Copper is required as a redox cofactor in many enzymes involved in vital metabolisms. Only traces of copper are needed to fulfill its role. Copper is highly toxic even at low concentrations and promotes oxidative damage or degradation of solvent-exposed 4Fe-4S centers (1), leading to impaired cell functioning and cell death. Accumulation of copper in the cytoplasm may also interfere with the formation of iron-sulfur clusters, as suggested in reference 2. In Gram-negative bacteria, all cuproenzymes are located within the inner membrane and the periplasm, which implies transport of copper from the medium or from the cytoplasm to these cell compartments. Copper diffuses through nonspecific outer membrane porins into the periplasm and transits to the cytoplasm (3). Copper can also be supplied from the cytoplasm to the periplasmic and inner membrane proteins through specific copper ATPases (4–6). In all subcellular compartments, copper presents a threat to the cell integrity; therefore, it has to be handled by specific copper homeostasis systems to avoid its toxicity. For that purpose, bacteria have acquired different systems to tightly control the copper concentration. Among these systems, a well-studied copper induced efflux system was described in Escherichia coli. The system includes the transcriptional regulator CueR, which senses Cu ions in the cytoplasm and triggers the response by inducing the expression of copA and cueO genes, coding for the P1B-1-type ATPase (CopA) and the periplasmic multicopper oxidase (CueO), respectively (7, 8). CopA is required for removing excess Cu from the cytoplasm to the periplasm (9), where CueO oxidizes Cu(I) under aerobic conditions to less toxic Cu(II) (10). Excess copper can also be removed from the periplasm and released outside the cell by the CusF/CusCBA system, which has been identified in only few species and studied solely in E. coli (11, 12). In contrast, the P1B-1-type ATPase CopA is found in almost all proteobacteria, and copA mutations result in increased sensitivity to copper (13).

In addition to these systems, other periplasmic proteins that...
are or may be involved in copper handling were found in many bacteria, such as PcoC and PcoE in E. coli (14), CopG, CopK, and CzcE in Cupriavidus metallidurans (15, 16), CueP in Salmonella enterica serovar Typhimurium (17, 18), CuaC and Cina in Pseudomonas putida (19), and CopM in Synechocystis strain PCC 6803 (20, 21). This variety and multiplicity of proteins highlight their essential role to protect cells from the periplasmic highly toxic reduced Cu(I). To avoid the toxic effect of copper ions in the periplasm, some of these proteins can either (i) oxidize copper (CueO and CuiD in S. Typhimurium or CueO in Rhodobacter capsulatus) (10, 22, 23), (ii) sequester copper ions (PcoC and CopC in E. coli and CueP in S. Typhimurium) (14, 18), or (iii) deliver copper ions to outer membrane exporter to remove the metal outside the cell (CusF in E. coli) (7, 12).

In the purple photosynthetic bacteria R. capsulatus and Rhodobacter sphaeroides, the mechanisms and the whole copper efflux system are not yet characterized in detail (23, 24). In Rubrivivax gelatinosus, the roles of the P18 ATPases CtpA and CopA as a copper delivery protein to membrane cuproproteins and as a copper efflux pump required for copper tolerance, respectively, were reported (5, 25). In the copA-null mutant, excess copper affects the cytoplasmic pathway of heme and chlorophyll biosynthesis, by decreasing heme- and/or chlorophyll-containing complexes within the membrane (25, 26). It was reported that in R. gelatinosus (25) and in the human pathogen Neisseria gonorrhoeae (27), copper seems to poison HemN, likely by degradation of its exposed 4Fe-4S cluster.

To further characterize the copper tolerance system in R. gelatinosus, we performed protein profile analyses and identified the copper-induced periplasmic protein CopI. Genetic and physiological analyses of the ΔcopI mutant showed that CopI plays a major role in copper tolerance. Over a span of decades, intensive research provided evidence supporting excess-Cu-induced disorders in all organisms (28). DNA damage and reactive oxygen species generation are among the mechanisms by which copper inhibits or kills overloaded cells (28). Our findings pinpoint cytochrome c assembly as a new target of copper that could contribute to its toxicity and demonstrate the crucial role of copper detoxification within the periplasm.

RESULTS

CopI is a copper-induced putative periplasmic protein. To identify new copper-handling proteins involved in copper homeostasis in R. gelatinosus, the wild-type (WT) strain was subjected to copper stress (1.2 mM CuSO4) under anaerobic photosynthetic (PS) conditions. Copper-dependent induction of soluble proteins was assessed under PS conditions in the absence of copper (see Fig. S1).CopI is located in the periplasm. Since mass spectrometry analysis showed that CopI protein is N-terminally processed and

protein coverage (CopI, 44%; CopJ, 69%) were acceptable, N-terminal characterization was rather poor. Since predicted signal peptides (SignalP and SMART) for periplasm localization are present in sequences of both proteins, we reviewed the LC-MS/MS datasets using the Mascot Error Tolerant Search software to identify nontryptic peptides. N-terminally processed CopI peptides could not be found, whereas the CopI peptide 25-29DAGHDHGSAH AGAHAHDADTPYGRPGDAAK12 was unambiguously identified (Fig. 1B), thus confirming that CopI was N-terminal processed and that the protein is most probably localized in the periplasm.

Protein sequence analyses revealed a plastocyanin-like fold with a conserved copper binding motif (H70C122H130M133) within CopI and a C18XXC21 sequence within CopJ, a motif potentially involved in copper binding. CopI and CopJ are encoded by copI and copJ, located immediately downstream of the copAR locus, which is involved in copper tolerance in R. gelatinosus, suggesting that these proteins may be required for copper tolerance.

Because of the presence of five histidines within the mature CopI sequence (Fig. 1B), CopI expression could be detected specifically on Western blots using the horseradish peroxidase (HRP)-conjugated HisProbe. Thus, Western blotting of soluble fractions of WT strain grown anaerobically by photosynthesis under different copper concentrations was performed. CopI was not detected in the control (copper-depleted medium) or in the copI deletion strain (see below for descriptions of mutants); however, when the copper concentration was increased from 1.6 to 200 μM, CopI amount increased concomitantly (Fig. 1C) in agreement with the RT-PCR data (see Fig. S1 in the supplemental material). These data confirm that CopI is induced by excess copper. In addition, as for CopA expression (25), we showed that full induction of CopI requires the presence of the copper regulator CopR (see Fig. S1).

FIG 1 (A) Copper-dependent induction of soluble proteins in the WT strain grown under PS conditions in malate containing 1.6 μM CuSO4 or in malate supplemented with 1.2 mM CuSO4. (B) CopI sequence. The CopI peptides identified by MS are in red. The signal peptide and the His-rich sequence are highlighted in grey and in yellow, respectively. Residues of the CopI putative copper binding motif (H70C122H130M133) are blue and highlighted in yellow. (C) Expression of CopI in the WT soluble fraction. Cells were grown under PS conditions in the absence of copper (~Cu) and in the presence of increasing CuSO4 concentrations. Equal amounts of soluble protein were loaded on SDS-PAGE gels and then transferred for CopI detection using the HRP-HisProbe.
is likely a periplasmic protein, we isolated an enriched periplasmic fraction from intact WT *R. gelatinosus* and ΔcopI mutant (see below) cells grown under 1.6 μM or 200 μM CuSO₄ stress to localize CopI. As a control for the isolation procedure (see Materials and Methods), we analyzed membrane and soluble periplasmic c-type cytochromes in each isolated fraction by specific 3,3′,5,5′-tetramethylbenzidine (TMBZ) staining. In the periplasmic fractions of WT and the ΔcopI strain grown in the presence of 1.6 μM CuSO₄, two major cytochromes c′ corresponding to the soluble periplasmic c-type cytochromes c′ and the cytochrome c peroxidase CcpA were detected (Fig. 2A), whereas the membrane c cytochromes (CcpP, CcoO, and PufC) were present mainly in the membrane fraction. The two soluble cytochromes c were strongly reduced in cells grown in the presence of 200 μM CuSO₄. Interestingly, Coomassie blue staining revealed the presence of a 17-kDa protein band only in the periplasmic fractions of WT cells challenged with excess copper (Fig. 2B). The presence of CopI in this latter fraction was unambiguously confirmed by Western blotting (Fig. 2C), and a smaller amount of CopI was detected in the total fraction.

### CopI protein could bind copper.

Since CopI harbors a plastocyanin-like fold with a conserved copper binding motif and a cluster of 5 scattered histidines susceptible to bind transition metals like copper, we used the method of metal contaminant sweeping-blue native polyacrylamide gel electrophoresis (MICS-BN-PAGE) and a fluorescent probe (29) to map copper binding proteins in soluble fractions from WT cells grown microaerobically with 1.6 or 1,200 μM CuSO₄ compared with ΔcopI mutant cells grown with 200 μM CuSO₄. While no band was observed in the first BN-PAGE dimension for WT and ΔcopI mutant samples incubated, respectively, with 1.6 and 200 μM CuSO₄ (Fig. 3A and C), distinct protein bands were observed in the WT cells grown with 1,200 μM CuSO₄ (Fig. 3B). Subsequently, the copper content in each cut band was measured after acid extraction and addition of a fluorescence probe (FTC-ABDOTA); the resulting metal-probe complex was separated by PAGE (29). A strong copper signal was observed only in extracts of WT cells grown with 1,200 μM CuSO₄ (Fig. 3B). The percentage of copper in the selected cut band relative to total copper concentration in the entire lane of the gel was calculated as 76% ± 19% (n = 6). No copper ions were detected in the gel fractions corresponding to the same positions for ΔcopI and WT samples incubated with 200 and 1.6 μM CuSO₄, respectively. This strongly implies that the isolated protein at this position formed stable (inert) complexes with copper ions. To determine whether CopI was thus present in these bands, the proteins were electroeluted from the MICS-BN-PAGE and subjected to Western blotting for CopI detection. CopI was detected only in the gel fractions where a high concentration of copper was detected, i.e., in WT cells grown with 1,200 μM CuSO₄ (Fig. 3). Accordingly, these data strongly suggest that CopI strongly binds copper in the periplasm.

### CopI is required for copper resistance under both aerobic and anaerobic conditions. A copI deletion strain was constructed to analyze the role of CopI in copper homeostasis. The copper sensitivity of the ΔcopI strain was investigated by growing the strain with increasing CuSO₄ concentrations in comparison with the WT and the *copA* mutant. First, on solidified medium, growth of both ΔcopI and *copA* strains was affected at 120 μM and inhibited beyond 300 μM CuSO₄ with aerobic respiration, unlike the WT (Fig. 4A). Under anaerobic PS conditions, the growth of the ΔcopI mutant was inhibited at 80 μM CuSO₄, whereas growth of the *copA* mutant was unaffected by this CuSO₄ concentration (Fig. 4A). Second, growth inhibition by CuSO₄ of these strains was performed in liquid medium in the presence of increasing copper concentrations under both respiration and anaerobic PS conditions (Fig. 4B). Under both conditions, growth of ΔcopI and *copA*
mutants was reduced when they were exposed to CuSO₄, while the WT strain was unaffected (Fig. 4B). Under aerobic respiration \((Res)\) conditions, the \(\text{copI}^-\) mutant was less affected than the \(\text{copA}^-\) mutant, in contrast with the solid agar plate data. This may be due to the difference in oxygen tension between plates and shaken liquid cultures. Under PS anaerobic conditions, the \(\text{H}9004\copI^-\) mutant was extremely sensitive to copper, as its growth was inhibited at \(50\) \(\mu\)M CuSO₄, similar to the data obtained on solid agar plates. Growth of the \(\text{copA}^-\) strain was also reduced, as observed under aerobic conditions. Finally, WT copper tolerance could be restored in the \(\text{H}9004\copI^-\) mutant by an ectopic copy of \(\text{copI}\), showing that the phenotype was not due to polar effects of the cassette insertion on the downstream \(\text{copJ}\) gene (see Fig. S2 in the supplemental material). These data demonstrate that CopI is required for copper resistance under both aerobic and anaerobic conditions and that the \(\text{H}9004\copI^-\) strain is more sensitive to copper than the \(\text{copA}^-\) strain under anaerobic conditions. Moreover, disc diffusion assay results confirmed that the \(\text{H}9004\copI^-\) strain is extremely susceptible to copper under anaerobic photosynthesis conditions relative to the \(\text{copA}^-\) mutant (see Fig. S2). Altogether, the results showed that CopI is critical when oxygen tension drops.

Expression of CopI does not require the expression of the efflux pump CopA. The increased sensitivity to copper of the \(\Delta\text{copI}^-\) mutant originates from the deletion of \(\text{copI}\). However, this phenotype could also indirectly result from impaired expression or a complete lack of expression of the copper efflux pump ATPase CopA. To rule out this hypothesis, the \(\text{copI}^-\) gene was also deleted in a WT strain harboring the His-tagged \(\text{copA}\) construct on the chromosome to monitor the expression of CopA in the absence of CopI.

The expression of CopA-H₆ and CopI was monitored in WT and \(\text{H}9004\copI^-\) strains containing the CopA-H₆ construct and the \(\text{copA}\) strains grown microaerobically in malate medium (1.6 \(\mu\)M) or in 100 \(\mu\)M CuSO₄-supplemented medium. CopI and CopA-H₆ were not detected in the fractions from the respective mutants (Fig. 5A). CopA-H₆ was present in the membranes of the \(\Delta\text{copI}^-\) mutant under both experimental conditions, and the amount of
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CoppA-H₆ was even higher in the ΔcopI mutant than in the WT (CoppA-H₆) control strain. This indicates that the observed phenotype in the ΔcopI mutant was not related to a decrease in the expression of CoppA. The increase of CoppA protein within ΔcopI mutant membranes may be due to a loss of copper control within the periplasm, resulting in an overloading of the cytoplasm with copper, which in turn induces the expression of the efflux pump CoppA. Expression of CopI in the absence of the ATPase CoppA was also checked in the copA strain. The results showed a constitutive expression of CopI in the copA mutant, while in the WT (CoppA-H₆), CopI expression was induced by copper. Because in the copA strain, copper accumulates within the cytoplasm, the copper sensor CopR induces CopI expression (see Fig. S1 in the supplemental material).

To demonstrate that the expression of CopI in copA mutant and of CoppA in the ΔcopI mutant contributes to copper tolerance, a copA ΔcopI double mutant was generated, and copper resistance was assayed on agar plates in comparison with that of the copA and ΔcopI single mutants. Unlike the single mutants, the double mutant could not grow beyond 120 μM CuSO₄ under aerobic conditions and 20 μM CuSO₄ during anaerobic photosynthesis (see Fig. S3 in the supplemental material). The double mutant is extremely copper sensitive, indicating that the proteins expressed in the single mutants contribute individually to copper tolerance.

Mutations in copI and copA affect the photosystem and the cytochrome c oxidase cbb₃, but through different mechanisms.

In two recent reports, we showed that excess copper affects the tetrapyrrole biosynthesis pathway in copA mutant and thereby affects the assembly of heme- and chlorophyll membrane-containing complexes, such as the cbb₃ cytochrome c oxidase and the photosystem (25, 26). We therefore questioned whether deletion of copI can give rise to the same phenotype. First, we visually compared the photosynthetic pigment content of the cells grown under microaerobic conditions with two copper concentrations. In contrast to the WT and copA strains, the ΔcopI culture exhibited different pigmentation at 100 μM CuSO₄ (data not shown). Then, the pigment content of each culture was analyzed by UV-visible (UV-Vis) absorption. In the ΔcopI and copA cells, the bacteriochlorophyll (peak at 770 nm) and carotenoid (430 to 520 nm) contents were reduced when copper concentration was increased, in sharp contrast with the WT, where the photopigments were not affected (Fig. 5B). Interestingly, in ΔcopI cells and medium, no trace of coproporphyrinogen III (peak at 395 nm) was observed, in sharp contrast with copA cells (Fig. 5B and C), as previously reported by Azzouzi et al. (25). A decrease in the amount of the photosystem (RC-LH [reaction centre-light harvesting]) was also observed on BN-PAGE for both ΔcopI and copA strains grown in the presence of excess copper (Fig. 5D).

As for photosynthesis, excess copper results in slower microaerobic growth in the ΔcopI strain (data not shown). Since the cbb₃ oxidase is required for microaerobic respiration (30), we questioned whether the cytochrome c cbb₃ oxidase is affected when copper handling in the periplasm is deregulated in the ΔcopI mutant. Comparable DAB (3,3′-diaminobenzidine)-positive bands on BN-PAGE corresponding to the cbb₃ oxidase activity were observed in the WT native membranes regardless of the copper concentration (Fig. 6A). In contrast, in the copA and ΔcopI membranes, the amount of active cbb₃ was decreased when copper concentration increased to 100 μM. Concomitantly with the decrease in the oxidase activity, the catalytic CcoN and the two cytochrome c-type CcoO and CcoP subunits of cbb₃ oxidase were decreased, as shown on Western blots (Fig. 6B). Interestingly, solely in the membranes of the copA strain, additional bands corresponding to the proteolysis of CcoP (lower band) and CcoO (upper bands) were observed.

These results show that the mutation in the ΔcopI strain affects copper homeostasis within the periplasmic and membrane complexes. We also analyzed the presence of c-type cytochromes by TMBZ staining in membrane fractions. A significant

![Fig 6](image-url)
decrease of all membrane c-type cytochromes, including the tetraheme cytochrome c (PufC) subunit of the reaction center (RC) and the two cytochrome c subunits (CcoO and CcoP) of cbb₃, was observed for both ΔcopI and copA strains grown in the presence of excess copper (Fig. 6C). As expected, no c-type cytochrome could be detected in membranes of the ΔccmF strain affected in the cytochrome c biogenesis pathway, and only a slight effect was observed for the WT. We had also observed a decrease in periplasmic c-type cytochromes (Fig. 2A). These data suggest that excess copper affects both periplasmic and membrane c-type cytochromes in these mutants. Nevertheless, in the copA strain, copper accumulates within the cytoplasm, and the decrease of c-type cytochromes results from a decrease of heme synthesis; consequently, both heme b- and c-containing complexes should be affected. In contrast, in the ΔcopI strain, copper should accumulate within the periplasm and may affect only c-type cytochrome assembly because covalent binding of heme b to the apocytochromes takes place within the periplasm (31). To test this assumption, we subsequently analyzed the effect of excess copper on the activity of succinate dehydrogenase (Fig. 7A), a heme b membrane complex devoid of c-type cytochromes. Interestingly, in contrast to cbb₃, oxidase activity, the activity of this complex was not detectably diminished in the ΔcopI strain in the presence of excess copper, strongly suggesting that only heme c-type complex assembly is adversely affected by excess copper in this strain. The specificity of this signal was proved by loss of signal after addition of malonate (an inhibitor of succinate dehydrogenase) (data not shown). It is of note that in the copA mutant, the activity of succinate dehydrogenase was not affected, although the complex contains 4Fe-4S centers. These centers are protected from copper because they are occluded by polypeptides in the complex and thus are not exposed.

Therefore, these in-gel activities and the absence of heme intermediate accumulation in the ΔcopI strain strongly suggest that in the ΔcopI mutant, the heme b biosynthesis pathway is unaffected, in sharp contrast with the copA mutant.

To further confirm these data, we performed redox spectral analyses (reduced versus oxidized spectra) to assess the cytochrome c (peak at 552 nm) and b (peak at 560 nm) ratios present in these membranes (Fig. 7B). Comparable spectra were recorded for the WT, copA, and ΔcopI membranes from cells grown in 1.6 μM CuSO₄. Addition of 100 μM CuSO₄ resulted in a slight decrease of heme c and b in the WT. This decrease was more pronounced in the copA membranes, yet both heme c and b are present in these membranes, as judged by the 552- and 560-nm peaks. Interestingly, in the ΔcopI membranes, a significant decrease of the 552-nm peak was observed, demonstrating an overall decrease of c-type cytochromes within the ΔcopI cells. As controls, c- and b-type cytochromes were also monitored in membranes isolated from ΔccmF and ΔccoN strains, affected, respectively, in the cytochrome c biogenesis pathway and the cbb₃ catalytic subunit CcoN.

c-type cytochrome assembly is a primary target of copper toxicity within the periplasm. When low excess copper (100 μM) is present in the medium, the ΔcopI strain produces less c-type cytochromes. Because very high copper concentrations (1 to 2 mM) slow growth and also are toxic for the WT strain with no accumulation of heme intermediates, we assumed that high copper concentrations may also affect c-type cytochrome assembly in the WT background. To provide evidence, we monitored cytochrome c and b ratios present in total extract of WT cells exposed to increasing concentrations of CuSO₄ (1.6 to 1.500 μM) in comparison with ΔcopI and copA mutants (Fig. 7C and 8). The data clearly show a shift in the spectra, with cytochrome c present at low copper concentrations, while at high copper concentrations, the 552-nm peak decreased and the main peak detected corresponds to that of heme b (560 nm), indicating a gradual decline in the amount of c-type cytochromes in the WT membranes when cells are exposed to a high copper concentration. In the ΔcopI extracts, the effect of excess copper on cytochrome c content was observed early at a low CuSO₄ concentration (25 μM), and the effect was more drastic when cells were challenged with higher copper concentrations (Fig. 8). For the copA mutant, cells were challenged with increasing nonlethal concentrations up to 250 μM. As expected, the spectra show a decrease of both cytochrome c and b contents (Fig. 8), and the main peak detected corresponds to that of cytochrome c.

![FIG 7](image-url) (A) Succinate dehydrogenase (SDH) in-gel activity assay. The membranes on BN-PAGE assayed for cbb₃, oxidase activity (Fig. 6A) were subsequently assayed for SDH activity. (B) Difference (reduced minus oxidized) spectra of WT, ΔcopI, and Δcbb₃ membranes from cells grown in 1.6 μM (red) or 100 μM (blue) CuSO₄. Δcno and ΔccmF strains were grown only in 1.6 μM CuSO₄. (C) Difference spectra of total fractions from WT grown in the presence of increasing copper concentrations.
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Altogether, our data show that the amount of \(cbb_3\) oxidase and photosystem are similarly affected in \(copA\) and \(\Delta\text{copI}\) mutants by copper excess; nonetheless, the mechanisms are different. Indeed, in the \(\text{copA}\) mutant, the cytoplasmic copper accumulation affects heme and bacteriochlorophyll synthesis, while in the \(\Delta\text{copI}\) mutant, the periplasmic copper accumulation affects cytochrome \(c\) biogenesis.

DISCUSSION

The bacterial inner membrane and the periplasm are likely more exposed to copper than the cytoplasm in Gram-negative bacteria. Indeed, copper-requiring proteins in bacteria identified so far are located in these two cell compartments. Copper entry seems to occur via nonspecific importers, which may load the periplasm and cytoplasm with copper; in addition, the inner membrane copper efflux P-type ATPase CopA unloads excess cytoplasmic copper in the periplasm. Therefore, detoxification of Cu(I) within the periplasm is crucial to preserve the integrity of the membrane and the cell. In \(E.\ coli\), this is accomplished by the periplasmic CueO oxidase and the CusF chaperone, which delivers copper to the Cus efflux system (8). In \(Cupriavidus\), this involves many chaperones, such as CopH and CopK. In \(Salmonella\), the periplasmic protein CueP seems to be involved in copper sequestration within the periplasm. Finally, in \(P.\ aeruginosa\), periplasmic proteins such as PA2807, PA2808, and azurin are induced by copper and seem to be involved in copper handling within the periplasm (32, 33).

Copper can affect different pathways when it is accumulated in the cytoplasm (1, 25, 34). When present in the membrane or the periplasm, excess copper seems to affect proteins and lipids (35). In this study, we identified CopI, a periplasmic protein present in many bacteria (see Fig. S4 in the supplemental material), and used the corresponding null mutant to identify the pathway(s) damaged by accumulation of excess copper in this compartment. CopI is induced by excess copper and is required for copper tolerance. The mutant lacking CopI is even more sensitive to copper than the ATPase \(\text{copA}\) mutant under microaerobic and anaerobic photosynthetic conditions. This supports the idea that the accumulation of copper in the periplasm is more toxic and highlights the importance of copper handling within this compartment. In this study, we have shown that excess copper in the \(\Delta\text{copI}\) mutant affects periplasmic (CpA and cytochrome \(c'\)) and membrane (photosystem and cytochrome \(c\) oxidase \(cbb_3\)) \(c\)-type cytochromes. For \(cbb_3\) oxidase, this decrease was directly correlated with a decrease in the amount of cytochrome \(c\) subunits CcoP and CcoO within the membrane. A less pronounced reduction of the amount of the catalytic subunit CcoN was also observed, but probably as a consequence of CcoP and CcoO decrease. An effect on the photosystem was also observed and was explained by a decrease in the amount of the tetraheme cytochrome \(c\) subunit PuF. The high susceptibility to copper during photosynthetic growth in the \(\Delta\text{copI}\) mutant is consistent with the idea that periplasmic copper is especially toxic to the \(c\) type cytochromes. Indeed, photosynthetic growth depends, in addition to the photosystem, on the \(bc\) cytochrome complex and other soluble \(c\)-type cytochromes.

The specific decrease of \(c\)-type cytochromes within the \(\Delta\text{copI}\) mutant and even in the WT when challenged with a high copper concentration prompted us to conclude that copper may affect cytochrome \(c\) biogenesis within the periplasm. Because the cytochrome \(c\) biogenesis is a periplasmic pathway involving thiol oxidoreductases and the reduction of cysteine residues for the covalent binding of heme \(b\) (31), one can envision that copper, a potent oxidant, could interfere with cysteines and/or the reduction system of the cytochrome \(c\) biogenesis pathway in all organisms. In this respect, it was reported that copper stress in \(E.\ coli\) induces periplasmic disulfide bond formation under aerobic conditions and that copper catalyzes the formation of disulfide-bonded oligomers \textit{in vitro} (36). This is the first report that copper should affect a key process in all organisms: the cytochrome \(c\) biogenesis. Overall, we assume that CopI is an important defense against copper in the periplasm and may protect not only \(c\) type cytochromes but also other proteins with cysteine residues from copper ions that may catalyze nonnative disulfide bond formation.

In comparison with the \(\Delta\text{copI}\) mutant, the copper ATPase \(\text{copA}\) mutant exhibited a comparable phenotype, with a decrease in the photosystem and the cytochrome \(c\) oxidase \(cbb_3\). We have also found a decrease in the amount of the \(cbb_3\) subunits CcoP and CcoO. In contrast to the \(\Delta\text{copI}\) mutant, the tetrapyrrole biosynthesis pathway is affected only in the \(\text{copA}\) mutant, as evidenced by the release of coproporphyrinogen III, due to the accumulation of copper within the cytoplasm in this mutant (25) (Fig. 5B and C). Consequently, production of heme and chlorophylls is reduced in the \(\text{copA}\) mutant, which in turn affects the assembly and/or stability of membrane complexes requiring these cofactors. In the \(\Delta\text{copI}\) mutant, these cytoplasmic processes are unaffected and functional because excess copper is transferred to the periplasm by CopA. This is proved by the fact that the \(\Delta\text{copI}\) mutant grown in the presence of excess copper does not accumulate or release coproporphyrinogen III in the medium (Fig. 5B and C) and has diminished succinate dehydrogenase activity (Fig. 7A).
MATERIALS AND METHODS

Bacterial strains and growth media. *E. coli* was grown at 37°C in LB medium. *R. gelatinosus* was grown at 30°C, either microaerobically in the dark (250-ml flasks filled with 250 ml medium) or anaerobically in light (phototynthesis in filled and sealed tubes) on malate growth medium (30). Anaerobic growth on plates was performed using sealed bags with H₂ and CO₂-producing gas packs (Anaerocult P; Merck). Kanamycin, ampicillin, and trimethoprim were used at 50 µg/ml, and tetracycline was used at 1 µg/ml. The malate medium copper concentration is 1.6 µM, and exogenous CuSO₄₆ was added to the growth medium at different concentrations.

Membrane and soluble protein preparation. The cell extracts were prepared by cell disruption with a French press in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by differential ultracentrifugations as described in reference 5. The resulting pellet (membranes) was resuspended in the same buffer with the 0.7-kb SmaI-digested Tp or 1.2-kb MscI Km cassette. The resulting recombinant plasmids were designated pSA20 (copI::Tp) and pSA21 (copI::Tp).

Gene transfer and strain selection. Transformation of *R. gelatinosus* cells was performed by electroporation (38). Transformants were selected on plates supplemented with the appropriate antibiotic under aerobic conditions. Following selection, template genomic DNA was prepared from the ampicillin-sensitive transformants, and the antibiotic resistance marker’s presence at the desired locus was confirmed by PCR.

Membrane and soluble protein preparation. The cell extracts were prepared by cell disruption with a French press in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by differential ultracentrifugations as described in reference 5. The resulting pellet (membranes) was resuspended in the same buffer without PMSF, and the supernatant (soluble fraction) was also prepared by cell disruption with a French press in 0.1 M sodium phosphate buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), followed by differential ultracentrifugations as described in reference 5. The resulting pellet (membranes) was resuspended in the same buffer with the 0.7-kb SmaI-digested Tp or 1.2-kb MscI Km cassette. The resulting recombinant plasmids were designated pSA20 (copI::Tp) and pSA21 (copI::Tp).

RNA extraction and RT-PCR. Total RNA from WT cells grown photosynthetically under different copper concentrations was extracted according to (39). RT-PCR were performed with a Cary 500 spectrophotometer. For difference (reduced minus oxidized) spectra, the spectrum was collected on oxidized sample upon addition of 50 µM K₄Fe(CN)₆, the sample was reduced by addition of dithionite (few crystals), and the spectrum was collected to generate the reduced minus oxidized spectrum.

Blue-native gel electrophoresis and in-gel activities. Blue-native polyacrylamide gel (BN-PAGE) electrophoresis of β-dodecyl-maltoside (DDM)-solubilized membrane proteins and in-gel Cox activity assays were performed as described in reference 30. Succinate dehydrogenase in-gel activity assays were performed as described for the succinate-NBT (Nitroblue tetrazolium) reductase assay in reference 40.

Western blotting and immunodetection. Twenty micrograms (for cbb subunits detection) or 40 µg (for CopA_His, detection) of total protein was separated by SDS-PAGE (10, 12, or 13% polyacrylamide) and then transferred to an enhanced chemiluminescence polyvinylidene difluoride membrane (Hybond; GE Healthcare). Before loading, samples were treated 10 min at 37°C for CcoN detection, 5 min at 75°C for CcoO and CcoP detection, or 5 min at 95°C for CopA-His and CopP detection. Membranes were probed with different primary antibodies: anti-CcoN (dilution, 1:1,000), anti-CcoO (dilution, 1:10,000), anti-CcoP (dilution 1:5,000). Secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (BioRad) was used at a 1:5,000 dilution. For the detection of CopA-His, and CopP, HisProbe-HRP (Pierce) was used according to the manufacturer’s instructions. Reactive bands were detected using a chemiluminescent HRP substrate according to the method of Haan and Behrmann (41). Image capture was performed with a LAS-3000 cooled charge-coupled device (CCD) camera system (Fujifilm).

MICS-BN-PAGE and copper detection PAGE. The procedure for metal ion contaminant sweeping (MICS)-BN-PAGE and copper detection PAGE was performed as reported previously by Saito et al. (29). The procedure is described briefly as follows. A separation gel (10% polyacrylamide, 190 mM Tris-HCl [pH 8.8]) and an upper stacking gel (3.9% polyacrylamide, 65 mM Tris-HCl [pH 7.4]), with the addition of 10 µM EDTA, were prepared for MICS-BN-PAGE. Before sample injection, the gel was conditioned by applying voltage with an upper and lower migration buffer solution including 10 µM TPEN [N,N,N′,N′-tetraakis(2-pyridylmethyl)ethylenediamine] and 10 µM CyDTA (trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid monohydrate), respectively, in order to sweep all contaminant metal ions for preparing a metal-free stacking gel. Samples (soluble fractions from the WT and ΔcopI mutant incubated with the addition of 1.6 or 200 µM CuSO₄) were prepared by mixing with 6.3% glycerol, Tris-HCl buffer (pH 7.4), and 3.0 mM CBB (Coomassie Brilliant Blue) G-250. Samples were then loaded into the wells after a rinsing to remove all TPEN and exchange of the upper buffer solution with a Tris-Gly buffer solution without TPEN. A voltage of 600 V was then applied for 3 min to move the sample proteins into the concentration gel. Subsequently, electrophoresis (protein separation) was conducted after exchanging an upper buffer solution with 10 µM TPEN until CBB G-250 migrated to a distance of ~3 to 4 cm. The temperature of the slabs gel was kept constant at 273 K.

The separation gel was cut at intervals of 0.25 cm prior to acid extraction of copper ions bound to proteins. Extraction was achieved by shaking the gel fraction with 0.2 M pure hydrochloric solution. Thereafter, the extract was mixed with a solution of the fluorescent probe FTC-ABDOTA (50 µM, 1 µl) in Tris–HCl buffer, to form the Cu²⁺–FTC-ABDOTA emissive complex for copper detection PAGE. The prepared sample solution was loaded into the well in the second-dimension gel (10% and 30% polyacrylamide for stacking and separation gels, respectively), followed by electrophoresis for separation of copper complexes. The copper bands were detected by fluorescence using a CCD camera (Atto) with a photodiode excitation light source (Invitrogen).

In-gel cytochrome c detection. Detection of cytochrome c on nonreducing SDS-PAGE was performed using 3,3′,5,5′-tetramethylbenzidine (TMBZ) as described by Thomas et al. (42).

Spectrophotometric measurements. Absorption spectroscopy was performed with a Cary 500 spectrophotometer. For difference (reduced minus oxidized) spectra, total pigments were extracted from cell pellets or membranes with acetone-methanol (7/2 [vol/vol]). For each sample, the spectrum was collected on oxidized sample upon addition of 50 µM K₄Fe(CN)₆(N₃), the sample was reduced by addition of dithionite (few crystals), and the spectrum was collected to generate the reduced minus oxidized spectrum.

Mass spectrometry analyses. Peptide mixtures generated by standard in-gel digestion with trypsin (Gold; Promega) were SpeedVac treated for 10 min and then analyzed with a quadrupole time-of-flight (QToF) Premier mass spectrometer coupled to a Acquity nano-ultraperformance liquid chromatograph equipped with a trapping column (Symmetry C18; 180 µm by 20 mm; 5-µm particle size) and an analytical column (BEH130 C18; 75 µm by 100 mm; 1.7-µm particle size) (Waters). The aqueous solvent (buffer A) was 0.1% formic acid in water, and the organic phase...
(buffer B) was 0.1% formic acid in acetonitrile. A 2- to 40% B gradient was set for 25 min. For exact mass measurements, gluciferinopeptide reference (methionine); fixed modification, carbamido-methylation (cys-)

were submitted to Mascot searching using the following parameters: data bank, NCBI

taxonomy, bacteria; peptide tolerance, 20 ppm; fragment tolerance, 0.1 Da; digestion, reagent trypsin; variable modification, oxidation (methionine); fixed modification, carbamido-methylation (cy steine). Validation criteria for protein identification were two peptides with a Mascot individual ion score of >43.

**Nucleotide sequence accession number.** The copI and copP sequences reported in this paper were deposited in GenBank (accession number KP938002.1).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01007-15/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.5 MB.
Table S1, DOC file, 0.04 MB.

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