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Identification of Surf1 as an assembly factor of the cytochrome bc_1-aa_3 supercomplex of *Actinobacteria*

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ABSTRACT

Respiration in aerobic *Actinobacteria* involves a cytochrome bc_1-aa_3 supercomplex with a diheme cytochrome c_1, first isolated from *Corynebacterium glutamicum*. Synthesis of a functional cytochrome *c* oxidase requires incorporation of Cu_A, Cu_B, heme *a*, and heme a_3. In contrast to eukaryotes and α-proteobacteria, this process is poorly understood in *Actinobacteria*. Here, we analyzed the role of a Surf1 homolog of *C. glutamicum* in the formation of a functional bc_1-aa_3 supercomplex. Deletion of the *surf1* gene (cg2460) in *C. glutamicum* caused a growth defect and cytochrome spectra revealed reduced levels of cytochrome *c* and *a* and an increased level of cytochrome *d*. Membranes of the Δ*surf1* strain had lost the ability to oxidize the artificial electron donor *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine, suggesting that Surf1 is essential for the formation of a functional cytochrome aa_3 oxidase. In contrast to the wild type, a bc_1-aa_3 supercomplex could not be purified from solubilized membranes of the Δ*surf1* mutant. A transcriptome comparison revealed that the genes of the SigC regulon including those for cytochrome *bd* oxidase were upregulated in the Δ*surf1* strain as well as the copper deprivation-inducible gene *ctiP*. Complementation studies showed that the Surf1 homologs of *Corynebacterium diphtheriae*, *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* could at least partially abolish the growth defect of the *C. glutamicum* Δ*surf1* mutant, suggesting that Surf1 is a conserved assembly factor for actinobacterial cytochrome aa_3 oxidase.

1. Introduction

Aerobic bacterial respiratory chains are characterized by a large diversity including various types of terminal oxidases acting either as quinol oxidases or as cytochrome *c* oxidases [1,2]. Bacteria typically contain two or more terminal oxidases allowing adaptation to changing environmental conditions with respect to oxygen partial pressure, copper availability, or varying demands for respiratory activity. *Actinobacteria* form a large phylum within the Gram-positive bacteria and include a variety of species of high medical or biotechnological relevance, such as *Mycobacterium tuberculosis* or *Corynebacterium glutamicum*. Strains of *C. glutamicum* are used in the biotech industry for production of about five million tons of amino acids per year, mainly L-glutamate and L-lysine. These processes are performed under aerobic conditions and depend on respiration.

The respiratory chain of *C. glutamicum* is branched and contains two terminal oxidases, a cytochrome *c* oxidase of the aa_3-type and the menaquinol oxidase cytochrome *bd* [3]. A unique feature of the respiratory chain of *C. glutamicum* is the presence of a cytochrome bc_1-aa_3 supercomplex with a diheme cytochrome c_1 that represents the only *c*-type cytochrome of this species [4,5]. The supercomplex was purified in the active form by affinity chromatography with either Strep-tagged cytochrome *b* (QcrB) or with Strep-tagged subunit I (CtaD) of cytochrome aa_3 and included, besides the subunits of the bc_1 complex and the aa_3 oxidase, three additional proteins, a secreted lipoprotein (Cg2949), an integral membrane protein (Cg2211) and a cytosolic protein (Cg2444) [5]. Deletion of the genes cg2949, cg2211, and cg2444 caused no obvious growth phenotype under the conditions tested, suggesting that these accessory proteins are not essential for activity [5]. The complex was characterized with respect to the redox potential of the prosthetic groups and a structural model was built [6]. Moreover, the complex was characterized kinetically [7].

Cytochrome bc_1-aa_3 supercomplexes similar to the one of *C. glutamicum* were later also described for *Mycobacterium* species [8,9] and *Streptomyces coelicolor* [10]. With the availability of a large set of genome sequences it became clear that a bc_1-aa_3 supercomplex is presumably characteristic for all aerobic *Actinobacteria* [6]. Most recently, in two parallel studies the structure of the bc_1-aa_3 supercomplex of *Mycobacterium smegmatis* was solved by cryo-electron microscopy [11,12]. These studies confirmed key features of the model proposed

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for *C. glutamicum* and revealed new and interesting aspects, such as the presence of a periplasmic superoxide dismutase associated with cytochrome *b* (which is absent in *C. glutamicum*) or the presence of cytochrome c_1 in two different conformations [11,12]. Moreover, the cryo-EM structures revealed the presence of accessory proteins, including homologs of Cg2211 (PRSAF1) and Cg2949 (LpqE) previously found to be associated with supercomplex of *C. glutamicum*.

The formation of a catalytically active bc_1-aa_3 supercomplex requires an assembly process for insertion of the cofactors, which are Cu_A, Cu_B, heme *a*, and heme a_3 in the case of the aa_3 oxidase. Many proteins involved in these maturation events have been identified and characterized in mammals, *Saccharomyces cerevisiae*, and α-proteobacteria [13], but knowledge for Gram-positive bacteria and in particular *Actinobacteria* is scarce. By analyzing the transcriptional response of *C. glutamicum* to copper deprivation we recently identified two proteins presumably involved in copper insertion into the aa_3 oxidase, the membrane-integral copper transport and insertion protein CtiP and the secreted Cu-binding protein CopC, which were found to be conserved in aerobic *Actinobacteria* [14].

In the present study, we analyzed another protein, Cg2460, presumed to be a potential assembly factor for the aa_3 oxidase in *C. glutamicum*. The protein showed weak sequence similarity to Surf1 proteins from other organisms. Surf1 was identified as a protein that was able to complement cytochrome oxidase defects causing the Leigh syndrome in humans, a severe human neurodegenerative disease, and therefore proposed to be involved in cytochrome oxidase biogenesis [15]. The homolog from *S. cerevisiae* is Shy1p and deletion of the corresponding gene also led to reduced oxidase activity [16]. The role of Surf1 in mitochondrial cytochrome oxidase biogenesis is still unknown and the functions proposed include stabilization of subunit I and involvement in copper homeostasis [17,18]. A much better picture is available for the role of Surf1 homologs in α-proteobacteria (reviewed in [19]). Biochemical analysis of cytochrome aa_3 oxidase in *Rhodobacter sphaeroides* indicated that Surf1 plays a role in facilitating the insertion of heme a_3 into the oxidase [20]. *Paracoccus denitrificans* contains two Surf1 homologs, which were shown to be involved in oxidase assembly. Surf1q acts on the *ba*-type quinol oxidase and Surf1c acts on the aa_3-type cytochrome *c* oxidase [21]. Further studies revealed that the *P. denitrificans* Surf1 proteins bind heme *a* in a 1:1 stoichiometry with K_d values of 0.3–0.7 μM, indicating that they are involved in the transfer of heme *a* from the heme *a* synthase CtaA to subunit I of cytochrome aa_3 and its insertion [21,22].

In Gram-positive bacteria, the role of Surf1 proteins has not been studied until now. We therefore investigated the impact of the putative Surf1 homolog Cg2460 of *C. glutamicum* on the assembly and activity of the cytochrome bc_1-aa_3 supercomplex. Furthermore, to gain information about regulatory mechanisms, we compared global gene expression of a Δ*surf1* mutant and the wild type. Lastly, the conservation of Surf1 within actinobacterial species was examined.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used in this work are listed in Table 1. The *C. glutamicum* type strain ATCC13032 was used as wild type (wt). To start cultivation, 5 ml brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI) was inoculated with a single colony from an agar plate and incubated for 9 h at 30 °C and 170 rpm. When appropriate, 25 μg/ml kanamycin was added. The cells of this first preculture were used to inoculate a second preculture in 750 μl CGXII minimal medium [23] containing 30 mg l^{-1} 3,4-dihydroxybenzoate as iron chelator and 2% (*w/v*) glucose. The cultivation was performed overnight at 30 °C and 1200 rpm using a BioLector microcultivation system and a 48-well FlowerPlate (m2p-labs, Baesweiler, Germany). For larger cultivation volumes, 50 ml BHI medium was inoculated with 500 μl preculture in 500 ml-Erlenmeyer flasks that were incubated overnight at 140 rpm and 30 °C. The main cultures were performed in the same medium as the second preculture with the addition of 2% (*w/v*) glucose. For Biolector and shake flask cultivations, the initial optical density at 600 nm (OD_{600}) was set to 1 and 0.5, respectively. For complementation experiments, in which the cells carried expression plasmids with a *tac* promoter, 100 μM isopropyl-β-D-thiogalactoside (IPTG) was added to the medium immediately after inoculation. All cloning steps were performed with *Escherichia coli* DH5α as host, which was cultivated at 37 °C on LB agar plates or in liquid LB medium [24] with 50 μg/ml kanamycin.

2.2. Recombinant DNA work and construction of deletion mutants

Oligonucleotides used in this study are listed in Table S1. Routine methods such as PCR and DNA restriction were performed using established protocols [25]. Gibson assembly was used for plasmid construction [26]. DNA sequencing and oligonucleotide synthesis were performed by Eurofins Genomics (Ebersberg, Germany). In-frame gene deletion strains of *C. glutamicum* were constructed via a two-step homologous recombination protocol using 500 bp flanks [4]. Colony PCR using oligonucleotides annealing outside the deleted regions was performed to confirm the chromosomal deletions.

For complementation experiments with the *C. glutamicum* Δ*surf1* mutant, the expression plasmids pAN6-*surf1*, pAN6-CDC7B1688, pAN6-MSMEG4311 and pAN6-Rv2235 were constructed, which contain the *surf1* gene (cg2460) of *C. glutamicum* ATCC13032 and the homologs CDC7B1688 from *Corynebacterium diphtheriae* DSM44123, MSMEG4311 from *Mycobacterium smegmatis* MC^2155 (ATCC700084), and Rv2235 from *Mycobacterium tuberculosis* H37Rv (ATCC25618), respectively. The corresponding genes were amplified by PCR from chromosomal DNA of the corresponding strains using the oligonucleotides shown in Table S1 and cloned into the expression plasmid pAN6 under control of a *tac* promoter (Table 1). After sequencing of the inserts of the plasmids, they were transferred into the *C. glutamicum* Δ*surf1* mutant.

For construction of the reporter plasmids pJC1-P*cydA-venus* and pJC1-P*ctaD-venus*, approximately 500 bp upstream of the start codon and 30 bp of the coding region followed by an introduced stop codon were amplified from chromosomal DNA by PCR using the oligonucleotide pairs ctaD_venus_fwd/ctaD_venus_rev and cydA_venus_fwd/cydA_venus_rev, resulting in PCR products of 589 bp and 611 bp. Amplification of the *venus* gene including a ribosome binding site was performed with the oligonucleotide pair venus_3_fwd/venus_4_rev and pJC1-*venus*-term as template, resulting in a PCR product of 755 bp. The individual promotor and *venus* fragments were ligated into a BamHI/*Spe*I-linearized pJC1 vector using Gibson assembly [26]. After sequencing of the inserts of the plasmids, they were transferred into *C. glutamicum* wt, the Δ*surf1* mutant and the Δ*ctiP* mutant.

2.3. Global gene expression analysis

For a comparative transcriptome analysis of the *C. glutamicum* Δ*surf1* mutant and the parent wt, the cells were cultivated in shake flasks using CGXII medium with 2% (*w/v*) glucose as described above until they reached the early exponential phase (OD_{600} of about 5). Then RNA was isolated and used for DNA microarray analysis using custom-made 60mer DNA microarrays for genome-wide gene expression analysis (Agilent Technologies, Waldbronn, Germany). Four independent biological replicates were performed for comparison of the Δ*surf1* mutant and the wt and *p* values were calculated using Student's *t*-test (Excel, Microsoft). The experimental details and the data evaluation were performed as described previously [27]. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database and are accessible under the accession number GSE123974.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	F$^-$ Φ80*dlac*Δ(*lacZ*)M15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (r_K^-, m_K^+) *deoR thi-1 phoA supE44* λ$^-$ *gyrA96 relA1*; strain used for cloning procedures	[37]
C. glutamicum		
ATCC13032	Biotin-auxotrophic wild type	[38]
C. glutamicum Δ*ctiP*	Derivative of ATCC13032 with in-frame deletion of the *ctiP* (cg2699) gene	[14]
C. glutamicum Δ*ctaD*	Derivative of ATCC13032 with in-frame deletion of the *ctaD* (cg2780) gene	[4]
C. glutamicum Δ*surf1*	Derivative of ATCC13032 with in-frame deletion of the *surf1* (cg2460) gene	This work
C. glutamicum Δ*qcr*	Derivative of ATCC13032 with in-frame deletion of the *ctaE-qcrCAB* operon (cg2406–2404)	[4]
C. glutamicum Δ*qcr*Δ*ctiP*	Derivative of ATCC13032 with in-frame deletions of the *ctaE-qcrCAB* operon and *ctiP*	[14]
C. glutamicum Δ*qcr*Δ*surf1*	Derivative of ATCC13032 with in-frame deletions of the *ctaE-qcrCAB* operon and *surf1*	This work
C. glutamicum Δ*ctaD*Δ*ctiP*	Derivative of ATCC13032 with in-frame deletions of *ctaD* and *ctiP*	[14]
C. glutamicum Δ*ctaD*Δ*surf1*	Derivative of ATCC13032 with in-frame deletions of *ctaD* and *surf1*	This work
Plasmids		
pK19*mobsacB*	Kan^R; plasmid for allelic exchange in *C. glutamicum* (pK18 *ori*$V_{E.c.}$, *sacB*, *lacZ*α)	[39]
pK19*mobsacB*-Δ*surf1*	Kan^R; pK19*mobsacB* derivative containing a 1 kb PCR-product (*Eco*RI/*Pst*I) which covers the flanking regions of the *C. glutamicum surf1* gene	This work
pAN6	Kan^R; *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression using the P_{tac} promoter; derivative of pEKEx2	[40]
pAN6-*surf1*	Kan^R; pAN6 derivative containing *surf1* gene (cg2460) of *C. glutamicum* under control of P_{tac}	This work
pAN6-CDC7B1688	Kan^R; pAN6 derivative containing the CDC7B_1688 gene (*surf1* homolog) of *C. diphtheriae* DSM44123 under control of the *tac* promoter	This work
pAN6-MSMEG4311	Kan^R; pAN6 derivative containing the MSMEG4311 gene (*surf1* homolog) of *M. smegmatis* MC^2 155 under control of the *tac* promoter	This work
pAN6-Rv2235	Kan^R; pAN6 derivative containing the Rv2235 gene (*surf1* homolog) of *M. tuberculosis* H37Rv under control of the *tac* promoter	This work
pJC1	Kan^R; *E. coli*/*C. glutamicum* shuttle vector (pHM1519 ori_{Cg}, pACYC177 ori_{Ec})	[41]
pJC1-*venus*-term	Kan^R; pJC1 derivative harboring the *venus* gene and additional terminators	[42]
pJC1-$qcrB_{St}$	Kan^R; expression plasmid for purification of Strep-tagged QcrB; contains the *ctaE-qcrCAB* operon expressed from its native promotor; *qcrB* with 10 additional codons at the 3′-end (AAWSHPQFEK)	[5]
pJC1-$ctaD_{St}$	Kan^R; expression plasmid for purification of Strep-tagged CtaD; *ctaD* expressed from its native promotor with 10 additional codons at the 3′-end (AAWSHPQFEK)	[5]
pJC1-P*cydA-venus*	Kan^R; reporter plasmid for following expression of *cydABDC* operon; contains promoter region of *cydA* in front of the *venus* reporter gene	This work
pJC1-p*ctaD-venus*	Kan^R; reporter plasmid for following expression of *ctaD* gene; contains promoter region of *ctaD* in front of the *venus* reporter gene	This work

2.4. Purification of the cytochrome bc_1-aa_3 supercomplex

C. glutamicum strains carrying expression plasmids were cultivated in 5 l shake flasks with 500 ml BHI medium supplemented with 2% (*w*/*v*) glucose at 30 °C and 90 rpm and harvested at an OD_{600} of 8. Membrane isolation, solubilization of membrane proteins with *n*-dodecyl-β-D-maltoside and subsequent purification of Strep-tagged CtaD or Strep-tagged QcrB was performed as described previously [5]. Aliquots of the purified proteins were separated by SDS-PAGE using 12% polyacrylamide gels (BioRad, Munich, Germany) which were subsequently stained with Coomassie dye-based RAPIDStain solution (G-Biosciences, St. Louis, MO, USA). Protein bands were identified after in-gel trypsin digestion [28] by peptide mass fingerprinting using an Ultraflex III TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) as described previously [29].

2.5. Redox difference spectroscopy

Spectra were recorded at room temperature using 5-mm-light-path cuvettes with a Jasco V560 spectrophotometer. For recording a spectrum of dithionite-reduced intact cells, a suspension of *C. glutamicum* cells in 100 mM Tris-HCl, pH 7.5 with an OD_{600} of 100 and a silicon photodiode detector for turbid samples [30] was used. Attempts to record a spectrum of oxidized intact cells proved to be difficult due to rapid re-reduction caused by endogenous metabolism. Difference spectra of dithionite-reduced-minus-ferricyanide-oxidized isolated membranes (30 mg/ml) in 100 mM Tris-HCl, pH 7.5 were also recorded with the silicon photodiode detector. Difference spectra of dithionite-reduced-minus-ferricyanide-oxidized protein samples (0.5 mg/ml) obtained after affinity chromatography of $CtaD_{St}$ from solubilized membranes were recorded using the standard photomultiplier detector of the Jasco V560 spectrophotometer. A few grains of sodium dithionite or potassium ferricyanide were added to the samples to reduce and oxidize the samples, respectively.

Pyridine hemochrome spectra were performed according to a published protocol [31] using protein samples obtained after affinity chromatography of $CtaD_{St}$ from solubilized membranes of *C. glutamicum* Δ*ctaD*/pJC1-$ctaD_{St}$ and *C. glutamicum* Δ*ctaD*Δ*surf1*/pJC1-$ctaD_{St}$. For recording spectra of oxidized samples, 200 µl purified proteins (0.5 mg/ml) in elution buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM $MgSO_4$, 0.025% (*w*/*v*) *n*-dodecyl-β-D-maltoside, 2.5 mM *D*-desthiobiotin, and 10% (v/v) glycerol) were mixed with 200 µl of an oxidizing solution (0.2 M NaOH, 40% (v/v) pyridine, 500 µM potassium ferricyanide). Subsequently, 4 µl of a reducing solution (0.5 M sodium dithionite in 0.5 M NaOH) was added to the samples and spectra were recorded until no further signal increase was observed.

2.6. TMPD oxidase assay

N,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine (TMPD) oxidase activity of isolated membranes was measured spectrophotometrically at 562 nm in air-saturated 100 mM Tris-HCl buffer, pH 7.5, containing 200 µM TMPD, at 30 °C. For the calculation, an extinction coefficient of 10.5 $mM^{-1}\,cm^{-1}$ was used [32]. One unit of activity is defined as 1 µmol of TMPD oxidized per min. Autooxidation rates of TMPD were recorded using samples containing only buffer and TMPD and subtracted from the rates of the membranes.

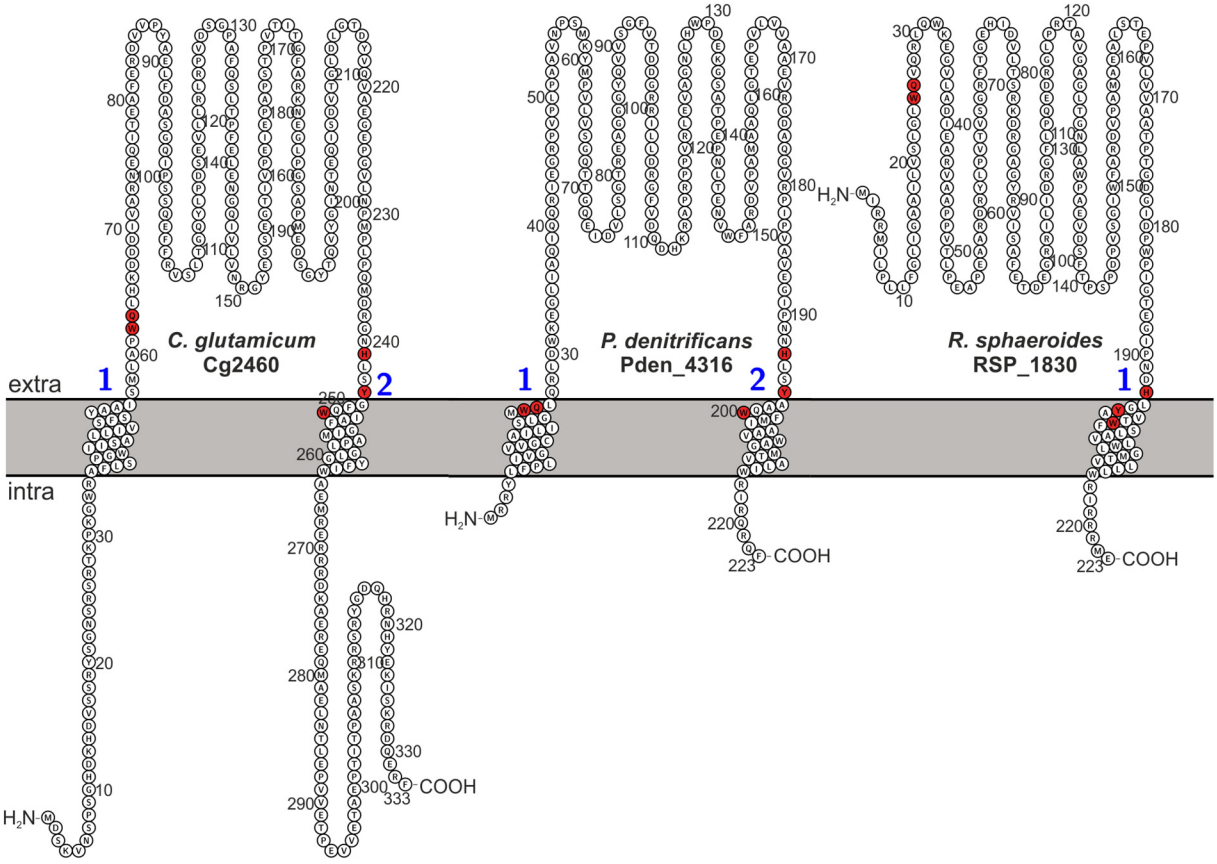

Fig. 1. Topology models of Surf1 proteins from *C. glutamicum* (Cg2460), *P. denitrificans* (Pden_4316) and *R. sphaeroides* (RSP_1830). Predictions of protein architectures were done using the PROTTER software [33]. Red shaded amino acid residues were shown to be involved in heme *a* binding in *P. denitrificans* Surf1 [19].

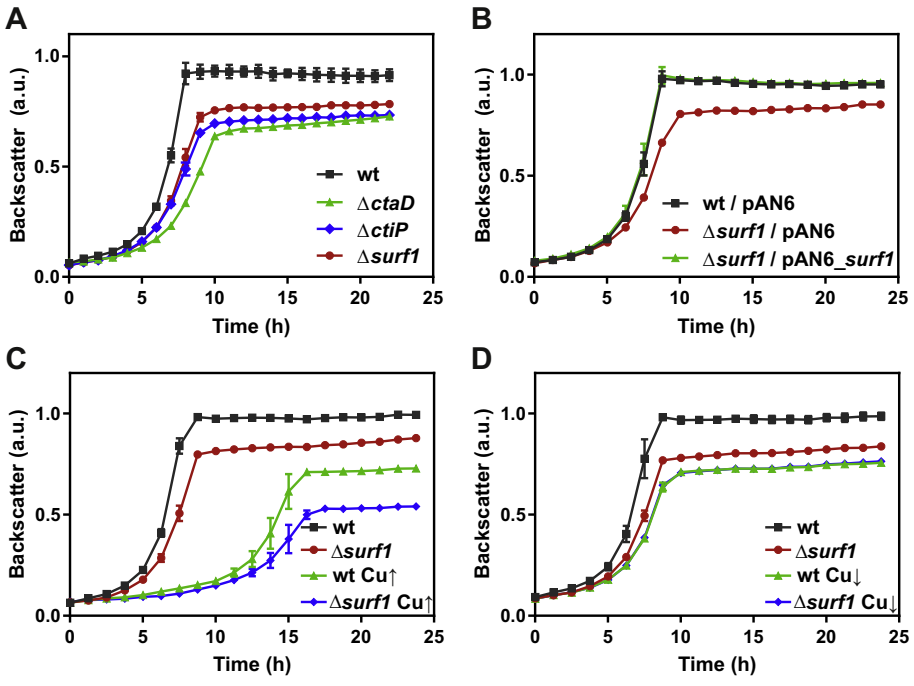

Fig. 2. Growth studies of the indicated *C. glutamicum* strains under different cultivation conditions. (A, B) Growth in standard CGXII medium supplemented with 2% (*w/v*) glucose and 1.25 µM $CuSO_4$. (C) Growth in CGXII medium with 2% (*w/v*) glucose supplemented with 100 µM $CuSO_4$ (Cu↑) and for comparison growth with 1.25 µM $CuSO_4$. (D) Growth in copper-deprived CGXII medium with 2% (*w/v*) glucose supplemented with 150 µM BCS and 1 mM ascorbate (Cu↓). For comparison, growth with 1.25 µM CuSO4 is shown. Cells were cultivated in a FlowerPlate using a Biolector microcultivation system (30 °C, 1200 rpm). Growth was measured online as backscatter at 620 nm every hour. All backscatter values were normalized by setting the maximal backscatter value of the wt used for comparison as 1. Depicted are mean values and standard deviation from three biological replicates. a.u., arbitrary units.

3. Results and discussion

3.1. In silico characterization of a Surf1 homolog in C. glutamicum

Studies in the α-proteobacteria *Paracoccus denitrificans* and *Rhodobacter sphaeroides* indicated that Surf1 proteins are involved in transferring the heme *a* group from heme *a* synthase, CtaA, to subunit I of cytochrome *c* oxidase and transiently bind heme *a* [19,20]. In the genome of *C. glutamicum*, the gene with the locus tag cg2460 encodes a protein of 333 amino acid residues (predicted molecular mass

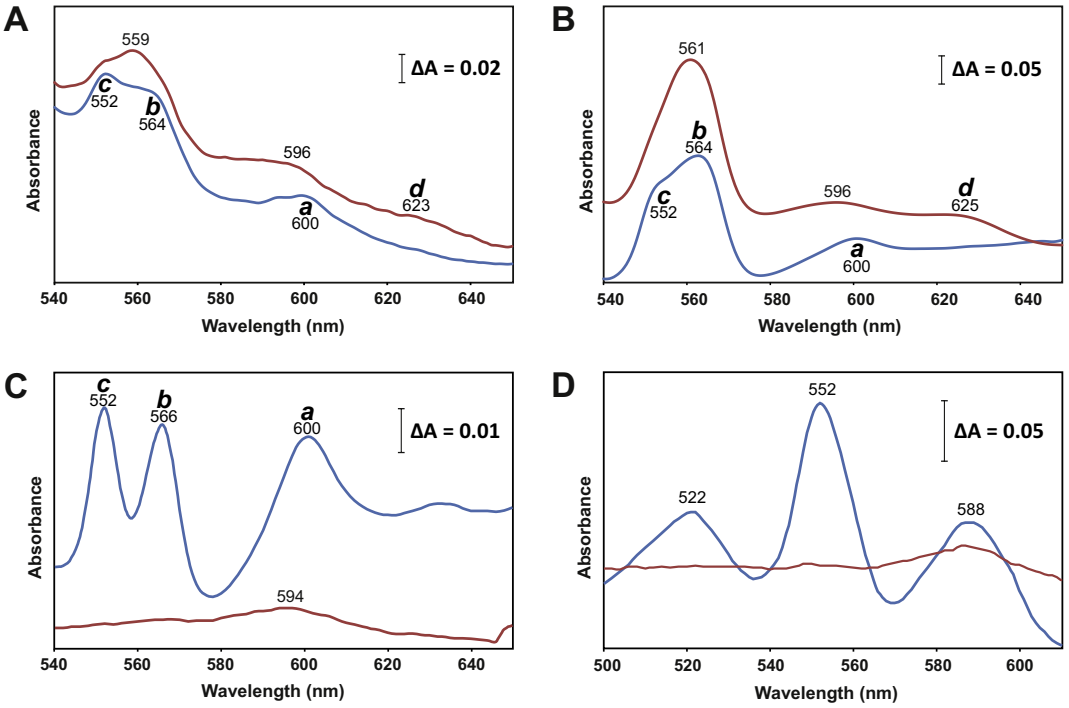

Fig. 3. Cytochrome and pyridine hemochrome spectra of *C. glutamicum* wt and its Δ*surf1* mutant. (A) Dithionite-reduced spectra of intact cells. (B) Dithionite-reduced-*minus*-ferricyanide-oxidized difference spectra of membranes isolated from the *C. glutamicum* Δ*surf1* mutant (red) and the wt (blue). (C) Dithionite-reduced-*minus*-ferricyanide-oxidized difference spectra of the proteins purified by StrepTactin affinity chromatography of $CtaD_{St}$ from strain Δ*ctaD*/pJC1-*ctaD*$_{St}$ (blue) and from strain Δ*ctaD*Δ*surf1*/pJC1-*ctaD*$_{St}$ (red). (D) Dithionite-reduced-*minus*-ferricyanide-oxidized pyridine hemochrome spectra of the proteins purified by StrepTactin-affinity chromatography of $CtaD_{St}$ from strain Δ*ctaD*/pJC1-*ctaD*$_{St}$ (blue) and from strain Δ*ctaD*Δ*surf1*/pJC1-*ctaD*$_{St}$ (red).

37.24 kDa) with 24% amino acid sequence identity to Surf1c of *P. denitrificans* [21] and 18% amino acid sequence identity to Surf1 of *R. sphaeroides* [20]. Bioinformatic analysis indicated that Cg2460, similar to previously characterized Surf1 proteins, possesses two transmembrane helices (residues 35–56 and 245–264) enclosing an extracytoplasmic SURF1 domain (Prosite PS50895) of 189 residues [33] (Fig. 1). An amino acid sequence alignment (Fig. S1) revealed the presence of the motifs $W^{62}Q^{63}$ and $Y^{244}xxxW^{248}$ in Cg2460, which were shown to be crucial not only for heme binding, but also for differentiation between heme types [22]. Furthermore, the histidine residue H^{241}, reported to serve as axial ligand of the heme iron in *P. denitrificans* Surf1, was conserved in *C. glutamicum* Surf1 (Figs. 1, S1). Unlike in other bacteria, such as *P. denitrificans*, the *surf1* gene of *C. glutamicum* is not located in the immediate neighborhood of genes coding for components of the respiratory chain or heme biosynthesis [34].

3.2. Phenotype of a surf1 deletion mutant of C. glutamicum

To analyze the role of Surf1 in *C. glutamicum,* a *surf1* deletion strain was constructed and analyzed regarding growth (note that growth data are also provided in the supplementary information). The loss of Surf1 caused a strong growth defect on BHI agar plates (data not shown) and in CGXII glucose minimal medium (Fig. 2A, Fig. S2, Table S3), which was similar to the growth defect of a Δ*ctaD* strain lacking subunit I of cytochrome aa_3 oxidase and of a Δ*ctiP* strain lacking the copper transport and insertion protein CtiP [14]. Thus, Surf1 is important for optimal growth of *C. glutamicum*. Under copper excess stress, the *surf1* mutant of *C. glutamicum* showed a growth defect comparable to the one observed under standard copper levels (Fig. 2C). In contrast, the Δ*surf1* mutant grew like the wt under copper deprivation (Fig. 2D), which is a clear evidence for the involvement of Surf1 in cytochrome aa_3 assembly. Under copper deprivation, cytochrome aa_3 oxidase is inactive due to the lack of copper and cytochrome *bd* oxidase serves as terminal oxidase. Therefore, if Surf1 is an assembly factor of cytochrome aa_3 oxidase, its absence should have no consequences under copper deprivation, as observed before for the assembly factor CtiP [14]. Complementation experiments with the native *surf1* gene restored wt-like growth (Fig. 2B), confirming that the growth phenotype is due to the lack of the *surf1* gene rather than to secondary mutations. Plasmid-based expression of *ctaA*, coding for heme *a* synthase, did not complement the growth defect of *C. glutamicum* Δ*surf1* (data not shown).

To study the impact of the *surf1* deletion on the cytochrome *c* content of the cell, an absorbance spectrum of a dithionite-reduced cell suspension was recorded and compared with the corresponding spectrum of the wt (Fig. 3A). The Δ*surf1* mutant differed from the wt by a decreased cytochrome *c* peak, loss of the 600 nm cytochrome *a* peak, and appearance of peaks at 596 nm and 623 nm. The latter is probably caused by cytochrome *d*. These results were confirmed by reduced-minus-oxidized difference spectra of isolated membranes (Fig. 3B) and showed that the lack of Surf1 has a strong effect on the cytochrome composition of the cell.

To test for the influence of Surf1 on cytochrome aa_3 oxidase activity, the oxidation of *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) was measured with isolated membranes. Whereas a specific activity of 88 nmol TMPD oxidized per min and mg protein (mean value of three biological replicates) was detected with membranes of the wt, no activity above the autooxidation rate of TMPD (1 nmol per min) was found for the Δ*surf1* mutant and for the Δ*ctiP* mutant. This suggests that both Surf1 and CtiP are required for the formation of an active cytochrome aa_3 oxidase.

3.3. Impact of the absence of Surf1 on the purification of the cytochrome bc_1-aa_3 supercomplex

To gain further insights into the possible function of Surf1 in the assembly of the cytochrome bc_1-aa_3 supercomplex, purification experiments by StrepTactin affinity chromatography were performed. In one series, C-terminally Strep-tagged QcrB was purified from *n*-

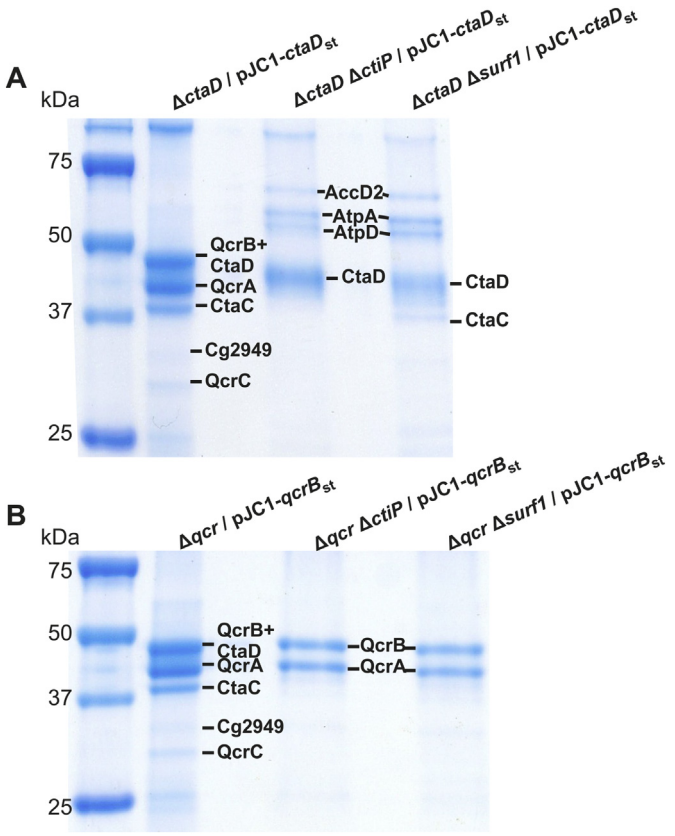

Fig. 4. Purification of cytochrome bc_1–aa_3 supercomplex subunits either with Strep-tagged QcrB (A) or with Strep-tagged CtaD (B) in the presence and absence of Surf1 or CtiP. The indicated *C. glutamicum* strains were cultivated in BHI medium with 2% (w/v) glucose at 30 °C in shaking flasks and harvested in the exponential phase at an OD_{600} of 8. Membrane proteins solubilized with *n*-dodecylmaltoside were used for Streptactin affinity chromatography. The protein fractions eluted with desthiobiotin were combined and separated by SDS-PAGE (12% separating gel). The gels were stained with Coomassie Blue. Identification of the protein bands was done by peptide mass fingerprinting after in-gel tryptic digestion and MALDI-ToF-MS analysis of the peptides.

dodecylmaltoside-solubilized membranes of strains Δ*qcr*/pJC1-$qcrB_{St}$, Δ*qcr*Δ*surf1*/pJC1-$qcrB_{St}$, and Δ*qcr*Δ*ctiP*/pJC1-$qcrB_{St}$ (Fig. 4A). In a second series, C-terminally Strep-tagged CtaD was purified from the *n*-dodecylmaltoside-solubilized membranes of strains Δ*ctaD*/pJC1-$ctaD_{St}$, Δ*ctaD*Δ*surf1*/pJC1-$ctaD_{St}$, and Δ*ctaD*Δ*ctiP*/pJC1-$ctaD_{St}$ (Fig. 4B). Strains Δ*qcr*Δ*ctiP* and Δ*ctaD*Δ*ctiP* were included as we recently had shown that the absence of CtiP also impaired the assembly of the bc_1-aa_3 supercomplex [14] and thus could follow differences in the effects of CtiP and Surf1.

Purification of $QcrB_{St}$ from strain Δ*qcr*/pJC1-$qcrB_{St}$ resulted in the purification of a complex composed of the subunits of the cytochrome bc_1 complex, $QcrB_{St}$, QcrA, and QcrC, of the cytochrome aa_3 oxidase subunits CtaD (subunit I) and CtaC (subunit II), and of the lipoprotein Cg2949 (Fig. 4A). The complex purified from strain Δ*qcr*Δ*surf1*/pJC1-$qcrB_{St}$ contained only QcrA besides $QcrB_{St}$, but neither QcrC nor subunits of the cytochrome aa_3 oxidase nor Cg2949 (Fig. 4A). The same pattern was observed for $QcrB_{St}$ purification from strain Δ*qcr*Δ*ctiP*/pJC1-$qcrB_{St}$, confirming the previous data [14]. It should be noted that the amount of proteins purified from the strains lacking Surf1 or CtiP was much lower (approx. 25%) than the amount purified from the strain containing these proteins, which might be due to an impaired stability of an incompletely assembled supercomplex.

Purification of $CtaD_{St}$ from strain Δ*ctaD*/pJC1-$ctaD_{St}$ resulted in the purification of a complex composed of $CtaD_{St}$, CtaC, QcrB, QcrA, QcrC, and Cg2949 (Fig. 4B), similar to the one obtained by purification of $QcrB_{St}$. The complex purified from strain Δ*ctaD*Δ*surf1*/pJC1-$ctaD_{St}$ contained $CtaD_{St}$ and CtaC, but no subunits of the cytochrome bc_1 complex or Cg2949. The complex purified from strain Δ*ctaD*Δ*ctiP*/pJC1-$ctaD_{St}$ showed a similar composition, except for the absence of CtaC (Fig. 4B), suggesting that CtiP but not Surf1 is required for complex formation of subunit I with subunit II.

Cytochrome spectra of the samples purified with $CtaD_{St}$ from different strain backgrounds revealed strong differences (Fig. 3C). Whereas the spectrum of the sample purified from Δ*ctaD*/pJC1-$ctaD_{St}$ contained peaks for *a*-, *b*-, *c*-type cytochromes with maxima at 600 nm, 566 nm, and 552 nm, the spectrum of the sample obtained from Δ*ctaD*Δ*surf1*/pJC1-$ctaD_{St}$ revealed only a very small peak at about 594 nm. In the case of the complex purified from Δ*ctaD*Δ*ctiP*/pJC1-$ctaD_{St}$, no cytochromes at all were observed (data not shown). To determine the heme type responsible for the 594 nm peak, pyridine hemochrome spectra were performed with the two samples. As shown in Fig. 3D, the sample purified from Δ*ctaD*/pJC1-$ctaD_{St}$ contained peaks at 588 nm, 552 nm, and 522 nm. According to reference [35], the peaks at 522 nm and 552 nm presumably represent mixtures of heme *b* and heme *c*, while the peak at 588 nm represents heme *a*. This suggests that the 594 nm peak observed in reduced-minus-oxidized spectra is due to some residual heme *a* that is bound to CtaD in the absence of Surf1.

3.4. Impact of the *surf1* deletion on global gene expression

To analyze consequences of *surf1* deletion at the transcriptional level, global gene expression of the Δ*surf1* mutant and the wt were compared using DNA microarray experiments. The two strains were cultivated in CGXII glucose medium and cells were harvested in the exponential growth phase and used for RNA isolation. The comparison revealed 13 genes upregulated ≥2.5-fold and 11 genes downregulated ≥2.5-fold (Table 2). Seven of the genes upregulated in the Δ*surf1* strain belong to the SigC regulon, which are the *cydABDC* operon and the cg1881-cg1883-*copC* cluster. Four further members of the SigC regulon also showed increased mRNA levels, but are not listed in Table 2 because the cut-off was set at a ratio of 2.5, namely *ctaA* (cg1769, 1.71-fold increased), *ctaB* (cg1773, 2.23-fold increased), cg2556 (2.39-fold increased) and cg2750 (2.07-fold increased). The *ctiP* gene, which is not belonging to the SigC regulon, also showed a 2.66-fold increased mRNA level. The other genes with an increased mRNA level encode metabolic enzymes (*mtlD*, *sucCD*), a transporter (*pacL*), and a transcriptional regulator (*fruR*). The genes found to have a ≥2.5-fold decreased mRNA level in the Δ*surf1* strain code for proteins with diverse cellular functions and an obvious relationship to the absence of Surf1 could not be deduced. The transcriptome data are in line with the presence of a defective bc_1-aa_3 supercomplex in the Δ*surf1* mutant, which was previously shown to activate the alternative sigma factor SigC [14,36]. Upregulation of the *ctiP* gene, which is not part of the SigC regulon, suggests the existence of another regulatory system sensing a defective cytochrome aa_3 oxidase.

To confirm the results of the DNA microarray data, the expression of the *cydABDC* operon and for comparison of *ctaD* was measured using the reporter plasmids pJC1-P*cydA-venus* and pJC1-P*ctaD-venus* in *C. glutamicum* wt, the Δ*surf*1 mutant and the Δ*ctiP* mutant (Table 3). The strains were grown in CGXII glucose medium either under standard copper conditions or under copper deprivation and the specific fluorescence corresponding to the expression level of *cydA* and *ctaD* was measured after 24 h. As expected from the DNA microarray data, the *cydA* promoter activity was 2-fold higher in the Δ*surf*1 mutant and also in the Δ*ctiP* mutant. Under copper deprivation, *cydA* promoter activity was two-fold increased in the wt and the activity in the Δ*surf1* mutant and in the Δ*ctiP* mutant was comparable under these conditions, i.e. no further increase was observed. In the case of *ctaD*, the promoter activity in the Δ*surf1* mutant and also in Δ*ctiP* mutant was slightly increased compared to the wt (factor 1.5) under standard copper conditions. Copper deprivation caused a small increase in *ctaD* promoter activity in

Table 2
Transcriptome analysis of the *surf1* deletion strain compared to the wt under standard CGXII conditions in four biological replicates during the early exponential phase. Depicted are upregulated genes with an at least 2.5-fold regulation (p-value ≤ 0.05). The locus tags of genes belonging to the SigC regulon are underlined.

Locus tag	Gene name	Annotated function	mRNA ratio	p
cg0143	*mtlD*	Mannitol-1-phosphate 5-dehydrogenase	9.74	0.00
cg1298	*cydC*	ABC-type transport system required for functional synthesis of cytochrome *bd* oxidase, ATPase and permease component	2.80	0.00
cg1299	*cydD*	ABC-type transport system required for functional synthesis of cytochrome *bd* oxidase, ATPase and permease component	5.76	0.00
cg1300	*cydB*	Cytochrome *bd* oxidase, subunit II	6.26	0.00
cg1301	*cydA*	Cytochrome *bd* oxidase, subunit I	6.62	0.00
cg1744	*pacL*	Cation-transporting ATPase	2.61	0.00
cg1881		Conserved protein of the DyP-type peroxidase family (PFAM PF04261) secreted via the Tat pathway	3.95	0.00
cg1883		Secreted copper(I)-binding lipoprotein, PCu_AC homolog	3.74	0.00
cg1884	*copC*	Membrane-bound copper(II)-binding protein C	4.12	0.00
cg2118	*fruR*	Transcriptional regulator of sugar metabolism	3.12	0.00
cg2699	*ctiP*	Copper transport and insertion protein	2.66	0.00
cg2836	*sucD*	Succinyl-CoA synthetase α subunit, ADP-forming	2.50	0.00
cg2837	*sucC*	Succinyl-CoA synthetase subunit β, ADP-forming	3.04	0.00
cg0693	*groEL*	60 kDa Chaperonin	0.38	0.00
cg0759	*prpD2*	2-Methycitrate dehydratase	0.31	0.00
cg0760	*prpB2*	2-Methylisocitrate lyase	0.31	0.00
cg0762	*prpC2*	2-Methylcitrate synthase	0.28	0.00
cg1290	*metE*	5-Methyltetrahydropteroyltriglutamate–homocysteine methyltransferase	0.29	0.00
cg2184	*oppD*	ABC-type peptide transport systems, ATPase component	0.25	0.00
cg2460	*surf1*	Assembly factor for cytochrome oxidase	0.06	0.00
cg2940	*siaI*	ABC-transporter for sialic acid	0.38	0.00
cg3141	*hmp*	Flavohemoprotein	0.33	0.00
cg3226		L-lactate permease	0.38	0.00
cg3395	*proP*	Proline/ectoine carrier	0.25	0.00

Table 3
Influence of the *surf1* deletion on the expression of the terminal oxidase genes *cydAB* and *ctaD*.[a]

Strain/reporter	pJC1-P*cydA-venus*		pJC1-P*ctaD-venus*	
	1.25 µM Cu	Cu deprivation	1.25 µM Cu	Cu deprivation
wt	1.00 ± 0.00	2.04 ± 0.08	1.00 ± 0.01	1.36 ± 0.06
Δ*surf1*	2.23 ± 0.01	2.08 ± 0.00	1.38 ± 0.02	1.36 ± 0.15

[a] The *C. glutamicum* strains with the reporter plasmids were grown in CGXII glucose medium either under standard copper conditions (1.25 µM $CuSO_4$) or under copper deprivation without added copper and supplemented with 150 µM BCS and 1 mM ascorbate. Expression of *cydA* was followed with the reporter plasmid pJC1-P*cydA-venus* (P_{cyd}) and expression of *ctaD* with the reporter plasmid pJC1-P*ctaD-venus* (P_{ctaD}). The strains were cultivated in FlowerPlates at 30 °C and 1200 rpm using the BioLector microcultivation system. Growth was followed by measuring the backscatter at 620 nm and Venus fluorescence was measured by excitation at 510 nm and emission at 532 nm. The specific fluorescence (ratio of absolute fluorescence and backscatter) was determined after 24 h of cultivation. Mean values and standard deviations of biological triplicates were normalized by setting the wt values cultivated under standard copper conditions for each promoter as 1.

the wt, which was not further increased in the two assembly mutants.

3.5. Conservation of Surf1 in Actinobacteria

As almost all aerobic *Actinobacteria* are expected to possess a cytochrome bc_1-aa_3 supercomplex with a diheme cytochrome c_1 [6], the assembly factors should also be conserved. In fact, Surf1 homologs were found to be present in all *Actinobacteria* with a diheme cytochrome c_1 (data not shown). In order to confirm the equivalent function of the Surf1 homologs of other *Actinobacteria*, the corresponding proteins of *Corynebacterium diphtheriae* DSM44123 (CDC7B_1688), of *Mycobacterium tuberculosis* H37Rv (Rv2235), and of *M. smegmatis* MC^2 155 (MSMEG_4311) were chosen, which show 53%, 44%, and 43% amino acid sequence identity to *C. glutamicum* Surf1 (Fig. S1). The corresponding genes were cloned into the expression plasmid pAN6 and then transferred into the *C. glutamicum* Δ*surf1* strain in order to test whether they can abolish the growth defect of this mutant. *C. glutamicum* Δ*surf1* with pAN6 served as negative control and the wt with pAN6 as positive control. Basal expression of the *C. diphtheriae surf1* gene in the absence of the inducer isopropyl-β-D-thiogalactoside (IPTG) allowed full complementation of the growth defect of the *C. glutamicum* Δ*surf1* mutant (Fig. 5A, Fig. S3, Table S3). In the case of the *surf1* homologs of *M. tuberculosis* and *M. smegmatis*, IPTG-induced expression allowed at least partial complementation (Fig. 5B). In addition to the growth experiments, we measured the TMPD oxidase activity of the Δ*surf1* strains expressing the heterologous *surf1* genes. As shown in Table S2, for all these strains activity was detectable, although the one observed with *M. tuberculosis* Surf1 was low. These results support an identical function of the Surf1 homologs in *Actinobacteria* in the assembly of the cytochrome bc_1-aa_3 supercomplex. Interestingly, in the phylum *Firmicutes*, only very few species were found to contain genes coding for putative Surf1 homologs (data not shown), suggesting that most *Firmicutes* species with a cytochrome aa_3 oxidase use other assembly proteins for heme *a* incorporation into subunit 1.

4. Conclusions

The results obtained in this study provide evidence for an involvement of Surf1 in the assembly of cytochrome aa_3 oxidase in *C. glutamium*. The presence of Surf1 homologs in other aerobic *Actinobacteria* and our complementation studies suggest a conserved function of these proteins in actinobacterial cytochrome aa_3 biogenesis. Several properties of the Δ*surf1* mutant of *C. glutamicum*, such as growth inhibition, altered cytochrome spectra, the inability to purify an intact cytochrome bc_1-aa_3 supercomplex, or the effects on global expression, were similar to those of the previously described Δ*ctiP* mutant lacking the copper transport and insertion protein CtiP. Considering that both copper and heme *a* insertion are essential for the formation of a functional cytochrome aa_3 oxidase, the similarities are not that surprising. However, the exact role and mode of action of Surf1 in heme *a* insertion and of CtiP in copper insertion into the CtaD apo-protein and the order of these processes have still to be clarified. Obviously, a correctly assembled, functionally active CtaD protein is a prerequisite for the formation of a stable cytochrome bc_1-aa_3 supercomplex. Preliminary studies by us indicated that CtaD formed in the Δ*ctiP* mutant is more sensitive to proteolytic degradation than intact CtaD, which agrees with the

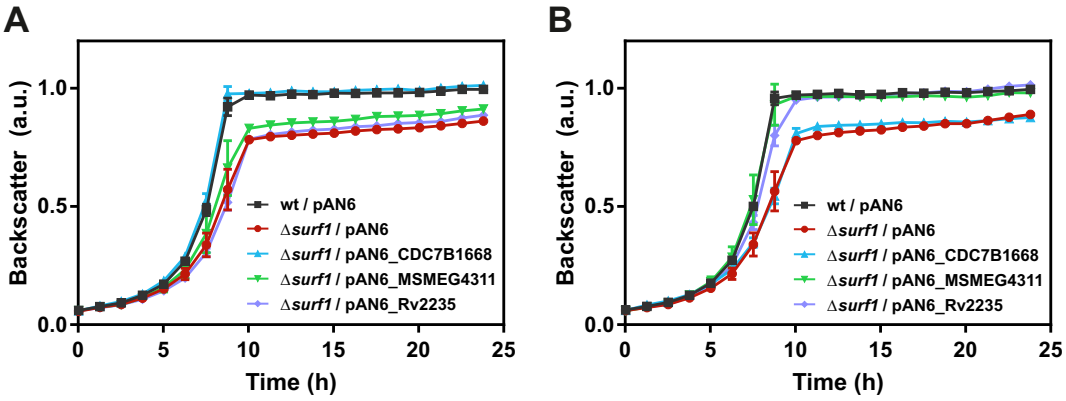

Fig. 5. Complementation of the growth defect of the *C. glutamicum* Δ*surf1* mutant with expression plasmids for genes encoding the Surf1 homologs of *Corynebacterium diphtheriae* (pAN6_CDC7B1688), *Mycobacterium smegmatis* (pAN6_MSMEG4311), and *Mycobacterium tuberculosis* (pAN6_Rv2235). The strains were cultivated in CGXII medium with 2% (w/v) glucose either in the absence of (A) or in the presence of 100 μM IPTG (B) at 30 °C and 1200 rpm using a BioLector microcultivation system (m2p-labs, Baesweiler, Germany). As reference, *C. glutamicum* wt and the Δ*surf1* mutant, both carrying the empty pAN6 plasmid, were used. Growth was followed as backscatter at 620 nm and the maximal value obtained for the wt was set as 1. Mean values and standard deviation from three biological replicates are shown. a.u., arbitrary units.

observation that the amount of $CtaD_{St}$ that can be purified from the Δ*ctiP* and Δ*surf1* mutants is much lower than from strains containing CtiP and Surf1. An open question is how the cell recognizes a defective CtaD protein and responds with an altered expression of genes, including those encoding the alternative cytochrome *bd* oxidase. Further studies are underway to tackle this question.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2019.06.005.

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