Identification and Characterization of the Novel Subunit CcoM in the cbb₃-Cytochrome c Oxidase from Pseudomonas stutzeri ZoBell

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ABSTRACT  Cytochrome c oxidases (CcOs), members of the heme-copper containing oxidase (HCO) superfamily, are the terminal enzymes of aerobic respiratory chains. The cbb₃-type cytochrome c oxidases (cbb₃-CcO) form the C-family and have only the central catalytic subunit in common with the A- and B-family HCOs. In Pseudomonas stutzeri, two cbb₃ operons are organized in a tandem repeat. The atomic structure of the first cbb₃ isoform (Cbb₃-1) was determined at 3.2 Å resolution in 2010 (S. Buschmann, E. Warkentin, H. Xie, J. D. Langer, U. Ermler, and H. Michel, Science 329:327–330, 2010, http://dx.doi.org/10.1126/science.1187303). Unexpectedly, the electron density map of Cbb₃-1 revealed the presence of an additional transmembrane helix (TMH) which could not be assigned to any known protein. We now identified this TMH as the previously uncharacterized protein CcoM. In order to identify the function of this new subunit in the cbb₃ complex, we generated and analyzed a CcoM knockout strain. The results of the biochemical and biophysical characterization indicate that CcoM may be involved in CcO assembly or stabilization. In addition, we found that CcoM plays a role in anaerobic respiration, as the ΔCcoM strain displayed altered growth rates under anaerobic denitrifying conditions.

IMPORTANCE The respiratory chain has recently moved into the focus for drug development against prokaryotic human pathogens, in particular, for multiresistant strains (P. Murima, J. D. McKinney, and K. Pethe, Chem Biol 21:1423–1432, 2014, http://dx.doi.org/10.1016/j.chembiol.2014.08.020). cbb₃-CcO is an essential enzyme for many different pathogenic bacterial species, e.g., Helicobacter pylori, Vibrio cholerae, and Pseudomonas aeruginosa, and represents a promising drug target. In order to develop compounds targeting these proteins, a detailed understanding of the molecular architecture and function is required. Here we identified and characterized a novel subunit, CcoM, in the cbb₃-CcO complex and thereby completed the crystal structure of the Cbb₃ oxidase from Pseudomonas stutzeri, a bacterium closely related to the human pathogen Pseudomonas aeruginosa.

In recent years, the central bacterial metabolic enzymes have become prime targets for the development of new antibiotic drugs (1). The heme-copper containing oxidase (HCO) superfamily comprises the terminal enzymes of the aerobic respiratory chain as well as the nitric oxide reductases (NOR) which are involved in anaerobic respiration. Terminal oxidases are present in the respiratory chains of eukaryotes and many prokaryotes and thus represent tempting candidates for drug development. They catalyze the four-electron reduction of molecular oxygen to water and couple this exergonic reaction to transmembrane proton pumping, thus contributing to the generation of the electrochemical proton gradient across the membrane. HCOs can be classified into three major families: A, B, and C (2). To date, at least one crystal structure has been published for each family (3–6). The C-family consists only of the cbb₃-type cytochrome c oxidases (cbb₃-COs) which are predominantly found in bacteria (7). In the genus Pseudomonas, the genes encoding the two isoforms of cbb₃-COs (Cbb₃-1 and Cbb₃-2) are organized as a tandem repeat (8, 9).

In Pseudomonas stutzeri, only the second cbb₃ operon contains a fourth gene (ccoQ) in addition to the ccoN, ccoO, and ccoP (ccoNOP) genes (6, 10). The crystal structure of Cbb₃-1 from P. stutzeri was determined at a resolution of 3.2 Å in 2010. Surprisingly, besides the known CcoNOP subunits, the electron density map revealed the presence of an additional fourth subunit consisting of a single transmembrane helix (TMH) (6). It is located in close proximity to helices VIII, IX, and XI of the catalytic subunit CcoN. Prior to this study, neither proteomic nor genomic information was available for this subunit.

In this work, we set out to identify and characterize this as-yet-undescribed subunit of cbb₃-CcO whose gene is located outside the main cbb₃ operon. We purified and subsequently sequenced the polypeptide using matrix-assisted laser desorption ionization time-of-flight tandem mass spectrometry (MALDI-TOF MS/MS) and propose to rename PstZoBell_05036 CcoM. To determine the physiological role of CcoM in the cbb₃ complex, we created a deletion strain for this gene and monitored its growth rates under...
different conditions. Furthermore, we compared the purified Cbb\(_3\)-1–ACcoM variant and the wild-type Cbb\(_3\)-1 using ultraviolet-visible light (UV-vis) spectroscopy, differential scanning calorimetry (DSC), and oxygen reductase activity measurements.

RESULTS

Identification of an uncharacterized CcO subunit. On the basis of the X-ray crystallographic electron density, we estimated that the unidentified subunit consists of one TMH with an overall size of 30 to 45 amino acids. We thus purified the full CcO complex by column chromatography (11) and immobilized the proteins on a c4 solid-phase extraction column. Sequential elution using organic solvent allowed us to generate a fraction enriched in a poly-peptide with a monoisotopic molecular mass of 3,986 Da (Fig. 1A). Since we were not able to annotate a matching protein in the \(P.\) stutzeri proteome, we performed tandem-MS measurements on a Bruker ultrafleXtreme MALDI-TOF mass spectrometer. The sequence tags derived from de novo sequencing matched the hypothetical \(PstZobell\_05036\) protein in an MS_BLAST search with a score of 134 (Fig. 1B). Upon matching the sequence to the MS/MS spectrum in BioTools, an N-terminal mass shift of 28 Da was assigned and explained as N-terminal formylation, caused by the presence of a starting N-formylmethionine. The result was confirmed by a direct protein search of the NCBI Nr database using Mascot 2.4 with N-terminal formylation as a variable modification and a Mascot score of 352.

Fitting of the identified peptide into the unassigned TMH of Cbb\(_3\)-1. A BLAST search of the identified peptide was performed against the NCBI “draft genome of \(P.\) stutzeri ATCC 14405” database (12). One matching protein with the locus tag of \(PstZoBell\_05036\) consisting of 36 amino acids and described as “putative uncharacterized protein” was found. This protein is predicted to contain one membrane spanning an \(\alpha\)-helix between Val7 and Trp29 as determined by the use of the TMHMM 2.0 server (http://www.cbs.dtu.dk/services/TMHMM/) (see Fig. S2 in the supplemental material). Residues 1 to 29 were fitted into the electron density map, and the model was subsequently refined. After refinement, the electron densities for the side chains were clearly improved. On the basis of these results, we renamed this protein CcoM. The residues located on the interface between CcoN and CcoM (e.g., T13, M17, F20, F24, and F27) match well with our structural template (Fig. 2A). Model building for the carboxy-terminal part of the peptide as well as the residues facing the exterior of the protein complex was not possible due to the absence of electron density. In summary, the majority of the amino acid side chains could be built into the electron density successfully, indicating that the observed TMH is correctly assigned to CcoM. On the basis of our determination of the placement into the crystal structure and due to the presence of a formylgroup at the N-terminal methionine, CcoM has an N-terminus-out and C-terminus-in topology. In this orientation, the positively charged residues enriched in the C-terminal region of CcoM reside in the cytosol, which is consistent with the “positive-inside” rule (13).

Interaction of CcoM with the cbb\(_3\) complex. Subunit CcoM is still tightly bound to the Cbb\(_3\)-1 complex after four stringent purification steps and the crystallization process (11). On the basis of our current structural model, we propose the idea of a ladder-like interaction between CcoM and the cbb\(_3\)-CcO complex. On the basis of our current structural model, we propose the idea of a ladder-like interaction between CcoM and helices VIII and IX of the catalytic subunit CcoN. The distances between these potentially interacting amino acids in each of the four cbb\(_3\) monomers, present in the asymmetric unit of the crystal structure, are listed in Table S3 in the supplemental material. Figure 2B exemplarily represents the proposed interaction ladder of CcoN and CcoM based on chains A and N of Protein Data Bank [PDB] file 5DJQ, respectively. The phenylalanine residue (F20) of CcoM shows hydrophobic interactions with W284 of CcoN and a \(\pi\)-\(\pi\) interaction with F322. The edge-to-edge dis-
stances between these residue pairs are 3.2 Å (F20-W284) and 3.7 Å (F20-F322), which corresponds to an optimal distance for a parallel sandwich arrangement of Phe-Phe pairs at 3.5 Å (14). In addition to the H9266-H9266 interactions, we observed multiple sulfur-H9266 interactions, including M17-M-F322N, F20-M-M287N, F24-M-M287N, and F24-M-M291N. The edge-to-edge distances of these interactions range from 3.8 to 4.2 Å and are comparable to the favorable distances of 3.6 and 5.5 Å for sulfur-H9266 interactions (15).

Furthermore, a Trp residue (W29) is present at the C-terminal end of the TMH in CcoM and is putatively oriented toward the lipid bilayer. Trp residues at the termini of transmembrane helices often interact with lipid headgroups and contribute to the stabilization of the position of the TMH in the lipid bilayer (16).

**Analysis of CcoM.** In *P. stutzeri* ZoBell, the ccoM gene is located far away from the two *cbb3* operons consisting of the structural genes (PstZoBell_18660 to PstZoBell_19488) that encode the two isoforms of Cbb3-Co. We found that the genes coding for a DNA repair system protein (PstZoBell_05026) and a type II secretory pathway protein (PstZoBell_05041) are located immediately upstream and downstream of ccoM, respectively (Fig. 3A). Promoter prediction indicated that a putative promoter is present upstream of ccoM (Fig. 3B). A putative arginine nitrate regulator

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**FIG 2** Interaction of CcoM and CcoN, exemplarily illustrated by chains N and A of PDB file 5DJQ, respectively. (A) Ribbon model of subunit CcoN (grey) and CcoM (red). The $2F_{o}-F_{c}$ electron density map of CcoM is shown in blue at a level of 1.5 $\sigma$. CcoM residues matching the electron density are indicated as sticks (M1, F2, D4, V6, T13, M17, F20, F24, and F27). (B) Closeup of the proposed ladder-like interaction between helices VIII and IX of CcoN (blue) and CcoM (red). Sulfur-aromatic interactions (M17-M-F322N, F20-M-M287N, F24-M-M287N, and F24-M-M291N) and aromatic-aromatic interactions (F20-M-W284N and F20-M-F322N) mainly contribute to the binding of CcoM to CcoN. The amino acids involved in interactions are shown as sticks, and their edge-to-edge distances are indicated. Sulfur atoms are shown in yellow.

**FIG 3** Location and upstream region of the ccoM gene. (A) Location of ccoM in *P. stutzeri* ZoBell genome. The ccoM gene (locus tag, PstZoBell_05036) is shown in red. Genes encoding an alkylated DNA repair protein (PstZoBell_05026), hypothetical protein (PstZoBell_05031), and type II secretory pathway protein (PstZoBell_05041) are shown in grey; PstZoBell_05026 and PstZoBell_05031 are positioned upstream of ccoM, and PstZoBell_05041 is positioned downstream. (B) Nucleotide sequence (5′ to 3′) of the ccoM upstream region. Sequences in the red boxes exhibit homology to an ANR (FNR) box (TTGAT-N4-gTCAA). Based on the data from *P. putida* (48), a putative $\sigma^{3}$-containing RNA polymerase recognition site with the −35 and −10 regions is shown in blue. A potential Shine-Dalgarno (SD) sequence located upstream of the ccoM transcription start site is indicated in orange. The consensus sequences are capitalized. The ccoM translation initiation site is indicated by a black arrow.
To confirm the absence of CcoM in Cbb3-1, the localization of this gene is similar to that of the gene in P. stutzeri (see Table S4 in the supplemental material). In other pseudomonad genomes, whereas F24 is sometimes replaced by valine or leucine residues M17 and F20 are conserved in all indicated CcoM homologues. Several key residues of CcoM that contribute to the proposed interaction with CcoN (M17, F20, and F24 [see numbering]) are highly conserved in CcoM homologues. Residues M17 and F20 are conserved in all indicated CcoM homologues, whereas F24 is sometimes replaced by valine or leucine (see Table S4 in the supplemental material). In other pseudomonads, the localization of this gene is similar to that of the ccoM gene in P. stutzeri. However, these CcoM-related proteins are partially annotated as ATP-dependent helicases, probably due to their annotated as ATP-dependent helicases, probably due to their function in heme incorporation and heme environments.

**Functional characterization of ΔCcoM strain.** In order to characterize the physiological function of CcoM in the Cbb3-2-CcO complex, we created a knockout strain (ΔCcoM variant) of P. stutzeri ZoBell. To confirm the absence of CcoM in Cbb3-1, MALDI-TOF MS measurements of the purified Cbb3-1–ΔCcoM variant were performed. As expected, the spectrum does not show any peak in the range from 2 to 15 kDa (see Fig. S3 in the supplemental material).

We subsequently tested bacterial cell growth under different conditions; the wild-type strain and the ΔCcoM strain did not show any observable differences concerning their growth behavior under microaerobic conditions (Fig. 4A). Under anaerobic denitrifying conditions, however, we observed a significant increase in the lag phase of the ΔCcoM P. stutzeri strain compared to the wild-type strain (Fig. 4B). The cultures were inoculated to an optical density at 600 nm (OD600) of 0.1, and the cells of the ΔCcoM P. stutzeri strain reached an OD600 of 0.3 after 25 h ± 1 h, whereas the wild-type cells already showed an OD600 of 0.3 after 10.1 h ± 1.7 h. However, in the log phase, the cells of the two strains had similar doubling times of 193 min ± 46 min (wild-type strain) and 201 min ± 24 min (ΔCcoM strain).

In addition, to gain insights into the significance of both cbb3 isoforms under different conditions, we compared the growth behaviors of the ΔCbb3-1 and ΔCbb3-2 P. stutzeri deletion strains to that of the wild-type strain (see Fig. S4 in the supplemental material). Under microaerobic conditions, the growth of the ΔCbb3-2 strain was slightly delayed compared to growth of the wild-type and ΔCbb3-1 strains, since the Cbb3-2 is particularly important under aerobic conditions which are present at the beginning of the cell growth. We observed that, under anaerobic denitrifying conditions, the ΔCbb3-1 cells had already grown to an OD600 of 0.3 after 5.2 h ± 0.18 h, whereas the ΔCbb3-2 cells had reached an OD600 of 0.3 only after 14.7 h ± 1.1 h. (versus an OD600 of 0.3 after 10.1 h ± 1.7 h for wild-type P. stutzeri).

The purified wild-type Cbb3-1 and Cbb3-1–ΔCcoM oxidases were compared using UV-vis spectroscopy, differential scanning calorimetry (DSC), and oxygen reductase activity measurements.

We used UV-vis spectroscopy to characterize the hemes in Cbb3-1–CcO. The states of the b– and c-type hemes can be analyzed using the absorption maxima at 411 nm (Soret band), 552 nm (α-band), and 522 nm (β-band) as well as the shoulders at 559 and 529 nm (Fig. 5A). We conclude from the identical Cbb3-1 and Cbb3-1–ΔCcoM spectra that there were no differences concerning heme incorporation and heme environments.

DSC was conducted to compare the stabilities of the recombinant (rec.) Cbb3-1 and rec. Cbb3-1–ΔCcoM variants by monitoring the melting temperature (Tm) profiles (Fig. 5B). Peaks 1 and 2 correspond to the denaturation of the Cbb3 complex. The shifts in the Tm values of peaks 1 and 2 between the wild type and the Cbb3-1–ΔCcoM variant indicate a decreased thermal stability of the Cbb3-1–ΔCcoM variant compared to the wild type. An identical trend was observed after 16 days, with the deletion variant displaying lower melting temperatures similar to those seen with the wild type (data not shown).

We determined the oxygen consumption rates of P. stutzeri membranes prepared from wild-type and ΔCcoM cells as well as the oxygen reductase activities of purified wild-type Cbb3-1 and the Cbb3-1–ΔCcoM variant. The measurements were performed...
with an artificial electron donor system consisting of sodium-ascorbate and TMPD (N,N,N’,N’-tetramethyl-p-phenylenediamine dihydrochloride) under optimized conditions (17). We did not observe significant differences between wild-type and ΔCcoM membranes in the oxygen consumption rates. In addition, the purified Cbb₃₋₁–ΔCcoM variant had a catalytic activity of approximately 1,000 e⁻/s and thus displayed wild-type-like activity (see Fig. S5 in the supplemental material).

DISCUSSION

Small subunits in membrane protein complexes. In this work, the CcoM subunit was identified as part of the cbb₃-Cco complex from P. stutzeri ZoBell after the crystallographic electron density map had indicated the presence of a fourth subunit. The situation resembles that of the aa₃-CO of Paracoccus denitrificans, where the small subunit IV also was identified after an X-ray crystallographic structure determination (20). For the cbb₃-Cco from P. stutzeri, our DSC data indicate reduced thermal stability of the purified CcoM-deficient variant (Cbb₃₋₁–ΔCcoM) compared to the wild type, suggesting that CcoM is probably involved in complex stability and/or assembly. The presence of small subunits that are essential for assembly, stability, and activity is a common feature of membrane protein complexes (21–24). For instance, the ba₃-CcoOs from Thermus thermophilus and Aquifex aeolicus contain one membrane-spanning helix, called subunit IIa (5, 25).

Prunetti and coworkers proposed that subunit IIa is involved in the stability and/or assembly of ba₃-Cco (25). Furthermore, the small protein CydX was found to be a substantial part of the bd-oxidase complex in Brucella abortus and E. coli, as it is essential for enzymatic catalytic activity (26–28). It was shown for the cbb₂-Cco from Rhodobacter capsulatus that CcoH stably associates with the cbb₂ complex and constitutes an assembly factor (29, 30). Beyond enzymes of the superfamily of HCOs, small transmembrane-spanning subunits were also identified as part of bc₁ complexes and NAD(P)H dehydrogenases, for instance (31–34).

Comparison of CcoQ and CcoM. In P. stutzeri ZoBell, the ccoQ gene is present only in the operon encoding Cbb₃₋₂. Similarly to CcoM, CcoQ has one predicted TMH. The function of this subunit is still under debate (35–37). We set out to investigate whether CcoQ is present also in Cbb₃₋₂ in addition to CcoQ. The results of our MALDI-TOF MS measurements suggest that CcoM interacts with Cbb₃₋₂ (data not shown). However, it remains unclear if CcoM and CcoQ are located at identical or different positions in Cbb₃₋₂. Theoretically, CcoM and CcoQ may be functionally redundant due to their secondary structure resemblances. Using multiple sequence alignments of CcoQ subunits from different γ-proteobacteria and CcoM from P. stutzeri (Fig. 6), we found that CcoQ and CcoM share several highly conserved residues (G10, T13, M17-F20, and W29 [CcoM numbering]) in the N-terminal region. Furthermore, the conserved M17⁵ and F20⁵ amino acids are part of the proposed interaction ladder with CcoN. In CcoQ, residue M17⁵ is replaced either by a phenylalanine (Vibrio cholerae) or by smaller hydrophobic residues like leucine (Azotobacter vinelandii) and valine (P. stutzeri). We are currently investigating the physiological roles of CcoM and CcoQ to determine if the two subunits perform distinct or similar functions.

Anaerobic growth of wild-type and P. stutzeri deletion strains under denitrifying conditions. In many pseudomonads, e.g., Pseudomonas aeruginosa, P. stutzeri, and P. putida, two cbb₃ operons are present in the genome (8, 17, 38). However, only one of the two operons is preceded by an ANR box in its promoter region. In P. aeruginosa, the expression of the ANR-dependent cbb₃ isofrom is highly upregulated under low-oxygen conditions and in the stationary phase. In contrast, the ANR-independent one is constitutively expressed and not directly dependent on oxygen concentrations (8, 39). Recently, Hamada and coworkers showed that the P. aeruginosa cbb₃-CcoOs accumulate nitric oxide and are involved in biofilm formation during the anaerobic denitrification process (40). In addition, cbb₂-CcoOs were reported to be expressed under anaerobic conditions in several other bacterial species, e.g., Shewanella oneidensis, Bradyrhizobium japonicum, and Rhodobacter capsulatus (19, 41, 42). In this work, the growth behaviors of wild-type, ΔCbb₃₋₁, ΔCbb₃₋₂, and ΔCcoM P. stutzeri
ZoBell strains were studied anaerobically under denitrifying conditions (Fig. 4B; see also Fig. S4B in the supplemental material). Compared to the wild type, the ΔCbb3-1 strain is characterized by a shortened lag phase. Results of previous studies on Rhodobacter sphaeroides suggest that the electron flow through cbb3-CO is inversely related to the level of expression of photosynthetic genes through the PrrBA two-component regulatory system (43–45). The PrrBA system is also involved in the regulation of the denitrification pathway and is an analog to the RoxSR system of Photobacterium profundum (46–48). The absence of Cbb3-1 in the ΔCbb3-1 P. stutzeri cells might have led to the loss of the inhibitory signal that potentially represses denitrification gene expression. As a consequence, an increased expression level of denitrification enzymes may explain why the growth of ΔCbb3-1 during the lag phase is faster than that of the wild-type P. stutzeri strain. In contrast to the ΔCbb3-1 results, we observed an extended lag phase of the ΔCbb3-2 strain compared to the wild type. However, the role of Cbb3-2 under anaerobic denitrifying conditions remains to be elucidated. Similarly to Cbb3-1, the promoter region of CcoM also contains an ANR box, indicating its significance under low-oxygen conditions. Beyond its functions as a bona fide subunit of the cbb3-CO complex, the extended lag phase of the ΔCcoM strain under anaerobic denitrifying conditions also suggests that CcoM may interact with proteins involved in the denitrification process. Further investigations, e.g., in vivo cross-linking experiments and whole proteome studies of the CcoM deletion strain under anaerobic conditions, are required to analyze the physiological role of CcoM in more detail.

In summary, we unambiguously identified the uncharacterized TMH in the Cbb3 crystal structure by MALDI-TOF MS/MS as PstZoBell_05036. The gene encoding this protein is located outside the cbb3 operon and was renamed ccoM. We fitted the CcoM sequence into the unknown TMH of the X-ray structure, successfully matching bulky amino acid side chains to the observed electron densities. In the structure, CcoM interacts with two helices of the catalytic subunit CcoN in a ladder-like conformation. Taken together, these data indicate that we have indeed identified the unknown subunit. Previously, this subunit had been assigned to the cbb3-CO biosynthesis protein CcoH (49). The assumption was based on the observation that CcoH can be found as a minor contaminant in the purified cbb3 isoforms (17) and CcoH’s secondary structure and on CcoH’s importance for the cbb3-CO function (50). The mode of interaction of CcoM with subunit CcoN also appears to be the existence that this subunit contributes to a transient proton-conducting network. While we observed no effect on heme incorporation and catalytic activity, the Cco complex lacking CcoM displayed significantly reduced melting temperatures in DSC, suggesting a putative role of CcoM in complex assembly and stability.

MATERIALS AND METHODS

Bacterial strains, media, and oligonucleotides. Pseudomonas stutzeri strain ZoBell (ATCC 14405) was used to construct a deletion strain of the ccoM gene (locus tag, PstZoBell_05036), resulting in the ΔccoM strain. The P. stutzeri ZoBell cells were grown on lysogeny broth (LB) agar plates and in asparagine minimal medium at 32°C (10, 11). Antibiotics were added to final concentrations of 100 µg/ml kanamycin and 170 µg/ml chloramphenicol. Escherichia coli strain DH1 was used for cloning purposes (51). DNA sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and synthetic oligonucleotides (Eurofins MWG Operon) prepared for this study are listed in Tables S1 and S2 in the supplemental material, respectively.

Construction of ΔccoM P. stutzeri strain. The lambda Red recombinase system (52) was used to replace ccoM (PstZoBell_05036) in the P. stutzeri ZoBell genome by a kanamycin resistance cassette. To construct a helper plasmid for the homologous recombination, genes araC, gam, bet, and exo were amplified from the pUCP18-RedS vector (53) into the linearized pBBR1MCS (54) vector, employing ligation-independent cloning techniques (In-Fusion cloning kit; Clontech, Mountain View, CA). The resulting pMK-RedS vector was electrotransformed into P. stutzeri ZoBell cells (55). The P. stutzeri ZoBell cells containing pMK-RedS were cultured in LB media at 32°C and 180 rpm to an optical density at 600 nm (OD600) of 0.5 to 0.6. Expression of proteins Gam, Bet, and Exo was then induced with 0.2% (wt/vol) L-arabinose. After 4 h of induction, the P. stutzeri ZoBell cells were electrotransformed with approximately 5 µg of linearized DNA and incubated in super-optimal-broth (SOC) medium without antibiotics at 37°C with shaking (180 rpm) for 2 h. The linearized DNA was generated by 3-step PCR as previously described (56) and contained a kanamycin resistance cassette with long (~500-bp) flanking regions upstream and downstream of the ccoM gene. The gene disruption was confirmed by kanamycin resistance selection, PCR, and sequencing (Eurofins MWG Operon, Ebersberg, Germany) (see Fig. S1 in the supplemental material). Curing of the pMK-RedS plasmid from the deletion strain was carried out using electroporation (57).

Cultivations of P. stutzeri and purification of Cbb3-CO. P. stutzeri ZoBell cells were cultured under microaerobic conditions and harvested as previously published (10). All cultivations were performed in 1-L asparagine minimal media (10, 11), unless stated otherwise. For anaerobic cultivation under denitrifying conditions, P. stutzeri was grown in 1-L asparagine minimal media with 11.8 mM KNO3 at 32°C and 260 rpm in a 2-L fermenter with continuously injection of nitrogen. KNO3 (11.8 mM) was additionally supplemented at OD600 values of 0.3 and 0.7.

FIG 6 Multiple sequence alignment of subunit CcoM from P. stutzeri and CcoQ subunits from γ-proteobacteria. Red boxes indicate the predicted TMH region. The intensity of the blue coloring of amino acids reflects the degree of conservation. Multiple sequence alignment was performed with Jalview (62). CcoQ and CcoM sequences from A. vinelandii Df (GenBank accession no. YP_002799181.1), P. aeruginosa PA01 (GenBank accession no. YP_00393610.1,YP_003936161.1), P. fluorescens P90-1 (GenBank accession no. YP_347553.1), P. putida KT2440 (GenBank accession no. NP_746368.1 and NP_746373.1), P. stutzeri ZoBell (accession no. ADJ00005.1 and EHY76787.1), P. syringae B728a (GenBank accession no. YP_236485.1), and V. cholerae O1 N16961 (GenBank accession no. NP_231083.1) strains were aligned.
Membrane preparation and solubilization were performed according to previously described procedures with cells grown under microaerobic conditions (10, 11). Genomically expressed Cbb3-1 was purified from membranes prepared from the wild-type and ΔCcoM P. stutzeri strains, yielding wild-type Cbb3-1 and Cbb3-1ΔΔcoM, respectively. The four-step chromatographic purification of both enzymes was carried as published previously (11), and both purified enzymes were analyzed using UV-vis spectroscopy and oxygen reductase activity measurements. For differential scanning calorimetry (DSC) measurements, plasmid-expressed recombinant Cbb3-1 was produced using expression vector pXH-22 (17) in the wild-type strain and the ΔCcoM strain. The purification of this streptavidin (Strep)-tagged recombinant Cbb3-1 was performed as previously described (17).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/dx.doi.org/10.1128/mBio.01921-15/-/DCSupplemental.

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