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Cooperation between two periplasmic copper chaperones is required for full activity of the *cbb*$_3$-type cytochrome *c* oxidase and copper homeostasis in *Rhodobacter capsulatus*

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Summary

Copper (Cu) is an essential micronutrient that functions as a cofactor in several important enzymes, like respiratory heme-copper oxygen reductases. Yet, Cu is also toxic and therefore cells engage a highly coordinated Cu uptake and delivery system to prevent the accumulation of toxic Cu concentrations. In the current work we analyzed Cu delivery to the *cbb*$_3$-type cytochrome *c* oxidase (*cbb*$_3$-Cox) of *Rhodobacter capsulatus.* We identified the PCu$_A$C-like periplasmic chaperone PccA and analyzed its contribution to *cbb*$_3$-Cox assembly. Our data demonstrate that PccA is a Cu-binding protein with a preference for Cu(I), which is required for efficient *cbb*$_3$-Cox assembly, in particular at low Cu concentrations. By using *in vivo* and *in vitro* crosslinking we show that PccA forms a complex with the Sco1-homologue SenC. This complex is stabilized in the absence of the *cbb*$_3$-Cox specific assembly factors CcoGHIS. In cells lacking SenC, the cytoplasmic Cu content is significantly increased, but the simultaneous absence of PccA prevents this Cu accumulation. These data demonstrate that the interplay between PccA and SenC is not only required for Cu delivery during *cbb*$_3$-Cox assembly, but that it also regulates Cu homeostasis in *R. capsulatus.*

Graphical abstract

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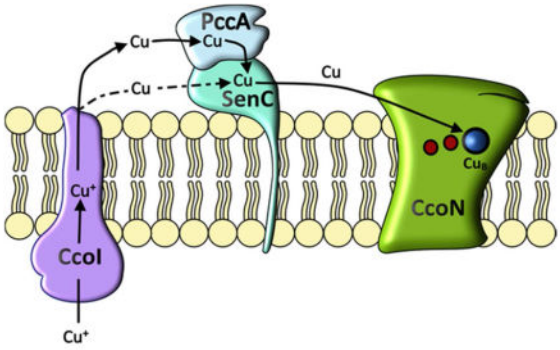

Keywords

cbb$_3$-type cytochrome *c* oxidase biogenesis; periplasmic copper chaperones; copper homeostasis; respiration; *Rhodobacter capsulatus*

Introduction

Cytochrome *c* oxidases (Cox) are ubiquitous multi-subunit enzyme complexes that terminate aerobic respiratory chains in prokaryotic and eukaryotic cells. They are members of the heme copper oxidase (HCO) superfamily and are characterized by the presence of a membrane integral catalytic subunit I, which contains a low-spin heme and a high-spin heme-Cu_B binuclear center (Ekici *et al.*, 2012a, Richter & Ludwig, 2003). Cox enzymes are generally classified into three groups: type A includes the mitochondrial-like *aa*$_3$-Cox, type B the *ba*$_3$-Cox and type C, the *cbb*$_3$-Cox, which is the most distant member of the HCO superfamily and present only in bacteria (Brzezinski & Gennis, 2008). Significant diversity in terms of subunit composition, co-factor content and proton transfer pathways is observed between these enzymes, yet their subunit I shows substantial amino acid sequence conservation.

The differences in subunit composition and co-factor content among the different Cox are also reflected in their assembly processes and their requirements for distinct assembly factors and chaperones. This is particularly evident for the process of copper delivery and insertion into Cox. The *aa*$_3$-Cox and some *ba*$_3$-Cox contain two redox-active copper centers: in addition to the membrane-embedded Cu_B center in the catalytic subunit I, they also have a binuclear Cu_A center in the surface-exposed and hydrophilic domain of subunit II (Brzezinski & Gennis, 2008). This Cu_A center is required for accepting electrons from electron donors, such as cytochromes, located in the periplasm of bacteria or in the inter-membrane space of mitochondria (Witt *et al.*, 1995). The delivery and insertion of Cu into both the Cu_A and Cu_B centers have been investigated in both bacteria and mitochondria, and seem to follow distinct pathways. The formation of the Cu_B center in *aa*$_3$-Cox and *ba*$_3$-Cox relies on the Cox11 protein, which is also termed CoxG or CtaG in some bacteria (Carr *et al.*, 2002, Carr *et al.*, 2005, Buhler *et al.*, 2010, Gurumoorthy & Ludwig, 2015). Cox11 has a periplasmically exposed Cu binding domain, and binds two Cu(I) atoms at the dimer interface of two Cox11 subunits (Carr *et al.*, 2002, Banci *et al.*, 2004). A direct interaction

between the *Paracoccus (P.) denitrificans* Cox11 homologue CtaG and the aa_3-Cox subunits I and II was recently shown (Gurumoorthy & Ludwig, 2015). In mitochondria, Cox11 receives Cu from the small soluble copper chaperone Cox17 (Horng *et al.*, 2004), but it is currently unknown whether copper loading onto Cox11 in bacteria requires additional proteins. The formation of the Cu_A center on the other hand requires the coordinated function of the Sco1 and PCu_AC proteins (Banci *et al.*, 2005). Sco1, also called SenC (Buggy & Bauer, 1995) or PrrC (Eraso & Kaplan, 2000, McEwan *et al.*, 2002) in some bacteria, is a single-spanning membrane protein, which contains a thioredoxin-like domain that protrudes into the periplasm (Balatri *et al.*, 2003). Sco1 binds either Cu(I) or Cu(II) via two cysteine residues, and Sco1 mutants are impaired in Cox assembly (Balatri *et al.*, 2003). The PCu_AC copper chaperones are located in the bacterial periplasm, but are absent in mitochondria (Banci *et al.*, 2010a). PCu_AC adopts a cupredoxin-like fold, and probably coordinates Cu(I) via two methionine and two histidine residues (Banci *et al.*, 2005). *In vitro* data show that PCu_AC inserts copper into the Cu_A site of subunit II of the *Thermus thermophilus* ba_3-Cox (Abriata *et al.*, 2008).

In the case of aa_3-Cox and ba_3-Cox, available data suggest Cox11 dependent formation of the Cu_B center and Sco1/PCu_AC-dependent formation of the Cu_A center. However, the formation of the Cu_B center appears to follow a different pathway in the cbb_3-Cox, which lacks the Cu_A center (Gray *et al.*, 1994, Ekici *et al.*, 2012a). The cbb_3-Cox consists of three to four subunits: CcoN is the catalytic subunit, and is homologous to subunit I of other members of the HCO superfamily. It contains a Cu_B-heme *b* binuclear center and a low-spin heme *b* (Gray *et al.*, 1994). Importantly, instead of a Cu_A containing subunit, cbb_3-Cox contains two membrane-bound *c*-type cytochrome subunits, CcoO and CcoP (Gray *et al.*, 1994). Electrons are probably transferred from the di-heme cytochrome CcoP via the mono-heme cytochrome CcoO to CcoN (Buschmann *et al.*, 2010). The fourth subunit, CcoQ, is a small membrane protein without any known cofactor and is required for the stability of cbb_3-Cox (Peters *et al.*, 2008). In addition, at least in *Rhodobacter (R.) capsulatus*, the assembly factor CcoH remains stably associated with cbb_3-Cox (Pawlik *et al.*, 2010).

Copper delivery to cbb_3-Cox was mainly studied in *Bradyrhizobium (B.) japonicum* and in *Rhodobacter* species. In particular, *R. capsulatus* is an attractive model organism for studying cbb_3-Cox assembly, because in this organism cbb_3-Cox is the only Cox present (Marrs & Gest, 1973, Gray *et al.*, 1994), while many other species (*e.g. B. japonicum* or *R. sphaeroides)* contain several Cox enzymes (Bott *et al.*, 1992, Hosler, 2012). Unlike in mitochondria, Cox11 seems not always required for Cu_B assembly of cbb_3-Cox, as *R. capsulatus* lacks a Cox11 homologue, and cbb_3-Cox assembly studies in *B. japonicum* and *R. sphaeroides* support Cox11-independent Cu_B insertion into cbb_3-Cox (Buhler *et al.*, 2010, Thompson *et al.*, 2010). Copper trafficking to *R. capsulatus* cbb_3-Cox is a highly complex and coordinated process. Copper uptake is mediated by CcoA, a member of the major-facilitator superfamily of transporters (Ekici *et al.*, 2012b). The intracellular copper homeostasis is controlled by the antagonistic activities of CcoA and the P_{1B}-type ATPase CopA, which exports excess Cu (Ekici *et al.*, 2014). A second P_{1B}-type-type ATPase, CcoI, is absolutely required for cbb_3-Cox assembly and appears to export Cu from the cytoplasm to the periplasm for insertion into the Cu_B center of this enzyme (Koch *et al.*, 2000, Preisig *et al.*, 1996). Moreover, under low Cu concentrations, the Sco1 homologue SenC is required

for *cbb*$_3$-Cox assembly (Lohmeyer *et al.*, 2012), and it was suggested that CcoI and SenC could cooperate in the assembly of the Cu_B center of *cbb*$_3$-Cox (Thompson *et al.*, 2012). Sco1 homologues were furthermore suggested to cooperate with PCu_AC-type copper chaperones during Cu_B center assembly of *cbb*$_3$-Cox (Serventi *et al.*, 2012, Thompson *et al.*, 2012). However, the individual effects of SenC/ScoI and PCu_AC on *cbb*$_3$-Cox assembly and on cellular copper distribution/availability are largely unexplored.

In the current study, the *R. capsulatus* PCu_AC homologue PccA was identified. The data showed that it is indeed a copper binding protein and required for *cbb*$_3$-Cox assembly and steady-state activity. The phenotype of a Δ*pccA* strain on *cbb*$_3$-Cox assembly was less pronounced than that of a Δs*enC* strain, even though both single mutants and a Δ*pccA*/Δ*senC* double mutant were rescued by copper supplementation. This indicated that both proteins are required for *cbb*$_3$-Cox assembly under conditions of low copper availability. Analyses of the cellular copper content and distribution showed an increased intracellular Cu content in the Δ*senC* strain, while the Cu content in the Δ*pccA* strain remained similar to that of a wild type. Finally, by using chemical crosslinking, we provide evidence for the formation of a SenC-PccA complex during *cbb*$_3$-Cox assembly.

Results

The periplasmic chaperone PccA is required for cytochrome cbb$_3$-Cox activity

Recent data have demonstrated that the Sco1 homologue SenC is required for *cbb*$_3$-Cox assembly in *R. capsulatus* (Lohmeyer *et al.*, 2012). As Sco1 proteins have been shown to cooperate with PCu_AC-like copper chaperones during Cox assembly (Thompson *et al.*, 2012), the genome of *R. capsulatus* was searched for PCu_AC-homologues and a putative ORF (Rcc0246), encoding for a hypothetical protein with a predicted signal-sequence (amino acids (aa) 1–23) and a potential signal sequence cleavage site between positions 23–24 (Fig. 1A). The candidate protein showed strong homology to PCu_AC proteins from other species (Fig. 1A) and contained the '$HX_{10}MX_{21}HXM$' motif, which is typical of PCu_AC-like chaperones (Abriata *et al.*, 2008). As *R. capsulatus* contains only cytochrome *cbb*$_3$-Cox, which lacks a Cu_A center, we termed the protein PccA (for ***p****eriplasmic* ***c****opper* ***c****haperone* ***A***) rather than PCu_AC. In the *R. capsulatus* genome, *PccA* is located in a cluster of several genes associated with amino acid metabolism (Fig. 1B), while in *B. japonicum*, the gene for the PCu_AC-like chaperone PcuC is located in the *pcuABCDE* operon, which is transcribed in response to copper limitation (Serventi *et al.*, 2012). Yet another genetic organization of PCu_AC is observed in *R. sphaeroides*, where the PCu_AC-gene (RSP_2017) is sandwiched between the gene encoding the 50S ribosomal protein L28 (RSP_2016) and a gene encoding a 102 amino acids long hypothetical protein (RSP_2018) of no significant homology to known proteins. It therefore appears that there is no synteny of PCu_AC-encoding genes and those surrounding it among different bacterial genomes.

For analyzing the function of PccA in *R. capsulatus*, a strain carrying a chromosomal deletion of *PccA* was constructed. Immune detection using polyclonal α-PccA antibodies detected a band of approx. 17 kDa in MT1131 wild type cells, but not in the Δ*pccA* strain (Fig. 1C). When a His-tagged PccA derivative was expressed in the Δ*pccA* strain, the PccA band was again detected but it migrated slightly slower due to the presence of the His-tag.

Since the PccA homologues presumably function as Cu chaperones, it was analyzed whether *pccA* expression was affected by addition of Cu. Cells were grown on MPYE medium, which contains approx. 250 nM Cu (Koch *et al.*, 2000) and on MPYE medium supplemented with an additional 10 μM $CuSO_4$. However, the level of PccA was not significantly affected by the addition of Cu (Fig. 1C), indicating that *pccA* expression does not respond to Cu-supplementation.

Next, cbb_3-Cox activity and stability was analyzed in the Δ*pccA* strain. Oxygen-uptake measurements with intracytoplasmic membranes of the deletion strain revealed an approx. 40% reduction of cbb_3-Cox activity (Fig. 2A) compared to the wild type. Intriguingly, when the Δ*pccA* strain was grown on Cu-supplemented media (+10 μM $CuSO_4$), its intracytoplasmic membranes displayed only a slightly reduced cbb_3-Cox activity (Fig. 2A). Cu supplementation of wild type cells did not significantly influence their cbb_3-Cox activity in membranes.

For correlating the reduced activity of cbb_3-Cox in the Δ*pccA* strain with the steady state amounts of cbb_3-Cox, heme staining and western blotting was performed. Heme staining, which detects the two membrane bound *c*-type cytochrome subunits of cbb_3-Cox, CcoP and CcoO, showed decreased amounts of both subunits (Fig. 2B, upper left panel), but also a slight decrease in the cytochrome c_1 subunit of the cytochrome bc_1 complex. These phenotypes were restored when cells were grown in the presence of 10 μM $CuSO_4$ (Fig. 2B, upper left panel). The steady state presence of the catalytic subunit CcoN was also reduced in the Δ*pccA* strain, and restored when cells were grown on Cu-supplemented media (Fig. 2B, lower left panel). A Coomassie-stained SDS-PAGE of the samples served as loading control (Fig. 2B, right panel). Finally, cbb_3-Cox assembly was analyzed by BN-PAGE and western blot analyses. In *R. capsulatus*, cbb_3-Cox assembles into an active 230 kDa complex, and in wild type membranes, formation of this complex was independent of Cu supplementation (Fig. 2C). A Cu-dependent cbb_3-Cox assembly has been observed previously in a Δs*enC* deletion strain. The Sco1-homologue SenC is a single spanning membrane protein with a periplasmically exposed Cu binding motif, which is required for cbb_3-Cox activity (Lohmeyer *et al.*, 2012). In agreement with previous data, no 230 kDa cbb_3-Cox complex was detectable in Δ*senC* membranes, unless the cells were grown in presence of supplementary Cu (Fig. 2C). In Δ*pccA* membranes, the 230 kDa complex was significantly reduced but still weakly detectable (Fig. 2C). In membranes of the Δ*pccA* strain grown on Cu supplemented media, the amount of the 230 kDa cbb_3-Cox complex almost reached wild type level (Fig. 2C). Finally, when the Δ*pccA* strain carried a copy of *pccA* on a low-copy number plasmid (pRK415), the 230 kDa complex formation was restored independently of Cu supplementation during growth (Fig. 2C).

In summary, these data indicate that PccA is required in particular for cbb_3-Cox assembly or stability under low Cu concentrations, which is in agreement with data in *R. sphaeroides* (Thompson *et al.*, 2012) or *B. japonicum* (Serventi *et al.*, 2012, Buhler *et al.*, 2010). However, the lack of PccA appears to cause a less severe cbb_3-Cox defect than the lack of SenC, which suggests that the function of PccA can be partially bypassed even in the absence of supplementary Cu.

PccA is a copper binding protein

PccA contains the signature motif of Cu-binding PCu_AC chaperones and Cu binding to PccA was analyzed by using radioactive ^{64}Cu. A signal sequence-less variant of PccA containing a C-terminal Strep-tag was expressed in *E. coli* and purified. Purified PccA was incubated with ^{64}Cu at different molar ratios, separated on native PAGE and analyzed by phospho-imaging. At a molar protein:Cu ratio of 1:5 and 1:10, two radioactive bands were observed, indicating Cu binding to PccA (Fig. 3A). The band with the lower molecular mass corresponds to PccA lacking the Strep-tag, as it was not recognized by the α-Strep antibody (data not shown). As a control, ^{64}Cu binding to the *R. capsulatus* Sco1 homologue SenC was also analyzed, for which copper binding has previously been shown (Lohmeyer *et al.*, 2012) (Fig. 3B). Similar to PccA, a SenC variant lacking its N-terminal transmembrane domain was expressed in *E. coli* and purified. The Cu binding motif in SenC includes two conserved cysteine residues, which are required for Cu binding (Lohmeyer *et al.*, 2012). We therefore reasoned that for *in vitro* Cu binding, SenC probably had to be in its reduced form. Purified SenC was incubated with ^{64}Cu with and without prior reduction using Tris(2-carboxyethyl)phosphine (TCEP). Without prior reduction, no significant ^{64}Cu binding to SenC was observed. In contrast, after reduction, a Cu concentration dependent binding of ^{64}Cu to SenC was detected (Fig. 3B).

^{64}Cu binding to PccA and SenC was further quantified using a scintillation counter. Purified proteins were incubated with ^{64}Cu, re-purified via their Strep-tag, and the radioactivity in the samples was measured. For both PccA and SenC, a Cu concentration-dependent increase of the radioactive signal was observed (Fig. 3C), whereas ^{64}Cu-binding to a mutant variant of PccA, lacking the two methionine residues of its '$HX_{10}MX_{21}HXM$' motif ($PccA_{Mut}$), was insignificant (Fig. 3C). These data showed that PccA is a Cu-binding protein, which binds Cu via the canonical '$HX_{10}MX_{21}HXM$' motif of PCu_AC-type chaperones. These data also support a specific binding of ^{64}Cu to SenC and PccA, and exclude significant non-specific Cu binding to these proteins.

Cu displays two oxidation states, and the reduced Cu(I) form appears to be the preferred substrate for P_{1B}-type Cu-exporting ATPases (Gonzalez-Guerrero & Arguello, 2008) and for PCu_AC-type chaperones (Banci *et al.*, 2005). We determined binding of Cu(I) or Cu(II) to PccA by following the intrinsic protein fluorescence of PccA. The aromatic amino acids tryptophan, tyrosine and phenylalanine display intrinsic fluorescence, and changes in their fluorescence emission have been used to monitor conformational changes or ligand binding to proteins. PccA lacks tryptophan but contains a single tyrosine residue at position 51 (Fig. 1A), which in the NMR structure of *D. radiodurans* PCu_AC is located close to the Cu binding 'HxM' motif (Banci *et al.*, 2005), and is expected to respond to Cu binding. When excited at 276 nm, purified PccA displayed an emission maximum at approx. 310 nm (Fig. 4A). For analyzing Cu binding to PccA, purified protein was incubated in an anaerobic chamber with either Cu(I) or Cu(II). The emission was readily quenched by the addition of Cu(I) (Fig. 4A), indicative for changes in the local environment of the tyrosine residue. Quenching was also observed when Cu(II) was added, but the effect was weaker than in the presence of Cu(I) (Fig. 4A). Quantification of several independent experiments revealed that Cu(II) reduced emission by approx. 30%, while Cu(I) reduced it by approx. 60% (Fig. 4B).

As control, Cu binding to $PccA_{Mut}$ was also analyzed, but neither Cu(I) nor Cu(II) significantly reduced fluorescence emission of the mutant protein. The specificity of Cu(I) binding to PccA was further verified by analyzing tyrosine fluorescence in the presence of different metals and at different metal concentrations. When purified PccA was incubated under anaerobic conditions with increasing concentrations of Cu(I), we observed a rapid decrease in fluorescence (Fig. 4C). Cu(II) also quenched fluorescence, however only at higher concentrations as compared to Cu(I). No significant quenching was observed for Pcc_{Mut}. Fe^{2+} had also only a weak quenching effect and Mn^{2+} did not quench fluorescence of PccA (Fig. 4B). In summary, our data demonstrate that the *R. capsulatus* PCu_AC homologue PccA is a Cu binding protein, possibly with a preference for Cu(I).

PccA interacts with SenC in vivo and in vitro

PCu_AC-like chaperones were suggested to interact with the SenC-homologue ScoI during the assembly of the Cu_A center of aa_3-Cox (Banci *et al.*, 2010b, Banci *et al.*, 2005, Thompson *et al.*, 2012, Serventi *et al.*, 2012). Thus, both *in vitro* and *in vivo* chemical crosslinking approaches were used to probe whether PccA and SenC interact during Cu transfer to cbb_3-Cox. In the first approach, the cysteine-specific cross-linker BMH was used. SenC contains two cysteine residues within its copper binding motif, but PccA has only one cysteine residue at its position 10, which is within the predicted cleaved signal sequence. Therefore, a PccA derivative (PccA-4C) containing four cysteine residues at positions 76, 93, 109 and 117, respectively was constructed (Fig. 1A). These residues are very close to the putative Cu-binding motif of PccA. PccA-4C was expressed as a Strep-tagged protein in *E. coli* and purified. Spheroplasts derived from wild type *R. capsulatus* cells (MT1131), expressing *senC* from a low copy plasmid, were incubated with purified PccA-4C in the presence or absence of BMH. PccA-4C and potential crosslinked proteins were subsequently purified and separated on SDS-PAGE followed by western transfer using α-Pcc1 antibodies. As a control, native PccA lacking these cysteine residues was also incubated with MT1131 spheroplasts. In the presence of PccA-4C and the crosslinker BMH, a strong band at approx. 43 kDa, was observed, which was only weakly detectable in the absence of BMH (Fig. 5A). Furthermore, this band was not detected when native PccA lacking cysteine residues was incubated with MT1131 spheroplasts in the presence of BMH (Fig. 5A). Unlike the native PccA, PccA-4C migrated on SDS-PAGE as multiple bands at approx. 25 kDa and 16 kDa (Fig. 5A), which could reflect intramolecular disulfide-bridges or disulfide-bridge linked dimers, but this was not further analyzed.

The crosslinked product of approx. 43 KDa would be consistent with a crosslink between PccA (16 kDa) and SenC (25 kDa). In support of this, the crosslink product was also detected by using α-SenC antibodies. However, the α-SenC antibodies also cross-reacted with purified PccA (Fig. 5A). To validate that the 43 kDa band represents a SenC-PccA complex, PccA-4C was incubated with spheroplasts derived from the Δ*senC* strain. Here, α-SenC antibodies did not recognize the 43 kDa band, indicating that the 43 kDa crosslinked product corresponded to a PccA-SenC complex (Fig. 5A, Pcc1-SenC XL (crosslink)).

The BMH crosslinking was further controlled by incubating either PccA-4C or native PccA with spheroplasts from cells expressing a SenC variant with a mutated Cu-binding motif

(SenCΔC), in which cysteine residues 83 and 89 were replaced by serine). Although the crosslinking product was still weakly detectable, probably due to the presence of additional cysteine residues within SenC, it was significantly weaker than with spheroplasts expressing wild type SenC (Fig. 5B). Incubating either SenC or SenCΔC spheroplasts with wild type PccA did not result in any specific crosslinked products.

A periplasmic PccA-SenC complex could potentially link Cu-export via CcoI with the subsequent Cu insertion into the catalytic CcoN subunit of cbb_3-Cox. Thus, the PccA-SenC interaction was analyzed in strain CW1 (Δ*ccoGHIS*), which lacks the CcoGHIS proteins required for cbb_3-Cox assembly. The PccA-SenC crosslink product was also detected in the absence of the CcoGHIS proteins and present even in slightly higher amounts than in wild type cells (Fig. 5B).

As the BMH crosslinking experiments used a modified derivative of PccA (PccA-4C), it could be argued that the observed interaction between PccA and SenC might be due to the non-native cysteine residues added to PccA. To address this possibility, the crosslinking experiment was repeated in living cells using para-formaldehyde (PFA). Wild type cells and wild type cells expressing PccA with a C-terminal His-tag were incubated with PFA, and PccA was subsequently purified via metal affinity chromatography. Purified PccA and potential crosslinked products were then separated on SDS-PAGE and analyzed using α-PccA and α-SenC antibodies. Both antibodies recognized again a band at approx. 43 kDa in the PFA treated sample, which was not observed in the absence of PFA or in cells not expressing $PccA_{His}$ (Fig. 6). This provides *in vivo* verification for the existence of a PccA-SenC complex.

The increased crosslinking between PccA and SenC in the Δ*ccoGHIS* strain was also observed with PFA *in vivo* (Fig. 6). In the absence of *ccoGHIS*, cbb_3-Cox is not assembled, raising the possibility that the increased interaction between SenC and PccA could result from the absence of cbb_3-Cox. This was analyzed by testing the PccA-SenC interaction in the cbb_3-Cox deletion strain GK32 (Δ*ccoNO*). The PccA-SenC crosslinking product was observed in GK32 cells, but the crosslink was not stronger than in wild type cells (Fig. 6). In summary, the crosslink data show that PccA and SenC form a complex of 43 kDa. The data also suggested an increased interaction between SenC and PccA in the absence of the CcoGHIS proteins. However, considering that the *senC* mRNA and protein levels also increase in a Δ*ccoI* strain (Lohmeyer *et al.*, 2012) (*c.f.* Fig. 6), we cannot completely exclude that this increased crosslinking results from increased levels of SenC.

A SenC-PccA double knock-out lacks cbb_3-Cox

The cbb_3-Cox defect seen in both the Δ*pccA* and the Δ*senC* strain is rescued by increasing copper concentrations in the growth medium. This finding suggested that either the delivery of Cu to cbb_3-Cox via the SenC-PccA pathway is completely bypassed at high Cu concentrations, or that either SenC or PccA is sufficient to deliver Cu to cbb_3-Cox at high Cu concentrations. For differentiating between these two possibilities, a *senC-pccA* double knock-out strain was constructed. cbb_3-Cox activity assays and BN-PAGE analyses revealed that the phenotype of the Δ*senC*-Δ*pccA* strain was identical to that of the Δ*senC* single deletion mutant (Fig. 7A & 7B); *i.e.* basically no cbb_3-Cox activity was detectable and the

230 kDa cbb_3-Cox complex was absent, unless the cells were grown in Cu-supplemented media. Thus, the SenC-PccA copper delivery pathway to cbb_3-Cox can be bypassed by a currently unknown SenC/PccA-independent pathway when cells are grown in the presence of high Cu concentrations. These data furthermore indicate that SenC and PccA cannot substitute for each other at low Cu concentrations, rationalizing why over-expression of either PccA or SenC is unable to rescue the cbb_3-Cox deficiency of the Δ*senC or* Δ*pccA* strains, respectively (Lohmeyer *et al.*, 2012).

Differential effects of PccA and SenC on copper homeostasis in Rhodobacter capsulatus

As the addition of Cu rescued the cbb_3-Cox defect seen in the Δ*pccA,* the Δ*senC* and the Δ*senC*-Δ*pccA* strains, the total Cu content and its distribution between the cytoplasm and the periplasm was examined in these mutants. The Cu concentrations were determinend by atomic absorption spectroscopy using the manganese and the zink concentration as internal references.

In whole cells grown on MPYE medium without Cu supplementation, the total Cu content of all three mutants was comparable to the Cu content of wild type cells (Fig. 8A, black bars). For testing whether the Cu distribution between the cytoplasm and the periplasm was changed, the Cu content in spheroplasts, which reflects the cytoplasmic Cu pool (*i.e.* the Cu content in the cytosol and in the cytoplasmic membrane), and the Cu content in the periplasm were determined. In wild type cells and in all three mutants, most of the copper was present in the spheroplast fraction (Fig. 8A, grey bars) and only a small amount was found in the periplasmic fraction (Fig. 8A, white bars). Thus, the cbb_3-Cox defect observed in the Δ*pccA,* the Δ*senC* and the Δ*senC*-Δ*pccA* strains cannot be attributed to a decrease of the total cellular Cu concentration or to a different Cu distribution between cytoplasm and periplasm.

The Cu content and the Cu distribution of cells grown in Cu-supplemented media were also analyzed. Surprisingly, the total and the cytoplasmic Cu content of the Δ*senC* cells was signficantly increased, as compared to wild type cells or the Δ*pccA* cells. This enhanced Cu accumulation suggested that either Cu import is increased in the absence of SenC, or Cu export is diminished. An increased Cu content was not observed in Δ*senC*-Δ*pccA* cells (Fig. 8A), which indicates that the increased Cu content seen in the Δ*senC* cells requires the presence of PccA. These data point to a so far unrecognized role of both proteins in regulating Cu homeostasis in response to high Cu concentrations.

So far, Cu uptake via the MFS-type transporter CcoA is the only known Cu uptake system in *R. capsulatus* (Ekici *et al.*, 2012b). Hence, it was analyzed whether the increased cellular Cu content in the Δ*senC* strain was caused by an increased expression of *ccoA.* CcoA was fused to the alkaline phosphatase (PhoA)-moiety and used as translational fusion protein to determine the expression of CcoA by alkaline phosphatase activity. As a control, the intrinsic PhoA activity in two wild type strains, MT1131 and SB1003, was determined, which revealed no significant background activity. PhoA activities in MT1131 and in the Δ*senC,* Δ*pccA* and Δ*senC*-Δ*pccA* strains expressing the CcoA-PhoA-fusion were comparable, and largely independent of Cu supplementation to the growth media (Fig. 8B). This demonstrated that the increased Cu content seen in the Δ*senC* strain is not caused by

enhanced expression of the Cu-importer CcoA, although an enhanced import activity of CcoA in the absence of SenC or a CcoA-independent Cu import at high Cu availability cannot be excluded. In any event, our data demonstrate that periplasmic Cu-chaperones are not only required for providing Cu for *cbb*$_3$-Cox assembly, but they are also crucial determinants for maintaining cellular Cu homeostasis.

Discussion

The acquisition of Cu is a crucial step during the biogenesis of heme copper oxidases and both eukaryotic and prokaryotic cells have developed sophisticated pathways for uptake and delivery of this potentially toxic metal (Arguello *et al.*, 2012, Arguello *et al.*, 2013). In bacteria, periplasmic Cu chaperones of the PCu$_A$C family and the Sco1-family have been suggested to cooperate during the assembly of the Cu$_A$ centers of *aa*$_3$-Cox and *ba*$_3$-Cox (Banci *et al.*, 2007, Khalimonchuk *et al.*, 2010, Rigby *et al.*, 2008, Abriata *et al.*, 2008, Banci *et al.*, 2006). This cooperation also appears to be required for the assembly of the Cu$_B$ center of *cbb*$_3$-Cox (Thompson *et al.*, 2012), even though the latter is deeply buried within the membrane, while the Cu$_A$ center is exposed to the periplasm. Cu transfer between PCu$_A$C and Sco1 is likely to require a physical interaction between both proteins, but so far a direct interaction between PCu$_A$C-like and ScoI-like proteins has not been demonstrated in any organism. This was achieved in the present study by using *in vitro* and *in vivo* cross-linking. Complex formation between the PCu$_A$C homologue PccA and the Sco1 homologue SenC was observed in wild type *R. capsulatus* cells. Importantly, the detected amounts of this complex increased in the absence of the assembly proteins CcoGHIS (Koch *et al.*, 2000, Kulajta *et al.*, 2006). Within the *ccoGHIS* gene cluster, *ccoI* codes for a putative Cu-transporting P-type ATPase that is absolutely essential for *cbb*$_3$-Cox assembly (Koch *et al.*, 2000). CcoI is thought to export Cu(I) from the cytoplasm to the periplasm (Gonzalez-Guerrero *et al.*, 2010, Raimunda *et al.*, 2011) and its deletion cannot be bypassed by increasing the Cu concentrations in the growth medium (Koch *et al.*, 2000). A functional link between CcoI and SenC is suggested by the observation that SenC is up-regulated in the absence of CcoI (Lohmeyer *et al.*, 2012). This is also reflected by increased co-purification of SenC with PccA when PFA-crosslinking is performed in the absence of *ccoGHIS* (Fig. 6). Up-regulation of periplasmic Cu-binding proteins when Cu-transport to the periplasm is impaired has also been observed in *P. aeruginosa* (Raimunda *et al.*, 2013) and might be a general strategy for coping with perturbed Cu transport. The increased SenC levels could explain why more of the SenC-PccA complex is observed in the absence of *ccoGHIS*. However, SenC appears to be present in *R. capsulatus* cells at a higher concentration than PccA (data not shown), suggesting that SenC is unlikely to be limiting for complex formation. It rather seems that the stability of the SenC-PccA complex is increased when CcoI-dependent Cu delivery to the periplasm is impaired. In any event, the available data likely position the SenC-PccA complex between CcoI and *cbb*$_3$-Cox during Cu delivery to CcoN (Fig. 9). It is conceivable that PccA might also convey Cu to other periplasmic proteins; however, this is currently unknown.

Interestingly, in contrast to the Δ*senC* strain, which does not assemble *cbb*$_3$-Cox, partial assembly occurs in the absence of PccA, indicating that the PccA function can be partially bypassed at low Cu concentrations. This observation is not related to differences in the total

cellular Cu pool, or to differences in the Cu distribution between cytoplasm and periplasm, because we did not observe significant changes in both mutants compared to wild type cells. It rather argues that SenC can deliver Cu to cbb_3-Cox even in the absence of PccA. In *R. sphaeroides* and *B. japonicum* it was suggested that the PccA-homologues work upstream of the SenC-homologues during Cu_B center assembly (Serventi *et al.*, 2012, Thompson *et al.*, 2012). This would be in line with the hypothesis that PCu_AC-like chaperones in bacteria are functionally related to mitochondrial Cox17 and donate Cu to either Sco1 or to Cox11 (Banci *et al.*, 2010a). PccA might prevent re-oxidation of Cu(I) to Cu(II) and accelerate Cu(I) loading onto SenC, which then delivers it to cbb_3-Cox. This assumption is supported by the observation that both proteins have a preference for Cu(I) and the observation of a direct interaction between SenC and cbb_3-Cox (Lohmeyer *et al.*, 2012) (Fig. 9).

The cbb_3-Cox defect of the Δ*senC* and the Δ*pccA* strains is rescued by Cu supplementation to the medium, which was previously also observed in *B. japonicum* and *R. sphaeroides* (Serventi *et al.*, 2012, Thompson *et al.*, 2012). The dispensability of PccA and SenC at high Cu concentrations could indicate that under these conditions Cu insertion into CcoN occurs unassisted directly from the periplasmic Cu pool. However, this appears unlikely for two reasons: (1) Cu supplementation primarily increases the cytoplasmic Cu pool, while the Cu concentrations in the periplasm of the Δ*senC* and the Δ*pccA* strains remain rather low and are not higher than in wild type cells. (2) The cbb_3-Cox defect in the Δ*ccoI* mutant is not rescued by supplementary Cu (Koch *et al.*, 2000, Ekici *et al.*, 2014), suggesting that Cu transport from the cytoplasm to the periplasm is an essential step during Cu delivery to cbb_3-Cox.

Unexpectedly, Cu supplementation increases the cytoplasmic Cu pool in the Δ*senC* strain more than in the wild type, the Δ*pccA* or the Δ*senC*-Δ*pccA* double knock-out strains. This indicates that SenC is not only required for cbb_3-Cox, but also regulates Cu homeostasis. A regulatory role of Sco1 and Sco2 on Cu homeostasis has already been proposed for humans (Leary, 2010, Leary *et al.*, 2007). Here, Sco1 transfers Cu(I) to the Cu_A site of CoxII, while Sco2 works as Cu-dependent thiol reductase for the cysteine ligands of CoxII (Morgada *et al.*, 2015). Conceptually, the absence of SenC could either stimulate Cu import or reduce Cu export. *R. capsulatus* imports Cu primarily via the MFS-type Cu-importer CcoA (Ekici *et al.*, 2014, Ekici *et al.*, 2012b), but we did not find indications that CcoA expression is increased in the absence of SenC. A very slow CcoA-independent Cu uptake by an unknown protein is also detectable in *R. capsulatus* cells (Ekici *et al.*, 2014), and we can currently not exclude that this CcoA-independent uptake system is stimulated by the absence of SenC. Cu export in *R. capsulatus* is mediated by the P_{1B}-type ATPases CopA and CcoI (Ekici *et al.*, 2014, Koch *et al.*, 2000) and their deletion leads to a weak (*ccoI*, (Lohmeyer *et al.*, 2012)) or strong (*copA* (Ekici *et al.*, 2014)) increase in the cellular Cu concentration, which reflects their different Cu export kinetics (Raimunda *et al.*, 2011, Gonzalez-Guerrero *et al.*, 2010). Thus, Cu accumulation is observed in the absence of either CopA/CcoI or SenC. In humans, Sco1/Sco2 appear to regulate Cu efflux from mitochondria (Briere & Tzagoloff, 2007), and our data are also consistent with a role of SenC in Cu export regulation. Whether this involves CopA or CcoI or both, is currently unknown. CcoI appears to be more likely, because it was functionally linked to SenC (Lohmeyer *et al.*, 2012) and is essential for cbb_3-

Cox assembly (Kulajta *et al.*, 2006), while CopA does not seem to influence cbb_3-Cox assembly (Ekici *et al.*, 2014).

The Δ*senC*-Δ*pccA* double knock-out strain accumulates much less cytoplasmic Cu than the Δ*senC* strain, suggesting that the absence of PccA somehow counterbalances the Cu accumulation seen in the Δ*senC* strain. The underlying molecular mechanisms are currently unknown and need to be further explored. A possibility is that the Cu occupancies of PccA or SenC are important for regulating Cu homeostasis. If PccA works upstream of SenC, deletion of PccA would probably result in the accumulation of Cu-free SenC, while the deletion of SenC would result in the accumulation of Cu-loaded PccA. Cu-loaded PccA could repress Cu-export via the CcoI or CopA pathways, explaining the accumulation of intracellular Cu. In the absence of both proteins, this repression would fail, explaining why in the double knock-out the Cu content is normal. It has been shown that P_{1B}-type ATPases are activated by their cognate cytosolic chaperones (Gonzalez-Guerrero & Arguello, 2008) and it is conceivable that periplasmic chaperones also influence the activity of these Cu-exporting proteins.

In summary, our data illustrate that an intricate interplay between two periplasmic Cu-binding chaperones is required for both the assembly of the Cu-containing cbb_3-Cox and maintenance of cellular Cu homeostasis.

Experimental Procedures

Bacterial strains and growth conditions

The strains and plasmids used are listed in Table S1. The *E. coli* strains harboring plasmids were grown in LB medium suplemented with appropriate antibiotics (ampiciline [Amp] 100 μg/ml, kanamycine [Kan] 50 μg/ml and tetracycline [Tet] 10 μg/ml) at 37° C in liquid medium or on LB agar plates. *R. capsulatus* strains were grown in Sistrom's minimal medium A, or in enriched MPYE medium at 35° C with the apropriate antibiotics (10 μg/ml of Kan and 2,5 μg/ml of Tet) (Daldal, 1988, Jenney & Daldal, 1993). For semi-aerobic growth, 500 ml of liquid culture were grown in 1000 ml flask at 35° C and 110 rpm shaking in the dark. Metal-free MPYE medium and metal-free water was obtained by mixing 100 ml MPYE medium/water with 5 g of Chelex-100 resin and stirring for 1 hour at room temperature. The Chelex-100 resin was then removed by filtration.

Molecular genetic techniques

Standard molecular genetic techniques were performed as described previously (Sambrook & Russel, 2001, Koch *et al.*, 1998b). To construct chromosomal knockout alleles of desired genes, the gene transfer agent (GTA) was used as described earlier (Koch *et al.*, 1998b). Genomic DNA of *R. capsulatus* was extracted from 10 ml cultures of wild type (MT1131) or Δ*PccA* strains, which were grown in MPYE medium by using the Dnasy Blood & Tissue kit (Qiagen Inc.). The isolated DNA was then analysed qualitatively and quantitatively by Nanodrop spectrophotometer and by agarose gel electrophoresis.

To construct a deletion-insertion allele of *PccA* (*R. capsulatus* RCC02646), a DNA fragment including ~500 bp 5′ upstream and 3′ downstream of *PccA* was PCR amplified using wild-

type (MT1131) chromosomal DNA as a template and the primers PccA_SacI_for and PccA_KpnI_rev containing 5′-SacI and 3′-KpnI restriction sites (Table S2). This DNA fragment was digested with appropriate restriction enzymes and cloned into pBlueScriptIIKS+ and into pRK415 to yield the plasmids pBS-PccA and pRK415-PccA, respectively (Table S1). *PccA* was deleted from pBS-PccA using the primers pB_circ_upll (forward primer) and pB_circ_downl (reverse primer) containing a 5′-NdeI and a 3′-XhoI restriction site, which amplified the remaining flanking regions of PccA and the vector. The Kan cassette of pKD4 vector was amplified using the primers pKD_Kan_for and pKD_Kan_Rev containing NdeI and XhoI restriction sites, and appropriate digestions ligated between the two flanking regions of *PccA* to yield pBS-Δ*PccA* carrying the Δ(*PccA::kan*) allele. The fragment between SacI and KpnI of pBS_ΔPccA was cloned into the respective cloning sites of pRK415 to yield pRK415_ΔPccA. This plasmid was used to generate the Δ*PccA* knockout by GTA crosses with the wild-type (MT1131) and a Δ*senC* (Swem *et al.*, 2005) mutant, to yield the strains IT1 and IT10, respectively.

Plasmid pBS-PccA was used as a template with the PccA_Strep_For and PccA_Strep_Rev primers (Table 2), and the PCR product thus obtained was cloned into pET22b plasmid to yield pET22b-PccAStrep carrying a Strep-tagged version of PccA (Table 1). This latter plasmid was also used as a template for constructing variants of PccA in which the amino acid residues at positions 76, 93, 109 and 117 were replaced by cysteines (PccA-4C; Fig. 1). This plasmid was also used to replace the two methionine residues of the Cu-binding motif by leucine residues ($PccA_{Mut}$).

Construction of ccoA::phoA fusions

For the *ccoA::phoA* fusions, the CcoA-XhoIFor primer located 400bp upstream the ATG of CcoA was used as a forward primer (Table S2). This forward primer was used in conjunction with BamHI249Rev and BamHI281Rev reverse primers to amplify the DNA fragments of *ccoA* encoding its N-termini up to His_{249} and Arg_{281} residue of CcoA, respectively. The PCR products obtained were digested with XhoI and BamHI restriction enzymes and cloned into the similarly digested pTFD342 to yield pBK86 (*ccoA249::phoA*) and pBK88 (*ccoA281::phoA*).

Protein purification, fluorescence measurements and ^{64}Cu binding assays

For fluorescence measurements and Cu binding assays, PccA-Strep and PccA-4CStrep variants of the PccA protein were expressed in *E.coli* BL21 (DE3) carrying the plasmids pET22b-PccAStrep and pET22b-PccA-4Cstrep, respectively, and were purified via their C-terminal Strep-Tag. *E.coli* cells were grown aerobically in LB medium with apropriate antibiotics, and when they reached an OD_{600} of 0.6, freshly prepared IPTG was added to the culture at a final concentration of 1mM. After 2 hours incubation at 30° with 170 rpm shaking, cells were harvested and washed once with 40 ml washing buffer (100 mM Tris/HCl pH 7.5, 150 mM NaCl and 1 mM EDTA). Following centrifugation cells were resuspended in 15 ml washing buffer, and disrupted by using a French pressure cell at 8000 psi (pounds per square inch). Cell lysates were centrifuged at 28,720 g for 30 min using the SS-34 rotor (Evolution RC6 centrifuge, Thermo Scientific Inc.) to remove cell debris. The supernatant was ultracentrifuged for 1 hour at 174,000 g in the Ti50 rotor (Beckmann-

Coulter, Krefeld, Germany), then loaded onto a polypropylene column containing Strep-Tactin matrix, equilibrated with the washing buffer. After five washing steps with 1 column volume of washing buffer each, elution was initiated using the elution buffer (100 mM TrisHCl pH 7.5, 150 mM NaCl, 1mM EDTA and 2.5 mM desthiobiotin), and fractions were collected.

For measuring intrinsic tyrosine fluorescence of PccA, purified protein (10 μM final concentration) was incubated with different concentrations of Cu(II) or Cu(I) for 10 min at room temperature (25°C) under anaerobic conditions (3.8% H_2 and 96.2% N_2) in an anaerobic chamber (Coy Laboratory products). Cu(II) was added as $CuSO_4$, which was dissolved in Cu-free washing buffer lacking EDTA. Cu(I) was added as Tetrakis(acetontrile)Cu(I) hexafluorophosphate ($[Cu(CH_3CN)_4]PF_6$; Sigma Aldrich, Munich, Germany) and was dissolved under anaerobic conditions in acetonitrile to a final concentration of 100 mM. Further dilutions were performed in Cu-free washing buffer lacking EDTA under anaerobic conditions. Samples were subsequently transferred under anaerobic conditions to a TECAN infinite M200 fluorescence microplate reader and were excited at 276 nm. Fluorescence emmision intensity was recorded between 280 and 380 nm. For quantification, the intrinsic fluorescence observed in the absence of metals at 310 nm was set to 1.0 and the values observed in the presence of different metals and different metal concentrations were normalized in respect to this value.

For measuring ^{64}Cu binding to SenC, PccA or $PccA_{Mut}$, 1 μM purified protein was incubated for 10 min at room temperature with 1, 5 and 10 μM radioactive copper and then precipitated with TCA for 30 min on ice (final TCA concentration 5%). After 10 min centrifugation at 13000 rpm, the pellet was resuspended in scintillation liquid and its radioactivity was measured using a scintillation counter to determine copper binding to these proteins. Alternatively, the pellet was resuspended in 25 μl TCA loading buffer (150 mM TRIS, 3.3% (w/v) SDS, 12% (w/v) glycerol, 2 mM EDTA, and 0.1 M DTT) and run on a 15 % native PAGE. Subsequently the gel was dryed using a gel dryer (BioRad Inc.) and visualised by phosphor imaging. ^{64}Cu was obtained as $CuCl_2$ from the National Centre for Nuclear Research, Otwock, Poland and delivered by Hartmann Analytic GmbH (Braunschweig, Germany). It was dissolved in washing buffer lacking EDTA to the appropriate concentrations.

Preparation of intracytoplasmic membranes (ICMs) and Blue Native Page analyses

Inverted cytoplasmic membranes from wild-type *R. capsulatus* (MT1131) and its mutants were prepared as described previously (Pawlik *et al.*, 2010, Koch *et al.*, 1998a). For Blue Native (BN) PAGE analyses ICM's (~100 μg of total protein) were incubated at 25° C for 15 minutes with continous shaking (550 rpm Eppendorf Thermomixer), in lysis buffer (50 mM NaCl, 50 mM imidazole/HCl, pH 7.0, 5 mM 6-aminohexanoic acid) containing 1% *n*-dodecylmaltoside (Thermo Scientific Inc.). After 15 min centrifugation at 70000 rpm in a TLA 100.3 rotor, 15 μl of the supernatant was mixed with 2 μl loading dye (5% Coomassie blue G250 in 0.5 M 6-aminohexanoic acid) and 50% glycerol and then analyzed using a 4–16% gradient BN-PAGE.

Oxygen uptake assays

Ascorbate-TMPD (*N,N,N,N*-tetramethyl-*p*-phenylendiamine) oxidase activity was measured with a fiber optic oxygen meter (Fibox 3; PreSens GmbH, Regensburg, Germany) at 28° C in a closed reaction chamber. *R. capsulatus* membranes were disolved in ICM buffer (50 mM triethanolamine acetate (TeaAc) pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF) to a final concentration of about 0.1 μg/μl. The reaction was initiated by the addition of 10 μl of 1 M sodium ascorbate (10 mM final concentration) and 5 μl of 24 mM TMPD (*N,N,N,N* – tetramethyl-*p*-phenylendiamine, final concentration 0.2 mM). Oxygen consumption was recorded at 28° C using the OxyView 3.5.1 software (PreSens GmbH) and terminated after several minutes of recording by the addition of 2 μl NaCN (0.1 mM final concentration). cbb_3-Cox activity was determined by substraction of the respiration rate remaining after addition of NaCN from that initiated by ascorbate-TMPD.

Alkaline phosphatase measurements

The alkaline phosphatase activities of strains carrying the *ccoA::phoA* fusions were measured *in vitro* using membranes (100 μg of total proteins) and *p*-nitrophenyl phosphate as a substrate, as described in (Brickman & Beckwith, 1975). The absorbance at 420 nm resulting from the pigments of *R. capsulatus* chromatophore membranes was recorded before adding the substrate, and subtracted from the final absorbance obtained at 420 nm after incubation with *p*-nitrophenyl phosphate. Alkaline phosphatase activity was calculated as follows: [A_{420} (after addition of substrate) – A_{420} (before addition of substrate)] x 1000/min/mg of total proteins.

In-gel heme staining

To visualize the c-type cytochromes, ICMs (100 μg of protein) were disolved in 20 μl Läemmli buffer for 5 minutes at 25° C with shaking a 1000 rpm, and denaturated at 37° C for 15 minutes. After separation, SDS-Tris-Tricine polyacrylamide gels were treated with 3,3,5,5-tetramethylbenzidine (TMBZ) to reveal the peroxidase activity associated with *c*-type cytochromes (Thomas *et al.*, 1976) as performed earlier (Koch *et al.*, 1998b).

Measurement of cellular copper accumulation

R. capsulatus cells were grown in MPYE media under aerobic conditions (100 ml of growth medium in 250 ml flask) at 35° C, 110 rpm to an OD_{685} of 1. Cells were harvested by centrifugation at 4° C, 5,000 rpm for 20 min, and the pellets were washed with 50 ml of metal-depleted MPYE and re-centrifuged. The pellet was then resuspended in 1 ml of metal-depleted H_2O, and a small aliquot (about 10 to 20 μl) was taken for protein concentration determination by the Lowry assay. Exactly 1 ml of cell suspension was transferred to a new 15 ml tube to which 0.75 ml of 53% nitric acid was added and incubated at 80 °C for 1 hour followed by overnight incubation at 60 °C to yield a clear liquid. The reaction was terminated by the addition of 300 μl H_2O_2. Cu-free water was added up to a final volume of 10 ml, and samples were measured by using an atomic absorption spectrophotometer Perkin-Elmer 4110 ZL Zeeman. In case any insoluble impurities were observed within the sample, they were filtered through a 0.45 μm filter.

R. capsulatus spheroplasts were essentially prepared as described previously (Norell *et al.*, 2014) with the following modifications: 10 ml of cells were grown at 35° C, 110 rpm up to OD_{685} of 0.8 – 1, and centrifuged at 5,000 rpm for 10 min at 4° C. The pellet was resuspended in 5 ml of 100 mM Tris/HCl pH 7.5 containing 0.5 M sucrose. Subsequentely, 0.1 mg/ml lysosyme dissolved in 8 mM EDTA, pH 8.0 was added, and the sample was incubated for 15 min on ice. Formation of spheroplasts was monitored microscopically, using a Olympus BX51 microscope at 100x magnification with a numerical aperture of 1.4. Spheroplasts were pelleted by centrifugation at 5,000 rpm for 20 min, and copper contents of the pellet and the supernatant were determined after acid digestions, as described above for intact cells.

Formaldehyde or cysteine cross-linking

In vivo formaldehyde cross-linking was performed as described previously (Lohmeyer *et al.*, 2012). Cysteine crosslinking was performed with PccA and PccA-4C proteins that were purified using the Strep-Tag purification protocol of the supplier (IBA GmbH, Göttingen, Germany) from appropriate *E. coli* strains carrying the pET22b-PccAStrep and pET22b-PccA-4CStrep plasmids, respectively,. Spheroplasts of *R. capsulatus* cells carrying pRK415-$SenC_{His}$ (Lohmeyer *et al.*, 2012) were prepared as described above. 500 µl of spheroplasts resuspended in 100 mM Tris/HCl pH 7.5, 150 mM NaCl was incubated with ~ 0,3 mg/ml of purified PccA or PccA-4C protein, followed by incubation for 1 hour at room temperature with bis(maleimido) hexane (BMH) (Thermo scientific Inc.), added to a final concentration of 0.5 mM. The reaction was stopped by quenching BMH via the addition of 10 mM 1,4-dithiothreitol (DTT). Strep-tagged PccA and PccA-4C were purified from the incubation mixture via Strep-Tag, and the elution fractions were analyzed by SDS-PAGE.

Immune detection methods

For specific visualization of proteins on SDS- or BN-PAGE, proteins were electro-blotted onto different types of membranes as follows. Proteins from BN-PAGE were transferred a PVDF Immobilon-P membrane (GE Healthcare). Proteins from SDS-PAGE were transferred to a nitrocellulose membrane (GE Healthecare). Polyclonal antibodies against PccA, CcoN, CcoP were used with either peroxidase-conjugated goat anti-rabbit (Caltag Laboratories, Burlingame, CA) or alkaline phosphatase-conjugated goat anti-rabbit antibodies (Sigma, St. Louis, USA) antibodies as a secondary antibodies with ECL (GE Helthcare, Munich, Germany) or NBT/BCIP (Roche, Germany) as detection substrate, as approriate. Peptide antibodies against SenC were generated by GeneScript (New Jersey, USA) using the following peptide CLQTPGDTPAAGNGN. Antibodies against PccA were raised in rabbits (Gramsch Laboratories, Schwabhausen, Germany) against the mature protein purified from *E. coli*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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A

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R.c. ------MIGKLSAPLCALMLALMPMSG QAHEAHAGALTII HPAIPAVMPGAQAAAGYFTI 54
T.t. ---MRRWIGLLF--LLALASA--------------QVVREG-WV-RF-SPGPNAAAYLTL 38
R.s. MTPFKTLLAAA---AAAVTFA---LP----AFADSNIAVRDAYARVSSPAAKSGGIFMVI 50
B.j. ---MKTMLKDVL---LAAFALTLCATPAAAEDVKAGDLVISQAWSRATPGGAKVAGGYLTI 55

R.c. VNAGDSADRLTAVEVDFAAMAMMHSSETD---AAGVTRMIDVGAVEVPAGATVKLAPGGL 111
T.t. ENPGDLPLRLVGARTPVAERVELHETFMREVEGKKVMGMRPVPFLEVPPKGRVELKPGGY 98
R.s. ENHGSTADRLVSAASEAAAKVELHTHLAGE---DGIMRMVEVPEGFEVPAQGSHALARGG 108
B.j. ENKGTTADRLVSVSADIAGKTEVHEMAMD----NGVMKMRPLDKGLVIDAGKTVKLAPGG 112

R.c. HVMFMGLTAPIAEGVMLPGTLVFDHAGKVPVEFMVTTAGKA -PDMSGHKMTP-------- 162
T.t. HFMLLGLKRPLKAGEEVELDLLFAGGKVLKVVLPVEAR ---------------------- 136
R.s. HVMLMGLTRPLKEGDEVALTLTFESGARVDLAVPVDLTR ---EDAGAHQMQGMDHAH--- 162
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B

C

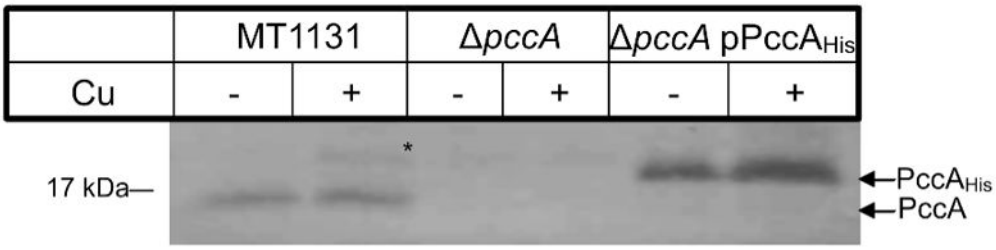

Figure 1. Sequence alignment and genetic organization of the periplasmic copper chaperone PccA in *R. capsulatus*

(A) The predicted amino acid sequence of PccA of *R. capsulatus* (R.c.) was aligned to other members of the PCu_AC copper chaperone family using the Clustal sequence alignment algorithm: *T. thermophilus* PCu_AC (T.t.); *R. sphaeroides* RSP_2017 (R.s.); and *B. japonicum* PcuC (B.j.). Indicated are the predicted cleavable signal sequence of *R. capsulatus* PccA (cyan) and the conserved residues of the putative Cu-binding site (red). The single tyrosine residue used for measuring the intrincisc fluorescence of PccA is indicated in blue and the positions used for cysteine substitutions in PccA are indicated in bold. (B) Genetic organization of *pccA* in *R. capsulatus*. The nucleotide sequence of *R. capsulatus* was retrived from NCBI and analyzed using the NCBI tool box (www.ncbi.nlm.nih.gov). Displayed is the *R. capsulatus* genomic region encompassing *pabB*, p-amino-benzoat

synthase (Rcc02644); *frc*, CoA transferase (Rcc02645); *pccA* (Rcc02646) *putA*, proline dehydrogenase (Rcc02647) and *putR*, proline dehydrogenase regulator (Rcc02648). (C) Wild type *R. capsulatus* cells (MT1131), cells of the Δ*pccA* strain and of the Δp*ccA* strain carrying *pccA* on a low copy plasmid (Δ*pccA*/pPccA$_{His}$) were grown on MPYE medium or on MPYE medium supplemented with 10 μM $CuSO_4$. Cells were harvested, and after washing, TCA precipitated and separated on SDS-PAGE. After western blotting, the samples were decorated with α-PccA antibodies. *indicates a band that cross-reacted with the α-PccA antibody in wild type cells grown in the presence of Cu.

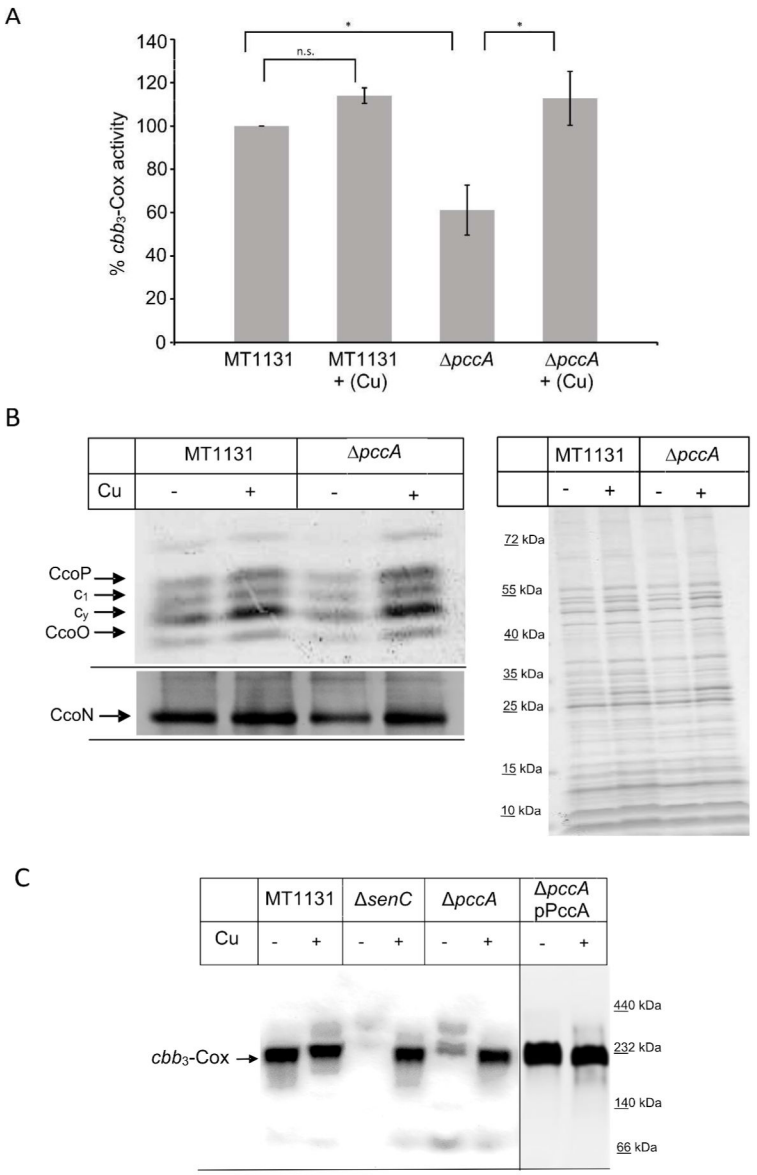

Figure 2. PccA is required for *cbb*$_3$ Cox assembly in *R. capsulatus*

(A) Oxygen uptake activity of intracytoplasmic membranes of *R. capsulatus* MT1131 (wild type) and Δ*pccA* strains was measured using a fiber optic oxygen meter. Cells for membrane preparation were grown either on regular MPYE medium or on MPYE medium supplemented with 10 μM $CuSO_4$ (+Cu). Oxygen uptake activity in MT1131 corresponds to 20 μmol O_2/min per mg protein and was set to 100%. Values shown are mean values of at least three independent experiments and the standard deviations are as indicated by error bars. * $p<0.01$; n.s. not significant. (B) The presence of *cbb*$_3$-Cox subunits was determined by either heme staining, which visualizes the membrane-bound *c*-type cytochrome profile of *R. capsulatus* membranes (upper left panel) or by western blotting using antibodies against the catalytic subunit CcoN (lower left panel). CcoP and CcoO correspond to the membrane-bound *c*-type cytochrome subunit of *cbb*$_3$-Cox, while c_1 is subunit of the cytochrome bc_1

complex and c_y a membrane bound electron carrier. Approximately 50 μg of protein were loaded per lane and separated on a 16.5% Tris-Tricine SDS-PAGE. When indicated, cells were grown in the presence of 10 μM $CuSO_4$ for membrane preparation. Right panel: Coomassie-brilliant blue stained samples after separation on SDS-PAGE (50 μg membranes). (C) Membranes of the indicated strains containing approximately 100 μg of protein, each, were solubilized with dodecyl-maltoside, separated on a Blue-Native PAGE (BN-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane, and decorated with antibodies against the CcoN subunit. The fully assembled *cbb*$_3$-Cox migrates at about 230 kDa on BN-PAGE. Δ*pccA* pPccA$_{His}$ corresponds to the Δ*pccA* strain carrying a plasmid borne copy of *pccA* with a C-terminal His-tag.

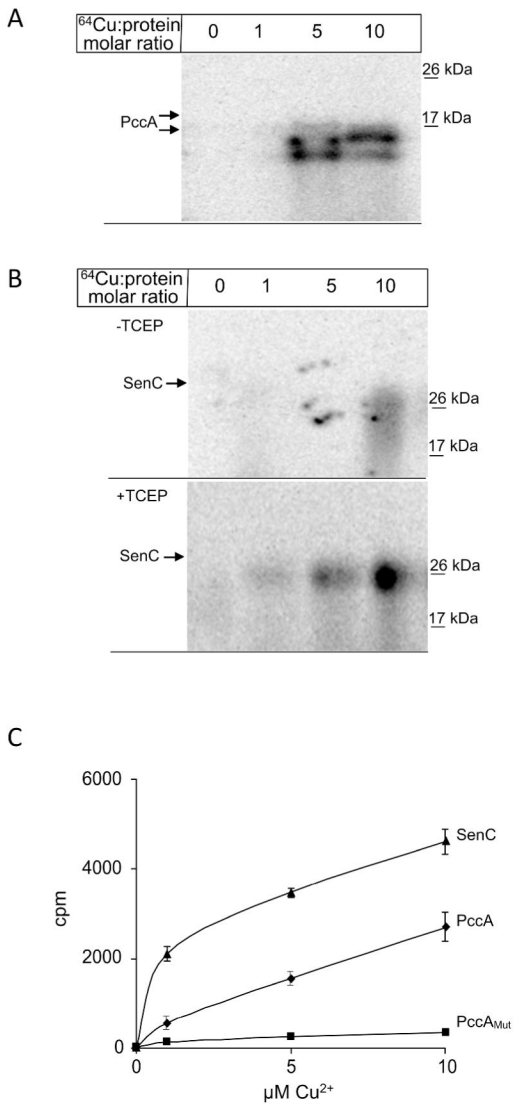

Figure 3. PccA is a copper binding protein

(A) PccA was purified via a C-terminal Strep-tag and 10 μM of purified proteins were incubated with increasing concentrations of $^{64}CuCl_2$. Samples were subsequently separated on native PAGE and analyzed by phosphor-imaging. (B) Purified SenC (10μM) was incubated with increasing concentrations of $^{64}CuCl_2$ with and without prior reduction of the cysteine-containing copper binding motif by TCEP. Samples were then analyzed as in (A). (C) Quantification of the ^{64}Cu binding to SenC (▲), or PccA (◆), or $PccA_{Mut}$, a mutant lacking the two conserved methionine residues of the Cu-binding motif of PCu_AC chaperones (■). After ^{64}Cu binding for 10 min at room temperature, samples were purified via Strep-tag and Cu binding was analyzed using a scintillation counter. The data displayed are the mean value of four independent experiments and the error bars represent the standard deviation.

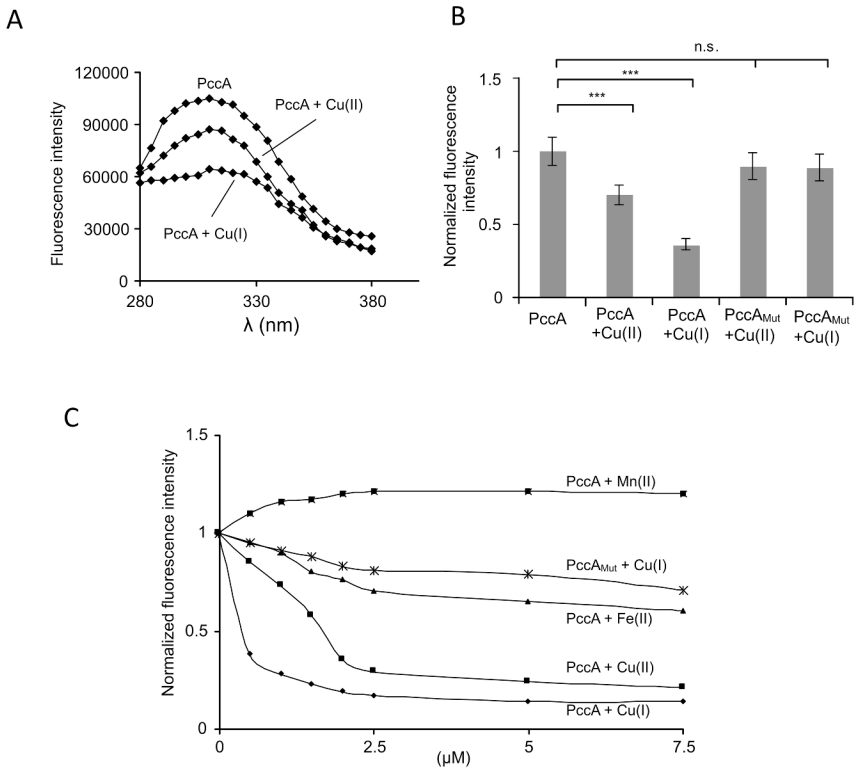

Figure 4. PccA preferentially binds Cu^{+}

(A) Intrinsic tyrosine fluorescence of PccA (10 μM protein) was determined under anaerobic conditions after excitation at 276 nm and emission was monitored between 280 and 380 nm. When indicated Cu(I) or Cu(II) was added at a final concentration of 0.5 μM. (B) Intrinsic fluorescence emission of PccA at 310 nm in the presence of either Cu(I) or Cu(II) is plotted relative to the initial signal in the absence of Cu, which was set to 1.0. In addition, the intrinsic fluorescence emission of the $PccA_{Mut}$ variant was determined in the presence of either Cu(I) or Cu(II). The error bars indicate the SD (n=4). *** $p<0.001$; n.s. not significant. (C) The intrinsic fluorescence emission of PccA or $PccA_{Mut}$ was plotted against different metal concentrations. Plotted are the mean values of three experiments. The SDs were ≤ 10% and are not displayed for clarity.

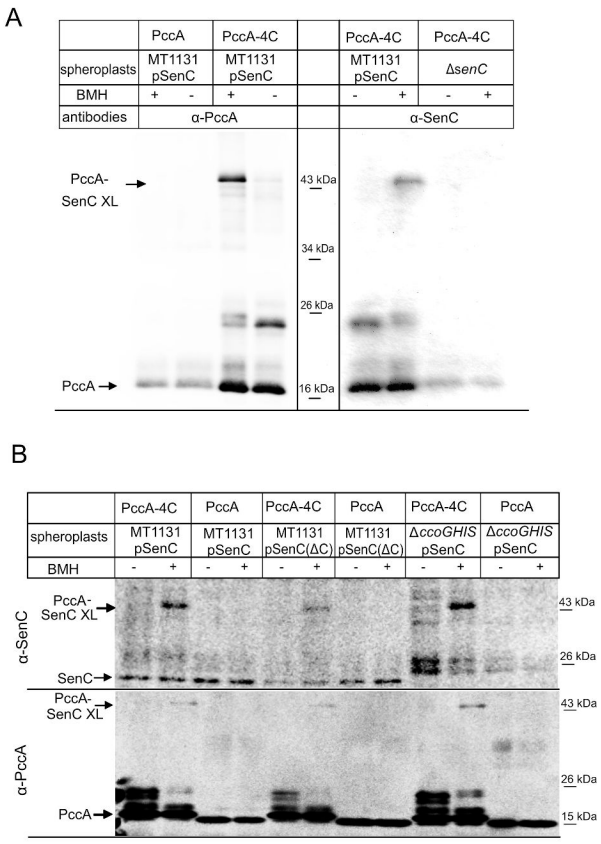

Figure 5. PccA interacts with SenC *in vitro*

(A) Spheroplasts of MT1131 cells expressing SenC or from a Δ*senC* strain were incubated with Strep-tag purified wild type PccA or PccA-4C, which contained four engineered cysteine residues close to the metal binding site. When indicated, the cysteine-specific crosslinker BMH was added. Samples were subsequently analyzed under non-reducing PAGE, transferred to PVDF membranes and decorated with antibodies against PccA or SenC. PccA-SenC XL indicates the crosslinking product. (B) Spheroplasts were prepared from MT1131 cells expressing either SenC or SenCΔC, which corresponds to a SenC derivative, in which the two cysteine residues of the Cu-binding motif were replaced by alanine. In addition, spheroplasts of the Δ*ccoGHIS* strain expressing SenC were analyzed. In the Δ*ccoGHIS* strain, the cbb_3-Cox assembly factors CcoGHIS are absent. The upper panel was decorated with antibodies against SenC and the lower panel with antibodies against PccA.

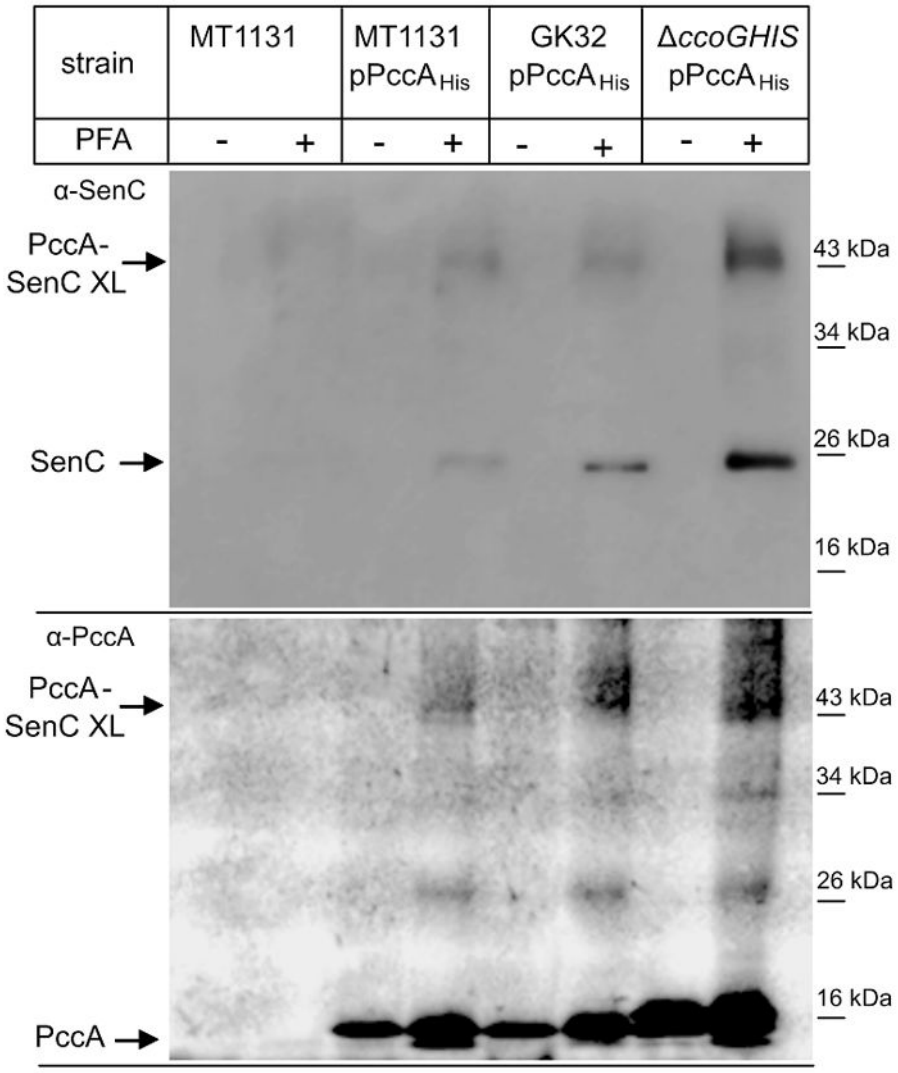

Figure 6. PccA interacts with SenC *in vivo*

The indicated strains were incubated with para-formaldehyde (PFA) (+) or the reaction buffer only (−). After membrane preparation, cells were solubilized with DDM, and $PccA_{His}$ was purified via metal affinity chromatography. Purified protein was then separated on SDS-PAGE, transferred to a PVDF membrane and decorated with antibodies against SenC (upper panel) or PccA (lower panel). GK32 carries a *ccoNO* deletion and lacks cbb_3-Cox. In the Δ*ccoGHIS* strain, the cbb_3-Cox assembly factors CcoGHIS are absent.

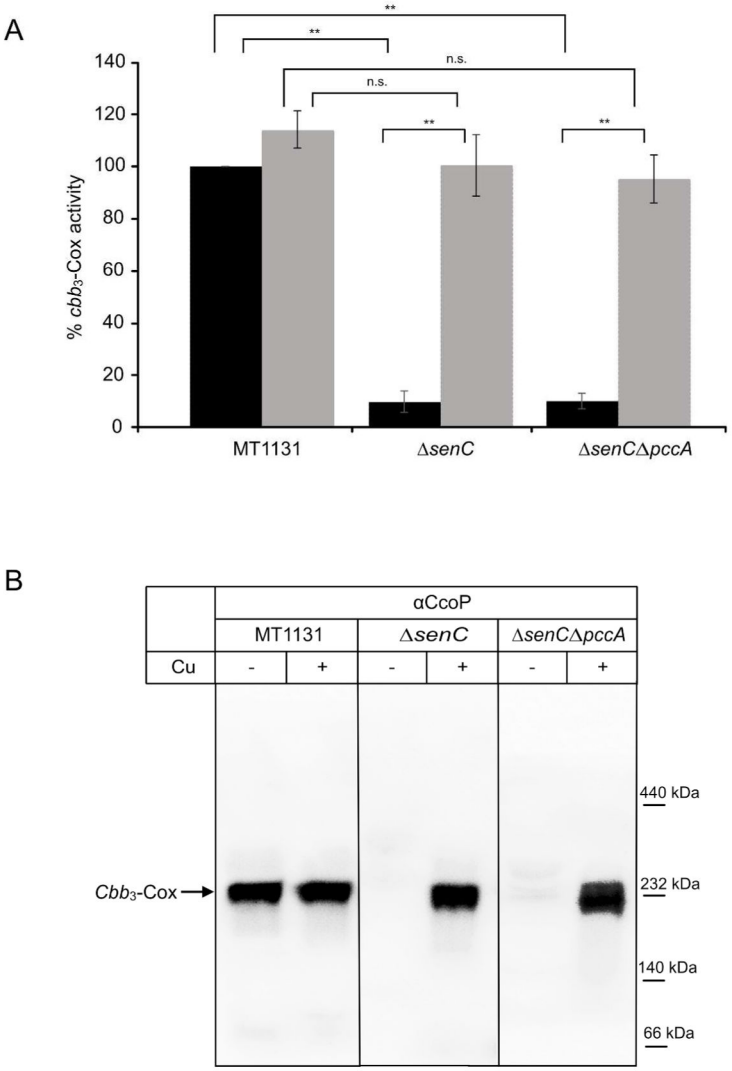

Figure 7. The phenotype of a *senC-PccA* double knock-out mimics that of the Δ*senC* strain

(A) *cbb*$_3$-Cox activity was determined in membranes of the indicated strains grown on MPYE medium (■) or on MPYE medium supplemented with 10 μM $CuSO_4$ (□). The activity observed in membranes isolated from cells grown on MPYE medium was set to 100%. Displayed are the mean values of three independent experiments and the error bars represent the SD. ** $p < 0.001$; n.s. not significant. (B) BN-PAGE of the membranes described in (B). After western transfer, the blot was decorated with α-CcoN antibodies.

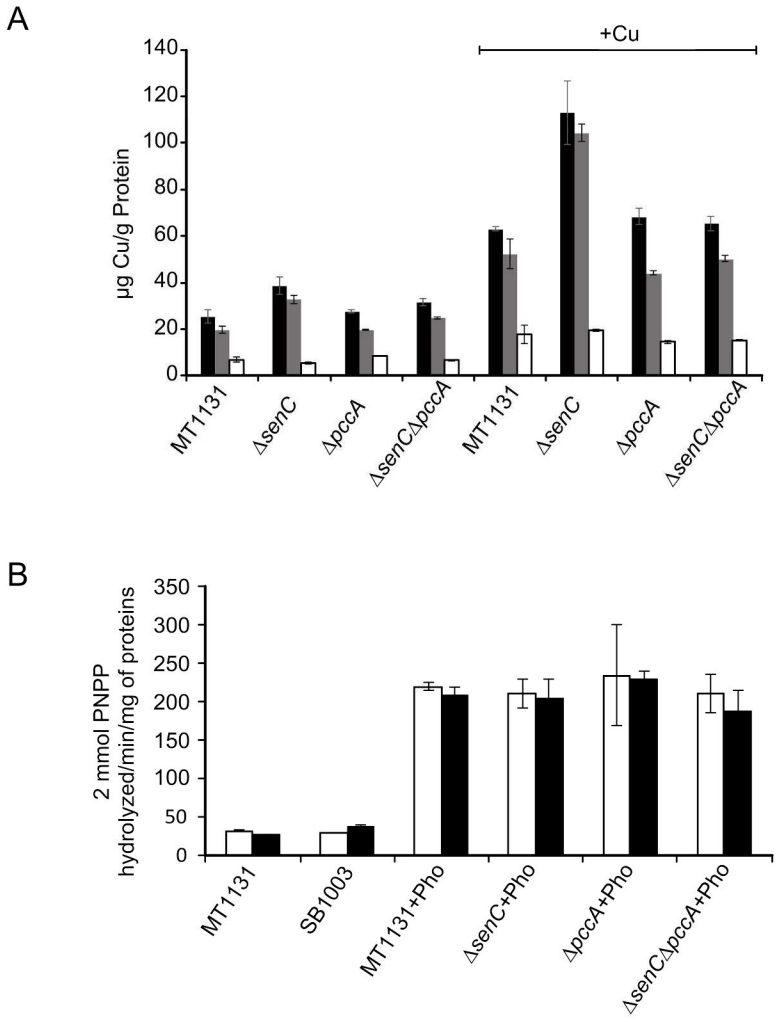

Figure 8. Copper distribution in *R. capsulatus* cells is influenced by the deletion of *SenC*

(A) Total copper concentration (black bars) of MT1131 (wild type), Δ*senC,* Δ*pccA* and Δ*senC*-Δ*pccA* cells was monitored by atomic absorption spectroscopy after growth on MPYE medium or MPYE medium supplemented with 10µM $CuSO_4$ (+) as described in Material and Methods. The manganese and zink concentrations were determined as internal reference. In addition, the copper content of spheroplasts (grey bars) and the supernatants of the spheroplast preparations (periplasmic fraction) (white bars) were also analyzed. The values are the mean of at least three independent experiments and the error bars indicate the SD. Significant p-values (p<0.01) were only observed for the Cu content in the Δ*senC* strain (+Cu) compared to the wild type (+Cu). (B) Alkaline phosphatase (PhoA) activity of a *ccoA-phoA* translational fusion was determined in cell extracts of the indicated strains grown either in the absence of presence of supplementary Cu. The values are the mean of at least two experiments and the error bars indicate the SD.

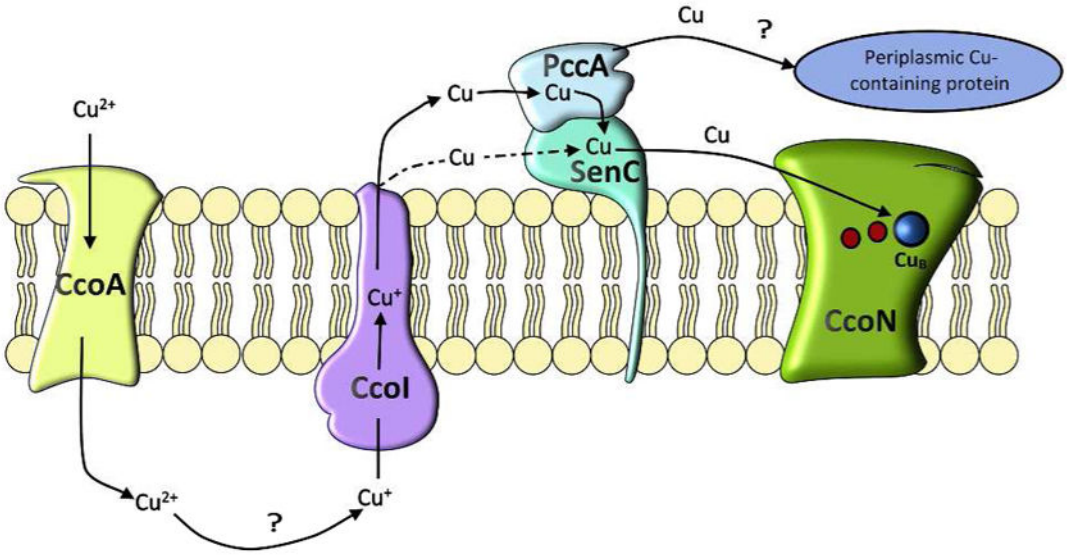

Figure 9. A putative Cu delivery pathway for *cbb*$_3$-Cox assembly in *R. capsulatus*

At low Cu concentrations, the MFS-type Cu transporter CcoA imports Cu^{2+}, which is reduced to Cu^{+} in the cytoplasm by an unknown mechanism. The P_{1B}-type ATPase CcoI transports Cu^{+} into the periplasm, where it is bound to PccA, which transfers it to the Sco1-homologue SenC and possibly to other periplasmic Cu-dependent proteins. SenC then delivers Cu^{+} to the CcoN subunit of *cbb*$_3$-Cox (red dots indicate the two heme groups in CcoN). In the absence of PccA, Cu^{+} loading onto SenC and subsequent *cbb*$_3$-Cox assembly is significantly reduced but still detectable (dashed arrows). At high Cu concentrations, *cbb*$_3$-Cox assembly proceeds independently of CcoA, SenC and PccA, but still requires CcoI. Although SenC and PccA are dispensable for *cbb*$_3$-Cox assembly at high Cu concentration, they are very important for regulating Cu homeostasis under these conditions. Absence of SenC leads to increased cytoplasmic Cu concentrations, which return to normal by the simultaneous elimination of PccA. A plausible explanation is the accumulation of Cu-loaded PccA in the absence of SenC, repressing Cu-export via CcoI and leading to increased cytoplasmic Cu pool.