Novel Transporter Required for Biogenesis of cbb_3-Type Cytochrome *c* Oxidase in *Rhodobacter capsulatus*

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ABSTRACT **The acquisition, delivery, and incorporation of metals into their respective metalloproteins are important cellular processes. These processes are tightly controlled in order to prevent exposure of cells to free-metal concentrations that could yield oxidative damage. Copper (Cu) is one such metal that is required as a cofactor in a variety of proteins. However, when present in excessive amounts, Cu is toxic due to its oxidative capability. Cytochrome *c* oxidases (Coxs) are among the metalloproteins whose assembly and activity require the presence of Cu in their catalytic subunits. In this study, we focused on the acquisition of Cu for incorporation into the heme-Cu binuclear center of the cbb_3-type Cox (cbb_3-Cox) in the facultative phototroph *Rhodobacter capsulatus*. Genetic screens identified a cbb_3-Cox defective mutant that requires Cu^{2+} supplementation to produce an active cbb_3-Cox. Complementation of this mutant using wild-type genomic libraries unveiled a novel gene (*ccoA*) required for cbb_3-Cox biogenesis. In the absence of CcoA, the cellular Cu content decreases and cbb_3-Cox assembly and activity become defective. CcoA shows homology to major facilitator superfamily (MFS)-type transporter proteins. Members of this family are known to transport small solutes or drugs, but so far, no MFS protein has been implicated in cbb_3-Cox biogenesis. These findings provide novel insights into the maturation and assembly of membrane-integral metalloproteins and on a hitherto-unknown function(s) of MFS-type transporters in bacterial Cu acquisition.**

IMPORTANCE **Biogenesis of energy-transducing membrane-integral enzymes, like the heme copper-containing cytochrome *c* oxidases, and the acquisition of transition metals, like copper, as their catalytic cofactors are vital processes for all cells. These widespread and well-controlled processes are poorly understood in all organisms, including bacteria. Defects in these processes lead to severe mitochondrial diseases in humans and poor crop yields in plants. In this study, using the facultative phototroph *Rhodobacter capsulatus* as a model organism, we report on the discovery of a novel major facilitator superfamily (MFS)-type transporter (CcoA) that affects cellular copper content and cbb_3-type cytochrome *c* oxidase production in bacteria.**

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Cells require copper (Cu) as a cofactor for many metalloproteins, including cytochrome *c* oxidases (Coxs) (1), superoxide dismutases (2), and multicopper oxidases (3). Among them, Cox enzymes terminate the electron transfer chains of aerobic organisms by catalyzing the four-electron reduction of dioxygen (O_2) to water during respiration. Cu is an essential catalytic and structural cofactor of this important energy transduction enzyme (1), but an excess amount of it is toxic, as it can activate O_2 via the Fenton reaction to generate dangerous reactive oxygen radicals (4, 5). Indeed, its shortage or surplus leads to severe human illnesses, like Menkes, Wilson's, and Alzheimer's diseases (6–9). Thus, cells need to precisely control Cu acquisition, trafficking, and incorporation into the target proteins.

Cox enzymes have a conserved catalytic subunit (i.e., subunit I) that contains a low-spin heme and a binuclear metal center, composed of a high-spin heme Fe and a Cu atom (i.e., Cu_B) (10). They also have additional subunits: the mammalian mitochondrial enzyme contains 13, whereas the bacterial Cox has 3 or 4 subunits in total (11). Subunit II is the primary electron acceptor and harbors extra cofactors, like another binuclear Cu center (Cu_A) in the mitochondrial aa_3-type Cox (10, 11). In the bacterial cbb_3-type Cox (cbb_3-Cox), which is important for aerobic respiration, the onset of photosynthesis, rhizobial symbiosis, and pathogenesis in various species (12–14), subunit II contains covalently bound hemes instead of a Cu_A center. The presence of various cofactors renders Cox biogenesis a complex process. Synthesis and maturation of individual subunits, their insertions into the membrane, and assembly of mature subunits into active enzymes are coordinated temporally and spatially (15). Regulated expression of the subunits and accurate insertion of the cofactors during biogenesis are required to control the concentration of potentially toxic, free redox-active cofactors or reactive assembly intermediates (5).

The purple nonsulfur facultative phototrophic bacterium *Rhodobacter capsulatus* contains only two terminal O_2 reductases, a cbb_3-Cox and a hydroquinone oxidase (Qox), that allow cells to

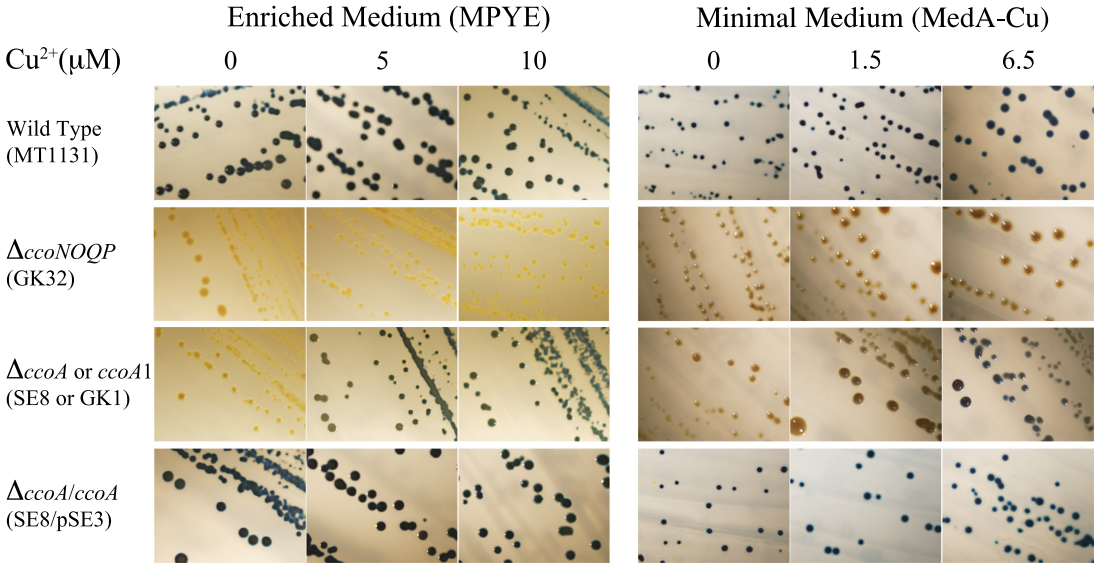

FIG 1 Growth medium-dependent NADI phenotypes of *R. capsulatus* mutants. The wild-type (MT1131), the Δ*ccoNOQP* (GK32), the Δ*ccoA* or *ccoA1* (SE8 or GK1, respectively), and the complemented Δ*ccoA*/*ccoA*+ (SE8/pSE3) strains were grown at 35°C under respiratory conditions on MPYE or MedA media supplemented with 0, 5, or 10 μM Cu^{2+}, as indicated. Colonies that contain an active cbb_3-Cox ($NADI^+$) turn blue immediately (<0.5 min) when exposed to a NADI stain, and those that have no ($NADI^-$) or low ($NADI^{slow}$) cbb_3-Cox activity remain green or become bluer upon longer (>10-min) exposure times. MedA with no Cu addition (MedA-Cu) was prepared by omitting 1.5 μM $CuSO_4$ from the regular MedA (indicated as 1.5 μM) and MedA with 6.5 μM by adding 5 μM $CuSO_4$ to the regular MedA (indicated as 0, 1.5, and 6.5, respectively).

grow under respiratory conditions even in the absence of an active Cox (16). Unlike in many other bacterial species, cbb_3-Cox is the only Cox in this species, making it an organism of choice for investigating cbb_3-Cox biogenesis. The *R. capsulatus* cbb_3-Cox has four subunits encoded by the *ccoNOQP* operon and, like all Cox enzymes, contains a heme Cu_B catalytic center located in subunit I (CcoN). It naturally lacks a Cu_A center; hence, the only Cu atom is that of the Cu_B center. Instead of a Cu_A center, cbb_3-Cox contains two membrane-bound *c*-type cytochrome subunits: the monoheme cytochrome *c* (CcoO or cytochrome c_o) and the diheme cytochrome *c* (CcoP or cytochrome c_p) as subunits II and III, respectively (17). Both CcoO and CcoP are required for electron transfer from the electron donor cytochromes *c* (i.e., cytochromes c_2 and c_y) to the heme Cu_B center at CcoN. The enzyme also has a fourth subunit (CcoQ) without any cofactor, but its absence hardly affects the enzymatic activity (18, 19).

Currently, a number of components involved in mitochondrial Cox biogenesis are known, and several of them are linked to human diseases (20–22). A smaller number of biogenesis components have been identified so far in the case of bacterial cbb_3-Cox. Earlier genetic screens for loss of Cox activity identified several genes affecting cbb_3-Cox biogenesis, including *ccoGHIS* (23), *olsAB* (24), and *senC* (25, 26). Recent proteomic approaches also associated *dsbA* and *degP* (27) with this process. However, many of the components and steps governing biogenesis of an active cbb_3-Cox still remain undefined. In particular, those involved in forming the universally conserved heme Cu_B binuclear center of Cox enzymes are unknown. In this work, we report on the molecular characterization of a cbb_3-Cox-defective mutant that requires Cu^{2+} supplementation to produce an active cbb_3-Cox. This mutant unveiled a novel transporter, CcoA, of the major facilitator superfamily (MFS), whose absence greatly diminishes the levels of intracellular Cu content and active cbb_3-Cox in *R. capsulatus*. To our knowledge, CcoA is the first example of an MFS-type transporter required for efficient Cu acquisition and cbb_3-Cox production in bacteria.

RESULTS

Cu^{2+} supplement-dependent cbb_3-Cox mutants. Previously, *R. capsulatus* mutants (e.g., GK1 [see Table S1 in the supplemental material]) that exhibited very low or no cbb_3-Cox activity in a growth medium-dependent manner were isolated following mutagenesis with ethyl methanesulfonate (28). These mutants had a NADI (α- naphthol plus dimethylphenylene diamine → indophenol blue plus H_2O)-negative (i.e., no cbb_3-Cox activity) phenotype when grown on enriched peptone yeast extract (MPYE) medium (23) but exhibited a $NADI^{slow}$ (i.e., very low cbb_3-Cox activity) phenotype on minimal (MedA) medium (Fig. 1). Subsequently, additional similar mutants (e.g., HY70 [see Table S1 in the supplemental material]) were also obtained. Two of these mutants (GK1 and HY70) were retained for further studies.

Testing of the chemical constituents of different growth media indicated that the growth medium-dependent NADI phenotype of GK1 correlated with the Cu^{2+} content of the medium used (Fig. 1). Earlier inductively coupled plasma mass spectrometry (ICP-MS) analyses indicated that, in the absence of $CuSO_4$ supplementation or chemical chelation of Cu, both MPYE and Med A lacking Cu (MedA-Cu) media contained approximately 150 nM Cu^{2+} as a "contaminant," but the level of bioavailable Cu was much lower in MPYE medium (23). When Cu^{2+} was omitted from MedA, which contains 1.5 μM $CuSO_4$, GK1 became NADI minus. Conversely, when MPYE that has no Cu^{2+} supplement contained 5 μM or more $CuSO_4$, GK1 regained a $NADI^{slow}$ phenotype (Fig. 1). Membranes of GK1 exhibited ~2% or 15% of the wild type's cbb_3-Cox activity (monitored as O_2 consumption activity in the presence of ascorbate and tetramethylphenylene di-

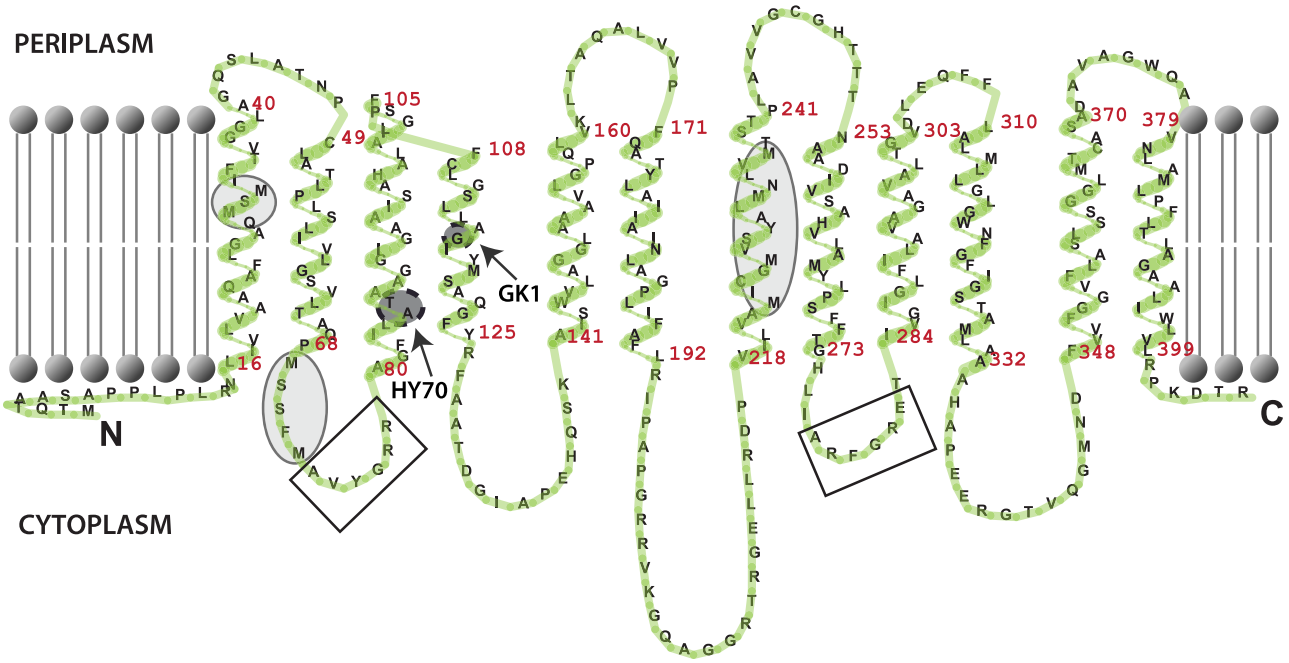

FIG 2 Topological model of the CcoA (RCAP_RCC02192) protein. The CcoA protein is predicted to have 12 transmembrane helices, like the MFS-type transporters. The topology model was drawn by using the TmRPres2D program (64). The mutations present in GK1 and HY70, the motifs similar to the conserved motifs found in MFS transporters (rectangles), and the metal-binding Mets motifs (ellipses) are also indicated.

amine [TMPD]) when cells were grown on MPYE (i.e., lacking Cu) and MedA (i.e., containing Cu) media, respectively (28). Addition of metal ions other than Cu^{2+}, including Fe^{3+}, Zn^{2+}, and Mn^{2+}, or redox-active chemicals, such as cysteine/cystine or oxidized/reduced glutathione, did not affect the NADI phenotypes of GK1, indicating that cbb_3-Cox activity in GK1 responded specifically to increased exogenous Cu^{2+} availability. Moreover, *R. capsulatus* structural (*ccoNOQP*) or assembly (*ccoGHIS*) genes of cbb_3-Cox (23, 28) or other genes (*dsbAB, senC*, and *olsAB*) known to affect its biogenesis (23, 24, 27) were unable to complement GK1 or HY70 for a $NADI^+$ phenotype in the absence of a Cu^{2+} supplement. We therefore surmised that these mutants might reveal a novel component(s) involved in cbb_3-Cox biogenesis.

New gene responsible for active cbb_3-Cox production. The gene that was defective in GK1 was identified by complementation with chromosomal libraries that were constructed using either the EcoRI or HindIII restriction enzyme. These crosses yielded the plasmids pSE1 and pSE2, which complemented GK1 to a $NADI^+$ phenotype without Cu^{2+} supplementation and carried 8.0-kb EcoRI and 4.8-kb HindIII fragments, respectively (see Fig. S1A in the supplemental material). DNA sequence determinations of the end portions of these fragments and their alignments with the *R. capsulatus* reference genome (http://www.ncbi.nlm.nih.gov) identified the chromosomal region that complemented GK1. The EcoRI fragment contained six intact open reading frames (ORFs), annotated as follows: a protein of unknown function (DUF88, GenBank accession no. RCAP_RCC02189), a heavy-metal-translocating P-type ATPase (RCAP_RCC02190), a transcriptional regulator of the MerR family (RCAP_RCC02191), a major facilitator superfamily member (RCAP_RCC02192), DNA-3-methlyadenine glycosylase II (RCAP_RCC02193), and a phospholipase/carboxylesterase family protein (RCAP_RCC02194). The HindIII fragment, which was contained within the EcoRI fragment, carried only the ORFs RCC02190, RCC02191, and RCC02192 (Fig. S1A). Three derivatives of pSE2 were constructed, namely, pSE201, pSE202, and pSE203, which contain deletions in RCC02190, in RCC02191, and in both, respectively. All three plasmids complemented GK1 to $NADI^+$ in the absence of a Cu^{2+} supplement. Concurrently, the knockout mutants SE4, SE5, and SE6, with inactive copies of RCC02190, RCC02191, and both RCC02190 and RCC02191, respectively, were constructed by interposon mutagenesis (see Text S1 in the supplemental material). All three mutants showed a $NADI^+$ phenotype (Fig. S1A). The plasmids pSE204 and pSE3 contained only RCC02192 (see Fig. S1A and -B in the supplemental material) and complemented both GK1 and HY70, whereas its derivatives with internal deletions in RCC02192, pSE5 and pSE6, were unable to do so (see Fig. S1B and Table S1 in the supplemental material). These data showed that RCC02192 was defective in GK1 and HY70.

Chromosomal knockout allele of RCC02192. A chromosomal deletion-insertion allele of RCC02192 was obtained using the gene transfer agent with pSE5 (Δ*RCC02192*::*spe*) as a donor (Text S1), to yield the mutant SE8 (Fig. S1B). Like GK1 and HY70, SE8 was $NADI^-$ on media lacking a Cu^{2+} supplement and $NADI^{slow}$ on Cu-containing media. It was fully complemented to a $NADI^+$ phenotype in the absence of Cu^{2+} supplementation by pSE3 but not by pRK-GK1 (Table S1), which carries an identical chromosomal DNA fragment derived from GK1. Thus, the defect in cbb_3-Cox activity seen in GK1 and HY70 was confined to RCC02192, which we subsequently named *ccoA* to recognize its role in cbb_3-Cox biogenesis. DNA sequencing of appropriate chromosomal regions encompassing *ccoA* defined the molecular bases of the mutation(s) in GK1 and HY70. A single base pair change (C to T) at nucleotide position 345 of *ccoA*, converting

glycine 116 of CcoA to aspartate, and a 6-base-pair-long insertion at nucleotide position 257, resulting in an in-frame insertion of a threonine-alanine dipeptide between positions 86 and 87 of CcoA, were found in GK1 and HY70, respectively (Fig. 2).

CcoA belongs to the major facilitator superfamily of transporters. In the *R. capsulatus* reference genome, *ccoA* (RCC02192) is annotated as a protein of the major facilitator superfamily (MFS). Like the MFS-type transporters, CcoA is an integral membrane protein (predicted to be 405 amino acids), with 12 putative transmembrane helices split into two subdomains of six helices each, separated by a large cytoplasmic loop (Transporter Classification Database [TCDB], http://www.tcdb.org) (29, 30) (Fig. 2). These transporters contain two highly conserved DRXGRR motifs between transmembrane helices two-three and eight-nine (AVYGRR and ARFGRE in CcoA) (31). Remarkably, mutations that inactivated *ccoA* in GK1 and HY70 are located near the first motif (Fig. 2), suggesting that this portion of CcoA is important for its function. A striking feature of CcoA is the presence of several Mets motifs (M_{30}SM, M_{70}SSFM, M_{223}ICGM, and M_{233}NLVM) associated with Cu binding and transport in Ctr-type Cu importers (32, 33). Earlier, mutating a conserved and functionally important tyrosine residue located in the same transmembrane helix as a Mets motif (YFLMLIFMT) was shown to decrease Cu transport by a Ctr-type Cu importer (33). Remarkably, a similar sequence (Y_{230}ALMNLVMT) is also present on the seventh helix of CcoA (Fig. 2).

Properties of a mutant lacking CcoA. In order to gain mechanistic insights into how the absence of CcoA decreases the activity of cbb_3-Cox in *R. capsulatus*, we compared the transcription levels of the *ccoNOQP* gene cluster between a wild type (MT1131) and its Δ*ccoA* derivative (SE8) in the presence and absence of a Cu^{2+} supplement. Total cellular RNA isolated from appropriate strains grown in MPYE medium with or without a Cu^{2+} supplement were subjected to reverse transcription-PCR (RT-PCR) using *ccoN*-specific primers (see Materials and Methods). Analyses of the amplification products (Fig. 3A) indicated that the amount of *ccoN* mRNA was slightly lower (~74% of the wild-type amount) in a mutant lacking *ccoA*. In the presence of a Cu^{2+} supplement, these amounts further decreased slightly and comparably in the *ccoA* mutant and the wild-type strain. The data indicated that the lack of CcoA had no significant effect on *ccoN* transcription and thus most likely on the entire *ccoNOQP* cluster, which is initiated from a promoter located immediately upstream of *ccoN* (28).

Next, a transcriptional-translational *ccoN*::*lacZ* fusion construct (pXG1) that carried the 220 bp 5′ of the ATG start codon and the first 13 amino-terminal codons of *ccoN* (28) was conjugated to the wild-type strain MT1131 and its Δ*ccoA* derivative SE8. The absence of CcoA decreased roughly 2-fold the amounts of β-galactosidase activity produced in these strains grown in MPYE medium without any Cu^{2+} supplement (Fig. 3B). Upon 5 μM Cu^{2+} supplementation, this activity increased slightly and comparably in both strains. Thus, neither the transcription nor the translation initiation of *ccoN* in *R. capsulatus* was abolished by the absence of CcoA, and Cu^{2+} supplementation enhanced it only marginally.

The steady-state amounts of cbb_3-Cox subunits were examined in membranes of appropriate strains grown with or without Cu^{2+} supplementation. Subunit I of cbb_3-Cox, CcoN, was monitored by immunoblot analyses using polyclonal anti-*R. capsulatus* CcoN antibodies (28). Subunits II and III, CcoO and CcoP, were visualized using SDS-PAGE with tetramethylbenzidine (TMBZ) staining, which reveals specifically membrane-bound *c*-type cytochromes (see Materials and Methods). The amount of CcoN in SE8 lacking CcoA was much lower than that seen in the wild-type strain, MT1131, and addition of 5 μM Cu^{2+} supplement increased this amount in both strains (Fig. 4A). Similarly, the amounts of CcoO and CcoP (cytochromes c_o and c_p, respectively) were lower in the absence of CcoA than in the wild-type strain. Cu^{2+} supplementation increased these amounts (Fig. 4B), even though no effect was seen with other cbb_3-Cox-unrelated membrane-bound *c*-type cytochromes (e.g., cytochromes c_1 and c_y). Thus, in the absence of CcoA, the steady-state amounts of the structural subunits of cbb_3-Cox in membranes were highly decreased, and Cu^{2+} supplementation palliated this defect(s) partially.

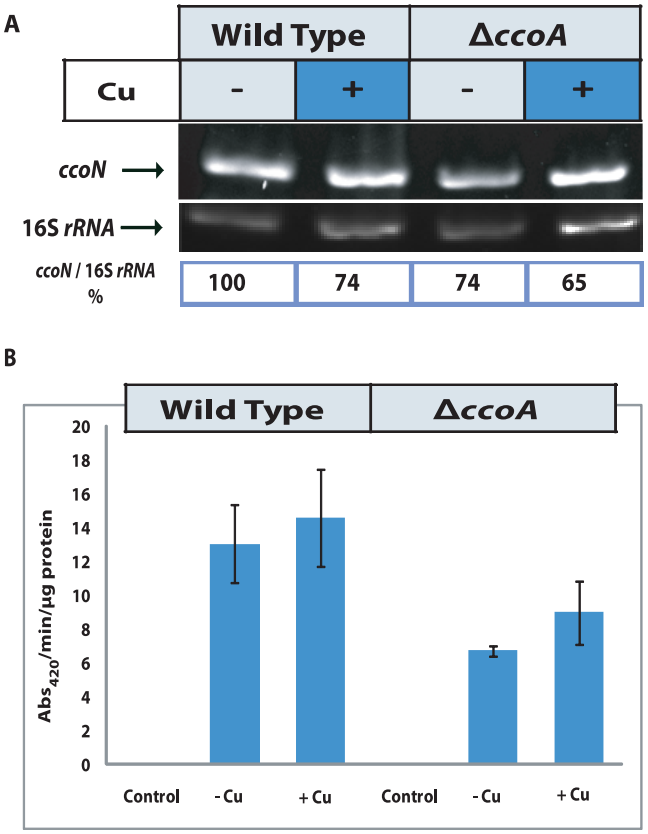

FIG 3 Effects of *ccoA* mutation on the expression of *ccoNOQP* structural genes of cbb_3-Cox. (A) The top row shows the results of RT-PCR of total RNA from wild-type (MT1131) and Δ*ccoA* mutant (SE8) strains grown in MPYE media supplemented with 0 or 5 μM Cu^{2+}. The middle row shows 16S rRNA gene expression, which is not affected by the *ccoA* mutation, and the bottom row shows the ratios of *ccoN* to 16S rRNA gene expression for comparison. The intensities of the bands were determined by ImageJ software (NIH). A control PCR omitting reverse transcriptase enzyme was performed in each case to check for DNA contamination (data not shown). (B) β-Galactosidase activities measured in cell extracts derived from the wild type (MT1131) and Δ*ccoA* mutant (SE8) strain carrying the *ccoN*::*lacZ* gene fusion, grown by respiration on MPYE media without Cu^{2+} (−Cu) or with (+Cu) 5 μM Cu^{2+} supplementation. Control reactions refer to the assays performed with cell extracts from the wild type and Δ*ccoA* mutant carrying the pXCA601 plasmid, which contains a promoterless *lacZ* gene used to construct the *ccoN*::*lacZ* fusion. The assays were performed at least in duplicate, and the activities are presented in nanomoles of ONPG hydrolyzed per minute per microgram of total proteins.

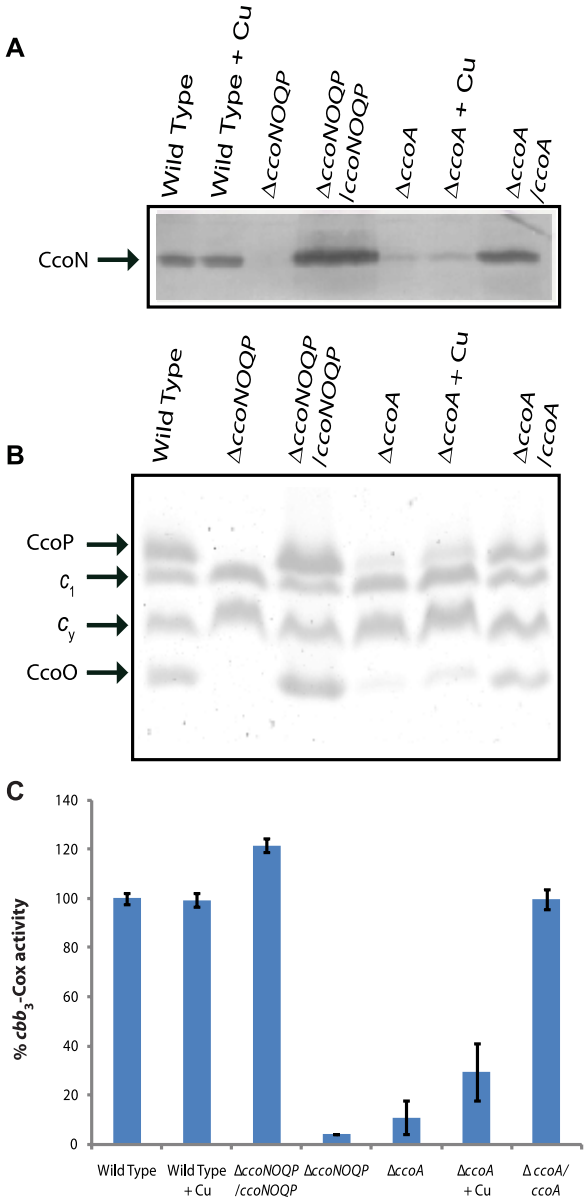

FIG 4 cbb_3-Cox subunit profiles and enzyme activity in membranes of various *R. capsulatus* strains. Chromatophore membranes derived from the wild-type (MT1131), Δ*ccoNO* (GK32), Δ*ccoNO/ccoNOQP* (GK32/pOX15), Δ*ccoA* (SE8), and Δ*ccoA/ccoA* (SE8/pSE3) strains grown at 35°C under respiratory conditions on MPYE medium supplemented (+Cu) or not supplemented with 5 μM Cu were prepared as described in Materials and Methods. (A) Immunoblot analysis to define the amounts of cbb_3-Cox subunit I CcoN. Approximately 50 μg of chromatophore membranes were separated by 12% SDS-PAGE and treated as described in Materials and Methods, using anti-CcoN polyclonal antibodies. (B) Comparison of membrane-associated cytochrome *c* profiles of various strains. Approximately 50 μg chromatophore membranes prepared from appropriate strains, grown as described above, were separated using 16.5% SDS-PAGE, and the *c*-type cytochromes were visualized using TMBZ staining as described in Materials and Methods. CcoO and CcoP refer to subunits II and III of cbb_3-Cox, and c_1 and c_y correspond to the cytochrome c_1 subunit of the cytochrome bc_1 complex and membrane-attached electron carrier cytochrome c_y, respectively. (C) cbb_3-Cox activities of various strains determined by monitoring the rate of oxidation of reduced horse heart cytochrome *c*, as described in Materials and Methods. The Cox activity exhibited by the wild-type strain, MT1131 (1.5 μmol of cytochrome *c* oxidized per min and per mg of total proteins), was taken as 100 to determine the relative amounts of Cox activities of appropriate strains. At least two independent duplicates were performed for each assay.

TABLE 1 Total Cu contents of the *R. capsulatus* wild type and of the Δ*ccoA* strain and its revertants as determined by ICP-DRC-MS[b]

Strain	5 μM Cu^{2+}	Relative level of:			
		Cu	Mn	Zn	Fe
Wild type (MT1131)	−	100[a]	100[a]	100[a]	100[a]
	+	363	111	ND	ND
Δ*ccoA* mutant (SE8)	−	79	107	101	97
	+	146	118	ND	ND
Δ*ccoA*Rev1 (SE8R1)	−	126	102	101	96
	+	490	137	ND	ND
Δ*ccoA*Rev2 (SE8R2)	−	150	100	93	92
	+	433	129	ND	ND

[a] A mean value of 12 μg Cu, 6.3 μg Mn, 34.5 μg Zn and 268 μg Fe per gr of lyophilized cells was determined and is referred to as 100% for the wild-type strain grown in the absence of Cu^{2+} in MPYE medium under respiratory growth conditions.
[b] For each strain, two sets of independently grown cells were analyzed; for each measurement, at least two repeats were done, and differences of ~10 to 20% were observed between the measurements. For a given strain, the absolute amounts of metals determined varied from culture to culture in MPYE medium, but the trend of metal contents of different strains remained unchanged between the cultures. In each case, the mean value of all measurements was presented as a percentage of the value obtained with the wild-type cells treated under the same conditions, as described in Materials and Methods. + or − refers to cells grown in the presence or absence of 5 μM Cu^{2+} added to MPYE medium.

The cbb_3-Cox activity present in detergent-dispersed membranes from cells lacking CcoA was determined using reduced horse heart cytochrome *c* (see Materials and Methods). The total amount of cbb_3-Cox activity detected in SE8 lacking CcoA was ~5 to 10% of that seen in the wild-type strain, MT1131 (Fig. 4C). Upon addition of 5 μM Cu^{2+} supplement, the cbb_3-Cox activity of SE8 increased to ~20 to 30% of that of MT1131, which was unchanged under these conditions. As expected, upon complementation with a plasmid carrying *ccoA* (e.g., SE8/pSE3), the NADI phenotypes, steady-state amounts, and activities of cbb_3-Cox reached wild-type levels (Fig. 1 and 4). Therefore, the absence of CcoA affected a step(s) that was subsequent to transcription and translation initiation of *ccoN* during biogenesis of *R. capsulatus* cbb_3-Cox under respiratory growth conditions. This defect(s) decreased drastically the steady-state amount and activity of cbb_3-Cox, and Cu^{2+} supplementation alleviated it partially.

The absence of CcoA decreases the total Cu content of *R. capsulatus* cells. In order to determine whether the absence of CcoA affected the intracellular Cu content, cells grown in MPYE medium with or without 5 μM Cu^{2+} supplement were analyzed by inductively coupled plasma dynamic-reaction cell mass spectrometry (ICP-DRC-MS) (see Materials and Methods). In the absence of a Cu^{2+} supplement, washed and lyophilized cells of SE8 lacking CcoA contained ~20% less Cu than its wild-type parent, MT1131, whereas the amounts of Fe, Mn, and Zn found in these strains were unchanged (Table 1). Both wild-type and CcoA-lacking cells grown in the presence of 5 μM Cu^{2+} contained larger amounts of Cu, but the amount found in the absence of CcoA was again ~60% smaller than that seen in the wild-type parent, MT1131. Thus, in the absence of CcoA, the intracellular Cu accumulation in *R. capsulatus* cells grown by respiration was significantly decreased but not completely abolished.

The absence of CcoA does not affect the production of multicopper oxidase. *Rhodobacter* species contain, in addition to cbb_3-Cox, other Cu cofactor-containing enzymes, like Cu/Zn superoxide reductase (34) or multicopper oxidase (laccase or CutO)

(35). Unlike *Rhodobacter sphaeroides*, *R. capsulatus* does not contain a Cu/Zn superoxide dismutase but has a twin-arginine translocation (TAT) signal sequence containing periplasmic CutO, which confers resistance to Cu^{2+}. Mutants lacking CutO exhibit increased sensitivity to Cu^{2+} toxicity, and its protective effect is readily observed under anoxygenic photosynthetic growth conditions (35). In order to test whether CutO enzyme was defective in the absence of CcoA, the *R. capsulatus cutO* gene (RCC02110) was cloned and an insertion-deletion allele (*cutO*::*kan*) was constructed and introduced into both wild-type *R. capsulatus* strain MT1131 and its Δ*ccoA* derivative SE8 (see Text S1 in the supplemental material). The sensitivity to Cu^{2+} of a Δ*cutO* single (SE15) and a Δ*ccoA* Δ*cutO* double (SE16) mutant (Table S1) was determined by a plate growth inhibition assay (see Materials and Methods) and compared to the Cu^{2+} sensitivity of SE8. The sizes of growth inhibition zones surrounding filter disks soaked with various concentrations of Cu^{2+} were determined. As expected, a Δ*cutO* mutant (SE15) was sensitive to Cu^{2+} under photosynthetic growth conditions, unlike the wild-type strain (MT1131). Unlike the Δ*cutO* mutant, a Δ*ccoA* mutant (SE8) was not sensitive to Cu^{2+}, like the wild-type strain (MT1131) (Fig. 5A). Similarly, a Δ*cutO* Δ*ccoA* double mutant was not more sensitive to Cu^{2+} than a Δ*cutO* mutant (not shown). Thus, the data inferred that CutO must still be functional to confer Cu^{2+} tolerance in the absence of CcoA in *R. capsulatus*.

Bypass suppressors of *ccoA* are hypersensitive to Cu^{2+}. During the complementation experiments using genomic libraries, we noticed that GK1 reverted to the $NADI^+$ phenotype at unusually high frequencies (~10^{-3} to 10^{-4}) when grown by respiration without the Cu^{2+} supplement. DNA sequence analyses of the *ccoA* locus in several such revertants indicated that these revertants still retained the initial mutation (a C-to-T change at position 345 of *ccoA*) carried by GK1 and produced cbb_3-Cox enzyme (Fig. 5B). Similar high frequencies of reversion to the $NADI^+$ phenotype were observed with SE8, which carried a deletion-insertion allele of *ccoA*, suggesting that the revertants restored the ability to produce cbb_3-Cox activity without any need for Cu^{2+} supplementation, thus bypassing the role of CcoA. Unexpectedly, when tested for their response to the Cu^{2+} supplement, the *ccoA* suppressors SE8R1 [Δ(*ccoA*::*spe*)*rev1*] and SE8R2 [Δ(*ccoA*::*spe*)*rev2*] (see Table S1 in the supplemental material) showed hypersensitivity to Cu^{2+} under both respiratory (Res) (Fig. 5B and C) and photosynthetic (Ps) (not shown) growth conditions. The *R. capsulatus* wild-type strain MT1131 and its derivative SE8, lacking CcoA, are tolerant up to a millimolar concentration of Cu^{2+}, but the *ccoA* suppressors SE8R1 and SE8R2 were sensitive to micromolar amounts of a Cu^{2+} supplement for respiratory growth inhibition. Indeed, these mutants were partially growth inhibited in MedA medium containing 1.5 μM Cu^{2+} and completely inhibited by addition of ~25 μM Cu^{2+} supplement to MPYE medium. This hypersensitivity was specific to Cu^{2+} only, as no similar effect was seen with other metals, including Fe^{3+}, Mn^{2+}, Zn^{2+}, and Ag^+, and oxidants, such as cystine or glutathione. We therefore concluded that SE8R1 and SE8R2 regained the ability to produce cbb_3-Cox at the expense of decreased tolerance to Cu^{2+} toxicity.

Total intracellular Cu contents of SE8R1 and SE8R2 cells grown in MPYE medium with and without 5 μM Cu^{2+} supplement were also determined using ICP-DRC-MS analyses (see Materials and Methods). In the absence of a Cu^{2+} supplement, the total intracellular Cu contents of these mutants were greater than

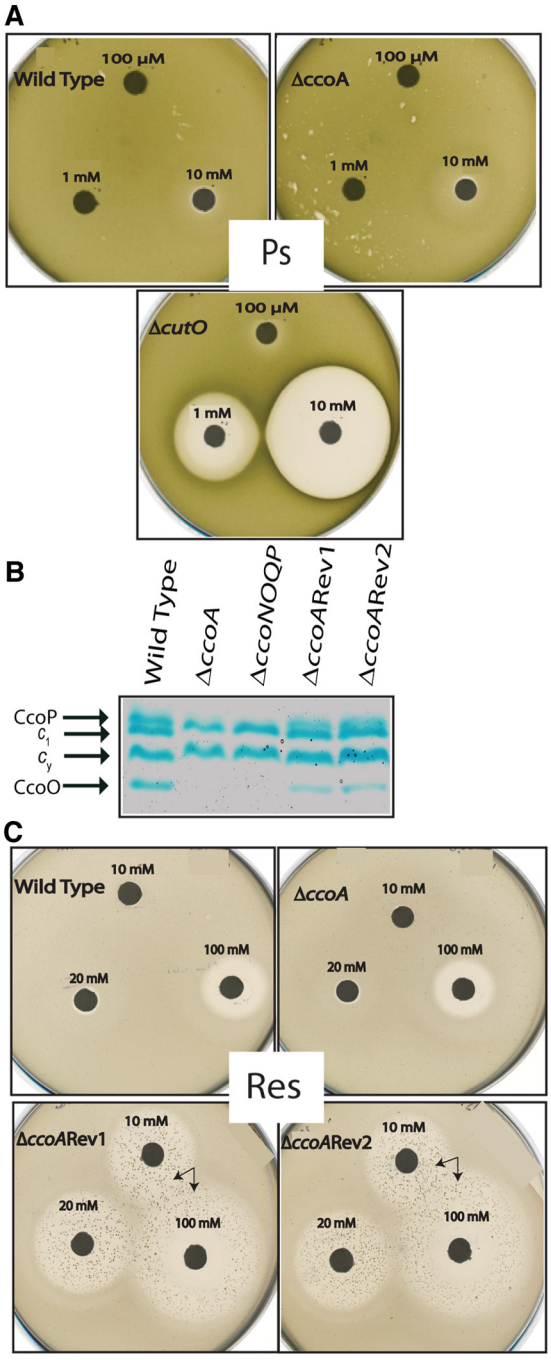

FIG 5 Cu^{2+} response of *R. capsulatus* strains lacking CcoA and its derivatives. (A) Cu^{2+} sensitivity or resistance phenotypes of the *R. capsulatus* wild-type (MT1131), Δ*ccoA* (SE8), and Δ*cutO* (SE15) strains under anaerobic photosynthetic growth conditions on MPYE medium were determined as described in Materials and Methods. Filter paper discs were soaked in 100 μM, 1 mM, and 10 mM Cu^{2+}, as indicated, and plates were incubated for 2 days under photosynthetic growth conditions (Ps) to visualize the growth inhibition zones. (B) Cytochrome *c* profiles of appropriate *R. capsulatus* strains grown in enriched MPYE medium. See Fig. 4B for details. (C) The Cu^{2+} sensitivity or resistance phenotypes of the *R. capsulatus* wild-type (MT1131) and Δ*ccoA* (SE8) strains and Δ*ccoA* revertant 1 (Δ*ccoA*Rev1; SE8R1) and 2 (Δ*ccoA*Rev2; SE8R2) under respiratory growth conditions (Res) on MPYE medium were determined as described above for panel A, except that filter paper discs were soaked in 10 mM, 20 mM, and 100 mM Cu^{2+}, as *R. capsulatus* is more tolerant to Cu^{2+} under aerobic growth conditions. Arrows indicate Cu-resistant derivatives of SE8R1 and SE8R2.

in their parent, SE8, which lacks CcoA, and similar or greater than in the wild-type strain, MT1131 (Table 1). In the presence of a Cu^{2+} supplement, all strains accumulated larger amounts of intracellular Cu. The levels found in SE8R1 and SE8R2 were much higher than those seen in the wild-type strain MT1131, although the intracellular amounts of Mn used as an internal control were unchanged in all cases (Table 1). Thus, the suppressor mutation(s) bypassed the absence of CcoA by increasing specifically intracellular Cu accumulation at the expense of compromised cellular tolerance to this toxic metal. The molecular basis of this suppression, which is beyond the scope of this work, remains to be identified.

DISCUSSION

The impetus behind this work was to understand how cells assemble catalytic metal cofactors into the heme Cu_B binuclear center of Cox, a process that is largely uncharacterized. Using *R. capsulatus*, we initiated a genetic approach to investigate bacterial Cox biogenesis and isolated various cbb_3-Cox-defective mutants. In this work, we focused on mutants that produced an active cbb_3-Cox only upon exogenous Cu^{2+} supplementation. Studies of these mutants uncovered a novel gene, *ccoA*, which was distinct from *ccoGHIS* (23), *senC* (26), *olsAB* (24), and *dsbA* and *degP* (27), known to affect this process. Mutants lacking CcoA were unable to produce normal amounts of cbb_3-Cox activity because the steady-state amounts of the subunits of this enzyme were drastically decreased in membranes. However, neither the transcription of the structural genes *ccoNOQP* nor the translation initiation of CcoN was abolished in the absence of CcoA, indicating that cbb_3-Cox assembly was defective. Whether *ccoA* affects only cbb_3-Cox is not known. Its absence does not inactivate the periplasmic Cu-containing multicopper oxidase CutO in *R. capsulatus*. The putative TAT signal sequence of CutO suggests that this enzyme might acquire its Cu cofactor in the cytoplasm prior to translocation, in a manner similar to that of its *Escherichia coli* homologue, CueO (3). Investigation of bacterial species that have CcoA homologues, like *R. sphaeroides* or *Bradyrhizobium japonicum*, and that also produce aa_3-Cox might further elucidate its role in the biogenesis of other Cox enzymes.

A major finding of this work was that the *ccoA* gene encodes a member of the MFS-type transporters, which have not been implicated hitherto into cbb_3-Cox biogenesis in bacteria. Remarkably, CcoA homologues with Mets motifs are present in most bacteria that contain cbb_3-Cox (except the epsilonproteobacteria and the *Cytophaga-Flexibacter-Bacteroides* [CFB] group) (see Table S2 in the supplemental material), suggesting that they are important for the production of this enzyme. How CcoA affects cbb_3-Cox assembly is intriguing. The MFS-type secondary transporters use the electrochemical potential difference generated by ion or solute gradients and transport a diverse range of substrates in and out of cytoplasm (36). Some MFS proteins have been implicated as importers or exporters of siderophores, including *E. coli* EntS (37), *Vibrio parahaemolyticus* PvsC (38), and *Sinorhizobium meliloti* RhtX (39), which secrete enterobactin, vibrioferrin, and rhizobactin, respectively. *R. capsulatus* mutants lacking CcoA produce various *c*-type cytochromes that rely on efficient siderophore trafficking and Fe supply for heme production, indicating that CcoA is not involved in Fe uptake. This is also supported by the unchanged intracellular Fe concentration in the CcoA knock out strain (Table 1). Instead, several findings suggest that CcoA is involved in cellular Cu acquisition for cbb_3-Cox assembly. First, mutants lacking CcoA exhibit enhanced cbb_3-Cox activity upon increased exogenous Cu^{2+} supplementation. This enhancement is specific to Cu^{2+} only, as Zn^{2+}, Mn^{2+}, or Fe^{3+} addition has no similar effects. Mass spectrometry measurements indicated that in cells lacking CcoA, only the total Cu (and not Zn, Mn, or Fe) content is lower than that of a wild-type cell under normal growth conditions. Upon Cu^{2+} supplementation, cellular Cu content increased in mutants lacking CcoA, although it never reached wild-type levels, whereas the cellular contents of metals other than Cu, like Zn, Mn, or Fe, remained unaffected. These findings are consistent with CcoA being involved in a Cu influx rather than a Cu efflux pathway. It is noteworthy that *R. capsulatus* mutants lacking CcoA still contain cellular Cu, indicating that unrelated and currently unknown Cu acquisition pathways also exist in this species.

Second, mutants lacking CcoA are not more sensitive to Cu^{2+} supplementation than a wild-type *R. capsulatus* strain under various growth conditions, suggesting that CcoA is not involved in Cu detoxification, unlike, for example, the P1B-type Cu exporters (40–42). Moreover, suppressor mutants that bypass the need for CcoA to recover cbb_3-Cox activity are extremely sensitive to very small amounts (~25 μM) of Cu^{2+} supplement in the medium. Again, this sensitivity is specific to Cu^{2+}, as these revertants exhibit normal tolerance towards Zn^{2+}, Mn^{2+}, Fe^{3+}, and even Ag^{+}, known to mimic Cu^{+} (42, 43). Mass spectrometry measurements indicated that in these suppressor mutants, Cu content was similar to that seen in the wild-type cells, suggesting that the suppressors overcame the function of CcoA by enhancing Cu acquisition or retention. Indeed, upon Cu^{2+} supplementation, the suppressor mutants accumulated much larger amounts of intracellular Cu (and not Mn) than wild-type cells. Based on the overall findings, it is therefore compelling to rationalize that the absence of CcoA induces an intracellular Cu^{2+} shortage to decrease cbb_3-Cox production (Fig. 6). Consequently, the availability of an increased exogenous Cu^{2+} supply or the occurrence of an additional mutation(s) overcomes this shortage to yield normal amounts of cbb_3-Cox at the expense of a cell's tolerance of Cu, which is compromised.

Bacteria utilize multiple transporters to achieve the movement of metals across the membrane and maintain homeostasis with metals in nontoxic concentrations (44). In the case of Cu, homeostatic pathways are multiple and complex (45, 46). For example, energy-dependent primary transporters of the P1B-type ATPases are involved in Cu^{+} efflux from the cytoplasm to the periplasm. Of these, CopA1-type transporters have high efflux rates and are involved in Cu detoxification. Their expressions are induced by an excess of Cu, and their absence induces Cu sensitivity (40, 42, 47, 48). In contrast, CopA2-type transporters, like *R. capsulatus* CcoI of the *ccoGHIS* gene cluster, have low efflux rates and no role in Cu toxicity but are involved in cbb_3-Cox biogenesis (23, 40). RND (resistance-nodulation-cell division protein family)-type transporters are also involved in the efflux of Cu ions from both the cytoplasm and the periplasm to the extracellular milieu to detoxify cells (49). Clearly, while Cu efflux and detoxification pathways are elaborate, how Cu is imported into the bacterial cytoplasm is less well known (41, 46). Only a few proteins, including *Enterococcus hirae* CopA (48), *Pseudomonas aeruginosa* HmtA (50), *Bacillus subtilis* YcnJ (51), *Pseudomonas syringae* CopCD (52), the cyanobacterial P1B-type ATPases (CtaA and PacS located in the cytoplasmic and thylakoid membranes, respectively) (53), and two

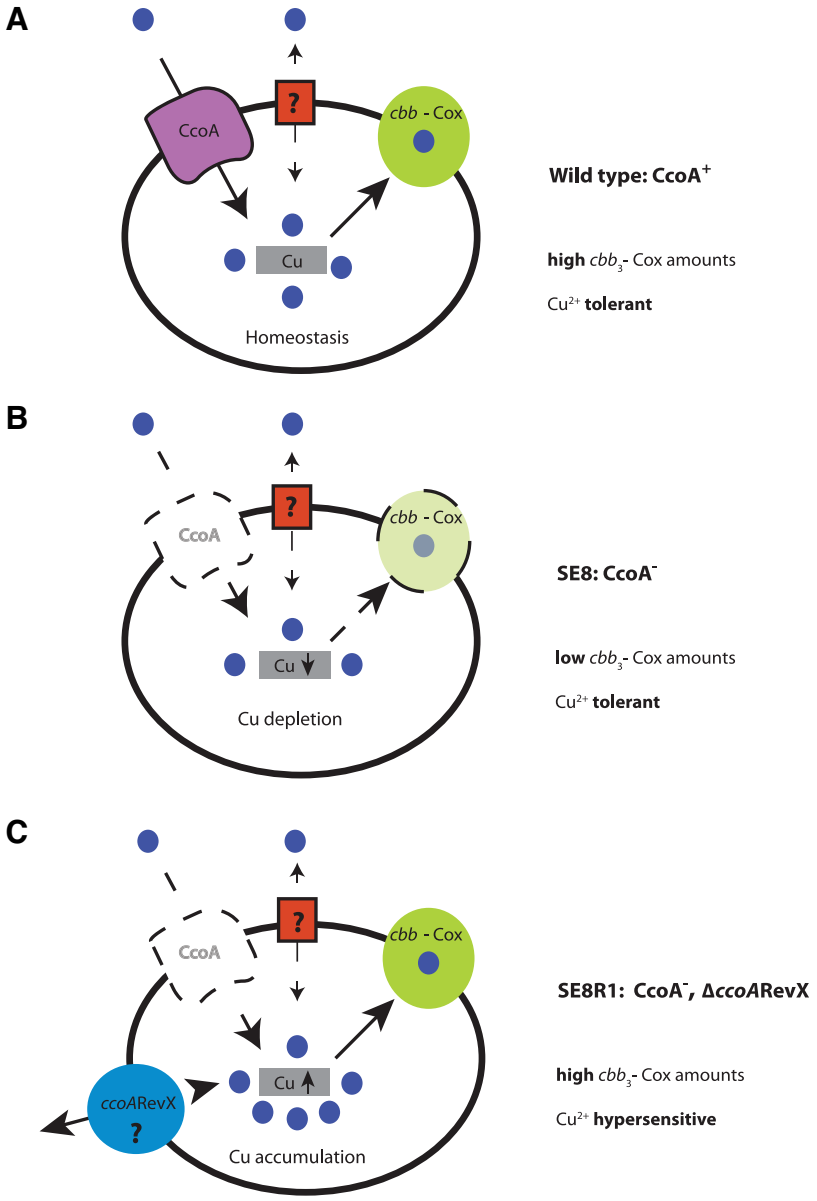

FIG 6 Hypothetical model of how CcoA affects cellular Cu content and the production of cbb_3-Cox in *R. capsulatus*. (A) A wild-type strain (MT1131) requires CcoA to produce normal amounts of active cbb_3-Cox and exhibits tolerance to Cu^{2+}. (B) In the absence of CcoA (SE8), cbb_3-Cox formation is abolished and intracellular Cu amounts are decreased, but cells remain tolerant to Cu^{2+}. (C) The absence of CcoA can be suppressed by a second mutation(s), referred to as Δ*ccoA*RevX (e.g., SE8R1 or SE8R2), that restores the production of active cbb_3-Cox at the expense of increased intracellular Cu amounts and renders cells sensitive to Cu^{2+}.

plant chloroplast P1B-type ATPases (PAA1/HMA6 and PAA2/HMA8 of the inner and thylakoid membranes, respectively) (54), have been implicated in Cu import. In eukaryotic microbes like *Saccharomyces cerevisiae*, mainly the plasma membrane-located Ctr-type transporters are known to import Cu with very high affinity into the cytoplasm (45, 46). So far, no bacterial Ctr homologue has been reported, and until very recently, no MFS-type Cu transporter was known. Very recently, Beaudoin et al. (56) reported a novel forespore membrane Cu transporter, Mfc1, which is distinct from the Ctr-type transporters (55) and involved in meiotic and sporulating cells of *Schizosaccharomyces pombe*. Mfc1 is a member of the MFS-type transporters, and it functions as a specific Cu importer during meiotic differentiation under Cu-limiting conditions in *S. pombe* (56). Excitingly, *S. pombe* Mfc1 is homologous to *R. capsulatus* CcoA, has a topology similar to CcoA's (with 12 transmembrane helices with cytoplasmic amino- and carboxyl-terminal ends), and contains other landmarks of MFS-type transporters. Moreover, both Mfc1 and CcoA contain the Mets motifs (MXM, MXXM, and MXCXM) involved in Cu transport. The Cu^{2+} importer function of Mfc1 was established by direct-transport assay using radioactive ^{64}Cu and by observation of its ability to complement appropriate *S. cerevisiae* mutants lacking the known Cu-importing Ctr-type transporters. The similarities found between CcoA and Mfc1 suggest that CcoA also acts as

a Cu importer, although direct proof of this is lacking. Finally, as in some methane-oxidizing bacteria (methanotrophs), Cu is imported into the cytoplasm, which is associated with siderophore-like molecules (called chalkophores or methanobactins) (57, 58), and whether CcoA-mediated Cu acquisition involves additional compounds (59) needs investigation.

In summary, our findings establish for the first time that the MFS-type transporter CcoA is required for maintaining normal amounts of intracellular Cu and cbb_3-Cox in *R. capsulatus* and possibly in other bacterial species. Future work will hopefully elucidate the links between CcoA, cellular Cu acquisition, and cbb_3-Cox biogenesis.

MATERIALS AND METHODS

Strains, culture conditions, phenotypes, and molecular genetic techniques. Detailed descriptions of strains, culture conditions, phenotypes, and molecular genetic techniques used in this study are presented in the supplemental material (Text S1).

Cu^{2+} sensitivity assays. Strains to be tested for Cu^{2+} sensitivity were grown to the exponential phase (optical density at 630 nm [OD_{630}] of ~0.5) in MPYE medium under appropriate growth conditions. Cells for respiratory (1.7×10^7 cells) and photosynthetic (2.6×10^7 cells) growth conditions (estimated with 1.0 OD_{630} unit equal to 7.5×10^8 *R. capsulatus* cells per ml) were added to 4 ml of the same medium containing 0.7% top agar and poured on top of regular plates containing 10 ml medium. Whatman 3 MM paper discs (3 mm in diameter), soaked with 8 μl per disc of desired concentrations of $CuSO_4$ solution, were placed on the surfaces of the plates after solidification of the top agar. Plates were incubated under the desired growth conditions and scanned at the end of incubation period, and the sizes of the growth incubation zones exhibited by different mutants were measured to estimate their responses to $CuSO_4$ toxicity. Each assay was repeated at least three times.

RNA isolation and RT-PCR. *R. capsulatus* cultures were grown semiaerobically in MPYE medium until mid-log phase (OD_{630} of approximately 0.5), and total RNA was isolated from about 2×10^8 cells using the Qiagen RNeasy minikit, digested with DNase I for 25 min at room temperature, and ethanol precipitated. Fifty nanograms of total RNA was used per RT-PCR with the Qiagen OneStep RT-PCR kit, and the *ccoN* BHK20B (5′ CCAGTCGGGCAGCGCGGTAT 3′) and N3-RT (5′ CGGC AACGGGATGCTGAACTTC 3′) primers amplified a 652-bp-long region internal to *ccoN* that corresponds to positions 299 and 972 of *ccoN*. The primers 16SrRNA-F (5′ ATATTCGGAGGAACACCAGTGGC 3′) and 16SrRNA-R (5′ CAGAGTGCCCAACTGAATGATGG 3′) were used as a control for amplification of a 450-bp region of the 16S rRNA gene. In each case, RT-PCR controls were prepared by omitting the reverse transcriptase enzyme from the reaction mixture, the amplification products were separated using 1% agarose gels, and their intensities were compared by ImageJ (NIH).

Cell extract preparation. Cells were grown in 10 ml of MPYE medium by respiration and harvested at 4,000 pm for 10 min. Pellets were resuspended in 200 μl of CellLytic B 2× cell lysis solution (Sigma Inc.) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 50 μg lysozyme, 20 μg DNase, and 10 mM $MgCl_2$, incubated at room temperature for 15 min, and centrifuged at 14,000 rpm for 10 min. Supernatants thus obtained were taken as whole-cell extracts. Intracytoplasmic membrane vesicles (chromatophore membranes) were prepared in 50 mM MOPS (pH 7.0) containing 100 mM KCl and 1 mM PMSF as described earlier (17). Protein concentrations were determined using the bicinchoninic acid assay according to the supplier's recommendations (Sigma Inc.; procedure TPRO-562).

Enzyme activity measurements. Cytochrome *c* oxidase activity was measured spectrophotometrically using reduced horse heart cytochrome *c* (Sigma, St. Louis, MO) in a stirred cuvette at 25°C. Horse heart cytochrome *c* was reduced by incubation for 15 min at room temperature with a 1 mM final concentration of fresh sodium dithionite (100 mM stock solution), which was then removed using a PD10 desalting column (GE Healthcare Life Sciences). *R. capsulatus* chromatophore membranes were detergent solubilized with 1 mg (wt/wt) dodecyl β-d-maltoside per mg of membrane proteins added to the assay buffer (10 mM Tris-HCl, pH 7.0, 120 mM KCl, and 25 μM reduced cytochrome *c*). The enzymatic reaction was started and stopped by the addition of solubilized membranes and 100 μM KCN, respectively. The linear range of the assay was controlled by using different amounts of solubilized membranes, and KCN-sensitive Cox activity was calculated as micromoles of cytochrome *c* oxidized per milligram of membrane protein per minute using an absorption coefficient (ε_{550}) of 20, as described earlier (17, 60). The β-galactosidase activities of whole-cell extracts prepared using 10-ml cultures of appropriate strains were measured spectrophotometrically at 420 nm using *o*-nitrophenyl galactoside (ONPG), as described earlier (23), and specific activity in nanomoles of ONPG hydrolyzed per minute per milligram of protein was determined using an absorption coefficient (ε_{420}) of 21,300 M^{-1} cm^{-1}.

SDS-PAGE, immunoblotting, and heme staining. For CcoN immunodetection, chromatophore membrane proteins (50 μg) in 62.5 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 25% (vol/vol) glycerol, 0.01% (wt/vol) bromophenol blue, and 5% β-mercaptoethanol were incubated at room temperature for 15 min prior to being loaded and were separated by 12% SDS-PAGE (61). The gels were electroblotted onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) and probed with *R. capsulatus* CcoN rabbit polyclonal antibodies (28). Alkaline phosphatase-conjugated monoclonal anti-rabbit IgG (clone RG-16) was used as the secondary antibody (Sigma-Aldrich, Saint Louis, MO), with BCIP (5-bromo-4-chloro-3-indolylphosphate)-nitroblue tetrazolium (NBT) as a substrate (Sigma-Aldrich, Saint Louis, MO) for the detection. For detection of the *c*-type cytochromes, ~50 μg of total membrane proteins was separated by 16.5% SDS-PAGE (62), and the gels were stained for endogenous peroxidase activity of the *c*-type cytochromes by using 3,3′,5,5′-tetramethylbenzidine (TMBZ) and H_2O_2 (63).

Determination of cellular Cu content by ICP-DRC-MS. The cellular Cu contents of various strains were determined using inductively coupled plasma-dynamic reaction cell-mass spectrometry (ICP-DRC-MS). In this technique, aliquots of sample digestions are introduced into a radio frequency plasma where energy transfer processes cause desolvation, atomization, and ionization. The ions thus formed are extracted from the plasma via a differentially pumped vacuum interface and travel through a pressurized chamber (DRC) containing a specific reactive gas that preferentially reacts with interfering ions of the same target mass-to-charge ratios (m/z). A solid-state detector detects ions transmitted through the mass analyzer on the basis of their mass-to-charge ratios, and the resulting current is processed by a data handling system. For sample preparation, at least 1 h prior to use, all containers, glassware, and tubes were washed with 2% nitric acid and rinsed with metal-free Milli-Q water to prevent metal contamination. Metal-free water and buffers were prepared by stirring them for 1 h at room temperature with 5 g Chelex 100 per liter. For each strain, a 1-liter culture was grown by respiration in MPYE medium to an OD_{630} of 0.8 to 0.9, and cells were harvested by centrifugation and washed three times with a metal-free buffer of 20 mM Tris-HCl, pH 8.0, and once with metal-free Milli-Q water. Cell pellets were lyophilized until complete dryness and shipped to Applied Speciation and Consulting, LLC (WA), for determination of total Cu, Zn, Mn, and Fe contents. Fifty milligrams of lyophilized cells was digested completely with aliquots of concentrated HNO_3 and H_2O_2 at 95°C. The digests were diluted to a known final volume (50 ml) with metal-free reagent water and analyzed via ICP-DRC-MS according to a standard procedure of this company. The data were provided in μg of metal of interest per g of cells (ppm).

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

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00293-11/-/DCSupplemental.

Text S1, DOCX file, 0 MB.
Figure S1, DOCX file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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