The copper-deprivation stimulon of Corynebacterium glutamicum comprises proteins for biogenesis of the actinobacterial cytochrome $b_{c_1} - a_{a_3}$ supercomplex

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Aerobic respiration in Corynebacterium glutamicum involves a cytochrome $b_{c_1} - a_{a_3}$ supercomplex with a diheme cytochrome $c_1$, which is the only $c$-type cytochrome in this species. This organization is considered as typical for aerobic Actinobacteria. Whereas the biogenesis of heme–copper type oxidases like cytochrome $a_{a_3}$ has been studied extensively in $c$-proteobacteria, yeast, and mammals, nothing is known about this process in Actinobacteria. Here, we searched for assembly proteins of the supercomplex by identifying the copper-deprivation stimulon, which might include proteins that insert copper into cytochrome $a_{a_3}$. Using gene expression profiling, we found two copper starvation–induced proteins for supercomplex formation. The Cg2699 protein, named CtiP, contained 16 predicted transmembrane helices, and its sequence was similar to that of the copper importer CopD of Pseudomonas syringae in the N-terminal half and to the cytochrome oxidase maturation protein CtaG of Bacillus subtilis in its C-terminal half. CtiP deletion caused a growth defect similar to that produced by deletion of subunit I of cytochrome $a_{a_3}$, increased copper tolerance, triggered expression of the copper-deprivation stimulon under copper sufficiency, and prevented co-purification of the supercomplex subunits. The secreted Cg1884 protein, named CopC, had a C-terminal transmembrane helix and contained a Cu(II)-binding motif. Its absence caused a conditional growth defect, increased copper tolerance, and also prevented co-purification of the supercomplex subunits. CtiP and CopC are conserved among aerobic Actinobacteria, and we propose a model of their functions in cytochrome $a_{a_3}$ biogenesis. Furthermore, we found that the copper-deprivation response involves additional regulators besides the ECF sigma factor SigC.

Actinobacteria represents one of the largest phyla within bacteria, with currently 57 families (1, 2). It includes several important human pathogens like Mycobacterium tuberculosis, Mycobacterium leprae, and Corynebacterium diphtheriae, and biotechnologically important species such as antibiotic-producing representatives belonging to the Streptomycesae or Corynebacterium glutamicum, a major host for amino acid production in multimillion ton scale. Due to its industrial importance, C. glutamicum has become a model organism for studying metabolism and regulation (3–6). It is a facultative anaerobic bacterium with the capability for limited growth either by nitrate respiration to nitrite (7, 8) or by mixed acid fermentation with l-lactate, succinate, and acetate as major products (9). However, the preferred way of growth is by aerobic respiration, which is performed by a branched respiratory chain composed of several dehydrogenases transferring electrons to menaquinone and two pathways for transferring electrons from menaquinol to oxygen, one involving a cytochrome $b_{c_1}$ complex and a cytochrome $a_{a_3}$ oxidase and the second one composed of cytochrome $b$ oxidase (10).

A unique feature of this respiratory chain is the presence of a cytochrome $b_{c_1} - a_{a_3}$ supercomplex, which was identified by co-purification of all subunits of the $b_{c_1}$ complex (QcrB, QcrA, and QcrC) and of cytochrome $a_{a_3}$ oxidase (CtaD, CtaC, CtaE, and CtaF) by affinity chromatography with either Streptagged QcrB (cytochrome $b$) or Strept-tagged CtaD (subunit I) (11). The existence of such a supercomplex had previously been suggested by us based on the observation that C. glutamicum contains a diheme cytochrome $c_1$, which is the only $c$-type cytochrome encoded in the genome (12–15). Mutational analysis confirmed that both heme groups of cytochrome $c_1$ are essential for the activity of the $b_{c_1} - a_{a_3}$ supercomplex, and the second heme group probably takes over the function of a separate cytochrome $c$, usually shuttling electrons from the $b_{c_1}$ complex to cytochrome $a_{a_3}$ oxidase (11).

Recently, studies have led to a structural model of the $b_{c_1} - a_{a_3}$ supercomplex, the determination of the redox potentials of the cofactors, and a detailed kinetic characterization of the partial reactions catalyzed by the supercomplex (16, 17). With respect to bioenergetics, the $b_{c_1} - a_{a_3}$ supercomplex is predicted to transport 6 $H^+$ per 2 e$^-$ from the cytoplasm to the outside, whereas the alternative cytochrome $bd$ oxidase is assumed to have a stoichiometry of only 2 $H^+/2$ e$^-$ (10). Analysis of defined mutants lacking either the genes of the $b_{c_1}$ complex (qcrCAB) or the gene for subunit I of the $a_{a_3}$ oxidase (ctaD) showed reduced growth rates and decreased biomass yields (12). A mutant lacking only the qcrA gene for the Rieske iron–sulfur protein, whose assembly was shown to be dependent on the TatABC translocase, showed the
same phenotype (18). In contrast, a mutant lacking the cydAB genes for the cytochrome bd oxidase showed no growth defects in the exponential growth phase, but only thereafter, leading to reduced biomass formation (19). These results support the key role of the bc1–aa3 supercomplex in aerobic respiration of C. glutamicum.

A comprehensive genetic analysis revealed that the cytochrome bc1–aa3 supercomplex is not restricted to Corynebacterium, but is probably characteristic for the majority of Actinobacteria (except for the anaerobic orders Actinomycetales and Bifidobacteriales) due to the absence of genes for a monoheme cytochrome c and the universal presence of the gene for diheme cytochrome c1 (17). This view is supported by studies in Mycobacterium (20–22), Rhodococcus (23), and Streptomyces (24).

The functionality of the cytochrome bc1–aa3 supercomplex depends on the correct assembly of the heme groups, of the iron–sulfur cluster, and of the copper centers. Currently, no studies have been reported on proteins involved in the biogenesis of the supercomplex. Here, we addressed this question with a focus on the proteins required for copper insertion. With respect to copper homeostasis in C. glutamicum, two regulatory systems involved in the response to copper excess stress have been identified, the two-component signal transduction system CopRS (25) and the one-component transcriptional regulator CsoR (26). The target genes of these regulators included genes presumably encoding copper exporters, but no obvious copper importers. As a starting point in our search for proteins involved in copper insertion into cytochrome aa3 oxidase, we determined the copper-deprivation stimulation of C. glutamicum and analyzed genes with increased expression. Copper deprivation is known to influence the content of cytochrome aa3 oxidase in various bacteria (27, 28). Using this approach, two candidate proteins were identified and characterized, CtiP (Cg2699) and CopC (Cg1884).

**Results**

**Copper consumption by C. glutamicum and influence of copper deprivation on growth and cytochrome composition**

The activity of the cytochrome bc1–aa3 supercomplex of Actinobacteria depends on the presence of the CuL and CuM centers in subunit II (CtaC) and subunit I (CtaD), respectively, of cytochrome aa3 oxidase. To search for proteins involved in the formation of the copper centers, we analyzed the transcriptional response of C. glutamicum to copper deprivation. We assumed that genes for such assembly factors might show an increased expression under these conditions. In initial studies, we analyzed copper consumption by C. glutamicum, established copper-deprivation conditions, and tested their influence on growth and cytochrome composition.

In standard CGXII glucose minimal medium, copper is supplied as a trace element in a concentration of 1.25 μM CuSO4. To analyze copper consumption in this medium, the concentration in freshly prepared medium just before inoculation and in the culture supernatant after 25 h of cultivation of C. glutamicum WT was determined via inductively coupled plasma MS. The copper concentration decreased from the measured 1.25 ± 0.26 μM copper before inoculation to 0.18 ± 0.02 μM copper after 25 h. Based on this difference and the cell density after 25 h (A600 = 60, corresponding to 1.68 × 1010 cells/ml), an average uptake of 3.8 × 1014 ions/cell was calculated.

To study the growth behavior of C. glutamicum under copper-deprivation conditions, we omitted CuSO4 from the CGXII medium and supplemented it with a 150 μM concentration of the copper-specific chelator bathocuproine disulfonate (BCS) and 1 mM ascorbate for reduction of Cu(II) to Cu(I) (29). When cultivated in shake flasks rinsed with 0.1 N HCl before the addition of medium to remove residual trace metal ions, the WT showed a reduced growth rate of 0.36 ± 0.01 h⁻¹ under copper deprivation compared with 0.40 ± 0.01 h⁻¹ under standard copper conditions. In contrast, the growth rate of a C. glutamicum ΔcydAB mutant lacking the copper-independent cytochrome bd oxidase showed a strongly reduced growth rate of 0.15 ± 0.01 h⁻¹ under copper deprivation compared with growth under copper conditions (μ = 0.40 ± 0.01 h⁻¹) (data not shown). As oxygen respiration of the C. glutamicum ΔcydAB mutant should be strictly dependent on the copper-dependent cytochrome aa3 oxidase (30), the residual growth of the ΔcydAB mutant under copper deprivation indicated that, under the experimental conditions applied, copper is still available to some extent and allows synthesis of some functional cytochrome aa3. To eliminate residual copper, the growth experiment was repeated in a Biolector microcultivation system using FlowerPlates™ made of high-purity polystyrene, and in this case, the ΔcydAB mutant showed no growth under copper deprivation (Fig. 1). After the addition of copper sulfate, growth could be recovered (data not shown). Thus, in the absence of copper aerobic respiration of C. glutamicum depends on cytochrome bd oxidase.

To obtain further insights into the consequences of copper deprivation on the composition of the respiratory chain, reduced spectra of C. glutamicum cells cultivated in shake flasks either under standard conditions or under copper deprivation were recorded (Fig. 2). Under standard conditions, C. glutamicum exhibits a classical spectrum with a peak at 550 nm that can be attributed to cytochrome oxidase CtaC. Under copper deprivation, the peak is strongly reduced and a shoulder at 605 nm becomes visible. This shoulder is characteristic for heme c1 of cytochrome bd oxidase, which is suggested to be present in the respiratory chain of C. glutamicum under copper deprivation.

**Figure 1. Influence of copper deprivation on growth of C. glutamicum WT and the ΔcydAB mutant**. Strains were cultivated either in standard CGXII medium with 2% (w/v) glucose and 1.25 μM CuSO4 or in copper-deprived CGXII medium with 2% (w/v) glucose without added CuSO4 and supplemented with 150 μM BCS and 1 mM ascorbate. The growth experiment was performed in FlowerPlates™ with 800 μl of medium using a Biolector microcultivation system (30 °C, 1200 rpm). Mean and S.D. (error bars) from three biological replicates are shown, a.u., arbitrary units.

The abbreviations used are: BCS, bathocuproine disulfonate; BHI, brain heart infusion; kb, kilobases; ECF, extracytoplasmic function.
Biogenesis of actinobacterial cytochrome c oxidase

limitation were recorded (Fig. S1). The spectra revealed that under copper deprivation, the peak at 630 nm was clearly increased, in line with an increased content of cytochrome bd oxidase. At the same time, the cytochrome a peak at 602 nm was reduced and slightly blue-shifted, and the cytochrome c peak at 552 nm was reduced. These changes are in agreement with previous data (28) and show that copper deprivation has a severe influence on the composition of the respiratory chain.

Determination of the copper-deprivation stimulus of C. glutamicum

To determine the copper-deprivation stimulus of C. glutamicum, we compared global gene expression of WT cells cultivated in shake flasks either under copper deprivation or under standard conditions using DNA microarrays. RNA was isolated from cells that had been harvested at an A600 of 20. 16 genes showed a ≳3-fold increased mRNA ratio under copper deprivation, and 10 genes showed a ≳3-fold lowered mRNA ratio (Table 1). The genes with the highest up-regulation under copper deprivation were those of the cydABDC operon encoding cytochrome bd oxidase and an ABC transporter required for functional synthesis of this terminal oxidase (19, 31). Other highly up-regulated genes encoded heme o synthase (ctaB), an ABC transporter with a binding protein for metal ions of the TroA_a family (cg1832 and cg1833), CopC, a secreted protein with a CopC domain and a C-terminal transmembrane helix (cg1884) (32), a secreted lipoprotein belonging to the PCuA_C family (cg1883) (33), a DyP-type heme peroxidase with a Tat signal peptide (cg1881), an integral membrane protein with a PepSY-associated TM region (cg2556), a large integral membrane protein (cg2699) with both a CopD (34) and a CtaG domain (35), and an integral membrane protein with a DUF3817 domain (cg2750). The gene displaying the strongest down-regulation under copper deprivation was cg2546, encoding an integral membrane protein of the DctM family of transporters. In addition, several other genes encoding transporter proteins and two genes of the ctaE-qcrCAB operon encoding subunit III of cytochrome aa3 oxidase and the cytochrome bc1 complex were found to be down-regulated. The up-regulation of the cydABDC operon and the concomitant down-regulation of the ctaE-qcrCAB operon are in agreement with the results of the cytochrome spectra reported above.

Several of the genes listed in Table 1 were also reported in a previous study to show altered expression in C. glutamicum strain R upon copper deficiency: cyaD, ctaA, ctaB, cgR_0179 (homolog of cg1884), cgR_2208 (homolog of cg2556), and cgR_2412 (homolog of cg2750) were found to be up-regulated 1 h after the addition of 0.5 mM BCS, whereas expression of ctaE was found to be down-regulated. It was shown that these alterations in gene expression were due to the ECF sigma factor SigC (36).

The target genes of the Cu(I)-regulated repressor CsoR (26) did not show reduced expression under copper deprivation, indicating that the intracellular concentration of "free" copper was comparable with growth with 1.25 μM CuSO4. Because members of the CsoR family have extremely high affinities for Cu(I) with Kd values in the range of 10−19 to 10−21 M (37, 38), we assume the “free” cytoplasmic Cu(I) concentration to be extremely low already under standard growth conditions with 1.25 μM CuSO4. Studies in yeast calculated less than one free copper ion per cell (39), supporting this assumption.

In silico characterization of CtiP (Cg2699)

Bioinformatic analysis of the genes up-regulated under copper deprivation revealed that the protein encoded by Cg2699 was a promising candidate for being involved in the biogenesis of the cytochrome bc1-aa3 supercomplex. It comprises 717 amino acid residues (calculated mass 79.2 kDa) and is predicted to contain 16 transmembrane helices. PFAM analysis (40) revealed that a region in the N-terminal half (residues 269–364) shows sequence similarity to the CopD family (PF05425), whereas the C-terminal region (residues 418–661) displays similarity to the Caa3-CtaG family (PF09678). The CopD integral membrane protein of Pseudomonas syringae was proposed to function together with the CopC protein, a periplasmic copper-binding protein (homologous to Cg1884 of C. glutamicum), in copper import, as overexpression of copCD led to copper hypersensitivity (41). The function of the ctaG gene was characterized until now only in Bacillus subtilis, where the results indicate that it is required specifically for the synthesis of a functional cytochrome c oxidase, cytochrome caa3 (35). Note that CtaG of B. subtilis is unrelated to CtaG/Cox11p of proteobacteria and eukaryotic cells. Cg2699 apparently represents a fusion protein that could combine the function of CopD in copper import and the function of CtaG in the biogenesis of cytochrome c oxidase. Based on results described below, we named the protein CtiP for “copper transport and insertion protein” and the gene accordingly ctiP. In Fig. 2, a topology model of CtiP is shown, which also highlights the regions assigned to the CopD family and the Caa3-CtaG family.

Impact of CtiP on growth, cytochrome content, copper resistance, and global gene expression

To test a possible involvement of CtiP in the maturation of the cytochrome bc1-aa3 supercomplex, the deletion mutant ΔctiP was constructed and analyzed for its growth behavior. In standard CGXII glucose medium, strain ΔctiP showed a lowered growth rate compared with the WT (Fig. 3A). This growth defect could be abolished by expression of ctiP with plasmid pPEKEx2-ctiP, confirming that it is caused by the deletion of ctiP (Fig. S2). A growth defect of the ΔctiP mutant was also clearly visible on brain heart infusion (BHI) agar plates, where the colonies were much smaller than those of the WT and similar in size to that of a ΔctaD mutant lacking subunit I of cytochrome aa3 oxidase (Fig. 3D). These results support the assumption that CtiP is a candidate for being involved in the biogenesis of the cytochrome aa3 oxidase.

To study the influence of CtiP on the composition of the respiratory chain, cytochrome spectra of dithionite-reduced cells were recorded. As shown in Fig. 2, the spectra of the ΔctiP strain grown either in standard CGXII glucose medium (1.25 μM CuSO4) or under copper deprivation were similar to those
of the WT grown under copper deprivation; the cytochrome d peak at 630 nm was increased compared with the WT grown with standard copper levels, the cytochrome a peak at 602 nm was reduced and slightly blue-shifted, and the cytochrome c peak at 552 nm was reduced. This result provided evidence for a role of CtiP in respiration and in particular suggested that the ΔctiP mutant behaves like the WT under copper deprivation. To support a role of CtiP in copper metabolism, we analyzed growth of the ΔctiP mutant under copper deprivation and under copper excess stress and observed WT-like growth under copper limitation but an improved growth rate in the presence of 100 μM CuSO₄ (Fig. 3, B and C). An increased copper tolerance could be explained by assuming that CtiP functions as a copper importer, as implied by the sequence similarity to CopD (see above).

The growth behavior and the cytochrome spectra of the ΔctiP mutant under regular copper concentrations (1.25 μM) were similar to those of the WT under copper deprivation. These observations prompted us to compare global gene expression of the ΔctiP mutant and the WT under standard copper levels (1.25 μM) using DNA microarrays. RNA was isolated from cells that had been harvested at an Abs₆₀₀ of 20.

In total, 19 genes showed a ≥3-fold increased mRNA ratio in the ΔctiP mutant, and 15 genes showed a ≥3-fold lowered mRNA ratio (Table 1). Remarkably, 12 of the 19 genes up-regulated in the ΔctiP mutant were also ≥3-fold up-regulated in of the WT grown under copper deprivation; the cytochrome d peak at 630 nm was increased compared with the WT grown with standard copper levels, the cytochrome a peak at 602 nm was reduced and slightly blue-shifted, and the cytochrome c peak at 552 nm was reduced. This result provided evidence for a role of CtiP in respiration and in particular suggested that the ΔctiP mutant behaves like the WT under copper deprivation. To support a role of CtiP in copper metabolism, we analyzed growth of the ΔctiP mutant under copper deprivation and under copper excess stress and observed WT-like growth under copper limitation but an improved growth rate in the presence of 100 μM CuSO₄ (Fig. 3, B and C). An increased copper tolerance could be explained by assuming that CtiP functions as a copper importer, as implied by the sequence similarity to CopD (see above).

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the WT under copper deprivation, clearly supporting the idea that the absence of CtiP results in copper deficiency or at least in a situation that elicits the copper-deprivation response. This is in line with a copper import function of CtiP. Of the 15 genes with a ≥3-fold lowered mRNA level in the ΔctiP mutant, only four also had ≥3-fold lowered mRNA levels in the WT under copper deprivation (i.e. the congruence was much lower than for the up-regulated genes).
Impact of CtiP on the assembly of the cytochrome bc$_{1}$–aa$_{3}$ supercomplex

The presence of a CtaG-like domain in the C-terminal part of CtiP pointed to an involvement of the protein in the assembly of cytochrome c oxidase. To address such a function, we tested whether the absence of CtiP has an influence on the ability to purify the cytochrome bc$_{1}$–aa$_{3}$ supercomplex. We constructed the double-deletion mutant ΔctaDΔctiP, transformed it with the plasmid pC1-ctaD$_{St}$ expressing C-terminally Strep-tagged CtaD from its native promoter, and purified CtaD$_{St}$ by Streptactin affinity chromatography as described (11). As a reference, we used strain ΔctaD/pC1-ctaD$_{St}$. The proteins eluted with desthiobiotin were analyzed by SDS-PAGE and identified by peptide mass fingerprinting. As shown in Fig. 4A, with desthiobiotin were analyzed by SDS-PAGE and identified

In an analogous approach, we constructed the strain ΔqcrΔctiP in which, besides ctiP, the qcrCAB genes are deleted and transformed it with plasmid pC1-qcrB$_{St}$ expressing the ctaE-qcrCAB operon from its native promoter, with QcrB containing a C-terminal StrepTag. Solubilized membrane proteins of strain ΔqcrΔctiP/pC1-qcrB$_{St}$ and, for comparison, of strain Δqcr/pC1-qcrB$_{St}$ were subjected to Streptactin affinity chromatography, and the eluates were analyzed as described above. Whereas the eluate of strain Δqcr/pC1-qcrB$_{St}$ contained not only QcrB$_{St}$, but also QcrA, QcrC, CtaD, and Cg2949, in the eluate of strain ΔqcrΔctiP/pC1-qcrB$_{St}$ only QcrB$_{St}$ and QcrA were detectable (Fig. 4B).

These results indicate that the formation of the cytochrome bc$_{1}$–aa$_{3}$ supercomplex is disturbed in the absence of CtiP. The co-purification of QcrB$_{St}$ and QcrA from strain ΔqcrΔctiP/pC1-qcrB$_{St}$ is comparable with the purification result obtained with a ΔctaD mutant carrying pC1-qcrB$_{St}$ (11), indicating that the lack of CtiP has no influence on the interaction of QcrB with QcrA. However, the absence of CtiP prevented co-purification of the other subunits of cytochrome aa$_{3}$ oxidase with CtaD$_{St}$, which was previously shown to be independent of the presence of the cytochrome bc$_{1}$ complex (11). This suggests that in the absence of CtiP, either CtaD itself or at least one of the subunits CtaC, CtaE, or CtaF is altered in a way that prevents formation of a stable oxidase complex that can be purified with CtaD$_{St}$. Possible reasons could be an insufficient metallation of either the Cu$_{A}$ center in CtaD or the Cu$_{A}$ center in CtaC, as delineated under “Discussion.”

Growth behavior of deletion mutants lacking individual genes of the copper-deprivation stimulon

To search for additional genes of the copper-deprivation stimulon that might be involved in the biogenesis of the cytochrome bc$_{1}$–aa$_{3}$ supercomplex, nine C. glutamicum deletion mutants were constructed lacking cg0569, cg1744, cg1832, cg1833, cg1881, cg1883, copC (cg1884), cg2556, or cg2750. All of the genes could be deleted, showing that none of them is essential. The growth properties of the resulting mutant strains were analyzed in standard CGXII medium, in copper-deprived CGXII medium, and in CGXII medium supplemented with 100 μM CuSO$_{4}$, besides the 1 complex (11). This suggests that in the absence of CtiP, either CtaD itself or at least one of the subunits CtaC, CtaE, or CtaF is altered in a way that prevents formation of a stable oxidase complex that can be purified with CtaD$_{St}$. Possible reasons could be an insufficient metallation of either the Cu$_{A}$ center in CtaD or the Cu$_{A}$ center in CtaC, as delineated under “Discussion.”

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in the absence of CopC, the stability of the cytochrome 5 sequence alignment (Fig. S6) revealed that the Cu(II)-binding motif might also be a copper-binding protein involved in copper residues. As shown in Fig. 5 Met51, and Tyr79 of pJC1, CtaC, QcrB, QcrA, and QcrC were co-purified with CtaD, in the WT, whereas in the eluate of the ΔcopC mutant, only CtaD was found. This result suggests that in the absence of CopC, the stability of the cytochrome aa₃ oxidase complex is impaired. In view of the likely function of CopC as a copper-binding protein, a role in the metallation of the copper centers appears to be a possible reason.

Discussion
Numerous studies have addressed the biogenesis of respiratory cytochromes in bacteria, yeast, and mammals and a multitude of proteins involved in this process have been identified (43, 44). In bacteria, the majority of experimental work was performed with α-proteobacteria, such as Rhodobacter sphaeroides and Paracoccus denitrificans. Knowledge on the assemblage of respiratory oxidases in Gram-positive bacteria is sparse, and this is particularly true for the large phylum of the Actinobacteria. This study aimed at the identification of proteins involved in the insertion of copper ions into cytochrome c oxidase of C. glutamicum.

Our approach was based on the hypothesis that proteins involved in copper insertion into cytochrome aa₃ oxidase might show an elevated expression under copper deprivation. Therefore, we determined the copper-deprivation stimulon of C. glutamicum and identified 16 genes with an at least 3-fold elevated expression. Interestingly, all 12 genes previously shown to be transcriptionally activated by the ECF sigma factor SigC in C. glutamicum strain R were included in the copper-deprivation stimulon of strain ATCC13032, except for cgR_0144, which appears to be a paralog of cgR_1719 (cg1884) only present in strain R (36). Our results are in full agreement with those obtained previously in strain R by quantitative RT-PCR showing that cydA, ctaA, and ctaB were up-regulated under copper deprivation in a SigC-dependent manner (36).

Expression of the sigC gene itself, however, was not up-regulated by copper deprivation, neither in strain R nor in our studies with strain ATCC13032, indicating that increased expression of the genes of the SigC regulon is not due to increased sigC transcription. It was suggested that SigC is activated by an impaired electron transfer via cytochrome aa₃ oxidase (36), but the mechanism is still unknown. Based on these results, it can be assumed that SigC is responsible for up-regulation of its 11 target genes included in the copper-deprivation stimulon of C. glutamicum ATCC13032. The observation that the genes of the cydABDC operon exhibited the highest up-regulation under copper deprivation is in line with the fact that cytochrome bd oxidase is copper-independent and therefore can replace cytochrome aa₃ oxidase under these conditions.

Besides the known genes of the SigC regulon, however, six additional genes were found to be up-regulated at least 3-fold under copper deprivation in strain ATCC13032. Inspection of their promoter regions revealed no obvious SigC-binding motifs (data not shown). Four of these are likely involved in metal ion transport. The genes cg0569 and cg1744 were both annotated as cation-transporting ATPases, whereas cg1832 and cg1833 encode a permease and a secreted substrate-binding lipoprotein of an ABC transporter. The binding protein belongs to the TroA_a family of proteins that are predicted to function as initial receptors in ABC transport of metal ions in eubacteria. The up-regulation of these four genes under copper deprivation points to a role in copper uptake. The lysE gene (cg1424) encodes a secondary exporter for lysine and arginine (45, 46). Its expression is activated by the transcriptional regulator LysG, which senses elevated levels of lysine, arginine, and histidine (46–48). We assume that copper deprivation and the concomitantly reduced growth rate presumably results in increased intracellular concentrations of one or several of these amino acids and activation of lysE expression. The protein encoded by cg2699 has been intensively studied in this work and will be discussed below.

Except for lysE, the mechanisms responsible for increased expression of the genes not belonging to the SigC regulon under
copper deprivation are unclear at present. In the case of cg1832 and cg1833, the gene cg1831 located upstream and divergent to cg1832 encodes an ArsR-type transcriptional repressor, which could be responsible for derepression under copper starvation. Overall, the results of the transcriptome comparison suggest that expression control of the genes up-regulated under copper deprivation involves not only SigC, but also other transcriptional regulators and possibly different stimuli.

Our studies of the ΔctiP mutant indicate that the CtiP protein is involved in the biogenesis of cytochrome aa₃ oxidase and the cytochrome bc₁–aa₃ supercomplex. The ΔctiP mutant had a clear growth defect in BHI medium, on BHI agar plates, and in standard glucose minimal medium with 1.25 μM CuSO₄, but it grew like the WT under copper-deprived conditions and even better than the WT in medium containing 100 μM CuSO₄ (Fig. 3). The increased copper tolerance indicates an involvement of CtiP in copper import, which is supported by the fact that almost the entire copper-deprivation stimulus was induced in the ΔctiP mutant during growth under standard conditions with 1.25 μM CuSO₄. Thus, in the absence of CtiP, the mechanisms signaling copper deprivation are activated although sufficient copper ions are present in the medium. The growth defect of the ΔctiP mutant under standard copper concentrations is most likely due to an impaired cytochrome aa₃ biogenesis, which was indicated by the failure to purify the cytochrome bc₁–aa₃ supercomplex (Fig. 4). An impaired formation of the CuA or the CuB center is a possible explanation for this result, assuming that these copper centers are necessary for the formation of a stable supercomplex.

The CuA center is located in the extracytoplasmic domain of CtaC and involves two copper ions that are bridged by two sulfur ligands from cysteine residues (Cys₂⁸⁵ and Cys₂⁸⁹ in the case of C. glutamicum CtaC). It is formed after the translocation of the domain by the Sec machinery. In bacteria, three proteins have been described to be involved in the formation of the CuA center, the Sco protein (synthesis of cytochrome oxidase), the PCuA₃C protein (protein CuA₃ chaperone), and the TlpA protein (thioredoxin-like protein A). Sco proteins are membrane-bound copper chaperones belonging to the thioredoxin family. They are located in the periplasm and contain a CXXC motif involved in copper binding. Sco proteins can function both as disulfide reductases that reduce the cysteines of apo-CuA and as copper donor to the CuA center (44). Whereas B. subtilis contains an Sco protein presumably involved in CuA formation of the cytochrome caa₃ oxidase (49), C. glutamicum does not possess an Sco homolog.

The TlpA protein was identified in Bradyrhizobium japonicum in a screen for tetramethyl-p-phenylenediamine oxidase-negative transposon mutants (50) and is a membrane-anchored periplasmic protein with the active site sequence WCVPC (51). It was recently shown that TlpA is a specific reductant for the copper chaperone ScoI and the apo-Cu₃ in CoxB of B. japonicum (52). In C. glutamicum, two TlpA candidates were found. Cg0354 has the motif WCEPC and may contain a signal peptide, but no transmembrane helix. Therefore, a function of this protein as a reductant for apo-Cu₃ in CtaC seems questionable. Cg0520 is a secreted lipoprotein with the active site sequence WCAPC and is encoded in a gene cluster involved in cytochrome c biogenesis (10). A function of Cg0520 as CtaC reductant appears possible, but this requires further experimental studies.

PCuA₃C proteins are copper chaperones that bind a single Cu(I) in a cupredoxin-like fold (33, 53, 54). In Thermus thermophilus, PCuA₃C was shown to serve as copper donor to the CuA center (53). Also, C. glutamicum possesses a PCuA₃C homolog encoded by cg1883. This gene is part of an operon including copC (cg1884) and cg1881, all of which were found to be up-regulated under copper deprivation and are part of the SigC regulon. Cg1883 contains a signal peptide with the lipobox VMAACS (55) and thus presumably is a lipoprotein. The copper-binding motif in the structurally and functionally analyzed PCuA₃C homologs was found to be (H/M)X₁₀MX₂₁HXM. The Cg1883 protein and its homologs from other actinobacteria contain a slightly different motif with the sequence HX₂MX₂₁HXM. As the Δcg1883 mutant constructed in our study showed no growth phenotype under the conditions tested, the function of Cg1883 remains unclear. It might support Cu₃ assembly of actinobacterial cytochrome aa₃ oxidase but apparently is not essential for this process.

The CuB center, which is an invariable characteristic of heme–copper oxidasases, is located within the transmembrane region closer to the positive side of the membrane (56). In yeast, mammals, and several bacteria, the Cox11 protein was shown to be involved in formation of the CuB center. In R. sphaeroides, lack of Cox11 led to a cytochrome aa₃ oxidase containing all centers except Cu₄, even when copper was in excess, indicating that in this species, Cox11 is essential for Cu₄ formation (57). Cox11 is a membrane-anchored protein with a periplasmic domain containing a CFCF motif that binds Cu(I) and donates it to the CuB center. As C. glutamicum does not contain a Cox11 homolog, other mechanisms and proteins must be involved in the formation of the CuB center.

Based on the presence of a CtaG domain and our data, the CtiP protein is a good candidate for CuB formation. However, for B. subtilis CtaG, a function in the assembly of the CuA center or another feature only present in the cytochrome c oxidase caa₃ was proposed based on the observation that a mutant lacking ctaG and cydABCD was still able to grow on agar plates (35). Respiratory growth of this mutant was assumed to be dependent on cytochrome caa₃, a heme–copper type menaquinol oxidase, which does not possess a CuA center, but the heme a₃-CuB center (35). If CtaG would be required for Cu₄ formation, not only cytochrome caa₃, but also cytochrome aa₃ should be defective, and the mutant lacking ctaG and cydABCD should not be able to grow. This assumption, however, might be wrong, as B. subtilis possesses a second bd-type oxidase encoded by the ythAB genes (58), which could allow growth of the mutant even if cytochrome caa₃ and cytochrome aa₃ are inactive due to the lack of CtaG. Alternatively, a protein different from CtaG could be responsible for Cu₄ formation in the cytochrome aa₃ menaquinol oxidase.

The data available for the C. glutamicum ΔctiP mutant suggest that CtiP might couple copper import via its N-terminal part with copper insertion into the CuB center via its C-terminal part. This would imply that insertion of copper into CuB does not proceed from the extracytoplasmic side of the
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In summary, our study has provided the first data on the biogenesis of respiratory enzymes in Actinobacteria, a topic that has been neglected in the past. Based on the determination of the copper-deprivation stimulus of *C. glutamicum*, we identified two proteins involved in the maturation of cytochrome *aa*₃ oxidase. A model of this process based on our data is shown in Fig. 6. Obviously, further studies are required to validate this model and to identify additional proteins involved in the assembly process.

**Experimental procedures**

**Bacterial strains, media, and growth conditions**

Bacterial strains and plasmids used or constructed in this work are listed in the Table 2. *C. glutamicum* strains were cultivated aerobically at 30 °C either in CGXII minimal medium containing 2 or 4% (w/v) glucose as carbon and energy source and 30 mg/liter 3,4-dihydroxybenzoate as iron chelator (61) or in BHI medium supplemented with 2 or 4% (w/v) glucose or on BHI agar plates. Cultivations were performed either in 500-ml glass shake flasks containing 50 ml of medium that were incubated on a rotary shaker at 120 rpm or in a Biolector microcultivation system using FlowerPlates™ with 800 μl of medium that were shaken at 1200 rpm (m2p-labs, Baesweiler, Germany). For cultivation under copper deprivation, copper (regular concentration 1.25 μM) was excluded from the CGXII medium in the preculture and the main culture. The latter was supplemented with a 150 μM concentration of the copper-specific chelator BCS. Because BCS binds Cu(I), the medium was additionally supplemented with freshly prepared ascorbate (1 mM) for reduction of Cu(II) to Cu(I), as described (29). For cultivation under copper excess stress, CGXII medium was supplemented with 100 μM CuSO₄. Copper concentrations in the medium before and after cultivation were determined via inductively coupled plasma MS at ZEA, Forschungszentrum Jülich. *E. coli* DH5α was used as host for cloning purposes. The *E. coli* strains were cultivated aerobically in lysogeny broth medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) at 37 °C. When appropriate, kanamycin was

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membrane, as assumed for the Cox11-mediated process in *R. sphaeroides*, but from the cytoplasmic side or intramembranously. Alternatively, an additional copper chaperone might be involved, which is loaded by CtiP in the cytoplasm or intramembranously, and then transfers the copper to Cu₄₈ on the extracytoplasmic side, as suggested in a recent study for the assembly of the Cu₄₈ center of the *cbb*₃-type cytochrome *c* oxidase in *Rhodobacter* (59). In this example, the secondary copper importer CcoA is responsible for copper import and required for biogenesis of cytochrome *cbb*₃ oxidase (60).

As shown by the examples described above, different routes for the formation of the Cu₄₈ center of heme–copper type terminal oxidases have been evolved. In the case of *C. glutamicum*, CtiP is likely to be involved in this process, which implies that the protein should be conserved in Actinobacteria, most of which harbor the genes for the cytochrome *bc*₁–*aa*₃ supercomplex (17). Bioinformatic analysis confirmed that CtiP homologs are encoded in all actinobacterial orders except for most species of the predominantly anaerobic members of the Actinomycetales and Bifidobacteriales (data not shown). This distribution supports the proposed function of CtiP.

A second protein that we identified in this study to be presumably involved in the biogenesis of cytochrome *aa*₃ oxidase of *C. glutamicum* was CopC. It contains a Sec-type signal peptide, a C-terminal transmembrane helix anchoring the protein in the cytoplasmic membrane, and a Cu(II)-binding site. In contrast to *ctiP*, the *copC* gene is part of the SigC regulon. A Δ*copC* mutant grew like WT in standard glucose minimal medium but showed a growth defect on BHI agar plates similar to the Δ*ctiP* mutant and a growth advantage under copper excess stress. The latter phenotype suggests a role of CopC in copper import. Furthermore, impaired purification of the cytochrome *bc*₁–*aa*₃ supercomplex from membranes of the Δ*copC* mutant suggested disturbed or at least weakened complex formation. CopC might serve as an extracytoplasmic copper chaperone supporting copper import under copper deprivation (e.g. by donating Cu(II) to CtiP). Like CtiP, CopC was also found to be highly conserved in Actinobacteria containing the genes for the cytochrome *bc*₁–*aa*₃ supercomplex (data not shown).
added to a concentration of 50 µg/ml (E. coli) or 25 µg/ml (C. glutamicum).

Recombinant DNA work

The enzymes for recombinant DNA work were obtained from Roche Diagnostics (Mannheim, Germany) or New England Biolabs (Frankfurt am Main, Germany). All oligonucleotides (Table S1) were synthesized by Eurofins (Ebersberg, Germany). Routine methods like PCR, restriction, or ligation were carried out according to standard protocols (62). The generation of all PCR products was performed with High Fidelity DNA polymerase (Roche Diagnostics). Plasmids were isolated from E. coli with the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). E. coli was transformed by the RbCl method (63). Transformation of C. glutamicum was performed as described previously (64). All plasmid constructs described in this work were controlled by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).

Table 2

Bacterial strains and plasmids used in this study

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| C. glutamicum ATCC13032 | Biotin- auxotrophic WT strain | Ref. 69 |
| C. glutamicum ΔctaD | Derivative of ATCC13032 with in-frame deletion of the ctaD gene (cg2780) | This work |
| C. glutamicum Δacr | Derivative of ATCC13032 with in-frame deletion of the acr gene | Ref. 12 |
| C. glutamicum ΔctiP | Derivative of ATCC13032 with in-frame deletion of the ctiP gene (cg2699) | This work |
| C. glutamicum ΔctiPΔactiP | Derivative of ATCC13032 ΔactiP with in-frame deletion of the ctiP gene | This work |
| C. glutamicum ΔacrΔctiP | Derivative of ATCC13032 Δacr with in-frame deletion of the ctiP gene | This work |
| C. glutamicum Δcg0569 | Derivative of ATCC13032 with in-frame deletion of the cg0569 gene | This work |
| C. glutamicum Δcg1744 | Derivative of ATCC13032 with in-frame deletion of the cg1744 gene | This work |
| C. glutamicum Δcg1832 | Derivative of ATCC13032 with in-frame deletion of the cg1832 gene | This work |
| C. glutamicum Δcg1833 | Derivative of ATCC13032 with in-frame deletion of the cg1833 gene | This work |
| C. glutamicum Δcg1881 | Derivative of ATCC13032 with in-frame deletion of the cg1881 gene | This work |
| C. glutamicum Δcg1883 | Derivative of ATCC13032 with in-frame deletion of the cg1883 gene | This work |
| C. glutamicum ΔcopC | Derivative of ATCC13032 with in-frame deletion of the copC gene (cg1884) | This work |
| C. glutamicum Δcg2566 | Derivative of ATCC13032 with in-frame deletion of the cg2566 gene | This work |
| C. glutamicum Δcg2750 | Derivative of ATCC13032 with in-frame deletion of the cg2750 gene | This work |
| E. coli DH5α | F′ <q80lacA3( lacZ)M15 Δ(lacZAM15) lacY1 Δ(lac-proAB) 16095thiA1 hsdR17 (rK− mB−) deoR recA1 araD139 endA1 supE44 hgal relA1 | Invitrogen |

Construction of C. glutamicum deletion mutants

In-frame deletion mutants of C. glutamicum (Table 1) were constructed via a two-step homologous recombination procedure as described previously (12).

Global gene expression analysis

DNA microarray analysis was used to compare (i) the genome-wide mRNA levels of C. glutamicum WT cultivated under copper-deprived conditions (see above) with those of C. glutamicum WT cultivated in standard CGXII medium (1.25 µM CuSO₄) and (ii) the genome-wide mRNA levels of the C. glutamicum ΔctiP mutant with those of C. glutamicum WT, both strains cultivated in standard CGXII medium (1.25 µM CuSO₄). RNA was prepared as described previously (65). For RNA isolation, the strains were cultured in CGXII medium with 4% (w/v) glucose and the indicated copper concentrations and harvested at an A₆₀₀ of 20 in the late exponential growth phase. All DNA microarray analyses were performed with custom-made DNA microarrays based on 70-mer oligonucleotides.
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obtained from Operon Biotechnologies and later from Agilent Technologies. For each of the two comparisons, three independent biological replicates were performed, and p values were calculated using Student’s t test (Excel, Microsoft). The experimental details and the data evaluation were performed as described previously (66). The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database and are accessible under accession numbers GSE117530 and GSE117566.

Purification of Strep-tagged CtaD and QcrB

The preparation of cell membranes, the solubilization of membrane proteins with n-dodecyl-β-D-maltoside, and the affinity chromatography with Strep-Tactin-Sepharose were performed as described (11). Isolated proteins were analyzed by SDS-PAGE and identified by peptide mass fingerprinting using MALDI-TOF MS (67).

Analysis of cytochromes

For recording dithionite-reduced spectra of intact cells, they were resuspended in 100 mM Tris-HCl buffer, pH 7.4, to an A450 of 100 and analyzed at room temperature using 5-mm light path cuvettes with a Jasco V560 spectrometer equipped with a silicon photodiode detector for turbid samples (68).

Author contributions—X. M., C. D., M. Brocker, M. Baumgart, and M. Bott designed the experiments and analyzed the data; X. M. and C. D. performed the experiments; X. M. and C. D. prepared the figures; and M. Bott wrote the manuscript.

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