: 11114333]
- Katzen F, Deshmukh M, Daldal F, Beckwith J. Evolutionary domain fusion expanded the substrate specificity of the transmembrane electron transporter DsbD. *Embo J.* 2002; 21:3960–3969. [PubMed: 12145197]
- Keen NT, Tamaki S, Kobayashi D, Trollinger D. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene.* 1988; 70:191–197. [PubMed: 2853689]
- Koch HG, Hwang O, Daldal F. Isolation and characterization of *Rhodobacter capsulatus* mutants affected in cytochrome *cbb3* oxidase activity. *J Bacteriol.* 1998; 180:969–978. [PubMed: 9473054]
- Kojima N, Yamanaka M, Ichiki S, Sambongi Y. Unexpected elevated production of Aquifex aeolicus cytochrome *c555* in *Escherichia coli* cells lacking disulfide oxidoreductases. *Biosci Biotechnol Biochem.* 2005; 69:1418–1421. [PubMed: 16041151]
- Kranz R, Lill R, Goldman B, Bonnard G, Merchant S. Molecular mechanisms of cytochrome c biogenesis: three distinct systems. *Mol Microbiol.* 1998; 29:383–396. [PubMed: 9720859]
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970; 227:680–685. [PubMed: 5432063]
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951; 193:265–275. [PubMed: 14907713]
- Metheringham R, Tyson KL, Crooke H, Missiakas D, Raina S, Cole JA. Effects of mutations in genes for proteins involved in disulphide bond formation in the periplasm on the activities of anaerobically induced electron transfer chains in *Escherichia coli* K12. *Mol Gen Genet.* 1996; 253:95–102. [PubMed: 9003292]

- Monika EM, Goldman BS, Beckman DL, Kranz RG. A thio-reduction pathway tethered to the membrane for periplasmic cytochromes c biogenesis; in vitro and in vivo studies. *J Mol Biol.* 1997; 271:679–692. [PubMed: 9299319]
- Onder O, Turkarslan S, Sun D, Daldal F. Overproduction or absence of the periplasmic protease DegP severely compromises bacterial growth in the absence of the dithiol:disulfide oxidoreductase DsbA. *Mol Cell Proteomics.* 2008
- Pittman MS, Robinson HC, Poole RK. A bacterial glutathione transporter (*Escherichia coli* CydDC) exports reductant to the periplasm. *J Biol Chem.* 2005; 280:32254–32261. [PubMed: 16040611]
- Poole RK, Gibson F, Wu G. The cydD gene product, component of a heterodimeric ABC transporter, is required for assembly of periplasmic cytochrome c and of cytochrome bd in *Escherichia coli*. *FEMS Microbiol Lett.* 1994; 117:217–223. [PubMed: 8181727]
- Prentki P, Krisch HM. In vitro insertional mutagenesis with a selectable DNA fragment. *Gene.* 1984; 29:303–313. [PubMed: 6237955]
- Ren Q, Ahuja U, Thony-Meyer L. A bacterial cytochrome c heme lyase. CcmF forms a complex with the heme chaperone CcmE and CcmH but not with apocytochrome c. *J Biol Chem.* 2002; 277:7657–7663. [PubMed: 11744735]
- Robertson IB, Stevens JM, Ferguson SJ. Dispensable residues in the active site of the cytochrome c biogenesis protein CcmH. *FEBS Lett.* 2008
- Sambrook, J.; Russel, DW. *Molecular cloning: a laboratory manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001.
- Sanders C, Wethkamp N, Lill H. Transport of cytochrome c derivatives by the bacterial Tat protein translocation system. *Mol Microbiol.* 2001; 41:241–246. [PubMed: 11454216]
- Sanders C, Deshmukh M, Astor D, Kranz RG, Daldal F. Overproduction of CcmG and CcmFH(Re) fully suppresses the c-type cytochrome biogenesis defect of *Rhodobacter capsulatus* CcmI-null mutants. *J Bacteriol.* 2005a; 187:4245–4256. [PubMed: 15937187]
- Sanders, C.; Turkarslan, S.; Daldal, F. Periplasmic oxidative folding and cytochrome c maturation: A mechanistic view of stereo-selective heme attachment. In: Van der Est, A.; Bruce, D., editors. *Photosynthesis: Fundamental Aspects to Global Perspectives.* 2005b.
- Sanders C, Boulay C, Daldal F. Membrane-spanning and periplasmic segments of CcmI have distinct functions during cytochrome c Biogenesis in *Rhodobacter capsulatus*. *J Bacteriol.* 2007; 189:789–800. [PubMed: 17122341]
- Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem.* 1987; 166:368–379. [PubMed: 2449095]
- Scolnik PA, Walker MA, Marrs BL. Biosynthesis of carotenoids derived from neurosporene in *Rhodospseudomonas capsulata*. *J Biol Chem.* 1980; 255:2427–2432. [PubMed: 7358679]
- Sistrom WR. A requirement for sodium in the growth of *Rhodospseudomonas spheroides*. *J Gen Microbiol.* 1960; 22:778–785. [PubMed: 14447230]
- Thomas PE, Ryan D, Levin W. An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal Biochem.* 1976; 75:168–176. [PubMed: 822747]
- Thony-Meyer L. Haem-polypeptide interactions during cytochrome c maturation. *Biochim Biophys Acta.* 2000; 1459:316–324. [PubMed: 11004446]
- Thony-Meyer L. Cytochrome c maturation: a complex pathway for a simple task? *Biochem Soc Trans.* 2002; 30:633–638. [PubMed: 12196152]
- Yen HC, Hu NT, Marrs BL. Characterization of the gene transfer agent made by an overproducer mutant of *Rhodospseudomonas capsulata*. *J Mol Biol.* 1979; 131:157–168. [PubMed: 490646]
- Zannoni D, Daldal F. The role of c-type cytochromes in catalyzing oxidative and photosynthetic electron transport in the dual functional plasmamembrane of facultative phototrophs. *Arch Microbiol.* 1993; 160:413–423. [PubMed: 8297207]



Fig. 1. Production of cyts *c* in the presence of the Ccm thio-redox loop

In a wild type background, the cysteine thiols of the C₁XXC₂H motif of reduced apocyts *c*, secreted through the general secretion pathway (Sec), are oxidized via the DsbA and DsbB dependent thio-oxidation pathway. Oxidized apocyts *c* are then reduced by the CcdA and CcmG dependent thio-reduction pathway, and conveyed to the CcmFHI heme ligation components that use heme from the heme delivery pathway (double arrows) to yield mature cyts *c*. Thick arrows show the route followed by apocyts *c*, and Cys, His, N and C refer to cysteine, histidine, N-terminal and C-terminal of apocyts *c*, respectively, and WWD the tryptophan rich motif of CcmF component of Ccm.

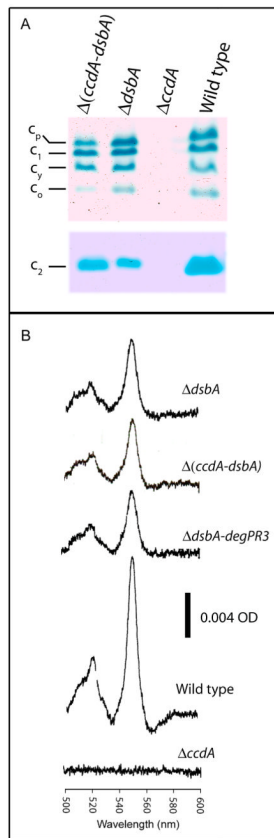


Fig. 2. Ccm thio-redox loop and related mutants

(A) Cyt *c* profiles of the Ccm thio-redox loop mutants. Chromatophore membranes were prepared from *R. capsulatus* wild type (MT1131), $\Delta ccdA$ (MD1), $\Delta dsbA$ (MD20) and $\Delta(ccdA-dsbA)$ (MD21) strains grown by respiration in minimal medium. Approximately 100 μg of total proteins were separated by SDS-PAGE, and cyts *c* profiles were visualized by TMBZ staining as described in Material and Methods. Upper and lower panels correspond to the membrane and soluble fractions, respectively. The membrane bound cyts c_p , c_1 , c_y , c_o and the periplasmic cyt c_2 are indicated on the left. (B) Cyt c_2 levels in the Ccm thio-redox loop mutants. Ascorbate-reduced minus ferricyanide-oxidized optical difference spectra of soluble fractions (protein concentration of 0.5 mg/ml) obtained from *R. capsulatus* wild type, $\Delta ccdA$, $\Delta dsbA$, $\Delta(ccdA-dsbA)$ and $\Delta dsbA-degPR3$ strains, as described in Materials and Methods and Table 2.

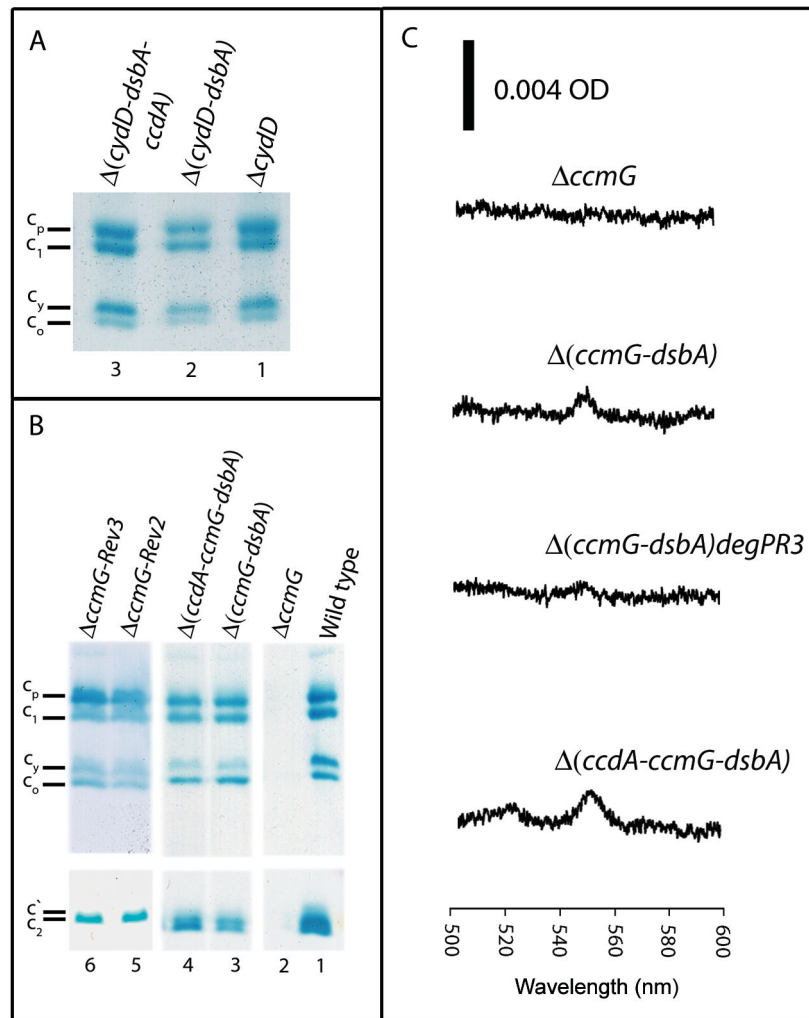


Fig. 3. Cyt *c* profiles of *R. capsulatus* thio-redox mutants

The cyts *c* production in the putative glutathione transporter mutant $\Delta cydD$ (STD1) and its derivatives $\Delta(cydD-dsbA)$ (STD3) and $\Delta(cydD-dsbA-ccdA)$ (STD5) (A), in the wild type (MT1131) and Ccm thio-reduction mutant $\Delta ccmG$ (MD11), its Ps^+ revertants ($\Delta ccmG-Rev2$) (MD11R2) and $\Delta ccmG-Rev3$) (MD11R3) and its derivatives $\Delta(ccmG-dsbA)$ (ST21) and $\Delta(ccdA-ccmG-dsbA)$ (ST34) (B), grown by respiration in minimal medium were determined by SDS-PAGE/TMBZ as described in Fig. 2 and Materials and Methods. Different cyts *c* are indicated on the left. (C) Cyt *c*₂ levels in the Ccm thio-reduction mutant CcmG and its derivatives: Ascorbate-reduced *minus* ferricyanide-oxidized optical difference spectra of soluble fractions (protein concentration of 0.5 mg/ml) obtained from *R. capsulatus* strains $\Delta ccmG$, $\Delta(ccmG-dsbA)$, $\Delta(ccmG-dsbA)degPR3$ (ST40) and $\Delta(ccdA-ccmG-dsbA)$ (Table 2) were determined as in Fig. 2.

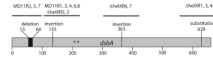


Fig. 4. Ps⁺ revertants of *R. capsulatus* CcmG-null mutants are located in DsbA

Ps⁺ revertants from a CcmG-null mutant (MD11Ri from MT1131 or ΔhelXRi from SB1003-ΔhelX) (Table 2) were isolated as described in the text, and DNA sequence of the chromosomal *dsbA* locus of each mutant was determined after PCR amplification. The mutant names, positions and type of the mutations found are indicated on top of the *dsbA* gene, and * indicates the active site cysteine residues of DsbA.

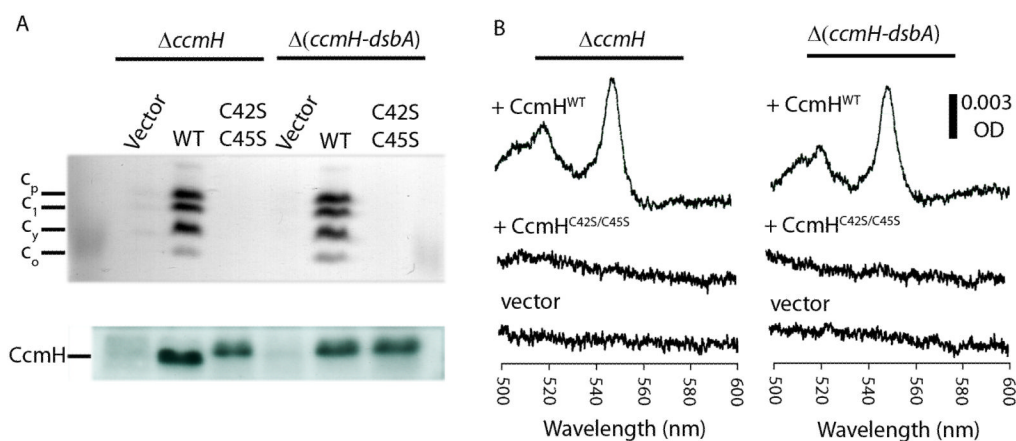


Fig. 5. Cyt *c* profiles and CcmH immunoblots of wild type and cysteineless CcmH mutants
 Cyt *c* profiles of *R. capsulatus* $\Delta ccmH$ (MD14) and $\Delta(ccmH-dsbA)$ (ST27) strains harboring empty plasmid (vector) or wild type (WT/pST6) or cysteineless (C42S C45S/pST14) alleles of *ccmH* grown in minimal medium were determined by (A) SDS-PAGE/TMBZ analyses (**top panel**) as in Fig. 2. The same preparations were also subjected to SDS-PAGE using 50 μ g total proteins per lane, and the amounts of CcmH were detected by immunoblot analyses (**lower panel**) using anti-CcmH polyclonal antibodies, as described in Materials and Methods. (B) Cyt *c*₂ levels in the CcmH-null mutant and its DsbA-null derivatives: Ascorbate-reduced *minus* ferricyanide-oxidized optical difference spectra of soluble fractions (protein concentration of 0.5 mg/ml) obtained from *R. capsulatus* $\Delta ccmH$ (left) and $\Delta(ccmH-dsbA)$ (right) mutants harboring empty plasmid (vector) or wild type (+ CcmH^{WT}) or cysteineless (+ CcmH^{C42S/C45S}) alleles of *ccmH* were determined as described in Fig. 2.

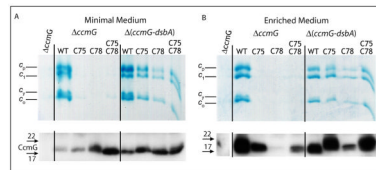


Fig. 6. Cyt *c* profiles and CcmG-immunoblots of cysteineless CcmG mutants

Cyts *c* profiles of *R. capsulatus* $\Delta ccmG$ and $\Delta(ccmG-dsbA)$ strains harboring wild type (pCcmG^{WT} (WT)) or different cysteineless (pCcmG^{C75S} (C75S), pCcmG^{C78S} (C78S) and pCcmG^{C75S/C78S} (C75S C78S)) alleles of *ccmG* grown in either minimal (A) or enriched (B) medium were determined by SDS-PAGE/TMBZ analyses (top panels), as described in Fig. 2. The same preparations were subjected to SDS-PAGE using 50 μ g total proteins per lane, and the amounts of CcmG were detected by immunoblot analyses (lower panels) using anti-CcmG polyclonal antibodies, as described in Materials and Methods.

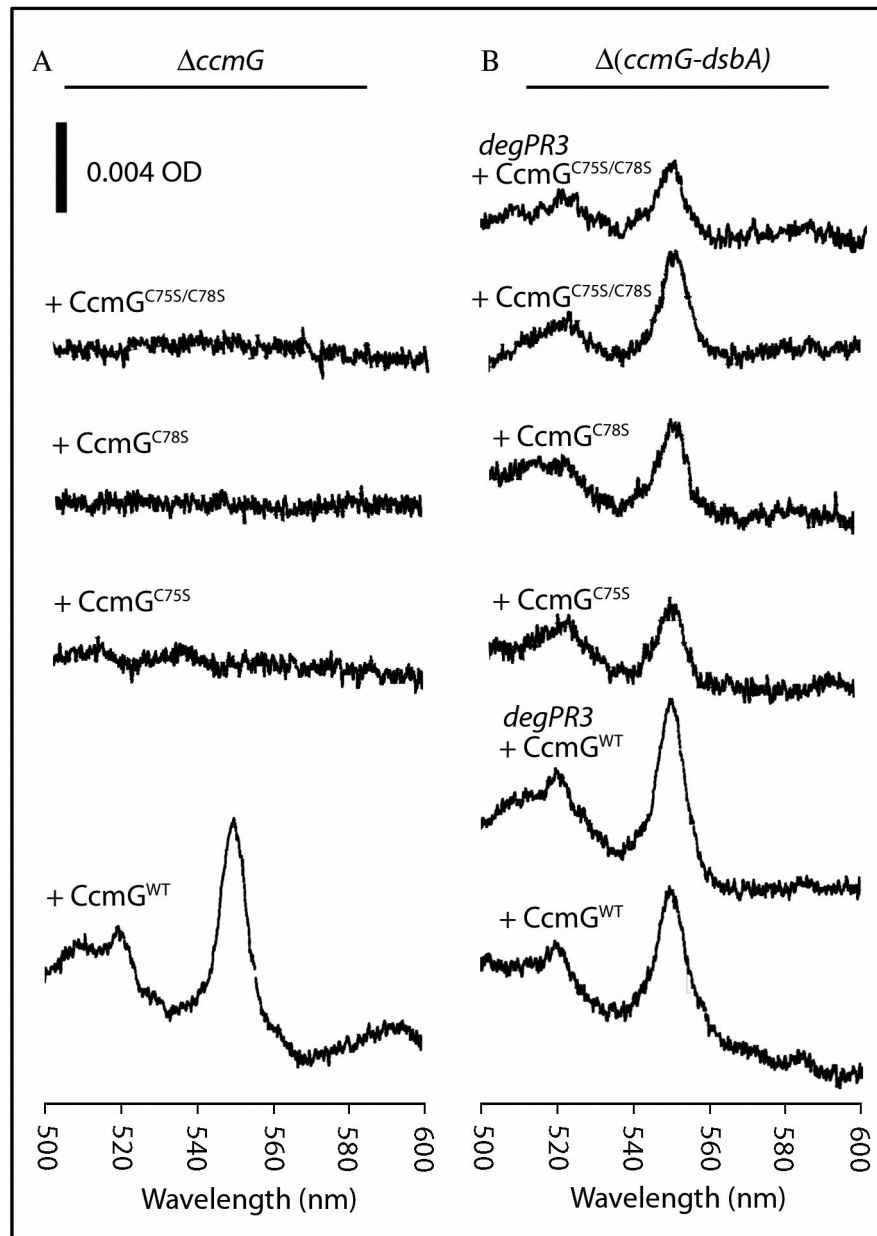


Fig. 7. Cyt c_2 levels in *R. capsulatus* cysteineless CcmG mutants and its DsbA-null derivatives Ascorbate-reduced *minus* ferricyanide-oxidized optical difference spectra of soluble fractions (0.5 mg of total proteins/ml) prepared from *R. capsulatus* $\Delta ccmG$ (A) and $\Delta(ccmG-dsbA)$ or $\Delta(ccmG-dsbA)degPR3$ (B) strains harboring (+ pCcmG^{WT} (WT)) or different cysteineless (+ pCcmG^{C75S} (C75S), + pCcmG^{C78S} (C78S) and + pCcmG^{C75S/C78S} (C75S C78S)) alleles of *ccmG*, grown on enriched medium, were determined as described in Fig. 2. In B, the spectra labeled *degPR3* were from a CcmG-null DsbA-null mutant carrying the $\Delta(ccmG-dsbA)degPR3$ mutations lowering the levels of DegP in this background (Onder *et al.*, 2008).

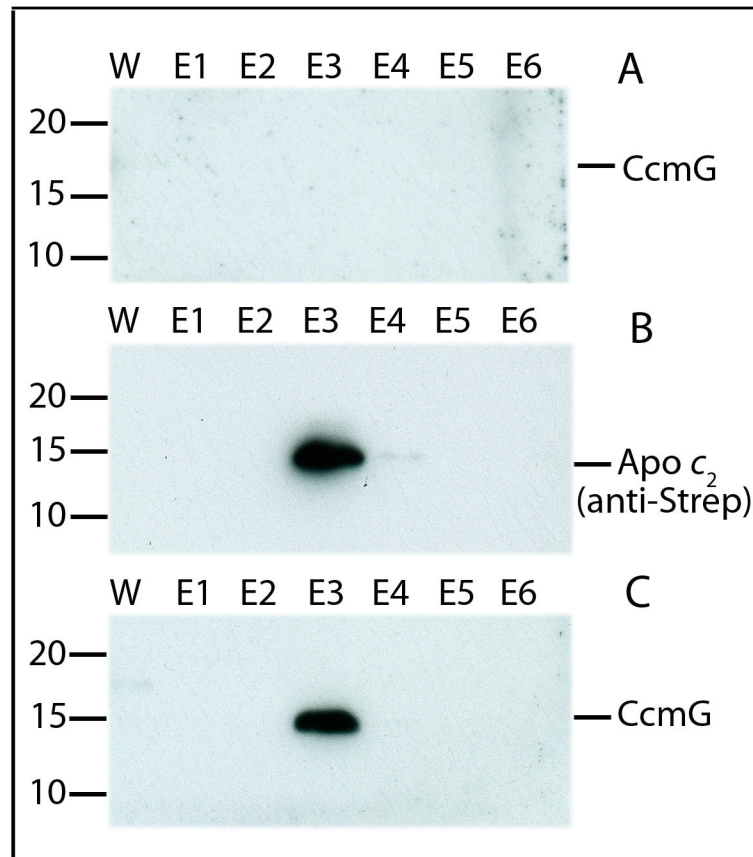


Fig. 8. Co-purification of CcmG* with apocyt *c*₂

Purified apocyt *c*₂ (50 μg) and CcmG* enriched extracts (40 μg) were incubated in the presence of 20 mM Tris-HCl pH = 7.9, 50 mM NaCl, 50 μg DTB and 10 mM imidazole for two hrs at 4 °C and loaded onto a StrepTactin® Sepharose affinity chromatography column, washed, and eluted with 2.5 mM DTB as described in Experimental Procedures. Aliquots (300 μl of 1 ml total) from various purification steps (W: column wash, and E1 to E6: DTB elution fractions 1-6) were precipitated with ice cold Acetone/Methanol and analyzed by SDS-PAGE and immunoblots. Antibodies against CcmG and Strep-tag are as indicated on the right, and molecular weight markers (in kDa) are as shown on the left of each panel. Panel A illustrates that CcmG*, which does not contain a Strep-tag, is not retained by the StrepTactin® Sepharose column in the absence of apocyt *c*₂. Panels B and C shows that in the presence of apocyt *c*₂, CcmG* is retained by the column and co-eluted with apocyt *c*₂, documenting that the two proteins interact with each other under the conditions used.

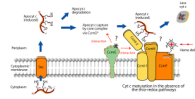


Fig. 9. Production of cyts *c* in the absence of the Ccm thio-redox loop

In the absence of the DsbA-DsbB, CcdA and CcmG thio-redox loop shown in Fig. 1, reduced apocyts *c*, although more prone to degradation, are matured at lower levels by the CcmFHI ligation components. The presence of a thio-reduction inactive CcmG derivative (CcmG* shown in dashed line) increases the amounts of mature cyts *c*, by acting as an apocyts *c* holdase improving delivery of the apocyts *c* to the CcmFHI heme ligation components. Thick arrows show the route followed by the apocyts *c*, dashed arrows indicate the interactions of apocyts *c* and heme ligation components via CcmG*, and Cys, His, N, C and WWD are as described in Fig. 1.

Table 1
Ps growth properties and cyt c₂ contents of *R. capsulatus* thio-redox mutants

Pathway	Strain	Plasmid	<i>I</i> _{Med A}		<i>Med A</i> *		MPYE		MPYE*		Cyt c ₂ (%)
			2Ps	Ps	Ps	Ps	Ps	Ps			
	Wild Type		+	+	+	+	+	+	+	+	100
Thio-oxidation mutants											
	$\Delta dsbA$		+	+	+	+	+	+	+	+	~44
	$\Delta dsbA$ - <i>degPR3</i>		+	+	+	+	+	+	+	+	~40
Thio-reduction mutants											
	$\Delta ccdA$ or $\Delta ccmG$ or $\Delta ccmH$		-	-	-	-	-	-	-	-	0
Thio-redox loop mutants											
	$\Delta(ccdA-dsbA)$		+	+	+	+	+	+	+	+	~50
	$\Delta(ccmG-dsbA)$		-	+	-	-	-	-	-	-	~10-15
	$\Delta(ccmH-dsbA)$		-	-	-	-	-	-	-	-	0
	$\Delta ccmG$ -Rev2		-	+	-	-	-	-	-	-	~10-15
	$\Delta(ccmG-dsbA)$ <i>degPR3</i>		-	+	-	-	-	-	-	-	~10
	$\Delta(ccdA-ccmG-dsbA)$		-	+	-	-	-	-	-	-	~10-15
	$\Delta(ccdA-ccmH-dsbA)$		-	-	-	-	-	-	-	-	0
	$\Delta ccmG$	pCcmG ^{WT}	+	+	+	+	+	+	+	+	100
	$\Delta(ccmG-dsbA)$	pCcmG ^{WT}	+	+	-	-	-	-	-	-	~65
	$\Delta ccmG$	pCcmG ^{C75S} or pCcmG ^{C78S} or pCcmG ^{C75S78S}	-	-	-	-	-	-	-	-	0
	$\Delta(ccmG-dsbA)$	pCcmG ^{C75S}	s	+	-	-	-	-	-	-	~40-50
		pCcmG ^{C78S}	-	+	-	-	-	-	-	-	~40-50
		pCcmG ^{C75S78S}	+	+	-	-	-	-	-	-	~48
	$\Delta(ccmG-dsbA)$ - <i>degPR3</i>	pCcmG ^{WT}	+	+	+	+	+	+	+	+	~70
		pCcmG ^{C75S78S}	+	+	+	+	+	+	+	+	~41
	$\Delta ccmH$	pCcmH ^{WT}	+	+	+	+	+	+	+	+	100
		pCcmH ^{C42S/C45S}	-	-	-	-	-	-	-	-	0

Pathway	Strain	Plasmid	<i>I</i> Med A	Med A*	MPYE	MPYE*	Cyt <i>c</i> ₂
			2Ps	Ps	Ps	Ps	(%)
	$\Delta(ccmH-dsbA)$	pCcmH ^{WT}	+	+	+	+	~90
		pCcmH ^{C42S/C45S}	-	-	-	-	0

*I*MedA and MPYE refer to minimal and enriched growth medium, respectively.

* indicates that they were supplemented with 0.33 mM cysteine and 0.165 mM cystine.

²Ps designate photosynthetic growth conditions, and +, s and – refer to normal, slow and no growth, respectively. All strains are Res⁺ in both Med A* and MPYE*, but those lacking DsbA often exhibit variable degrees of Res or Ps growth defects (slow to no growth) in the absence of the thio-reactive supplements.

Table 2

Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Relevant phenotype	Source or reference
Strains			
<i>E. coli</i>			
HB101	F ⁻ Δ(<i>gpt-proA</i>)62 <i>araC14 leuB6</i> (Am) <i>supE44 galK2</i> (Oc) <i>lacY1</i> Δ(<i>oncrC-mrr</i>) <i>rpsL20</i> (Str ^r) <i>xyiA5 mtl-1 thi-1</i>		(Sambrook, 2001)
XLI-Blue	F ⁻ ::Tn10 <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^q Δ(<i>lacZ</i>)M15/ <i>recA1 endA1 gyrA96</i> (Nal ^r) <i>thi hsdR17</i> (xg ⁻ m _g ⁺) <i>supE44 relA1 lac</i>		Stratagene
<i>R. capsulatus</i>			
MT1131 ^a	<i>ortD121</i> Rif ^r	Wild type, Res ⁺ , Ps ⁺ , cyts c ⁺	(Scolnik <i>et al.</i> , 1980)
MD1	Δ(<i>ccda</i> :: <i>spe</i>)	Res ⁺ /Ps ⁺ , cyts c ⁻	(Deshmukh <i>et al.</i> , 2000)
MD14	<i>ccmH</i> :: <i>spe ccmF</i> ⁺	Res ⁺ /Ps ⁻ , cyts c ⁻	(Deshmukh <i>et al.</i> , 2002)
MD20	Δ(<i>dsbA</i> :: <i>kan</i>)	Res ⁺ /Ps ⁺ on MedA, Res ⁺ /Ps ⁺ on MPYE, cyts c ⁺	(Deshmukh <i>et al.</i> , 2003)
MD20R3	Δ(<i>dsbA</i> :: <i>kan</i>) <i>degPR3</i>	Res ⁺ /Ps ⁺ , cyts c ⁺ , low DegP	(Onder <i>et al.</i> , 2008)
MD21	Δ(<i>ccda</i> :: <i>spe</i>) Δ(<i>dsbA</i> :: <i>kan</i>)	Res ⁺ /Ps ⁺ on MedA, Res ⁺ /Ps ⁺ on MPYE, cyts c ⁺	(Deshmukh <i>et al.</i> , 2003)
MD11	Δ(<i>ccmG</i> :: <i>kan</i>)	Res ⁺ /Ps ⁻ , cyts c ⁻	(Sanders <i>et al.</i> , 2007)
SB1003-ΔhelX	Δ(<i>ccmG</i> :: <i>kan</i>)	Res ⁺ /Ps ⁻ , cyts c ⁻	(Beckman and Kranz, 1993)
ST21	Δ(<i>ccmG</i> :: <i>kan</i>) Δ(<i>dsbA</i> :: <i>spe</i>)	Res ⁺ /Ps ⁻ on MPYE and MPYE [*] , Res ^{s/} Ps ⁻ on MedA, Res ⁺ /Ps ⁺ on MedA [*]	This work
ST38	Δ(<i>dsbA</i> :: <i>spe</i>) <i>degPR3</i>	Res ⁺ /Ps ⁺ , cyts c ⁺ , low DegP	This work
ST40	Δ(<i>ccmG</i> :: <i>kan</i>) Δ(<i>dsbA</i> :: <i>spe</i>) <i>degPR3</i>	Res ⁺ /Ps ⁻ on MPYE and MPYE [*] , Res ^{s/} Ps ⁻ on MedA, Res ⁺ /Ps ⁺ on MedA [*] , low DegP	This work
ST34	Δ(<i>ccmG</i> :: <i>kan</i>) Δ(<i>dsbA</i> :: <i>spe</i>) Δ(<i>ccda</i> :: <i>gen</i>)	Res ⁺ /Ps ⁻ on MPYE and MPYE [*] , Res ^{s/} Ps ⁻ on MedA, Res ⁺ /Ps ⁺ on MedA [*]	This work
ST27	<i>ccmH</i> :: <i>spe</i> Δ(<i>dsbA</i> :: <i>kan</i>)	Res ⁺ /Ps ⁻ , cyts c ⁻	This work
ST36	<i>ccmH</i> :: <i>spe</i> Δ(<i>dsbA</i> :: <i>kan</i>) Δ(<i>ccda</i> :: <i>gen</i>)	Res ⁺ /Ps ⁻ , cyts c ⁻	This work
MD11R _i or SB1003-ΔhelXR _i (i=1-10)	Δ(<i>ccmG</i> :: <i>kan</i>)-Rev(<i>i.e.</i> , <i>dsbA</i> _{<i>i</i>})	Res ⁺ /Ps ⁻ on MPYE and MPYE [*] , Res ^{s/} Ps ⁻ on MedA Res ⁺ /Ps ⁺ on MedA [*]	This work

Strain or plasmid	Description	Relevant phenotype	Source or reference
STD1	$\Delta(\text{cydD}::\text{gen})$	Res ⁺ /Ps ⁺ , cyts c ⁺	This work
STD3	$\Delta(\text{cydD}::\text{gen}) \Delta(\text{dsbA}::\text{kan})$	Res ⁺ /Ps ⁺ , cyts c ⁺	This work
STD5	$\Delta(\text{cydD}::\text{gen}) \Delta(\text{dsbA}::\text{kan}) \Delta(\text{ccdA}::\text{spe})$	Res ⁺ /Ps ⁺ , cyts c ⁺	This work
Plasmids			
pBluescript	pBluescript II KS(+) (pBSK)	Amp ^r	Stratagene
pRK2013	Broad host-range vector with <i>E. coli lacZ</i> promoter	Kan ^r , helper	(Ditta et al., 1985)
pRK415	Broad host-range vector with <i>R. capsulatus cycA</i> promoter	Tet ^r	(Keen et al., 1988)
pCHB500	Ω -spectinomycin (<i>spe</i>) cassette	Tet ^r	(Benning and Somerville, 1992)
pHP45Q-Spc	Phosphorylated 700 bp PCR product with <i>dsbA</i> cloned into the SmaI site of pBSK	Spc ^r	(Prentki and Krisch, 1984)
pBS-DsbA ^{wt}	4.2 kb insert carrying <i>dsbA</i> interrupted by kanamycin cassette in pRK415	Tet ^r , Kan ^r	(Deshmukh et al., 2003)
pTC4-1K	<i>ccdA</i> with engineered BglIII site at position 749 in pBluescript	Amp ^r	(Deshmukh et al., 2000)
pK6	570 bp PCR product containing <i>ccmG</i> cloned into XbaI-KpnI site of pCHB500	Tet ^r	(Sanders et al., 2005a)
pCS1540	cysteineless <i>ccmG</i> -C75S/C78S allele of <i>ccmG</i> in pCS1540	Tet ^r	(Sanders et al., 2005a)
pCS1545	2.82 kb fragment carrying <i>ccmFH</i> operon expressed from G488A up-promoter mutation in pBluescript	Amp ^r	(Deshmukh et al., 2002)
pYZ1	pRK415 with a DNA fragment containing the $\Delta(\text{ccmG}::\text{kan})$ [previously called $\Delta(\text{heIX}::\text{kan})$]	Tet ^r , Kan ^r	(Katzen et al., 2002)
pHX1	Expression vector (Qiagen)	Amp ^r , P _{T5}	Qiagen
pQE60	<i>R. capsulatus ccmG</i> without its signal peptide cloned at the NcoI and BamHI sites of pQE60 to yield a C-terminal His tagged derivative of CcmG	Amp ^r , P _{T5}	J. F. Collet, unpublished
pQE60-heIX	pET-3a derivative (Novagen) with T7 promoter region replaced by a DNA fragment encoding <i>lacI</i> and the <i>tac</i> promoter region	Amp ^r , P _{Tac} , LacI ⁺	(Sanders et al., 2001)
pCS905	pCS905 derivative, Strep-tag II sequence (IBA) fused to GFP, rendering GFP replaceable by cloning any gene of interest in frame into NdeI and BamHI sites	Amp ^r , P _{Tac} , LacI ⁺ , Strep-GFP ⁺	Sanders et al., submitted
pCS1302	<i>R. capsulatus cycA</i> derivative encoding the mature form of cyt c ₂ with a N-terminal Strep-tag cloned into pCS1302 using NdeI and BamHI sites	Amp ^r , P _{Tac} , LacI ⁺ , Strep-cyt c ₂ ⁺	Sanders et al., submitted
pCS1726	<i>R. capsulatus cycA</i> derivative encoding the mature form of cyt c ₂ with a N-terminal Strep-tag cloned into pCS1302 using NdeI and BamHI sites	Amp ^r , P _{Tac} , LacI ⁺ , Strep-cyt c ₂ ⁺	Sanders et al., submitted
pCS1757	<i>R. capsulatus cycA</i> derivative encoding the mature form of cyt c ₂ with a N-terminal Strep-tag and a C-terminal 6xHis-tag cloned into pCS1302 using NdeI and BamHI sites	Amp ^r , P _{Tac} , LacI ⁺ , Strep-cyt c ₂ ⁺ , 6xHis ⁺	This work
pCS1554	Cysteine 75 and 78 of <i>R. capsulatus ccmG</i> in pQE60-heIX mutated to serines	Amp ^r , P _{T5}	This work
pST6	<i>ccmH-Strep</i> from pST8 cloned into the XbaI-KpnI sites of pCHB500	Tet ^r	This work
pST7	PCR amplified 0.48 kb <i>ccmH</i> fragment from pYZ1 cloned into the XbaI-KpnI sites of pBluescript	Amp ^r	This work

Strain or plasmid	Description	Relevant phenotype	Source or reference
pST8	PCR amplified and 3' end Strep-tagged 0.53 kb <i>cmhH</i> fragment from pST7 cloned into the XbaI-KpnI sites of pBSK	Amp ^r	This work
pST14	<i>cmhH-C42S/C45S</i> allele in pCHB500	Tet ^r	This work
pKZ66	A 4.8 kb PCR fragment containing <i>cydDC</i> cloned into BamHI-EcoRI sites of pBSK	Amp ^r	This work
pKZ68	pKZ66 with Gen ^r cartridge replacing the 1.2 kb PstI fragment in <i>cydC</i>	Amp ^r Gen ^r	This work
pKZ69	XbaI-KpnI fragment of pKZ68 containing $\Delta(\text{cydC}::\text{gen})$ in pRK415	Tet ^r Gen ^r	This work
pKZ7	HindIII digested and religated pKZ66 without its BbsI site downstream from <i>cydC</i>	Amp ^r	This work
pKZ71	pKZ7 with Gen ^r cartridge replacing the 320 bp BbsI fragment in <i>cydD</i>	Amp ^r Gen ^r	This work
pKZ72	XbaI-KpnI fragment of pKZ71 containing $\Delta(\text{cydD}::\text{gen})$ in pRK415	Tet ^r Gen ^r	This work
pCHB::Gm	pCHB500 carrying a Gen ^r cassette (<i>gen</i>) driven by the <i>cydA</i> promoter	Tet ^r , Gen ^r	K. Zhang and F. Daldal
pBS-STA3	The Spe ^r cartridge of pHP45 Ω -Spe cloned into the BglII-SfiI sites of pBS-DsbA ^{wt}	Amp ^r , Spe ^r	This work
pSTA1	2.1 kb XbaI-KpnI fragment of pBS-STA3 cloned into the same sites of pRK415	Tet ^r , Spe ^r	This work
pST1	Phosphorylated 1.2 kb PCR fragment of Gen ^r cassette from pCHB::Gm cloned into pBluescript	Gen ^r , Amp ^r	This work
pST3	BamHI fragment of pST1 cloned into the BglIII sites of pK6	Gen ^r , Amp ^r	This work
pST4	$\Delta(\text{ccdA}::\text{gen})$ from pST3 cloned into pRK415	Gen ^r , Tet ^r	This work
pCS1543	<i>cmhG-C75S</i> allele of <i>cmhG</i> in pCS1540	Tet ^r	This work
pCS1544	<i>cmhG-C78S</i> allele of <i>cmhG</i> in pCS1540	Tet ^r	This work

^a *R. capsulatus* MT1131 is a derivative of SB1003 and referred to as wild type with respect to its growth properties and *cyts c* profile.

* refers to minimal (MedA) and enriched (MPYE) growth medium supplemented with 0.33 mM cysteine and 0.165 mM cystine.