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.
grow under respiratory conditions even in the absence of an active Cox (16). Unlike in many other bacterial species, cbb₃-Cox is the only Cox in this species, making it an organism of choice for investigating cbb₃-Cox biogenesis. The *R. capsulatus* cbb₃-Cox has four subunits encoded by the *ccoNOQP* operon and, like all Cox enzymes, contains a heme Cu₃ catalytic center located in subunit I (CcoN). It naturally lacks a Cu₄ center; hence, the only Cu atom is that of the Cu₃ center. Instead of a Cu₄ center, *cbb₃*-Cox contains two membrane-bound c-type cytochrome subunits: the monoheme cytochrome c (CcoO or cytochrome c₉) and the diheme cytochrome c (CcoP or cytochrome c₄₅) 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 c₂ and c₇) to the heme Cu₃ 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 *cbb₃*-Cox. Earlier genetic screens for loss of Cox activity identified several genes affecting cbb₃-Cox biogenesis, including *ccoGHIS* (23), *ol*-*sAB* (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 *cbb₃*-Cox still remain undefined. In particular, those involved in forming the universally conserved heme Cu₃ binuclear center of Cox enzymes are unknown. In this work, we report on the molecular characterization of a *cbb₃*-Cox-defective mutant that requires Cu²⁺ supplementation to produce an active *cbb₃*-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 *cbb₃*-Cox in *R. capsulatus*. To our knowledge, CcoA is the first example of an MFS-type transporter required for efficient Cu acquisition and *cbb₃*-Cox production in bacteria.

**RESULTS**

**Cu²⁺ supplement-dependent *cbb₃*-Cox mutants.** Previously, *R. capsulatus* mutants (e.g., GK1 [see Table S1 in the supplemental material]) that exhibited very low or no *cbb₃*-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 H₂O)-negative (i.e., no *cbb₃*-Cox activity) phenotype when grown on enriched peptone yeast extract (MPYE) medium (23) but exhibited a NADIslow (i.e., very low *cbb₃*-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 Cu²⁺ content of the medium used (Fig. 1). Earlier inductively coupled plasma mass spectrometry (ICP-MS) analyses indicated that, in the absence of CuSO₄ supplementation or chemical chelation of Cu, both MPYE and MedA lacking Cu (MedA-Cu) media contained approximately 150 nM Cu²⁺ as a “contaminant,” but the level of bioavailable Cu was much lower in MPYE medium (23). When Cu²⁺ was omitted from MedA, which contains 1.5 μM CuSO₄, GK1 became NADI minus. Conversely, when MPYE that has no Cu²⁺ supplement contained 5 μM or more CuSO₄, GK1 regained a NADIslow phenotype (Fig. 1). Membranes of GK1 exhibited ~2% or 15% of the wild type’s *cbb₃*-Cox activity (monitored as O₂ consumption activity in the presence of ascorbate and tetramethylphenylene di-
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-methyladenine 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 (ΔRCC02192::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 NA-DI$^-$ 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 between 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 between 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).

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 ΔccoA 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 ΔccoA 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$_1$ and c$_2$, 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$_2$). 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.
CcoA is Required for cbb\textsubscript{3}-Cox Production

The *cbb\textsubscript{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\textsubscript{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 \(\mu\)M Cu\textsuperscript{2+} supplement, the *cbb\textsubscript{3}-Cox* activity of SE8 increased to ~20 to 30% of that of MT1131, which was unchanged under these conditions. As expected, upon supplementation with a plasmid carrying *ccoA* (e.g., SE8/pSE3), the NADI phenotypes, steady-state amounts, and activities of *cbb\textsubscript{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\textsubscript{3}-Cox* under respiratory growth conditions. This defect(s) decreased drastically the steady-state amount and activity of *cbb\textsubscript{3}-Cox* and Cu\textsuperscript{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 \(\mu\)M Cu\textsuperscript{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\textsuperscript{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 \(\mu\)M Cu\textsuperscript{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\textsubscript{3}-Cox*, other Cu cofactor-containing enzymes, like Cu/Zn superoxide reductase (34) or multicopper oxidase (laccase or CutO).

### Table 1: Total Cu contents of the *R. capsulatus* wild type and of the ∆ccoA strain and its revertants as determined by ICP-DRC-MS\textsuperscript{a,b}

| Strain                   | 5 \(\mu\)M Cu\textsuperscript{2+} | Cu | Mn | Zn | Fe |
|-------------------------|-------------------------------|----|----|----|----|
| Wild type (MT1131)      | −                             | 100\textsuperscript{a} | 100\textsuperscript{a} | 100\textsuperscript{a} | 100\textsuperscript{a} |
| ∆ccoA mutant (SE8)      | +                             | 363 | 111 | ND | ND |
| ∆ccoAR1 (SE8R1)         | +                             | 79  | 107 | 101 | 97 |
| ∆ccoAR2 (SE8R2)         | +                             | 146 | 118 | ND | ND |

\textsuperscript{a} A mean value of 12 \(\mu\)g Cu, 6.3 \(\mu\)g Mn, 34.5 \(\mu\)g Zn and 268 \(\mu\)g Fe per g of lyophilized cells was determined and is referred to as 100% for the wild-type strain grown in the absence of Cu\textsuperscript{2+} in MPYE medium under respiratory growth conditions.

\textsuperscript{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 \(\mu\)M Cu\textsuperscript{2+} added to MPYE medium.

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**FIG 4** *cbb\textsubscript{3}-Cox* subunit profiles and enzyme activity in membranes of various *R. capsulatus* strains. Chromatophore membranes derived from the wild-type (MT1131), ∆ccoNO (GK32), ∆ccoNO/ccoNOQP (GK32/pOX15), ∆ccoA (SE8), and ∆ccoAMiscoA (SE8/pSE3) strains grown at 37°C under respiratory conditions on MPYE medium supplemented (+ Cu) or not supplemented with 5 \(\mu\)M Cu were prepared as described in Materials and Methods. (A) Immunoblot analysis to define the amounts of cbb\textsubscript{3}-Cox subunit 1 CcoN. Approximately 50 \(\mu\)g 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 \(\mu\)g chromatophore membranes prepared from appropriate strains, grown as described above, were separated using 16.5% SDS-PAGE, and the c\textsubscript{3}-type cytochromes were visualized using TMBZ staining as described in Materials and Methods. CcoO and CcoP refer to subunits II and III of cbb\textsubscript{3}-Cox, and c\textsubscript{x} and c\textsubscript{z} correspond to the cytochrome c\textsubscript{x} subunit of the cytochrome b\textsubscript{x} complex and membrane-attached electron carrier cytochrome c\textsubscript{z}, respectively. (C) cbb\textsubscript{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 pmol 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.

| Strain                   | Relative level of: |
|-------------------------|--------------------|
| Wild type (MT1131)      | Cu Mn Zn Fe |
| ∆ccoA mutant (SE8)      | + − + + |
| ∆ccoAR1 (SE8R1)         | + − + + |
| ∆ccoAR2 (SE8R2)         | + − + + |
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 ΔccoA derivative SE8 (see Text S1 in the supplemental material). The sensitivity to Cu$^{2+}$ of a ΔcutO single (SE15) and a ΔccoA ΔcutO 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 ΔcutO mutant (SE15) was sensitive to Cu$^{2+}$ under photosynthetic growth conditions, unlike the wild-type strain (MT1131). Unlike the ΔcutO mutant, a ΔccoA mutant (SE8) was not sensitive to Cu$^{2+}$, like the wild-type strain (MT1131) (Fig. 5A). Similarly, a ΔcutO ΔccoA double mutant was not more sensitive to Cu$^{2+}$ than a ΔcutO 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(cccoA::spe)rev1] and SE8R2 [Δ(cccoA::spe)rev2] (see Table S1 in the supplemental material) showed hypersensitivity to Cu$^{2+}$ under both respiratory (Res) (Fig. 5A) 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 mutants 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 cysteine 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...
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 SBR1 and SBR2 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 cbb$_3$-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 Mts 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, vibrioferin, 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 knockout 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 importers (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$^{2+}$ 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 CcoA 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

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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<sup>2+</sup> importer function of Mfc1 was established by direct-transport assay using radioactive 64Cu 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 specific Cu importer.
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 chalcolhores 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 $\times$ 10$^7$ cells/ml) and photosynthetic (2.6 $\times$ 10$^7$ cells/ml) growth conditions (estimated with 1.0 OD$_{630}$ unit equal to 7.5 $\times$ 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 $\mu$l 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 semi-aerobically in MPYE medium until mid-log phase (OD$_{630}$ of approximately 0.5), and total RNA was isolated from about 2 $\times$ 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 in RT-PCR with the Qiagen OneStep RT-PCR kit, and the ccoN BHK208 (5' CCAGTCCGGCCACGGCGGTAT 3') and N3-RT (5' CGGC AACCGGATCTGAATGATGG 3') primers amplified a 625-bp-long region internal to ccoN that corresponds to positions 299 and 972 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 extraction preparation. Cells were grown in 10 ml of MPYE medium by respiration and harvested at 4,000 rpm for 10 min. Pellets were resuspended in 200 $\mu$l of CellLytic B $\times$ 2 $\times$ cell lysis solution (Sigma Inc.) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 50 $\mu$g lysozyme, 20 $\mu$g 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 $\beta$-d-maltoside per mg of membrane proteins added to the assay buffer (10 mM Tris-HCl, pH 7.0, 120 mM KCl, and 25 $\mu$M reduced cytochrome c). The enzymatic reaction was started and stopped by the addition of solubilized membranes and 100 $\mu$M 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 ($e_{550}$) of 20, as described earlier (17, 60). The $\beta$-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 ($e_{420}$) of 21,300 M$^{-1}$ cm$^{-1}$.

SDS-PAGE, immunoblotting, and heme staining. For CcoN immunodetection, chromatophore membrane proteins (50 $\mu$g) 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% $\beta$-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$_3$-type cytochromes, ~50 $\mu$g of total membrane proteins was separated by 16.5% SDS-PAGE (62), and the gels were stained for endogenous peroxidase activity of the c$_3$-type cytochromes by using 3,3'5,5'-tetramethylbenzidine (TMBZ) and H$_2$O$_2$ (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 spectrometer (ICP-DRC-MS). In this technique, aliquots of sample digests 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$_2$O$_2$ 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 $\mu$g 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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