THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 6, Issue of February 7, pp. 4339–4346, 2003

Printed in U.S.A.

Purification of a Cytochrome bc_1-aa_3 Supercomplex with Quinol Oxidase Activity from *Corynebacterium glutamicum*

IDENTIFICATION OF A FOURTH SUBUNIT OF CYTOCHROME aa_3 OXIDASE AND MUTATIONAL ANALYSIS OF DIHEME CYTOCHROME c_1*

Received for publication, October 14, 2002, and in revised form, November 19, 2002
Published, JBC Papers in Press, November 20, 2002, DOI 10.1074/jbc.M210499200

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The aerobic respiratory chain of the Gram-positive *Corynebacterium glutamicum* involves a bc_1 complex with a diheme cytochrome c_1 and a cytochrome aa_3 oxidase but no additional *c*-type cytochromes. Here we show that the two enzymes form a supercomplex, because affinity chromatography of either strep-tagged cytochrome *b* (QcrB) or strep-tagged subunit I (CtaD) of cytochrome aa_3 always resulted in the copurification of the subunits of the bc_1 complex (QcrA, QcrB, QcrC) and the aa_3 complex (CtaD, CtaC, CtaE). The isolated bc_1-aa_3 supercomplexes had quinol oxidase activity, indicating functional electron transfer between cytochrome c_1 and the Cu_A center of cytochrome aa_3. Besides the known bc_1 and aa_3 subunits, few additional proteins were copurified, one of which (CtaF) was identified as a fourth subunit of cytochrome aa_3. If either of the two C*XX*CH motifs for covalent heme attachment in cytochrome c_1 was changed to S*XX*SH, the resulting mutants showed severe growth defects, had no detectable *c*-type cytochrome, and their cytochrome *b* level was strongly reduced. This indicates that the attachment of both heme groups to apo-cytochrome c_1 is not only required for the activity but also for the assembly and/or stability of the bc_1 complex.

Corynebacterium glutamicum is a non-pathogenic aerobic soil bacterium that has gained considerable interest because of its use in large scale biotechnological production of L-glutamate and L-lysine (1) and because of its emerging role as a model organism for the Gram-positive bacteria with high G+C content (2), which include a number of important pathogens, in particular *Corynebacterium diphtheriae* and *Mycobacterium tuberculosis*. In this context, the respiratory chain of *C. glutamicum* was also analyzed in recent years, both genetically and biochemically. It is composed of several dehydrogenases that transfer electrons to an intramembrane pool of menaquinone-9 (3) and at least two branches for reoxidation of menaquinol, one consisting of a cytochrome *bd*-type quinol oxidase (4) and the second one consisting of menaquinol:cytochrome *c* oxidoreductase (cytochrome bc_1 complex) and cytochrome aa_3 oxidase (5–7). The latter one is of primary importance for growth, because mutants lacking either the bc_1 complex or cytochrome aa_3 have severe growth defects (5) (see also Fig. 2).

The dehydrogenases include a non-proton-pumping NADH dehydrogenase encoded by the *ndh* gene (8, 9), malate:quinone oxidoreductase encoded by the *mqo* gene (8, 10), and succinate dehydrogenase encoded by the *sdhCAB* genes (Cgl0370, Cgl0371, Cgl0372). Succinate oxidase activity was shown to be inhibited by an uncoupler, indicating that electron transfer from succinate to menaquinone requires the electrochemical proton potential across the cytoplasmic membrane (11).

The cytochrome *bd* oxidase was purified and shown to consist of two subunits of 56 and 42 kDa encoded by *cydA* and *cydB*, respectively. It was proposed that this oxidase is predominant during the stationary phase of growth (4). The cytochrome bc_1 complex is encoded by the *qcrCAB* genes (Fig. 1) for cytochrome c_1, Rieske iron-sulfur protein, and cytochrome *b*, respectively (5, 6). The protein sequences deduced from these genes revealed a number of differences to classical representatives of the bc_1 complex, such as an extension of about 120 amino acids at the C terminus of cytochrome *b* and the presence of three putative transmembrane helices in the N terminus of the Rieske iron-sulfur protein rather than only one. Most remarkably, cytochrome c_1 was found to have two C*XX*CH motifs for covalent heme attachment, suggesting that it is a diheme *c*-type cytochrome (5, 6). Purification of cytochrome c_1 confirmed the presence of two heme groups in the protein (6). Upstream of *qcrC*, the genes encoding subunit II (*ctaC*) and III (*ctaE*) of cytochrome aa_3 oxidase were identified (5, 6), as was as an additional open reading frame located in between these two genes, which was designated *ctaF* in the course of this work (Fig. 1). Compared with "classical" subunit II representatives, CtaC of *C. glutamicum* contained an insertion of about 30 amino acid residues in the substrate binding domain, which was proposed to play a role in the interaction with cytochrome c_1 (7). The gene encoding subunit I of cytochrome aa_3 was found to be located separately at a different genomic site (5, 7). Cytochrome aa_3 oxidase was isolated by conventional chromatographic techniques as a complex consisting of CtaD, CtaC and CtaE (7). HPLC[1] and mass spectrometry of the isolated heme of subunit I indicated that it is presumably heme a_S, in which the farnesyl group ($C_{15}H_{25}-$) of heme *a* is replaced by a geranylgeranyl side chain ($C_{20}H_{33}-$). Subunit II contains a lipoprotein signal sequence, and in fact Cys-29, whose thiol group might be diacylglycerated, was identified as the N-terminal residue of the mature protein (7).

Staining of proteins separated by SDS-PAGE for covalently bound heme indicated that there is only a single *c*-type cytochrome with an apparent mass of 31 kDa present in *C. glu-*

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[1] The abbreviations used are: HPLC, high pressure liquid chromatography; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; DMN, 2,3-dimethyl-1,4-naphthoquinone.

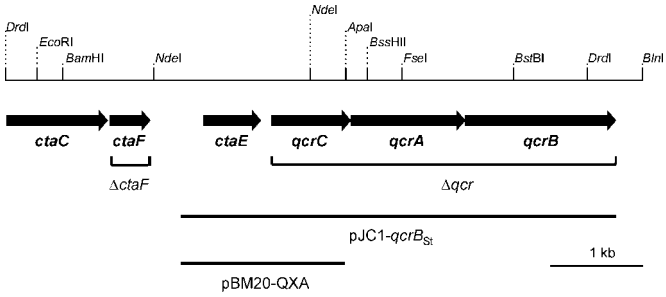

FIG. 1. **Physical map of the *C. glutamicum* genome region harboring the genes for the bc_1 complex and cytochrome aa_3 oxidase except *ctaD*.** The DNA regions deleted in strains 13032Δ*qcr* and 13032Δ*ctaF* are indicated, as well as the fragments present in plasmid pJC1-*qcrB*$_{St}$ and derivatives and in plasmid pBM20-QXA.

tamicum wild type (5, 6). This protein was missing in the mutant strain 13032Δ*qcr*, which lacks the *qcrCAB* genes, confirming that it represents cytochrome c_1 (5). The absence of additional *c*-type cytochromes in *C. glutamicum* indicated that the second heme group of cytochrome c_1 takes over the function of a separate cytochrome *c* in electron transfer to cytochrome aa_3 oxidase. Such a function would require an intimate contact between cytochrome c_1 and the Cu_A electron entry site in subunit II of cytochrome aa_3, and therefore we suggested that the bc_1 complex and cytochrome aa_3 might form a supercomplex (5). In this work, we were able to prove the existence of such a supercomplex by using a very gentle method for its purification, *i.e.* affinity chromatography with the StrepTag II/StrepTactin system (12). Moreover, a fourth subunit of cytochrome aa_3 oxidase was identified, and it was shown that incorporation of both heme groups into cytochrome c_1 is essential for the assembly and/or stability of the entire bc_1 complex.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—*C. glutamicum* strain ATCC 13032 (13) and derivatives were cultivated aerobically in Erlenmeyer flasks at 120 rpm and 30 °C in brain heart infusion medium (BHI; Difco) with 2% (w/v) glucose or in CGXII minimal medium with 4% (w/v) glucose as carbon and energy source (14). When appropriate, 25 μg of kanamycin/ml was added. The *C. glutamicum* strains and the plasmids used in this study are listed in Table I. For all cloning purposes, *Escherichia coli* DH5α (Invitrogen) was used and routinely grown at 37 °C in LB medium (15). When appropriate, 50 μg of kanamycin/ml or 100 μg of ampicillin/ml was added.

Recombinant DNA Work—All enzymes for recombinant DNA work were obtained either from Roche Diagnostics or New England Biolabs. The oligonucleotides used in this study were obtained from MWG Biotech (Ebersberg, Germany) and are listed in Table II. Standard methods were used for the cloning procedures (15).

For the purification of the cytochrome bc_1 complex, a QcrB derivative with a C-terminal StrepTag II (12) was constructed as follows. The entire *ctaE-qcrCAB* gene cluster including the putative promoter region was amplified by PCR (Expand high fidelity PCR system; Roche Diagnostics) using a reverse primer that included the codons for the StrepTag II (WSHPQFEK) preceded by two alanine codons. The resulting 5.0-kb fragment was cloned into the *E. coli-C. glutamicum* shuttle vector pJC1 using the *Xba*I and *Sal*I restriction sites introduced by the primers. The resulting plasmid pJC1-*qcrB*$_{St}$ encoded a QcrB derivative with ten additional residues at the C terminus (calculated mass, 61.1 kDa). A CtaD derivative with a C-terminal StrepTag II for the purification of the cytochrome aa_3 complex was constructed similarly except that only the monocistronic *ctaD* gene with its native promoter was amplified by PCR. The resulting 2.0-kb fragment was cloned via *Xba*I restriction sites into pJC1 yielding pJC1-*ctaD*$_{St}$. The modified CtaD protein contained ten additional residues at the C terminus (calculated mass, 66.3 kDa). Each of the two plasmids was transferred into the *C. glutamicum* strains 13032Δ*ctaD* and 13032Δ*qcr* by electroporation as described (16). For the synthesis of cytochrome c_1 derivatives defective in covalent binding of either the N-terminal or the C-terminal heme group, site-directed mutagenesis of *qcrC* was performed by a two-step PCR procedure according to Higuchi *et al.* (17). For that purpose, a 2.0-kb *Xba*I-*Apa*I fragment from pJC1-*qcrB*$_{St}$ was cloned into pUC-BM20. The resulting plasmid pBM20-QXA served as template for mutagenesis with the universal primers M13-forward/-reverse and the mutagenic primers C67S-forward/-reverse and C177S-forward/-reverse, respectively. The products obtained after crossover PCR were digested with *Xba*I and *Apa*I and cloned into pUC-BM20, yielding pBM20-QXA-C67S and pBM20-QXA-C177S. The presence of the desired mutations and the absence of additional mutations were confirmed by DNA sequence analysis (18). The mutated 2.0-kb *Xba*I-*Apa*I fragments were exchanged against the corresponding wild-type fragment of pJC1-*qcrB*$_{St}$ yielding pJC1-*qcrB*$_{St}$-C67S and pJC1-*qcrB*$_{St}$-C177S, respectively, which were transferred into *C. glutamicum* strain 13032Δ*qcr*. In the cytochrome c_1 variants encoded by these plasmids, residues Cys-67 and Cys-70 or Cys-177 and Cys-180 are replaced by serine residues. In-frame deletion mutants of *C. glutamicum* were constructed as described previously using crossover PCR and the suicide vector pK19*mobsacB* (5). The deletions were verified by PCR and by Southern blot analysis (data not shown).

Preparation of Cell Membranes—Cells (10 g wet weight) were suspended in 15 ml of 100 mM Tris-HCl buffer, pH 7.5, containing 5 mM $MgSO_4$ and 10 mg/ml lysozyme. After 45 min of incubation at 37 °C, 1 mM of the protease inhibitor phenylmethanesulfonyl fluoride was added, and the cells were disrupted by five passages at 207 mega Pascals through a French pressure cell (SLM Aminco). Cell debris was removed by centrifugation at 27,000 × *g* for 20 min, and the supernatant was ultracentrifuged at 150,000 × *g* for 90 min. The pellet containing the cytoplasmic membrane fraction was washed in 100 mM Tris-HCl, pH 7.5, and centrifuged again at 150,000 × *g* for 90 min. Then the membranes were resuspended in a small volume of the same buffer containing 10% (v/v) glycerol and stored at −20 °C.

Purification of the Strep-tagged Protein Complexes—Washed membranes were adjusted to a protein concentration of 5 mg/ml in 100 mM Tris-HCl, pH 7.5, containing 50 μg/ml egg white avidin (Sigma). The membrane proteins were solubilized by adding *n*-dodecyl-β-D-maltoside (Biomol, Hamburg, Germany) from a 10% (w/v) aqueous solution to a final ratio of 2 g of dodecyl maltoside/g of protein. After 45 min of incubation on ice with slow stirring the sample was ultracentrifuged at 180,000 × *g* for 20 min. The supernatant was applied to a StrepTactin-Sepharose column with a bed volume of 2 ml (IBA, Göttingen, Germany) equilibrated with 100 mM Tris-HCl buffer, pH 7.5, containing 0.025% (w/v) dodecyl maltoside. The column was washed with 9 ml of a buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgSO_4$, and 0.025% (w/v) dodecyl maltoside. Specifically bound proteins were eluted with the same buffer supplemented with 2.5 mM D-desthiobiotin (Sigma) and 10% (v/v) glycerol.

Protein Identification by Peptide Mass Fingerprinting—Proteins separated by SDS-PAGE and stained with Coomassie Blue were identified as described previously (19) by peptide mass fingerprinting after in-gel digestion with trypsin (Promega) or with cyanogen bromide (20). If required for reliable identification, post-source decay analysis of selected peptides was carried out (19). Peptide mass lists were used to search a local digest data base of 3312 *C. glutamicum* proteins, provided by the Degussa AG (Frankfurt, Germany).

Enzyme Assays—*N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity was measured spectrophotometrically at 562 nm in air-saturated 100 mM Tris-HCl buffer, pH 7.5, containing 200 μM TMPD, at 25 °C. For the calculation, an extinction coefficient of 10.5 $mM^{-1} \cdot cm^{-1}$ was used (7). One unit of activity is defined as 1 μmol of TMPD oxidized per min. Quinol oxidase activity was measured as oxygen consumption in a magnetically stirred 2-ml chamber with a Clark-type oxygen electrode (Rank Brothers, Cambridge, United Kingdom). The chamber was thermostatted at 25 °C and filled with 1 ml of 50 mM air-saturated sodium phosphate buffer, pH 6.5, supplemented with 200 μM dimethylnaphthoquinol ($DMNH_2$). After recording the rate of autoxidation, the measurement was started by adding the protein sample. One unit of activity refers to 1 μmol of O_2 reduced per min. Dimethylnaphthoquinone (DMN) was obtained initially as a gift from A. Kröger (Frankfurt, Germany) and later synthesized by mild oxidation of 2,3-dimethylnaphthaline with chromium(VI) oxide as described by Kruber (21). $DMNH_2$ was formed by adding a few grains of sodium borohydride and sodium dithionite to a 5 mM solution of DMN in 50% ethanol. Cytochrome *c* oxidase activity was measured spectrophotometrically at 550 nm with bovine heart cytochrome *c* (Sigma) or yeast cytochrome *c* (Sigma) as described (7). One unit of activity refers to 1 μmol of cytochrome *c* oxidized per min.

Difference Spectroscopy—Dithionite-reduced minus ferricyanide-oxidized difference spectra were recorded at room temperature with a

TABLE I
Strains of Corynebacterium glutamicum and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
ATCC13032	Wild type, biotin-auxotrophic	13
13032Δ*ctaD*	In-frame deletion of *ctaD* gene encoding subunit I of cytochrome aa_3 oxidase	5
13032Δ*qcr*	Deletion of the *qcrCAB* genes encoding the three subunits of the bc_1 complex	5
ΔC-D_{St}	13032Δ*ctaD* with pJC1-*ctaD*$_{St}$	This work
ΔC-B_{St}	13032Δ*ctaD* with pJC1-*qcrB*$_{St}$	This work
ΔQ-B_{St}	13032Δ*qcr* with pJC1-*qcrB*$_{St}$	This work
ΔQ-D_{St}	13032Δ*qcr* with pJC1-*ctaD*$_{St}$	This work
ΔQ-B_{St}-C67S	13032Δ*qcr* with pJC1-*qcrB*$_{St}$-C67S	This work
ΔQ-B_{St}-C177S	13032Δ*qcr* with pJC1-*qcrB*$_{St}$-C177S	This work
ΔCg2017	In-frame deletion of gene Cgl2017	This work
ΔCg2194 = Δ*ctaF*	In-frame deletion of gene Cgl2194	This work
ΔCg2226	In-frame deletion of gene Cgl2226	This work
ΔCg2664	In-frame deletion of gene Cgl2664	This work
pJC1	Kan^R; *E. coli-C. glutamicum* shuttle vector	53
pJC1-*qcrB*$_{St}$	Kan^R; expression plasmid for streptagged QcrB; *ctaE-qcrCAB* expressed from their native promoter; *qcrB* with 10 additional codons at the 3′-end (AAWSHPQFEK)	This work
pJC1-*qcrB*$_{St}$-C67S	Kan^R; expression plasmid for cytochrome c_1 lacking its N-terminal heme binding site through C67S and C70S mutations	This work
pJC1-*qcrB*$_{St}$-C177S	Kan^R; expression plasmid for cytochrome c_1 lacking its C-terminal heme binding site through C177S and C180S mutations	This work
pJC1-*ctaD*$_{St}$	Kan^R; expression plasmid for streptagged CtaD; *ctaD* expressed from its native promoter and with 10 additional codons at the 3′-end (AAWSHPQFEK)	This work
pUC-BM20	Amp^R; cloning vector for *E. coli*	Roche Molecular Biochemicals
pBM20-QXA	Amp^R; pUC-BM20 derivative with 2.0-kb *Xba*I-*Apa*I fragment from pJC1-*qcrB*$_{St}$; used for site-directed mutagenesis	This work
pBM20-QXA-C67S	Amp^R; pBM20-QXA derivative with C67S and C70S mutations in *qcrC*	This work
pBM20-QXA-C67S	Amp^R; pBM20-QXA derivative with C177S and C180S mutations in *qcrC*	This work
pWK0	Kan^R; low copy *E. coli-C. glutamicum* shuttle vector	54

TABLE II
Oligonucleotides used in this work

Restriction sites used for cloning and for verification of site-directed mutations are underlined. Bold letters indicate the Strep Tag II coding sequence or changed nucleotides in the mutagenic primers.

Primer	Sequence (5′ → 3′)
Primers for the construction of pJC1-*ctaD*$_{St}$ and pJC1-*qcrB*$_{St}$	
ctaD$_{St}$-for	ACTTCTAGATGACTGAACCTGGCAGCGACC
ctaD$_{St}$-rev	TGATCTAGATTA**CTTCTCGAACTGTGGGTGGGACCA**AGCTGCGCGGCTGGAGTCAGATGCAAG
qcrB$_{St}$-for	ACTTCTAGATAGGGTTGAGCATTTTGTC
qcrB$_{St}$-rev	AGTGTCGACTTA**CTTCTCGAACTGTGGGTGGGACCA**AGCTGCGTTCTTGCCCTCATTCTTGTC
Primers used for site-directed mutagenesis of *qcrC*	
C67S-for	CCTCTACGATGTCGCAT**CG**ATCACCT**CT**CACGGCGTAAACCTCC
C67S-rev	GGAGGTTTACGCCGTG**AG**AGGTGAT**CG**ATGCGACATCGTAGAGG
C177S-for	CTGTTCCGCCTGAA**TTCC**GCATCCT**CT**CACAACTTCACTGGTCG
C177S-rev	CGACCAGTGAAGTTGTG**AG**AGGATGC**GGAA**TTCAGGCGGAACAG
Primers used for the construction of deletion mutants	
ΔCg2017–1	GACTCTAGAATCTTCGCAGCATCGGTTCC
ΔCg2017–2	CCCATCCACTAAACTTAAACACGTGTGGGAGGAACCAGCCAC
ΔCg2017–3	TGTTTAAGTTTAGTGGATGGGCTGCGTGCTGCAGATTCTGCG
ΔCg2017–4	GATGTCGACGTTGTTGATGCCCAGGCACTG
ΔCg2194–1	GACTCTAGATAACCCAATTCACGGCAACTC
ΔCg2194–2	CCCATCCACTAAACTTAAACAGTACATGAGTTTTGCTAAGACTTC
ΔCg2194–3	TGTTTAAGTTTAGTGGATGGGCTCAACCTTCAGTACGGCGTGC
ΔCg2194–4	GATGTCGACCTTCCGGGAACTTTTCGTA
ΔCg2226–1	GACTCTAGAGGATTCCCGTGCGGCTGGTCT
ΔCg2226–2	CCCATCCACTAAACTTAAACATTTACACCCGAGACTACGTACCA
ΔCg2226–3	TGTTTAAGTTTAGTGGATGGGCCATTCGGTCCGGGATACCGC
ΔCg2226–4	GATGTCGACGGTTCTAACGATTTTCGCCGTC
ΔCg2664–1	GACTCTAGATAGCCAACGCTTCGCCCAAGTCATG
ΔCg2664–2	CCCATCCACTAAACTTAAACACTTCACGGATTCGTCCTCCATTGG
ΔCg2664–3	TGTTTAAGTTTAGTGGATGGGGAGGTCGGCGGAGACACTGCAG
ΔCg2664–4	GTCGTCGACAGCTGCGGTGCGCTCAGCG

Jasco V560 spectrophotometer. For turbid samples (intact cells and membranes) a special silicon photodiode detector was used (22). Heme contents were calculated from reduced minus oxidized spectra using the following wavelength pairs and absorption coefficients ($mM^{-1}cm^{-1}$): heme *a*, $\Delta\epsilon_{630-600\ nm}$ = 11.6 (23); heme *b*, $\Delta\epsilon_{562-577\ nm}$ = 22, and heme *c*, $\Delta\epsilon_{552-540\ nm}$ = 19.1 (24).

Miscellaneous—Protein concentrations were determined with the bicinchoninic acid protein assay (25) using bovine serum albumin as the standard. SDS-PAGE was carried out as described (26) except that the samples were incubated at 40 °C for 30 min before loading. Staining of *c*-type cytochromes in polyacrylamide gels was performed with 3,3′,5,5′-tetramethylbenzidine (27). For Western blotting, proteins were separated by Tricine-SDS-PAGE (28) and electroblotted onto a polyvinylidene difluoride membrane (Immobilon P; Millipore) using the semidry method according to Schägger and von Jagow (29). Strep-tagged QcrB was detected using streptavidin-alkaline-phospha-

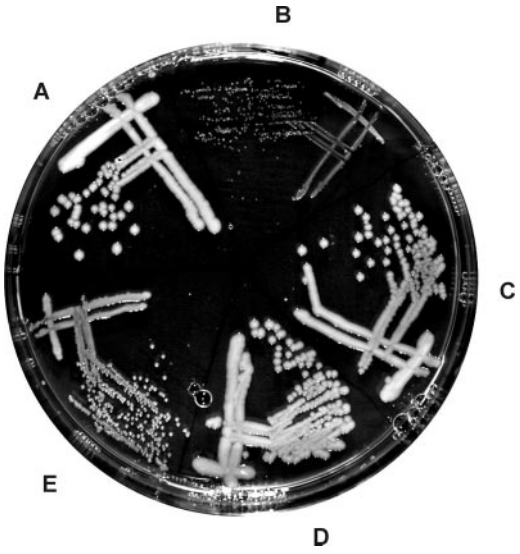

FIG. 2. **Complementation of the growth defects of the *C. glutamicum* strains 13032Δ*ctaD* and 13032Δ*qcr* by the plasmids pJC1-*ctaD*$_{St}$ and pJC1-*qcrB*$_{St}$ encoding strep-tagged CtaD and QcrB, respectively.** The *C. glutamicum* strains were cultivated for 2 days at 30 °C on brain heart infusion agar plates containing 0.5 M D-sorbitol and 25 μg/ml kanamycin. *A*, ATCC13032 (wild-type)/pWK0; *B*, 13032Δ*qcr*/pJC1; *C*, 13032Δ*qcr*/pJC1-*qcrB*$_{St}$ (ΔQ-B$_{St}$); *D*, 13032Δ*ctaD*/pJC1-*ctaD*$_{St}$ (ΔC-D$_{St}$); *E*, 13032Δ*ctaD*/pWK0.

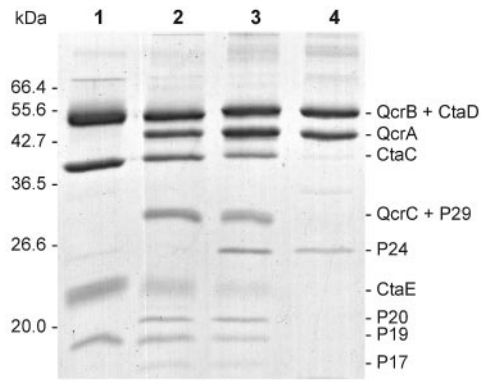

FIG. 3. **SDS-polyacrylamide gel analysis of cytochrome bc_1 and cytochrome aa_3 single complexes and supercomplexes from *C. glutamicum* purified by affinity chromatography on StrepTactin-Sepharose via QcrB$_{St}$ or CtaD$_{St}$.** The proteins were denatured, separated on an 8–16% Tris-HCl gradient gel (Bio-Rad), and stained with Coomassie Blue. *Lane 1*, cytochrome aa_3 oxidase (7.2 μg of protein) purified from strain ΔQ-D$_{St}$; *lane 2*, cytochrome bc_1-aa_3 supercomplex purified from strain ΔC-D$_{St}$ (4.7 μg); *lane 3*, cytochrome bc_1-aa_3 supercomplex purified from strain ΔQ-B$_{St}$ (5.7 μg); *lane 4*, cytochrome "bc_1" complex purified from strain ΔC-B$_{St}$ (4.3 μg).

tase conjugate and CDP-star (Roche Diagnostics, Mannheim, Germany) as described (12).

RESULTS

Construction and Functional Analysis of Strep-tagged Variants of Cytochrome b (QcrB) and of Subunit I (CtaD) of Cytochrome aa_3 Oxidase—To purify the cytochrome bc_1 complex and cytochrome aa_3 oxidase by affinity chromatography, plasmids pJC1-*qcrB*$_{St}$ and pJC1-*ctaD*$_{St}$ were constructed encoding QcrB and CtaD proteins elongated with a C-terminal StrepTag II, respectively. Plasmid pJC1-*qcrB*$_{St}$ contained the entire *ctaE-qcrCAB* gene cluster under control of its presumed native promoter and was able to complement the severe growth defect of *C. glutamicum* strain 13032Δ*qcr*, which contains a deletion of the chromosomal *qcrCAB* genes (Fig. 2). Plasmid pJC1-*ctaD*$_{St}$ contained the *ctaD* gene with its promoter region and complemented the growth defect of *C. glutamicum* strain 13032Δ*ctaD* (Fig. 2). Reduced minus oxidized difference spectra of the complemented strains ΔQ-B$_{St}$ (13032Δ*qcr* with plasmid pJC1-*qcrB*$_{St}$) and ΔC-D$_{St}$ (13032Δ*ctaD* with plasmid pJC1-*ctaD*$_{St}$) revealed a wild-type-like pattern with cytochromes of the *a*-, *b*-, and *c*-type (data not shown) whereas those of strains 13032Δ*qcr* and 13032Δ*ctaD* lacked cytochrome *c* and cytochrome *a*, respectively (5). Thus, pJC1-*qcrB*$_{St}$ and pJC1-*ctaD*$_{St}$ allowed the synthesis of a functional bc_1 complex and of a functional cytochrome aa_3 oxidase, respectively, and the presence of the StrepTag II did not interfere with the activity of the two respiratory complexes.

Isolation of a Cytochrome bc_1-aa_3 Supercomplex—For the purification of the bc_1 complex and cytochrome aa_3 oxidase, membranes of the complemented strains ΔQ-B$_{St}$ and ΔC-D$_{St}$ were isolated, and the proteins obtained after solubilization with dodecyl maltoside were subjected to affinity chromatography on StrepTactin-Sepharose. After washing, specifically bound proteins were eluted with desthiobiotin and analyzed by SDS-PAGE. Surprisingly, the protein pattern observed in the eluates from strains ΔQ-B$_{St}$ (Fig. 3, *lane 3*) and ΔC-D$_{St}$ (Fig. 3, *lane 2*) were highly similar and contained eight protein bands of identical apparent mass. The protein of 24 kDa (P24) was not only copurified with QcrB$_{St}$ but also appeared in some preparations obtained with CtaD$_{St}$ (data not shown). The identity of the proteins indicated in Fig. 3 except for the 17-kDa protein (P17) was determined by peptide mass fingerprinting using in-gel digestion with trypsin or cyanogen bromide and matrix-assisted laser desorption ionization-time of flight mass spectrometry. Because the bands with an apparent mass of 52 and 29 kDa were found to consist of two different proteins at a time, 10 proteins were identified in total. Besides the known subunits of the bc_1 complex (QcrA, QcrB, QcrC) and of cytochrome aa_3 oxidase (CtaC, CtaD, CtaE), the four additional proteins with an apparent mass of 29 kDa (P29), 24 kDa (P24), 20 kDa (P20) and 19 kDa (P19) were assigned to the hitherto hypothetical proteins Cgl2664, Cgl2226, Cgl2017, and Cgl2194, respectively.

The successful protein identification clearly showed that the eluate both of strain ΔQ-B$_{St}$ (Fig. 3, *lane 3*) and of strain ΔC-D$_{St}$ (Fig. 3, *lane 2*) contained the three subunits of the bc_1 complex (QcrA, QcrB, and QcrC) and the three subunits of cytochrome aa_3 oxidase (CtaD, CtaC, and CtaE). The fact that these proteins were copurified irrespective of whether the purification was performed via QcrB$_{St}$ or CtaD$_{St}$ strongly indicated that the bc_1 complex and cytochrome aa_3 oxidase form a supercomplex in *C. glutamicum*.

Heme Contents and Enzymatic Activities of the bc_1-aa_3 Supercomplex—Reduced minus oxidized difference spectra of the supercomplex purified either via QcrB$_{St}$ or via CtaD$_{St}$ showed that both preparations contained cytochromes of the *a*-, *b*-, and *c*-type but in different ratios (Fig. 4). The calculated contents of heme *a*, heme *b*, and heme *c* were 1.6, 6.1, and 2.8 μmol/g of protein in the QcrB$_{St}$ complex and 4.2, 2.6 and 3.0 μmol/g in the CtaD$_{St}$ complex. These values cannot be fit into a simple ratio of small integers, indicating that the preparations are stoichiometrically heterogeneous. In both cases the strep-tagged subunit was most abundant, *i.e.* cytochrome *b* in the QcrB$_{St}$ complex and cytochrome *a* in the CtaD$_{St}$ complex. Thus, the bc_1-aa_3 supercomplex was partially dissociated despite the gentle method used for purification.

A functional association of bc_1 complex and cytochrome aa_3 oxidase should possess quinol oxidase activity. Using the menaquinol analogon DMNH$_2$ as substrate, such an activity could be measured polarographically not only with membrane fractions but also with the purified supercomplexes as summarized in Table III. The turnover number decreased during the purifica-

TABLE III

Purification of a cytochrome bc_1-aa_3-supercomplex from membranes of the C. glutamicum strains ΔQ-B_{St} and ΔC-D_{St}

The quinol oxidase activity was determined with $DMNH_2$ as substrate by measuring the oxygen consumption rate with a Clark-type oxygen electrode. One unit corresponds to 1 μmol of O_2 consumed per min. The turnover numbers (TN) are calculated as electrons transferred per cytochrome aa_3 per second, except for the number in parentheses, which was calculated as electrons transferred per cytochrome *b* per second. ND, not determined.

Purification step	Protein	Quinol oxidase activity			
	mg	*units*	*units/mg*	*TN, s^{-1}*	*Yield, %*
Membranes from strain ΔQ-B_{St}	44.7	32.3	0.72	ND	100
Dodecylmaltoside extract	15.3	14.5	0.95	314	45
StrepTactin sepharose eluate	0.64	1.7	2.70	220	5.4
Membranes from strain ΔC-D_{St}	42.3	33.5	0.79	ND	100
Dodecylmaltoside extract	17.9	15.2	0.85	365	45
StrepTactin sepharose eluate	0.50	1.5	2.96	93 (152)	4.4

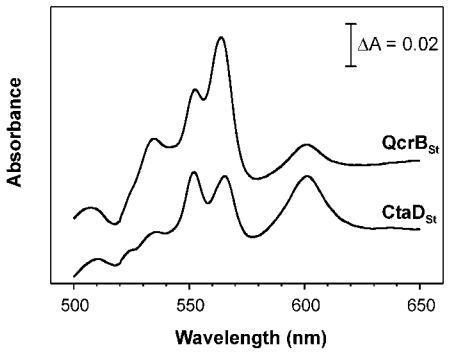

FIG. 4. **Reduced minus oxidized difference spectra of the bc_1-aa_3 supercomplex purified from the *C. glutamicum* strains ΔC-D_{St} and ΔQ-B_{St}.** The supercomplexes purified by affinity chromatography on StrepTactin-Sepharose were reduced with dithionite or oxidized with ferricyanide before recording the spectra at room temperature (protein concentration 0.5 mg/ml).

tion, most likely because of the partial dissociation of the supercomplex indicated above. Besides quinol oxidase activity of 1.5–1.7 units/mg protein, the preparations also possessed TMPD oxidase activity of 0.8 units/mg ($QcrB_{St}$ complex) and 1.0 units/mg ($CtaD_{St}$ complex) corresponding to turnover numbers (TMPD/aa_3 s^{-1}) of 16.1 and 7.6, respectively.

Purification of the bc_1 Complex and of Cytochrome aa_3 Oxidase as Single Complexes—To purify the bc_1 complex as a single complex rather than as a supercomplex, plasmid pJC1-*qcrB*$_{St}$ was transferred to *C. glutamicum* 13032Δ*ctaD*. The resulting strain ΔC-B_{St} had the same growth defect as strain 13032Δ*ctaD* because of the absence of CtaD and formed both wild-type and strep-tagged QcrB. Purification of strep-tagged proteins from dodecyl maltoside-solubilized membranes by StrepTactin affinity chromatography resulted in two dominant proteins that were identified as cytochrome *b* (QcrB) and Rieske iron-sulfur protein (QcrA; Fig. 3, *lane 4*). In addition, minor amounts of P24 were enriched. Most remarkably, cytochrome c_1 (QcrC) was not present in this preparation, indicating that the interaction between QcrC and the two other subunits of the bc_1 complex is quite weak.

For the purification of cytochrome aa_3 as a single complex, a similar approach was applied as described above for the bc_1 complex, *i.e.* plasmid pJC1-*ctaD*$_{St}$ was transferred into strain 13032Δ*qcr*. The resulting strain ΔQ-D_{St} had the same phenotype as strain 13032Δ*qcr* and formed both wild-type and strep-tagged CtaD. The eluate obtained after StrepTactin affinity chromatography of dodecyl maltoside-solubilized membranes contained four proteins (Fig. 3, *lane 1*), which were identified as CtaD, CtaC, CtaE, and P19. The TMPD oxidase activity of the cytochrome aa_3 oxidase preparation was 0.34 units/mg, corresponding to a turnover number of 1.1 TMPD oxidized/aa_3 s^{-1}. The 10-fold decreased TMPD oxidase activity compared with the supercomplexes is because of the absence of cytochrome c_1. The cytochrome *c* oxidase activity with bovine heart cytochrome *c* and yeast cytochrome *c* was 0.35 and 0.28 units/mg, respectively. This corresponds to turnover numbers of 1.2 and 0.9 cytochrome *c* oxidized/aa_3 s^{-1}.

Evidence for a Fourth Subunit of Cytochrome aa_3 by Phenotypic Analysis of Mutants Lacking P29, P24, P20, or P19—To determine the relevance of proteins P29, P24, P20, and P19 for respiration and formation of the bc_1-aa_3 supercomplex, the corresponding genes were deleted in-frame from the chromosome of *C. glutamicum*, resulting in strains ΔCg2664, ΔCg2226, ΔCg2017, and ΔCg2194, respectively. The former three strains showed no obvious phenotype regarding growth in rich medium and the formation of *a*-, *b*-, and *c*-type cytochromes (data not shown). Apparently, proteins P29, P24, and P20 are not essential for the formation and activity of the bc_1-aa_3 branch of the respiratory chain, and the functional significance of the interaction between these proteins and the bc_1-aa_3 supercomplex remains to be elucidated.

In contrast, deletion of the gene Cgl2194 encoding P19 led to a similar phenotype observed previously for the 13032Δ*ctaD* strain. Growth on rich medium agar plates was strongly impaired (data not shown), cytochrome *a* was almost absent in the spectrum of dithionite-reduced cells, and the level of cytochrome c_1 was markedly lower than in the wild-type (Fig. 5). Consequently, the P19 protein is essential for the formation of an active cytochrome aa_3 oxidase. P19 was enriched with the supercomplex and the isolated cytochrome aa_3 oxidase, but not with the isolated bc_1 complex (Fig. 3), showing that copurification is because of an interaction with the cytochrome aa_3 subunits. Based on these data, P19 has to be regarded as a fourth subunit of the *C. glutamicum* cytochrome aa_3 oxidase.

Protein P19 is composed of 143 amino acids and has a predicted mass of 15.5 kDa. It contains three hydrophobic regions extending from residues 7–27, 40–60, and 97–130, which presumably form three or four transmembrane helices. The first transmembrane helix may be part of a signal peptide. As shown in the alignment in Fig. 6, the primary sequence is well conserved in other species of the actinomycetales including *C. diphtheriae* (68% identity), mycobacteria (38–39%), *Streptomyces coelicolor* (39%), and *Thermobifida fusca* (33%). In all these organisms the corresponding gene is located immediately downstream of *ctaC* or a *ctaCD* gene cluster in the case of *S. coelicolor* and *T. fusca* and presumably is cotranscribed with these genes. This further supports the previous suggestion that the P19 homologues represent a fourth subunit of cytochrome aa_3 oxidase in the actinomycetes. Therefore, the corresponding genes were named *ctaF*.

Necessity of Heme Incorporation into Cytochrome c_1 for Assembly and/or Stability of the bc_1-aa_3 Supercomplex—According to our previous proposal that the second heme group of the *C. glutamicum* diheme cytochrome c_1 is involved in electron

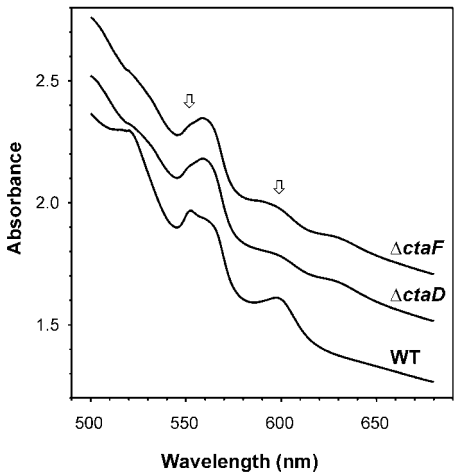

FIG. 5. **Absolute spectra of reduced intact cells of the *C. glutamicum* strains ATCC13032 (wild-type, *WT*), 13032Δ*ctaD*, and 13032Δ*ctaF*.** Cells were resuspended in 100 mM Tris-HCl buffer, pH 7.5, to an $A_{600\ nm}$ of 200 and reduced by the addition of dithionite before recording the spectra at room temperature. The relevant differences in the cytochrome pattern are indicated by *arrows*. The spectra of strains 13032Δ*ctaD* and 13032Δ*ctaF* are drawn with an offset of 0.2 and 0.4, respectively.

transfer from the first heme group of c_1 to the Cu_A center of cytochrome aa_3 oxidase, both heme groups should be essential for the activity of the bc_1-aa_3 branch of the respiratory chain. To test this assumption, both cysteine residues in each of the two C*XX*CH heme binding motifs of QcrC were converted to serine residues by site-directed mutagenesis of plasmid pJC1-*qcrB*$_{St}$. The effects of these mutations were analyzed after transformation of strain 13032Δ*qcr* with the resulting plasmids pJC1-*qcrB*$_{St}$-C67S and pJC1-*qcrB*$_{St}$-C177S, respectively. Both mutant strains showed strongly impaired growth similar to strain 13032Δ*qcr* (Fig. 7), indicating the absence of a functional bc_1 complex. The membranes of the two strains did not contain *c*-type cytochromes as judged by heme staining of SDS gels (Fig. 8*A*) and reduced minus oxidized difference spectra of membranes (data not shown). Obviously, both heme groups of cytochrome c_1 are essential for respiration via the bc_1-aa_3 branch of the respiratory chain, and no stable monoheme intermediate can be formed during the maturation of QcrC if the incorporation of the other heme group is blocked.

Western blot analysis with streptavidin-alkaline phosphatase conjugate was performed to check whether the disturbed cytochrome c_1 maturation in the mutant strains also influences the $QcrB_{St}$ content of the cytoplasmic membranes. As shown in Fig. 8*B*, both QcrC mutant strains had strongly decreased $QcrB_{St}$ levels of less than 10% compared with strain ΔQ-B_{St} as estimated from the signal intensities. This indicated that the presence of holo-cytochrome c_1 is highly important for the assembly and/or stability of the entire bc_1 complex. Besides $QcrB_{St}$, which was unequivocally identified with a sample of the purified $QcrB_{St}$ complex, additional proteins were detected by the streptavidin-alkaline phosphatase conjugate (Fig. 8), which represented the biotinylated proteins pyruvate carboxylase (not shown) (30) and the β-subunit of acyl CoA carboxylase (AccBC; see Ref. 31). The cytochrome *a* level of the cytochrome c_1 mutants was unchanged compared with the control strain ΔQ-B_{St} (data not shown), indicating that synthesis of cytochrome aa_3 is independent of an intact bc_1 complex.

DISCUSSION

Identification of a Cytochrome bc_1-aa_3 Supercomplex in C. glutamicum—In the present study we show that the bc_1 complex and cytochrome aa_3 oxidase of *C. glutamicum* are organized in a supercomplex with quinol oxidase activity. The approach used to isolate this supercomplex involved the modification of one subunit with a StrepTag II and subsequent affinity purification with StrepTactin-Sepharose. Similar approaches were used previously, *e.g.* to systematically define protein complexes in yeast (32, 33). Although this procedure can lead to the accidental copurification of proteins, this was not the case here, because all subunits of the bc_1 complex and of cytochrome aa_3 were isolated both with strep-tagged QcrB and with strep-tagged CtaD. The lack of evidence for a supercomplex in the previous purification of either cytochrome c_1 (6) or cytochrome aa_3 oxidase (7) shows that the interactions are relatively weak and require a very gentle purification procedure for preservation. Although we could also isolate the bc_1-aa_3 supercomplex using a hexahistidine-tagged QcrB and Ni^{2+}-chelate affinity chromatography (data not shown), the StrepTag II/StrepTactin system proved to be superior in our hands.

The formation of a bc_1-aa_3 supercomplex with quinol oxidase activity is not unique to *C. glutamicum*. In fact, such complexes were purified from several bacteria, *i.e. Paracoccus denitrificans* (34), the thermophilic *Bacillus* PS3 (35), or the thermoacidophilic archaeon *Sulfolobus* sp. strain 7 (36). In *Bradyrhizobium japonicum*, a bc_1-c_M-aa_3 complex was isolated from aerobically grown cells but not characterized for its quinol oxidase activity (37). From bacteroids of *B. japonicum* a complex of cytochrome bc_1 and a *cb*-type cytochrome oxidase, most probably cytochrome cbb_3 (38, 39), was isolated (40). It displayed cytochrome *c* oxidase and TMPD oxidase activity but no quinol oxidase activity, presumably because of the lack of the Rieske iron-sulfur protein.

A supramolecular organization of complexes III and IV was also shown in yeast and bovine mitochondria (41, 42). Thus, quinol oxidase supercomplexes were detected in Gram-positive and Gram-negative eubacteria, in archaea, and in eukaryotes, indicating that this highly organized state is a general feature rather than a specific character of certain species.

Identification of Subunit IV of Cytochrome aa_3 Oxidase—In contrast to the previous purification of cytochrome aa_3 oxidase from *C. glutamicum* by conventional column chromatography, which resulted in the isolation of subunits I, II, and III (7), our preparation contained an additional protein (CtaF) encoded by the gene downstream of *ctaC* (Fig. 3). The identification of this protein as a fourth subunit rests on the observation that a *C. glutamicum* mutant lacking *ctaF* showed the same growth defect as a *ctaD* deletion mutant, and like in this strain cytochrome *a* was almost undetectable. Although CtaF is essential for the formation of a functional cytochrome aa_3 oxidase, it is presumably not required for catalytic activity, because the turnover numbers of the four-subunit complex were in the same range as those of the three-subunit complex (7). Therefore, CtaF is probably involved in the assembly and/or stabilization of cytochrome aa_3 oxidase.

The composition of four subunits is common within the heme-copper family of bacterial terminal oxidases. Subunit IV (CtaH) of cytochrome aa_3 oxidase from *P. denitrificans* consists of a single transmembrane helix residing in a cleft between subunits I and III (43). Deletion of the *ctaH* gene had no consequences for the integrity of the complex and its spectral and enzymatic properties (44). Subunit IV (CyoD) of the *bo*-type ubiquinol oxidase from *E. coli* consists of three transmembrane helices and is located between subunits I and III. The third helix is in contact with helix VII of subunit I in the vicinity of the Cu_B-heme a_3 binuclear center (45). Deletion analyses indicated that subunit IV is essential for the synthesis of the functional bo_3 oxidase complex and for the Cu_B binding

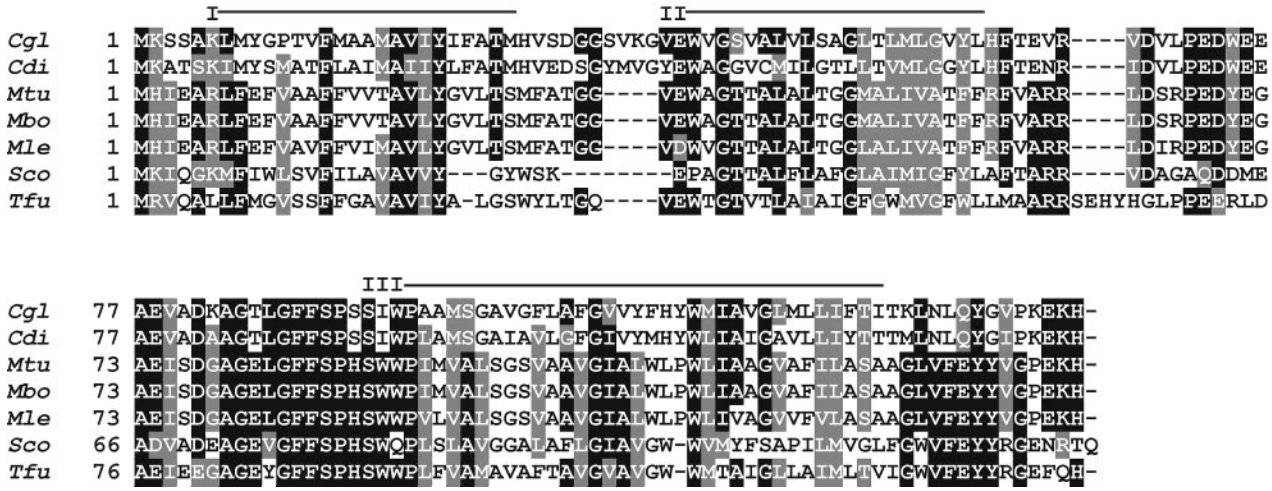

FIG. 6. **Sequence alignment of CtaF proteins from different species of actinomycetes.** Putative transmembrane helices are indicated by *lines* and are *numbered*. Amino acids identical in at least five sequences are shaded in *black*, and conservative exchanges are in *gray*. The bacteria have been abbreviated as follows: *Cgl*, *C. glutamicum* (Cgl2194); *Cdi*, *C. diphtheriae* (NC_002935); *Mtu*, *M. tuberculosis* (Rv2199c); *Mbo*, *M. bovis* (NC_002945); *Mle*, *M. leprae* (ML0876); *Sco*, *S. coelicolor* (NP_626410); *Tfu*, *T. fusca* (Tfus_p_278).

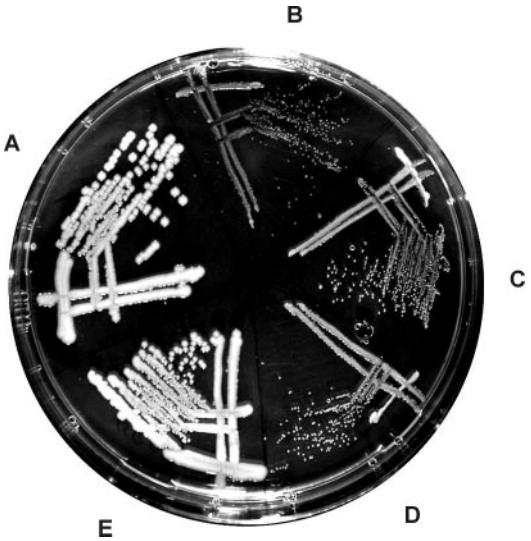

FIG. 7. **Effect of defective heme binding sites in cytochrome c_1 on growth of *C. glutamicum*.** The strains were cultivated for 2 days at 30 °C on brain heart infusion agar plates containing 0.5 M D-sorbitol and 25 μg/ml kanamycin. *A*, ATCC13032 (wild-type)/pWK0; *B*, 13032Δ*qcr*/pJC1; *C*, 13032Δ*qcr*/pJC1-*qcrB*$_{St}$-C67S; *D*, 13032Δ*qcr*/pJC1-*qcrB*$_{St}$-C177S; *E*, 13032Δ*qcr*/pJC1-*qcrB*$_{St}$.

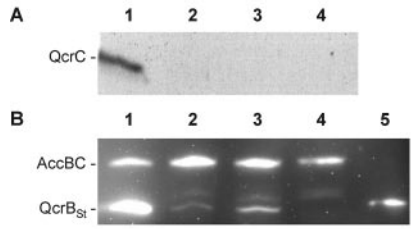

FIG. 8. **Analysis of cytochrome c_1 (*QcrC*) and strep-tagged cytochrome *b* (*QcrB*$_{St}$) in mutant strains of *C. glutamicum*.** *A*, membrane proteins (200 μg per *lane*) of the strains were separated by SDS-PAGE, and the gels were stained with tetramethylbenzidine for covalently bound heme in cytochrome c_1. *B*, Western blot analysis of QcrB$_{St}$ in membranes of the strains indicated below. The membrane proteins (50 μg per *lane*) were separated by SDS-PAGE on a 10% Tris-Tricine gel and subsequently blotted onto a polyvinylidene difluoride membrane. Detection was performed with a streptavidin-alkaline phosphatase conjugate and the CDP-Star substrate (both from Roche Diagnostics). *Lane 1*, 13032Δ*qcr*/pJC1-*qcrB*$_{St}$; *lane 2*, 13032Δ*qcr*/pJC1-*qcrB*$_{St}$-C177S; *lane 3*, 13032Δ*qcr*/pJC1-*qcrB*$_{St}$-C67S; *lane 4*, 13032Δ*qcr*/pJC1; *lane 5*, purified cytochrome bc_1-aa_3 supercomplex (4.6 μg of protein) from *C. glutamicum* ΔQ-B$_{St}$.

to the binuclear center, although it can be removed *in vitro* without a loss of the enzymatic activity (46). Subunit IV (QoxD) of the cytochrome aa_3 menaquinol oxidase from *B. subtilis*, like CyoD of *E. coli*, consists of three transmembrane helices. A mutant lacking the *qoxD* gene was reported to have decreased respiratory activity and proton pumping activity (47).

CtaF of *C. glutamicum* shows no significant sequence similarity to CtaH of *P. denitrificans*, CyoD of *E. coli*, QoxD of *B. subtilis*, and CtaF of *B. subtilis*, and homologs of these proteins were absent in the *C. glutamicum* genome. In contrast, all actinomycetes with known genome sequence contain homologs of *C. glutamicum* CtaF (Fig. 6), and the corresponding genes are always clustered with *ctaC*. Thus, CtaF represents the first member of subunit IV of cytochrome aa_3 oxidase in this group of bacteria.

Electron Transfer between the bc_1 Complex and Cytochrome aa_3—The identification of a bc_1-aa_3 supercomplex with quinol oxidase activity supports the assumption that the second heme group of the diheme cytochrome c_1 transfers electrons from the first heme group to the Cu_A center in subunit II of cytochrome aa_3 oxidase. The question whether further proteins are involved in this process remains open at present. The proteins P29, P24, and P20 can certainly be excluded, because *C. glutamicum* mutants lacking these proteins showed no obvious growth defects. In the case of subunit IV (CtaF) of cytochrome aa_3 oxidase, a role in electron transfer is also very unlikely (see above). However, the copurified protein P17 could not yet be identified, and therefore the effect of its absence on the formation and activity of the supercomplex could not be tested.

Besides two covalently bound heme groups, cytochrome c_1 of *C. glutamicum* has another unusual property, *i.e.* its weak interaction with the Rieske iron-sulfur protein and cytochrome *b*. Neither of these two proteins was copurified during the isolation of cytochrome c_1 by conventional chromatographic methods (6), and vice versa, the preparation isolated via strep-tagged cytochrome *b* lacked cytochrome c_1 (Fig. 3, *lane 4*). The presence of QcrC in the supercomplex, purified either via strep-tagged QcrB or via strep-tagged CtaD, therefore must be because of an interaction with cytochrome aa_3 oxidase or requires interaction with both the bc_1 complex and cytochrome aa_3 oxidase. Further studies are needed to discriminate between these possibilities.

Formation of Holo-cytochrome c_1 Is Essential for Assembly and/or Stability of the bc_1 Complex—Mutation of either the N-terminal or the C-terminal C*XX*CH motif in cytochrome c_1 to S*XX*SH led to a severe growth defect in the corresponding mutants similar to strain 13032Δ*qcr* (Fig. 7), which was because of the absence of holo-cytochrome c_1 and drastically reduced levels of cytochrome *b*. This shows that if any monoheme cytochrome c_1 is formed, it must be rapidly and completely degraded. Similar results have been reported for the diheme cytochrome *c* subunit (FixP) of the cbb_3 oxidase in *B. japonicum* (48). The strong effect of the cytochrome c_1 mutations on the cytochrome *b* level showed that holo-cytochrome c_1 is not only required for electron transfer but also for the maturation and/or stabilization of the entire bc_1 complex. The purification of an apparently stoichiometrical complex of cytochrome *b* and Rieske iron-sulfur protein from strain $\Delta C\text{-}B_{St}$ argues against a role of cytochrome c_1 in stabilization but certainly does not exclude this possibility.

The data from *C. glutamicum* are in accordance with results from other bacteria, *i.e.* that deletion of the cytochrome c_1 gene in *P. denitrificans* (49), *Rhodobacter capsulatus* (50), and *B. japonicum* (51), as well as mutation of the heme binding site of *B. japonicum* cytochrome c_1 (37), caused degradation of cytochrome *b* and, if tested, also of the Rieske iron-sulfur protein. According to these data the current model for bc_1 complex maturation predicts that formation of holo-cytochrome c_1 is an early and essential requirement for assembly of the whole complex (52).

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