

**DECODING CYTOCHROME C OXIDASE BIOGENESIS: NEW INSIGHTS
INTO COPPER TRAFFICKING**

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Dedicated to my family...

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ABSTRACT

DECODING CYTOCHROME C OXIDASE BIOGENESIS: NEW INSIGHTS INTO COPPER TRAFFICKING

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Dr. Fevzi Daldal

Acquisition, delivery and incorporation of metals to their respective metalloproteins are important cellular processes. These processes are tightly controlled so that cells are not exposed to free metal concentrations that would lead to harmful oxidative damages. Cytochrome *c* oxidases (Cox) are among these metalloproteins whose assembly and activity involves incorporation of Cu cofactor into their catalytic subunits in addition to the maturation of other subunits. In this study, we focused on the pathways of acquisition of Cu by the facultative phototroph *Rhodobacter capsulatus* for incorporation into the heme-Cu binuclear center of its *cbb*₃-type Cox (*cbb*₃-Cox). Genetic screens identified a *cbb*₃-Cox defective mutant that requires Cu²⁺ supplement to produce an active *cbb*₃-Cox. Complementation of this mutant using wild-type genomic libraries unveiled a novel gene (*ccoA*) required for *cbb*₃-Cox biogenesis in *R. capsulatus*. In the absence of CcoA, cellular content of Cu decreases, and *cbb*₃-Cox assembly and activity becomes defective. CcoA shows pronounced homology to Major Facilitator Superfamily (MFS) type transporter proteins. Members of this family are known to transport small solutes or drugs, but so far, no MFS protein was implicated in *cbb*₃-Cox biogenesis. In order to dissect the mechanism of Cu acquisition in the absence of CcoA, we isolated

$\Delta ccoA$ mutants that were *cbb₃-Cox* defective after addition of Cu. Characterization of these mutants by genetic complementations revealed mutations in cytochrome *c* maturation (CCM) genes. These mutants were able to grow photosynthetically on the contrary to the usual phenotype of CCM genes deletion mutants. Here we show that these mutations are not directly involved in the Cu trafficking to CcoN but involved in the production of membrane bound cytochrome *c* subunits of *cbb₃-Cox*. Although this study provides additional information about CCM system in *R. capsulatus*, the additional pathways of Cu acquisition to *cbb₃-Cox* in the presence of exogenous Cu still remains to be identified. In the future, determination of *ccoA* bypass mutations will provide novel insights on the maturation and assembly of membrane-integral metalloproteins, and on hitherto unknown function(s) of MFS type transporters in bacterial Cu acquisition.

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CHAPTER 1
INTRODUCTION

RESPIRATORY OXIDASES

Molecular oxygen is estimated to be accumulated in the biosphere about 2.4 billion years ago (Canfield et al. 1996; Rye et al. 1998). The two families of enzymes which are respiratory oxygen reductases and photosystem II of oxygenic photosynthesis is thought to be evolved due to the increase in the oxygen levels leading to the existence of eukaryotic organisms. Respiratory oxidases are the main players for the reduction of molecular oxygen in the biosphere. They terminate the electron transfer chains of aerobic organisms by catalyzing the four-electron reduction of dioxygen to water during respiration. These enzymes pump protons from the negative to the positive side of the membrane, contributing to the establishment of an electrochemical potential that is ultimately used by the ATP synthase for ATP production (Figure 1). Most of the atmospheric oxygen is predicted to be reduced by the bacterial respiratory oxidases. The emerging ease of whole genome sequencing approaches has revealed the existence of multiple terminal oxidases in bacteria, which allow them to utilize efficiently varying oxygen (O₂) concentrations in their environments.

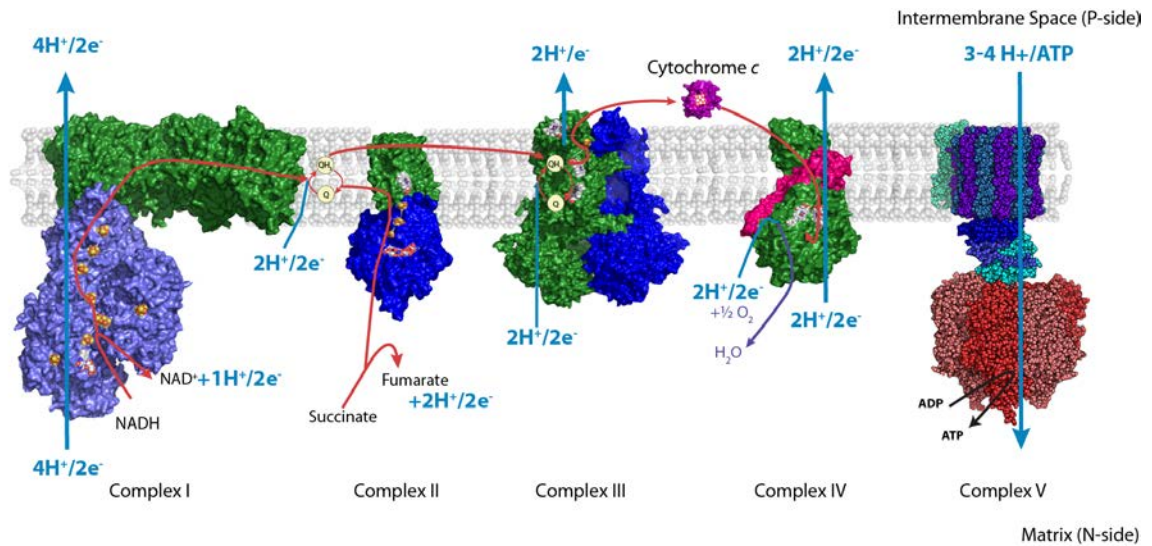


Figure 1: Electron transport chain in mitochondria

Schematic representation of the mitochondrial respiratory electron transport chain located in the inner membrane. Based on the structures from protein database (PDB IDs: 3RKO (Complex I membrane Domain from *Escherichia coli*), 3I9V (Complex I hydrophobic Domain from *Thermus thermophilus*), 1YQ3 (Complex II from *Gallus gallus*), 1BGY (Complex III from *Bos taurus*), 2B4Z (cytochrome *c* from *B. taurus*), 1QLE (Complex IV from *Paracoccus denitrificans*), 1Q01, 1C17, 1E79 (Complex V from *B. taurus*, *E. coli*, *B. taurus* respectively). Adapted from Ilya Belevic's Thesis 'Proton translocation coupled to electron transfer reactions in terminal oxidases' (Belevic 2007).

HEME-COPPER OXYGEN REDUCTASES

Generalities.

Most of the terminal oxidases belong to the heme-Cu: O₂ reductase superfamily (heme-Cu: O₂ reductases); they reduce O₂ to H₂O and couple electron transfer with vectorial proton translocation across the membrane (Pereira et al. 2001). Members of this superfamily are present in eukarya, archaea and eubacteria. They are thought to be of monophyletic origin, possibly related to nitric oxide reductases (NOR) (Castresana et al. 1994). NOR catalyze reduction of NO to N₂O during denitrification. Although they do not reduce O₂ and do not generate a proton gradient (Flock et al. 2006), their structural organization is similar to heme-Cu: O₂ reductases.

The heme-Cu: O₂ reductases can be divided into different subgroups in respect to their electron donors: those that use a *c*-type cytochrome (*i. e.*, cytochrome *c* oxidase, Cox) and those that use a quinol (*i. e.*, quinol oxidase, Qox). Cox are universally conserved oligomeric membrane proteins that terminate the respiratory chains of aerobic and facultative aerobic organisms. The structure and function of Cox enzymes have been studied intensely because of their central role in energy metabolism. Given the complexity of these multi-subunit, multi-cofactor enzymes, their assembly also attracted much attention. Indeed, assembly defects in the human Cox are major causes of mitochondrial disorders, and are crucial for neurodegenerative diseases, cancer and ageing (Bernstein et al. 2010; Matoba et al. 2006; Vesela et al. 2008; Won et al. 2011).

Among the Cox enzymes, the *aa*₃-type Cox (*aa*₃-Cox) of mitochondria and many bacterial species are highly abundant and the best-studied group. However, the heme-Cu: O₂ reductases represent a diverse ensemble of enzymes with significantly different subunit compositions and cofactors (Garcia-Horsman et al. 1994).

Classification

Members of heme-Cu: O₂ reductases are diverse in terms of their subunit composition, heme cofactor content, electron donor, and O₂ affinity (Ducluzeau et al. 2008; Garcia-Horsman et al. 1994; Pitcher et al. 2004). All members have a conserved core subunit (Subunit I), containing at least 12 transmembrane helices. This subunit contains a low spin heme (of *a*- or *b*-type), a binuclear metal center composed of a high spin heme (of *a*-, *o*-, or *b*-type heme, referred to as *a*₃, *o*₃ or *b*₃)-iron, and a Cu atom (Cu_B), as well as a tyrosine residue covalently linked to a histidine ligand of Cu_B (Garcia-Horsman et al. 1994; Pereira et al. 2001). Besides subunit I, which is the catalytic heart where O₂ is reduced to H₂O, heme-Cu:O₂ reductases have at least two other core subunits: Subunit II is the primary electron acceptor, and in some cases, it harbors extra cofactors like a binuclear Cu center (Cu_A), or is a *c*-type cytochrome. Subunit III in many cases does not contain any cofactor except in *cbb*₃-Cox where it is a diheme *c*-type cytochrome (Pereira et al. 2001). Most bacterial Cox are composed of these three core subunits, but mitochondrial Cox are more complex with 13 subunits of which only three (subunits I, II, and III) are encoded in the mitochondrial genome. An additional fourth subunit with a single transmembrane helix is also present in some bacterial Cox.

However, this subunit is unlike any of the ten nuclear encoded mitochondrial subunits (Herrmann et al. 2005).

A classification, based on common features of the core subunits, and key residues in proton transfer pathways, defines three (A, B and C) types of heme-Cu: O₂ reductases (Figure 2) (Pereira et al. 2001). Mitochondrial- *aa*₃-Cox (Type A), *ba*₃-Cox of *Thermus thermophilus* (Type B) and *cbb*₃-Cox of *Rhodobacter capsulatus* (Type C) are the most representative members of these types.

Available three-dimensional (3D) structures of the heme-Cu:O₂ reductase family members (*i. e.*, the *aa*₃-Cox from *P. denitrificans*, *R. sphaeroides* and bovine heart mitochondria; *ba*₃-Cox from *T. thermophilus*, *bo*₃-Qox from *E. coli* and the *cbb*₃-Cox from *Pseudomonas stutzerii*) (Figure. 2A) (Abramson et al. 2000; Buschmann et al. ; Iwata et al. 1995; Soulimane et al. 2000; Svensson-Ek et al. 2002; Tsukihara et al. 1996) reveal a remarkably conserved Cu and heme cofactors arrangement in these enzymes. Subunit I of type A enzymes contain two proton-transfer pathways referred to as K- and D- channels. The four electrons required for O₂ reduction at the binuclear heme-Cu_B center are conveyed sequentially via a non-covalently attached low spin heme, which itself receives electrons from the Cu_A center in subunit II (Iwata et al. 1995; Svensson-Ek et al. 2002). Type B enzymes are present only in bacteria and archaea, but not in eukaryotes, and constitute the least abundant group (Zimmermann et al. 1988). The catalytic center of type B enzymes is similar to that of type A, except that they lack the

D- channel for proton transfer (Chang et al. 2009). The prototypes of type C are the *cbb₃*-Cox, which are present only in bacteria, and considered to represent the most distant members of heme-Cu: O₂ reductases. They also lack the D-channel for proton transfer (Arslan et al. 2000; Hemp et al. 2007; Preisig et al. 1996), their subunits II and III are *c*-type cytochromes, and they exhibit higher O₂ affinity as compared with other types of Cox enzymes.

THE *cbb₃*-COX

Distribution of *cbb₃*-Cox in bacteria and its role in pathogenesis.

The *cbb₃*-Cox enzymes are common to proteobacteria and also found in the *cytophaga*, *flexibacter* and *bacteriodes* (CFB) group (Ducluzeau et al. 2008). They were first described in the facultative symbiotic N₂-fixing *rhizobiaceae* (Kahn et al. 1989; Preisig et al. 1993). In *B. japonicum* expression of *cbb₃*-Cox is required for symbiotic N₂ fixation under very low O₂ conditions in soybean root nodules (Preisig et al. 1993). Due to their importance for symbiotic N₂ fixation, the four structural genes of *cbb₃*-Cox were initially termed *fixNOQP* (Preisig et al. 1993). Subsequent studies identified these genes and their products also in non-symbiotic bacteria such as *R. capsulatus*, and referred to them as *ccoNOQP* (Garcia-Horsman et al. 1994; Gray et al. 1994; Thony-Meyer et al. 1994). A recent bioinformatics study, based on the occurrence of *ccoN* and *ccoO* genes, identified the *ccoNOQP* cluster in all bacterial species, except the *Thermotogales*, *Deinococcales* and *Firmicutes* (Ducluzeau et al. 2008). So far, no *cbb₃*-Cox coding sequences were identified in archaea (Hemp et al. 2008). Interestingly, *ccoNO* genes

were found recently in the last mitochondrial ancestor bacterium *Mitochondria* (Sassera et al. 2011).

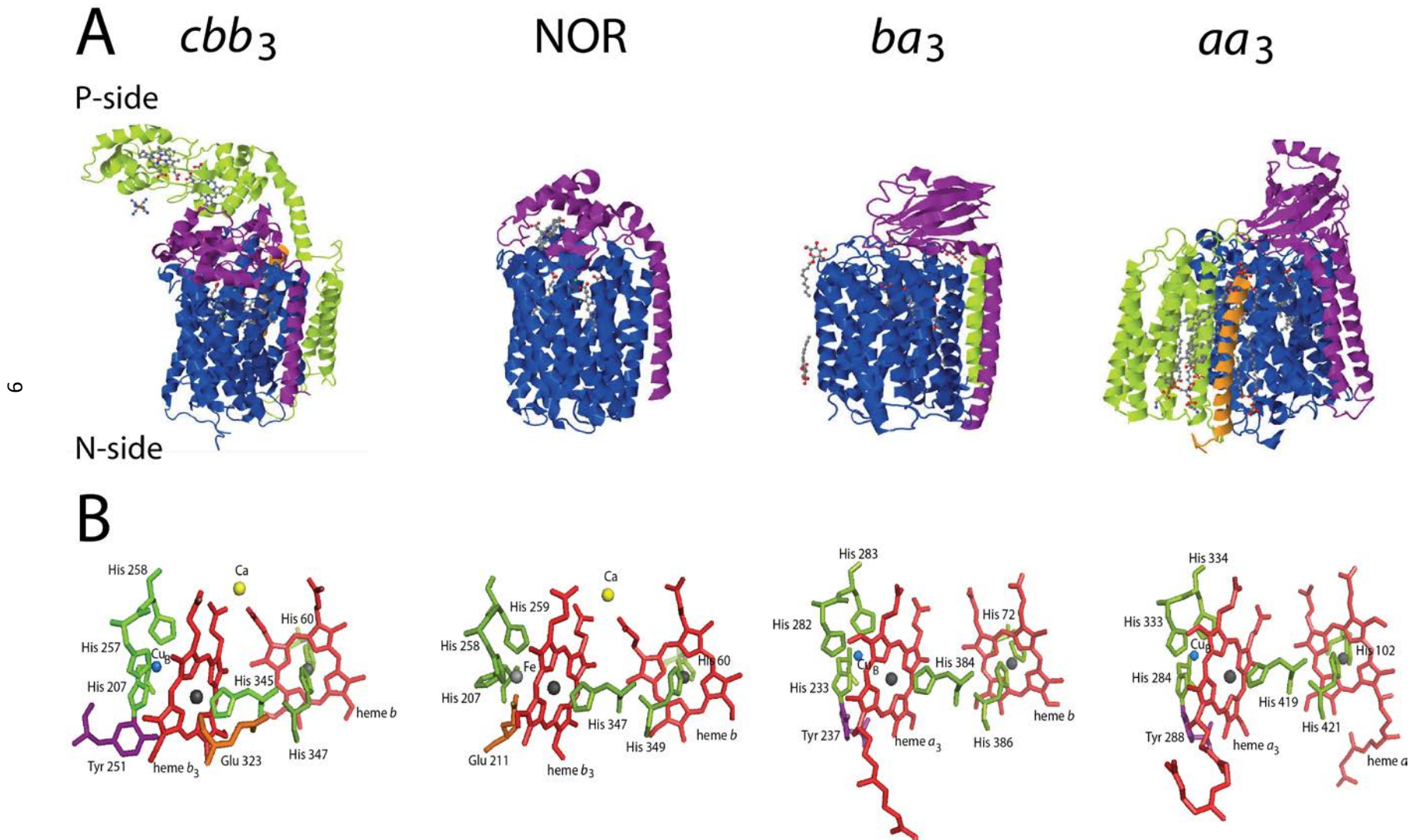


Figure 2: Structures of three types of heme-copper oxidases (*cbb*₃-Cox, *ba*₃-Cox, *aa*₃-Cox) and nitric oxide reductase (NOR)

Figure 2: Structures of three types of heme-copper oxidases (*cbb*₃-Cox, *ba*₃-Cox, *aa*₃-Cox) and nitric oxide reductase (NOR)

A. 3D structures of different types of heme-Cu:O₂ reductases and NO reductase: Catalytic subunit I – blue (*cbb*₃-Cox CcoN), subunit II (*cbb*₃-Cox CcoO) – magenta, subunit III (*cbb*₃-Cox CcoP) – green, additional subunit –orange. **B.** Architecture of the catalytic binuclear center of the different types of heme-Cu:O₂ reductases and NO reductase. Heme – red, His – green, Tyr – violet, Glu – orange. Spheres: copper – blue, heme iron - dark grey, non-heme iron – light grey, calcium – yellow. The structures depicted are taken from protein database (PDB) entries PDB ID: 3MK7 (*cbb*₃-Cox from *Pseudomonas stutzerii*) (Buschmann et al. 2010), PDB ID: 3O0R (NOR from *Pseudomonas aeruginosa*) (Hino et al. 2010), PDB ID: 1M56 (*aa*₃-Cox from *Rhodobacter sphaeroides*) (Svensson-Ek et al. 2002), PDB ID: 1EHK (*ba*₃-Cox from *Thermus thermophilus*) (Soulimane et al. 2000) using Jmol software (<http://www.jmol.org/>).

The *cbb₃*-Cox is present in many microaerophilic pathogenic bacteria and implicated in host colonization (Table 1). In some species including *Campylobacter jejuni* (Parkhill et al. 2000), *Helicobacter pylori* (Alm et al. 1999; Tomb et al. 1997), *Neisseria meningitidis* and *Neisseria gonorrhoea* (Li et al. 2010) *cbb₃*-Cox is the sole Cox, and it is suggested that its high O₂ affinity allows these pathogens to colonize low O₂ containing tissues (Pitcher et al. 2004). Correlations between *cbb₃*-Cox and host colonization have so far been examined mainly in *C. jejuni*, which is a non-fermenting microaerophile that colonizes the small and large intestines of humans and animals (Perez-Perez et al. 1996), causing acute gastroenteritis in humans. *C. jejuni* has a branched aerobic electron transport chain, with a *cbb₃*-Cox of high O₂ affinity and a quinol oxidase (CioAB) of low O₂ affinity. Gene expression studies have shown that CioAB was mainly expressed at high O₂ conditions (Jackson et al. 2007), while *cbb₃*-Cox was induced significantly in *C. jejuni* cells colonizing the chick caecum, which is a low O₂ environment (Woodall et al. 2005). *C. jejuni* mutants lacking *cbb₃*-Cox were unable to colonize the chick caecum, whereas mutants lacking CioAB were slightly impaired in colonization (Woodall et al. 2005). The caecum being primarily colonized by obligate anaerobic bacteria, *C. jejuni* is likely to be exposed to anaerobic conditions transiently. Probably, it tolerates anaerobiosis by using alternative electron acceptors like nitrate, nitrite, DMSO or TMAO (Weingarten et al. 2008), while its *cbb₃*-Cox would keep cellular O₂ concentrations low to protect its crucial O₂-labile enzymes and allow establishment of anaerobic respiration (Jones et al. 1973; Oelze 2000). Indeed, *C. jejuni* mutants lacking nitrate or nitrite reductases are significantly attenuated for colonizing the chick caecum (Weingarten et al. 2008). Thus, anaerobic respiration together with *cbb₃*-

Cox seems to allow *C. jejuni* to adapt successfully to environmental changes at their ecological niches.

A *cbb₃*-Cox is also present in *Brucella suis*, which is an intracellular Gram-negative pathogen and the causative agent of brucellosis (Loisel-Meyer et al. 2005). Under microaerobic conditions in liquid cultures, *B. suis* expresses mainly *cbb₃*-Cox. However inside macrophages, it relies almost exclusively on a *bd*-type quinol oxidase (Loisel-Meyer et al. 2005), which apparently has a higher O₂ affinity than *cbb₃*-Cox in this species. In *H. pylori*, which is responsible for stomach ulcers, *cbb₃*-Cox is the sole respiratory terminal oxidase recognizable in its genome (Table 1). Transposon insertion mutants in *ccoN*, *ccoO* and *ccoP* genes of this species were obtained under microaerobiosis, indicating that *cbb₃*-Cox is not essential under these conditions (Salama et al. 2004). Whether *H. pylori* needs *cbb₃*-Cox to colonize the intestine is unknown. The fact that *H. pylori* mutants that lack fumarate reductase fail to colonize the mouse stomach (Ge et al. 2000) indicates that anaerobic respiration is crucial for virulence of this and other microaerophilic pathogens.

Although not yet studied extensively, the role of *cbb₃*-Cox during pathogenesis might provide a potential antibacterial target for therapeutic interventions. Chemicals that interfere with *cbb₃*-Cox might act as powerful specific antibiotics with minimal side effects due to the absence of this type of Cox in mammals (Smith et al. 2000).

Table 1. Various terminal oxidases and NOR in some pathogenic bacteria (Pitcher et al. 2004)

	<i>cbb</i> ₃ -Cox	NOR	<i>aa</i> ₃ -Cox	<i>bd</i> -type Qox
<i>Campylobacter jejuni</i>	✓	✓		✓
<i>Helicobacter pylori</i> J99	✓			
<i>Neisseria gonorrhoea</i>	✓	✓		
<i>Neisseria meningitidis</i>	✓	✓		
<i>Pseudomonas aeruginosa</i> PAOI	✓	✓	✓	✓
<i>Vibrio cholera</i>	✓			✓

Regulation of *cbb*₃-Cox expression.

In *R. capsulatus* and most other bacteria the steady-state concentration and activity of *cbb*₃-Cox is dependent on the environmental O₂ concentrations. This activity is high under microaerobic, low under fully aerobic and even lower under fully anaerobic growth conditions (Swem et al. 2001). Several components, including RegA (or PrrA), RegB (or PrrB), FnrL, HvrA, and FixLJ-K that respond to different O₂ and redox conditions, are thought to coordinately regulate this process (Swem et al. 2002). RegA and RegB constitute a global two-component regulatory system that controls the expression of many genes involved in energy metabolism, including *cbb*₃-Cox, *bd*-quinol

oxidase, and various genes related to photosynthesis, hydrogen utilization, nitrogen fixation and carbon assimilation (Elsen et al. 2000; Mosley et al. 1994; Sganga et al. 1992; Swem et al. 2001; Vichivanives et al. 2000). RegA is a response regulator with a conserved helix-turn-helix DNA binding motif, whereas RegB is a sensor kinase proposed to contain a conserved region that monitors redox changes inside the cells. Under microaerobic and aerobic conditions the dephosphorylated form of RegA activates *cbb₃-Cox* expression. Conversely, under anaerobic conditions RegA is phosphorylated by the sensor kinase RegB, and phosphorylated RegA represses *cbb₃-Cox* and enhances photosynthesis genes expression (Swem et al. 2002). In *R. sphaeroides*, it was proposed that *cbb₃-Cox* monitors electron flow through its CcoN subunit (Eraso et al. 2000). When this electron flow is high, it sends an inhibitory signal to PrrA and PrrB (homologues of RegA and RegB) to repress photosynthesis genes expression (Eraso et al. 2000). Thus, in this species abolishing *cbb₃-Cox* results in activation of photosynthetic genes under aerobic conditions (O'Gara et al. 1998).

Besides RegA and RegB, some other genes also regulate *cbb₃-Cox* expression. FnrL-like genes are found upstream of *ccoNOQP* in *R. capsulatus* (Koch et al. 1998), and upstream of both *ccoNOQP* and *ccoGHIS* (involved in the assembly of *cbb₃-Cox*, see below) in other organisms including *R. sphaeroides*, *P. denitrificans*, *B. japonicum*. Studies performed in *E. coli* established that FnrL controls switching from aerobic to anaerobic respiration (Cotter et al. 1992). Inactivation of FnrL in *R. capsulatus* decreases *cbb₃-Cox* activity by about 80% under anaerobic growth conditions, suggesting that it

acts as an activator in the absence of O₂ (Swem et al. 2002). This effect is counter balanced by the trans-acting regulatory protein HvrA, which functions as a repressor of *ccoNOQP* under microaerobic and anaerobic conditions (Swem et al. 2002). Another major set of regulators of microaerophilic growth as well as photosynthesis is the FixLJ-K regulatory system. FixL is a histidine kinase that can bind O₂ via a heme *b* group, whereas FixJ is a transcription factor that undergoes phosphorylation by FixL under microaerophilic conditions. Phosphorylated FixL then activates the expression of FixK, a downstream transcription regulator that binds specifically upstream of *ccoN* gene to activate its expression (Fischer 1996; Gilles-Gonzalez et al. 2005; Rey et al. 2010). The FixLJ-K system regulating expression of *cbb₃-Cox* is also found in many other bacteria, including *B. japonicum*, *Caulobacter crescentus* and *Novosphingobium aromaticorans* (Cosseau et al. 2004).

Subunit composition and structure of *cbb₃-Cox*.

Usually, *cbb₃-Cox* enzymes are composed of CcoN (subunit I), CcoO (subunit II), CcoQ (subunit IV) and CcoP (subunit III) proteins (Ducluzeau et al. 2008). Excitingly, the 3D structure of *Pseudomonas stutzerii cbb₃-Cox* was solved recently at a resolution of 3.2 Å (Buschmann et al. 2010). The structure revealed that the overall shape and size of the membrane-embedded part of *cbb₃-Cox* is similar to those of *R. sphaeroides aa₃-Cox* (Svensson-Ek et al. 2002) and *T. thermophilus ba₃-Cox*, (Soulimane et al. 2000) whereas its periplasmic part, mainly formed by the heme containing domains of CcoO and CcoP, is more surface-exposed than in other Cox enzymes (Figure. 2A). Moreover, highlighting

the close evolutionary relationship between the *cbb*₃-Cox and NOR enzymes, the 3D structure of CcoN was found very similar to that of *Pseudomonas aeruginosa* NOR (Hino et al. 2010), even though the amino acid sequence conservation between these two proteins is below 40%.

CcoN forms a “clamshell”-like structure, with its the amino (N-) and carboxyl (C-) termini located close to each other on the cytoplasmic side. CcoN also has two β strands that form a hairpin loop between the helices V and VI. Two calcium atoms with a proposed stabilizing role are found in the structure (Figure. 2B). One of these atoms interacts directly with the heme *b*₃-Cu_B catalytic center, while the second one is located between the loops IV and V at the edge of the structure. The clamshell structure surrounds the catalytic center located near the outer membrane surface and consists of a low spin heme *b*, a high spin heme *b*₃ and a Cu (Cu_B) atom. The open edge of the clamshell is close to heme *b*, whereas heme *b*₃ and Cu_B are at its distal end. Like in NOR, the hemes *b* and *b*₃ are linked together by a calcium atom, which is coordinated to the carboxyl groups of pyrrole D rings of both hemes. Similar to other Cox enzymes, three histidine residues coordinate the Cu_B atom, and one of them is covalently ligated to a tyrosine residue on helix VII (Figure. 2B). This linkage is absent in NOR, and in *aa*₃-Cox the ligating tyrosine residue is located on helix VI (Svensson-Ek et al. 2002). Like the type B enzymes, *cbb*₃-Cox contains only one proton pathway that is positioned similar to the K-channel in the type A enzymes. Similar proton pathways have not been identified

in NOR which does not pump protons, but it is noteworthy that some *cbb*₃-Cox also exhibit low NOR activity (Forte et al. 2001).

CcoO is a mono-heme *c*-type cytochrome, and has an N-terminally located transmembrane helix as an anchor. It is thought to convey electrons to heme *b* of CcoN, and makes strong contacts with the CcoN α -helices. Of the two cavities that are visible on the 3D structure of *cbb*₃-Cox, one is close to the periplasmic face of the membrane, between CcoO and CcoN. This cavity is located at a position equivalent to the end of the D-channel of type A enzymes, and is proposed to provide an exit path for protons and water molecules from the catalytic site to the periplasm. The second cavity is membrane-embedded and connected by narrow and hydrophobic channels to the catalytic site, possibly providing an O₂ access pathway. An equivalent hydrophobic channel is also present in the NOR structure, and is suggested to allow access of NO to the catalytic site of this enzyme (Hino et al. 2010).

CcoP is a di-heme *c*-type cytochrome with both of its heme-groups solvent exposed. In the case of *P. stutzerii*, CcoP has two transmembrane helices connected via a long linker that makes multiple contacts to the cytoplasmic part of CcoN. In some other organisms, including *R. capsulatus*, CcoP probably contains only one transmembrane helix as its anchor. The distal heme group of CcoP is thought to accept electrons from a soluble or membrane bound electron donor (*e. g.*, cytochrome *c*₂ or cytochrome *c*_y in *R. capsulatus*) and transfers them to the proximal heme of this subunit. This latter heme then

conveys electrons to the heme group of CcoO, which transfers them to the catalytic center in CcoN.

CcoQ is a small subunit formed by a single transmembrane helix, and is not present in all *cbb*₃-Cox. It does not contain any cofactor, and its elimination does not completely abolish *cbb*₃-Cox activity. CcoQ is absent in *P. stutzerii* 3D structure, which instead contains an unassigned α -helix, located close to helices IX and XI of CcoN. The location of CcoQ in *R. capsulatus cbb*₃-Cox is unknown, but it can be cross-linked to CcoP, suggesting that it is associated with this subunit (Peters et al. 2008).

BIOGENESIS AND ASSEMBLY OF THE *cbb*₃-COX

In general, Cox assembly is an intrinsically complex process because this oligomeric enzyme is membrane embedded and contains multiple cofactors (Cobine et al. 2006). Membrane insertion and maturation of individual subunits, insertion of cofactors, and assembly of cognate partners have to be coordinated to produce an active enzyme (Cobine et al. 2006; Greiner et al. 2008). In eukaryotes, *aa*₃-Cox assembly involves more than 30 factors, which provide timely availability of mitochondrially-encoded subunits, or which associate transiently with various assembly intermediates (Herrmann et al. 2005; Mick et al. 2011). Assembly of bacterial *aa*₃-Cox is possibly less complex, but still relies on specific components that mediate heme and Cu insertion into the subunits. Assembly of *cbb*₃-Cox is particularly challenging because the maturation of CcoO and

CcoP require the *c*-type cytochrome maturation (CCM) machinery, whereas CcoN relies on heme *b* and Cu atom insertion processes. A set of genes, *ccoGHIS* are located immediately downstream of *ccoNOQP* operon in most *cbb₃*-Cox containing bacteria (Koch et al. 1998; Koch et al. 2000; Kulajta et al. 2006; Preisig et al. 1996). The roles of *ccoGHIS* products in the assembly of *cbb₃*-Cox are described below.

Multistep assembly pathway for the subunits of *cbb₃*-Cox

Assembly of *cbb₃*-Cox has been studied in *R. capsulatus* membranes and *cbb₃*-Cox was shown to form an active complex of 230 kDa, which contains all four (CcoNOQP) structural subunits (Kulajta et al. 2006). Later on, it was also found that the putative assembly factor CcoH is a part of the 230 kDa complex (Figure 3) (Pawlik et al. 2010). CcoH encodes a single-spanning membrane protein with an extended periplasmic domain that is suggested to serve as a dimerization domain (Pawlik et al. 2010). Unlike the mitochondrial *aa₃*-Cox, which forms together with other respiratory complexes a network of supercomplexes (Schagger et al. 1991), no similar large macromolecular assemblies were so far observed in the case of *cbb₃*-Cox in *R. capsulatus* membranes (Kulajta et al. 2006). In addition to the active 230 kDa complex, using BN-PAGE one large (210 kDa) and one small (~ 40 kDa) inactive assembly intermediates were also detected in *R. capsulatus* membranes (Figure. 3). The 210 kDa complex contained CcoN, CcoO and CcoH, but lacked CcoP (Kulajta et al. 2006; Pawlik et al. 2010), and the ~ 40 kDa complex had CcoP (Kulajta et al. 2006), CcoQ and CcoH (Pawlik et al. 2010; Peters et al. 2008). The CcoO subunit in the 210 kDa, and the CcoP subunit in the ~ 40 kDa

complexes contained their covalently attached heme groups as indicated by their peroxidase activity (Kulajta et al. 2006). Thus, maturation of the *c*-type cytochrome subunits occurred prior to their assembly into *cbb*₃-Cox. Genetic data supported these findings, as mutants expressing truncated CcoP derivatives contained only the 210 kDa, and not the 230 kDa complex (Kulajta et al. 2006), and mutations abolishing the *c*-type cytochrome maturation process also prevented *cbb*₃-Cox assembly (Deshmukh et al. 2000). Although not essential, CcoQ seemed to improve *cbb*₃-Cox assembly as in its absence active enzymes were produced at reduced amounts. More recent chemical cross-linking data suggest that interactions between CcoQ and CcoP favor assembly of the ~ 40 kDa complex with the 210 kDa complex (Peters et al. 2008). The surprising finding that CcoH is associated with the assembly intermediates of *cbb*₃-Cox (Pawlik et al. 2010) suggested that this protein behaved more like a *bona fide* subunit of *R. capsulatus* enzyme rather than an assembly factor. This is further supported by the observation that the steady-state stability of CcoH is strictly dependent on the presence of CcoNOQP (Pawlik et al. 2010). Available data suggest that *cbb*₃-Cox assembly might occur via a fusion between the CcoNOH and CcoQPH subcomplexes mediated by CcoH (Figure 3) (Kulajta et al. 2006; Pawlik et al. 2010). The 3D structure of *cbb*₃-Cox indicates that CcoO is sandwiched between CcoN and CcoP (Buschmann et al. 2010). A similar assembly pathway has also been proposed for *B. japonicum* *cbb*₃-Cox, although CcoH was not included in this model (Zufferey et al. 1996).

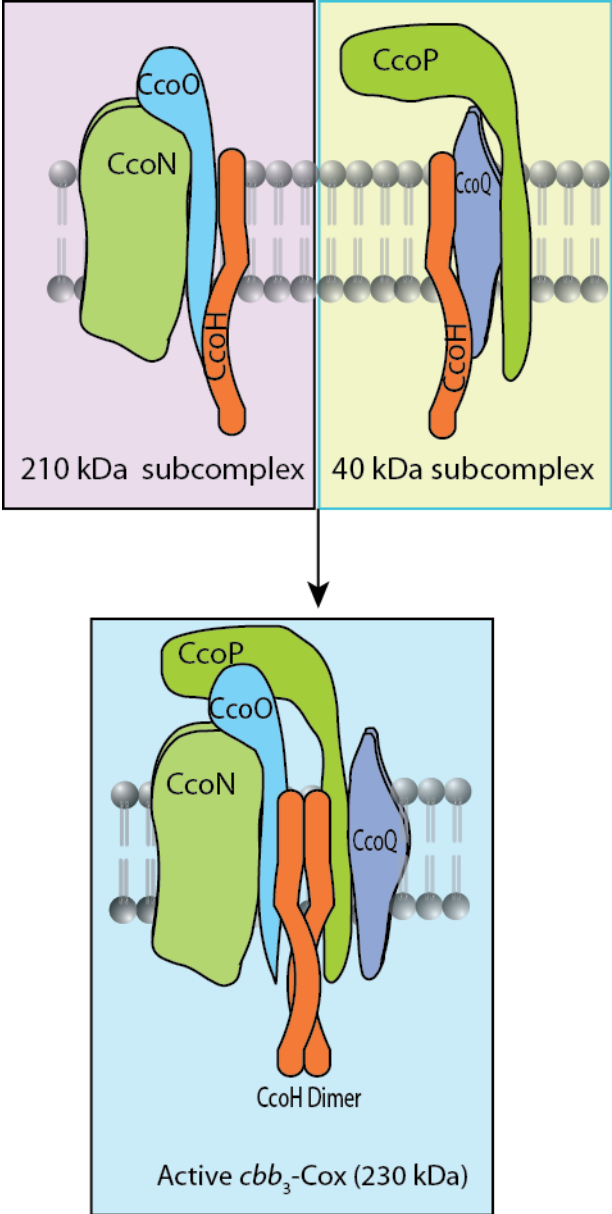


Figure 3: Multistep assembly pathway of *cbb*₃-Cox in *R. capsulatus*

Figure 3: Multistep assembly pathway of *cbb*₃-Cox in *R. capsulatus*.

Using BN-PAGE, active *cbb*₃-Cox is detectable in *R. capsulatus* membranes as a 230 kDa complex. Assembly of the 230 kDa complex proceeds via two inactive assembly intermediates: a 210 kDa subcomplex that contains the catalytic subunit CcoN, the mono-heme cytochrome *c* subunit CcoO and the single-spanning membrane protein CcoH, and a 40 kDa subcomplex composed of the di-heme cytochrome *c* subunit CcoP, the small subunit CcoQ and a second copy of CcoH. Full assembly of *cbb*₃-Cox is probably achieved by the dimerization of the cytoplasmic domains of the two CcoH. CcoH was originally considered to function as an assembly factor, but recent data demonstrate that it is a stable component of the fully assembled *cbb*₃-Cox. The *c*-type cytochromes in both assembly subcomplexes have their heme groups attached indicating that *c*-type cytochrome maturation preceded the formation of the assembly intermediates.

Maturation of *cbb*₃-Cox subunits

i) Maturation of CcoN

CcoN is a hydrophobic protein with 12 transmembrane helices, and is most likely co-translationally inserted into *R. capsulatus* cytoplasmic membrane (Hans-Georg Koch 2007; Koch et al. 2003). The two heme *b* and the Cu cofactor are deeply buried within CcoN, and their incorporation into this subunit might be critical for proper folding and stabilization of *cbb*₃-Cox.

a) Insertion of heme into CcoN

The heme molecule is an important player in the cellular energy production such as in the respiration and photosynthesis. Heme molecules are derived by iron (Fe) insertion into cyclic tetrapyrroles which can also give rise to other end products with different metal insertions such as the chlorophyll, bacteriochlorophyll (by insertion of magnesium), and Vitamin B12 (by insertion of cobalt) (Smart et al. 2004). The first three steps in this pathway are common to all tetrapyrroles. Glycine and succinyl CoA form 5-amino-levulinic acid which is then converted to uroporphyrinogen III in three steps. The branch of heme synthesis requires the formation of protoporphyrinogen IX from uroporphyrinogen III which would be later completed by the insertion of Fe (the same molecule becomes bacteriochlorophyll by the insertion of Mg). The synthesis of heme from protoporphyrin IX involves the enzyme ferrochelatase that inserts the iron into the tetrapyrrole.

In *R. capsulatus*, CcoS is a small membrane protein of 56 amino acids with no putative heme or Cu binding motifs. A mutant lacking CcoS assembles an inactive *cbb*₃-Cox variant of 230 kDa that lacks both of the heme *b* cofactors of CcoN, but contains properly matured CcoO and CcoP subunits (Koch et al. 2000). This finding indicates that *c*-type cytochrome maturation is not affected, and further supports that the CcoN and *c*-type cytochrome maturation processes occur independently of each other (Koch et al. 2000). Whether the inactive *cbb*₃-Cox variant present in a *R. capsulatus ccoS* knock out mutants contains Cu_B is unknown. Clearly, CcoS is required for proper maturation of CcoN, but defining its precise role in *cbb*₃-Cox assembly deserves further studies.

In both mitochondria and bacteria, heme insertion into *aa*₃-Cox is mediated by Surf1 (called Shy1 in yeast), which accepts heme *a* directly from heme *a* synthase (CtaA) and is thought to transfer it to subunit I (Hannappel et al. 2011). In the absence of Surf1, *aa*₃-Cox assembly is significantly reduced, but not completely abolished (Bundschuh et al. 2008; Smith et al. 2005; Tiranti et al. 1998) implying that Surf1 is important, but not essential for heme *a* insertion into the subunit I. Moreover, Surf1 homologues are not present in all species that contain heme *a* containing Cox (*e. g.* they are missing in *B. subtilis* and *T. thermophilus*). In the case of *T. thermophilus*, a protein called CbaX, which has no homology to Surf1, was implicated in heme *a* insertion into *ba*₃-Cox (Werner et al. 2010). A Surf1 homologue is absent in *R. capsulatus* genome, and a *R. sphaeroides* mutant that lacks SurfI is impaired only in the assembly of *aa*₃- but not *cbb*₃-Cox (Smith et al. 2005). Available data indicate that Surf1 is dedicated to heme *a*, and

not involved in heme *b* insertion. In fact, how heme *b* is inserted into any membrane protein is still enigmatic.

b) Insertion of Copper into CcoN

Metals are essential in the cell for the continuity of many cellular functions such as electron transport, oxidative phosphorylation, antioxidant defense and neuronal activities. Copper is an essential cofactor of many enzymes such as Cox, Cu-Zn superoxide dismutase, multicopper oxidase (or laccase) and tyrosinase. Like many metals, free Cu is basically undetectable in cells, minimizing the risk of reactive oxygen species production. Hence, Cu acquisition, trafficking, storage, and delivery to the target sites are strictly controlled processes (Banci et al. 2010). Cu can be found in two redox states: the reduced Cu^+ is considered to be the transported form while the oxidized Cu^{2+} is probably the catalytically active form.

The role of P-type ATPases (CcoI): In contrast to Cu import pathways, bacterial Cu efflux pathways are intensely studied, mainly motivated by the toxicity of Cu for all organisms. A major Cu efflux mechanism is provided by P-type ATPases (P1B subgroup). P-type ATPases are integral membrane pumps that hydrolyse ATP for maintaining ion homeostasis, electrochemical gradients and lipid asymmetry (Kuhlbrandt 2004). The P-type ATPase superfamily is composed of 11 distinct subgroups of which the P1B is one of the largest and most widespread. P1B-type ATPases use ATP

hydrolysis for Cu extrusion across the cytoplasmic membrane (Arguello et al. 2007; Linz et al. 2007; Lutsenko et al. 2007; Palmgren et al. 1998), and contain the characteristic motifs of P-type ATPases, including the ATP binding domain, the phosphorylation domain, and the phosphatase domain (Solioz et al. 1996). They also contain an N-terminal heavy metal binding domain (HMBD) with the conserved Cu-binding motif (MXCXXC), the membrane embedded ion translocation (CPX)- and the conserved histidine-proline (HP)-motifs (Solioz et al. 1996). The mechanism of loading cytosolic Cu to Cu-ATPases is not well understood, but HMBD domains are thought to interact with specific metallochaperones to deliver Cu atoms to these transporters (Gonzalez-Guerrero et al. 2008; Gonzalez-Guerrero et al. 2009). The recent resolution of the first 3D structure of a P1B-type ATPase from *Legionella pneumophila* revealed a putative docking platform for Cu chaperones (Gourdon et al. 2011). The P1B-type ATPases pump Cu^+ out of the bacterial cytoplasm, but their specific roles seem to be determined by their rate of Cu efflux (Raimunda et al. 2011). For example two of them, CopA1 and CopA2 of *Pseudomonas aeruginosa*, are highly homologous to each other (35% identity and 50% similarity), yet their functions differ. CopA1 has a faster Cu efflux rate and is required for Cu detoxification as its absence renders cells highly sensitive to Cu. CopA2 has a much slower Cu efflux rate and its absence does not affect cellular Cu sensitivity, but leads to the loss of *cbb*₃-Cox activity (Gonzalez-Guerrero et al. 2010).

CcoI is a homologue of CopA2, and is the product of *ccoI* gene located in the *ccoGHIS* cluster in many species (Koch et al. 1998; Preisig et al. 1996). In *B. japonicum*

and *R. capsulatus*, CcoI appears to be specifically required for *cbb*₃-Cox assembly, but a *Rubrivivax gelatinosus* mutant lacking the CcoI homologue CtpA seems to be defective in other Cu containing enzymes such as *caa*₃-Cox and N₂O reductase as well (Hassani et al. 2010). In *R. capsulatus*, absence of CcoI (Koch et al. 2000) or mutations in its N-terminal HMBD domain or CPC motifs, drastically reduce the steady-state amounts of *cbb*₃-Cox subunits (Figure 4) (Koch et al. 2000). Cu supplementation does not suppress the *cbb*₃-Cox defect of a mutant lacking CcoI, which is also not sensitive to Cu (Koch et al. 2000). Whether Cu_B is inserted into CcoN before or after heme *b* is not known. In case of *aa*₃-Cox, the availability of enzyme variants containing heme *a*, but lacking Cu_B suggests that heme *a* is likely to be inserted before Cu_B (Smith et al. 2005).

CcoG: Among the *R. capsulatus* mutants lacking any one of the *ccoGHIS* products, those devoid of CcoG exhibits the mildest effect on *cbb*₃-Cox assembly. In the absence of CcoG, all subunits are present at quasi wild type levels, and the enzyme activity is not significantly reduced (Koch et al. 2000). CcoG is an integral membrane protein, which has five predicted transmembrane helices and two putative [4Fe-4S] cluster-binding motifs, but whether it contains an iron-sulfur cluster has not been shown experimentally. The exact function of CcoG remains unknown (Figure 4). It has been proposed that it might be involved in intracellular oxidation of Cu⁺ to Cu²⁺ (Preisig et al. 1996).

SenC (ScoI): SenC is a membrane-anchored protein with a single transmembrane helix, and in its absence, *cbb₃-Cox* biogenesis in *R. capsulatus* (Swem et al. 2005) and in *P. aeruginosa* (Frangipani et al. 2009) is drastically decreased and *cbb₃-Cox* activity is regained upon addition of exogenous Cu. Its periplasmic domain contains a thioredoxin fold and a conserved Cu binding motif comprising CxxxC and a His ligand (Swem et al. 2005). SenC is a close homologue of the universally conserved ScoI protein, which has been implicated mainly into the assembly of the Cu_A center of subunit II of *aa₃-Cox*. Whether ScoI acts as a direct donor of Cu to subunit II (Glerum et al. 1996), or is a thiol:disulfide oxidoreductase reducing appropriate cysteines of subunit II for subsequent Cu delivery by another Cu chaperone (Abriata et al. 2008) is not yet clear (Banci et al. 2011). *B. japonicum* mutants lacking SenC were impaired in the assembly of *aa₃-* but not *cbb₃-Cox* and showed reduced symbiotic N₂ fixation (Buhler et al. 2011). Similarly, *R. sphaeroides* mutants lacking PrrC (a SenC homologue) had no effect on *cbb₃-Cox* assembly although they were defective in photosynthetic growth (Eraso et al. 2000). Like *R. capsulatus senC*, *R. sphaeroides prrC* is located next to *prrAB* (homologues of *R. capsulatus regAB*) genes that encode a two component regulatory system controlling energy processes, including photosynthesis (Masuda et al. 1999; Swem et al. 2001). Whether *prrC* mutations have any polar effect on downstream *prrB* gene, indirectly interfering with cellular amounts of PrrB, is not known.

Several studies have shown that the role of prokaryotic ScoI homologues is not restricted to their involvement in *aa₃-Cox* assembly. A role of these proteins in oxidative

stress response (Seib et al. 2003) or photosynthetic gene regulation (Eraso et al. 2000) has been proposed. Bioinformatics based genome surveys indicated that ScoI homologues are also present in many bacterial species, like *R. capsulatus*, which do not have a Cu_A containing Cox (Banci et al. 2011). *R. capsulatus* mutants lacking SenC produce very low amounts of *cbb*₃-Cox (Borsetti et al. 2005; Swem et al. 2005), a phenotype which was rescued by addition of exogenous Cu (Swem et al. 2005) (Figure 4).

Most eukaryotes have two homologs of Sco. Although yeast has two homologs it has been shown that the presence of only ScoI was enough to have active Cox suggesting that these proteins had additional and different functions in contrast to human that needs both Scos to have functional Cox (Glerum et al. 1996). In yeast ScoII could only rescue point mutations in *scoI* and rescue *cox17* defect in the presence of Cu pointing out that these proteins function differently (Glerum et al. 1996).

The role of the Sco proteins in the assembly of Cox remains obscure but studies showed that Sco is involved in functions other than Cu insertion into Cox such as in redox signaling in mitochondria and *R. sphaeroides* (Eraso et al. 2000), protection against oxidative stress in *Neisseria meningitis* and *Neisseria gonorrhoeae* (Seib et al. 2003), symbiosis in *B. japonicum* (Buhler et al. 2010) and maintaining the cellular copper homeostasis in humans (Leary et al. 2007).

PCu_AC (DR1885): The periplasmic Cu_A-chaperone (PCu_AC) was first identified in *Deinococcus radiodurans* and its structural gene is located near ScoI (Banci et al. 2005). In this species, PCu_AC is thought to provide Cu to the Cu_A center after ScoI reduces the appropriate disulfide bond at subunit II of *aa*₃-Cox. In the case of *T. thermophilus* PCu_AC is involved in Cu transfer to Cu_A of *ba*₃-Cox (Abriata et al. 2008). Homologues of PCu_AC are also found in many species that lack *aa*₃-Cox such as *R. capsulatus*. Unlike *D. radiodurans*, in *R. capsulatus* the structural gene of PCu_AC is not located near that of SenC, but its product contains similar conserved metal binding motifs. In this species whether PCu_AC affects *cbb*₃-Cox Cu_B center assembly is unknown as no chromosomal PCu_AC knockout mutant could be obtained so far (Figure 4).

c) Cu distribution and trafficking to Cox and Cu containing proteins in other organisms

Cu proteome (Cu containing proteins) is estimated to be less than the 1% of the total proteome of an organism (both eukaryotes and prokaryotes) (Andreini et al. 2008). Although this is less than the amount of total zinc or iron containing proteins, the organisms still need to develop highly organized Cu trafficking and transport systems to the copper containing proteins due to the toxic nature of the free Cu atoms. In addition to the Cox which is the main subject of this study, there are many other Cu containing proteins and the biogenesis of these enzymes are achieved by the synchronized function of many low affinity and high affinity transporters, metallochaperones and Cu binding compounds (Banci et al. 2010). Especially in eukaryotes this is an elaborate process due

to the locations of these proteins in different compartments (organelles). In case of multicellular eukaryotes, there is an additional level of complexity which is the distribution and homeostasis between different tissues and at a systemic level.

In the next section, we will present the mechanisms of Cu trafficking to the Cu containing enzymes in organisms such as the Gram positive bacteria, eukaryotes (human, yeast), plant chloroplasts and mitochondria and cyanobacteria.

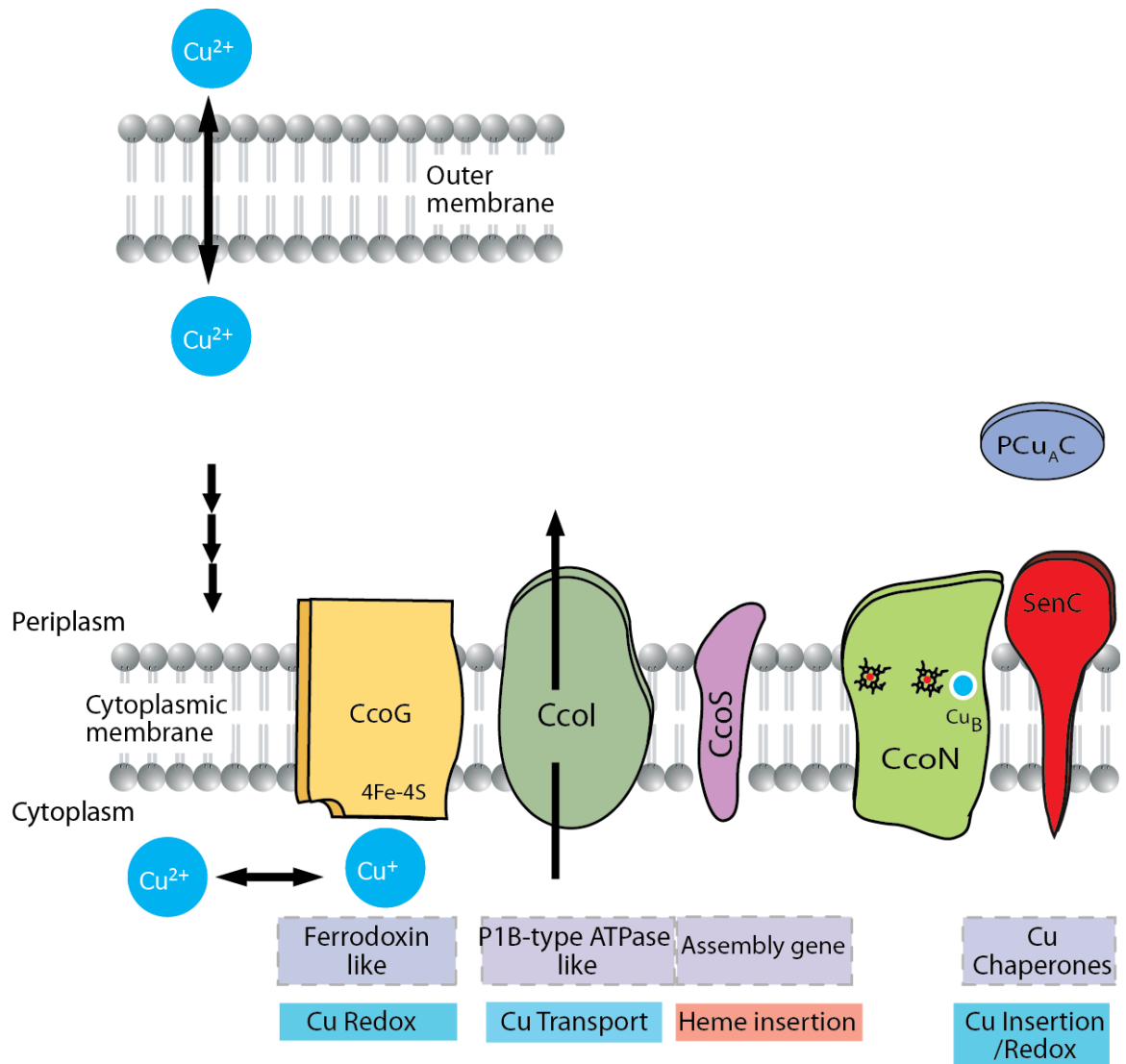


Figure 4: Insertion of Cu and heme cofactors into CcoN subunit of *cbb*₃-Cox

Figure 4: Insertion of Cu and heme cofactors into CcoN subunit of *cbb*₃-Cox

The maturation of CcoN requires Cu and heme *b* insertions. On the cytoplasmic face of the membrane Cu^{2+} is reduced to Cu^+ by the ferredoxin-like protein CcoG and transported back to the periplasmic face of the membrane by the P1B-type ATPase CcoI. CcoI then supplies Cu^+ to CcoN either directly within the membrane or from the periplasm, or via some putative periplasmic Cu binding proteins such as SenC and PCu_AC. The insertion of heme *b* and heme *b*₃ into CcoN is probably mediated by the assembly factor CcoS. This model is tentative.

Yeast/Humans

Eukaryotes need Cu to be transported to different compartments of the cells for the maturation of Cu containing enzymes such as Cox in mitochondria and tyrosinase or ceruloplasmin in the Golgi apparatus. Cu is imported into the eukaryotic cells by the family of high affinity copper transport protein (Ctr) transporters (Dancis et al. 1994). Ctr family are functionally conserved from yeast to humans. All members of Ctr family is composed of three transmembrane helices, an extracellular amino terminus rich in MX_xM (Met) motifs that were proposed to be needed for the binding of Cu⁺ for the uptake under highly limiting Cu conditions (Puig et al. 2002) and Cys/His conserved residue at the C-terminus in the cytoplasm implicated in metal binding in cytoplasm (De Feo et al. 2009) (De Feo et al. 2007). MXXM motif is also present in the second transmembrane helices which is required for the Cu uptake and provides ligands to Cu atoms. Ctr1 which was the first identified Ctr in *Saccharomyces cerevisiae* (Dancis et al. 1994) is found in the plasma membrane of both yeast and human cells and were shown to transport Cu⁺ rather than Cu²⁺ by the requirement of yeast cell-surface metalloreductase activity, stimulation of Cu uptake by the presence of an external reductant (Hassett et al. 1995) and the transport of Ag which is a Cu⁺ mimetic by these transporters (Lee et al. 2002; Martins et al. 1998).

To the Golgi apparatus: In human cells Atox1 delivers Cu to P-type ATPases ATP7A and ATP7B in the Golgi apparatus (Walker et al. 2002). In yeast cells Atx1 is required for delivery to P-type ATPase Ccc2. The P-type ATPases later secrete Cu into lumen for the insertion into Cu containing enzymes multicopper oxidase Fet3 in yeast

and ceruloplasmin in the human cells (Yuan et al. 1995). The human homologs of Cu transporting P1B type ATPases are ATP7A and ATP7B have been studied extensively to understand the copper transport in humans. The functions of both proteins are to export excess Cu from the cells, but their locations differs leading to different disorders when they are inactive. ATP7A is ubiquitously found in extrahepatic cells and export Cu into circulation to be distributed through the body, and the mutations in this protein results in Menkes disease (systemic copper deficiency) (Vulpe et al. 1993) in which Cu accumulates mainly in kidney and liver that leads to malabsorption of Cu that reduces the available Cu to the cuproenzymes (Horn et al. 1980; Kodama et al. 1999) ATP7B is expressed in liver, some regions of brain, kidney and is involved in the delivery of Cu to the bile (Malhi et al. 2002). Inactivation of this transporter leads to accumulation of Cu in the liver leading to Wilson's disease (copper toxicosis) (Bull et al. 1993). Although these transporters are characterized by their Cu pumping function, they are not located in the mitochondria and there is no evidence that they are involved in the Cu insertion into the Cox or other cuproenzymes located in mitochondria. Therefore it is clear that these proteins are involved in keeping the homeostasis and delivering Cu to the cells and protecting the cells from Cu stress, however there must be other transporters that would supply Cu for cuproenzymes and should have different features that would distinguish them.

To the mitochondria: In both human and yeast cells: Delivered by an unknown transporter to be inserted in Cox. In the mitochondrial intermembrane space (IMS),

another Cu chaperone Cox17 delivers Cu to either ScoI/ScoII needed for the insertion into CuA center in the subunit II of Cox, or to Cox11 for the insertion into CuB center of subunit I (for reviews (Banci et al. 2010; Cobine et al. 2006; Kim et al. 2008)).

Plant Chloroplast/Mitochondria and Cyanobacteria

Cu enters the plant cells via a Ctr like transporter Copper Transporter Protein family protein COPT1 (Sancenon et al. 2003). In plants, in addition to the need for Cox in the mitochondria, more than half of the Cu is present in the chloroplasts required for the two Cu containing enzymes plastocyanin and Cu/Zn SOD. In the plant chloroplast, PAA1/HMA6 and PAA2/HMA8 which are P-type ATPases import Cu into the inner and thylakoid membrane respectively. CCS Cu chaperone delivers the cytosolic Cu to the PAA2/HMA8 to be transported in the thylakoid. This uptake system of Cu is conserved in both cyanobacteria and plants. In cyanobacteria, Cu is imported through two P-type ATPases called CtaA and PacS which is localized at the inner and thylakoid membrane respectively and a Cu chaperone, Atx1 is carries Cu to the thylakoid. In cyanobacteria, thylakoids are the site of both photosynthetic and respiratory electron transport processes so both plastocyanin and Cox is also located here (Tottey et al. 2001). However it is not clear how Cu is transported to the mitochondrial Cox (Nouet et al. 2011; Pilon et al. 2006).

In plants, Cu has been found to have another novel role, on the biogenesis of molybdenum cofactor. Molybdenum is found as cofactor in many enzymes such as nitrate reductase, xanthine dehydrogenase. The structure of the protein CNX1 required

for the insertion of molybdenum into the molybdopterin revealed the presence of a Cu atom bound to the thiols of the molybdopterin moiety which was suggested to be there for the protection of thiols before the insertion of molybdenum, linking the Cu and molybdenum metabolism (Kuper et al. 2004).

Gram positive Bacteria

Cu transport in Gram positive (Gr^+) bacteria was extensively studied in *Enterococcus hirae* and is composed of an operon that consists of four genes *copA*, *copB*, *copY* and *copZ* (Solioz et al. 2010). CopA and CopB are P1B-type ATPases and CopA was suggested to import Cu into the cytoplasm (Odermatt et al. 1993). After Cu is in the cytoplasm it binds to CopZ a copper chaperone, which then delivers Cu to CopY repressor or CopB efflux transporter. CopY is one of the two types of Cu responsive regulators that is present in Gr^+ bacteria (Strausak et al. 1997). The other class is CsoR type, which is not only present in Gr^+ but also in proteobacteria (Liu et al. 2007). Another class called CueR type regulators that are homologous to MerR type transcriptional activators are only present in proteobacteria (Stoyanov et al. 2001).

ii) **Maturation of CcoO and CcoP subunits: *c*-type cytochrome maturation (CCM) system**

The CcoO and CcoP subunits of *cbb₃*-Cox are matured by a *c*-type cytochrome maturation (CCM) process, which operates independently from CcoN maturation. CCM is a post-translation and post-translocation process that occurs on the periplasmic face of the cytoplasmic membrane (Figure 5). During this process, heme *b* groups are covalently and stereo-specifically attached by thioether bonds formed between their vinyl groups and the cysteine thiols of conserved CXXCH motifs of apocytochromes (Barker et al. 1999; Thony-Meyer 2000). Several CCM systems are encountered in nature, and in α - and γ -proteobacteria and *Deinococcus* species maturation of the CcoO and CcoP subunits relies on CCM-system I, which was reviewed in detail recently (Kranz et al. 2009; Sanders et al. 2010). CCM-system I can be divided into three operation modules that are described briefly below.

Module 1: Transport and relay of heme: The function of this module is to translocate cytoplasmically synthesized heme *b* to the periplasm, and to prepare it for ligation to apo-cytochromes (Figure 5, right). This module consists of five proteins, named CcmABCDE. CcmABCD is suggested to form an ATP binding cassette (ABC)-type transporter involved in loading heme *b* to CcmE. CcmE has a single membrane-anchoring helix and a conserved histidine residue that covalently binds heme, which then acts as a heme donor to apocytochromes. CcmA has an ATP binding domain and Walker A and B motifs needed for ATPase activity. CcmB is an integral membrane protein with

six transmembrane helices, required for membrane localization of CcmA. CcmC contains a conserved tryptophan-rich WWD motif and is involved in loading heme to CcmE, and apparently CcmD enhances this process. A recent bioinformatics study grouped CcmC and its homologues as the “heme handling proteins” (HHP) (Lee et al. 2007). To what extent CcmAB and CcmCD function together or separately for loading heme onto CcmE and delivering heme-loaded CcmE to the heme ligation complex is unclear. A possibility is that CcmC and CcmD are necessary for attachment of heme to CcmE (Goldman et al. 1998; Goldman et al. 1997; Thony-Meyer 2003), and that CcmAB is required for ATP dependent release of heme-loaded CcmE from CcmCD (Feissner et al. 2006). On the other hand, whether CcmAB has another, yet to be defined, role in this process is unknown (Christensen et al. 2007; Schulz et al. 1999).

Module 2: Apocytochrome thio-oxidoreduction and chaperoning: Like most *c*-type apocytochromes, CcoO and CcoP are thought to be secreted via the SecYEG secretory pathway (Figure 5, left). Their signal-anchor sequences are not processed, and serve as their N-terminal membrane-anchors. Upon translocation across the cytoplasmic membrane, the DsbA-DsbB dependent oxidative protein-folding pathway (Kadokura et al. 2003; Nakamoto et al. 2004) is thought to rapidly oxidize the thiol groups of apocytochromes to possibly avoid their proteolytic degradation. The disulfide bonds formed between the cysteines at the conserved heme binding (CXXCH) motifs need to be reduced prior to heme *b* attachment. Thus, a Ccm-specific thio-reduction pathway involving CcdA, CcmG and CcmH proteins has been proposed (Beckman et al. 1993;

Monika et al. 1997). CcdA is an integral membrane protein with six transmembrane helices and is responsible for conveying from the cytoplasm to the periplasm reducing equivalents required for this process. CcmG and CcmH are membrane-anchored thioredoxin-like proteins that contain a single CXXC domain facing the periplasm (Monika et al. 1997), and thought to reduce the disulfide bonds at the heme binding sites. Of the three components, CcdA and CcmG are not required in the absence of DsbA-DsbB dependent thio-oxidation pathway, but CcmH is still required for *c*-type cytochrome production, suggesting that it has an additional role. Moreover, the 3D structure of CcmG showed that, in addition to its canonical thioredoxin fold, it has a cavity that might bind apocytochromes which is in accordance with a putative holdase role of CcmG as shown in *R. capsulatus* (Turkarlan et al. 2008)

Module 3: Apocytochrome and heme *b* ligation: In *R. capsulatus*, CcmF, CcmH and CcmI proteins have been proposed to form a heme ligation core complex (Figure 5, middle), bases on reciprocal co-purification experiments documenting protein-protein interactions between these components (Sanders et al. 2008). Of these proteins, CcmF is a large integral membrane protein with 11 transmembrane helices, with a tryptophan-rich (WWD) signature motif and four conserved histidine residues facing the periplasm proposed to bind heme *b* before its ligation to apocytochromes (Goldman et al. 1998). It interacts with heme-loaded CcmE (Ren et al. 2002), is a heme handling protein (HHP) like CcmC (Goldman et al. 1998). CcmI is a bipartite protein that contains a membrane embedded N-terminal region with two transmembrane helices and a

cytoplasmic leucine-zipper-like motif containing loop (CcmI-1 domain) and a large periplasmic C-terminal extension with tetratricopeptide repeat (TPR) motifs (CcmI-2 domain) (Lang et al. 1996; Sanders et al. 2007; Sanders et al. 2005). Unlike most other bacteria, in *E. coli* the homologue of CcmI-2 domain is fused to the C-terminal end of CcmH, leaving only CcmF and a modified “CcmH” as components of heme ligation core complex (Fabianek et al. 1999). *R. capsulatus*, mutants lacking CcmI can be suppressed by overproduction of CcmF and CcmH or CcmG. Complementation studies with two distinct domains of CcmI indicated that CcmF and CcmH overproduction relates to the functional role of CcmI-1 domain (Deshmukh et al. 2002), whereas CcmG overproduction to that of CcmI-2 (Sanders et al. 2005). These findings suggested that CcmI might be a junction point between the CcdA-CcmG dependent thio-reduction and CcmF-CcmH dependent heme ligation processes. Indeed, CcmI was initially proposed to chaperone apocytochromes to the heme ligation complex (Lang et al. 1996), and very recently *in vitro* studies provided strong biochemical evidence to support this proposal (Verissimo et al. 2011). Protein-protein interaction studies conducted using purified CcmI and purified apocytochrome *c*₂ indicated that the C-terminal CcmI-2 domain of CcmI recognizes and binds tightly the most C-terminal helix of apocytochrome *c*₂ in the absence of heme *b*. The folding process of cytochrome *c* (and cytochrome *c*₂) (Akiyama et al. 2000) indicates that their most C-terminal helix interacts with their most N-terminal heme binding helix to form a stable folding intermediate that can trap heme *b* non-covalently (Colon et al. 1996). Altogether, these observations led to the proposal that at least some *c*-type apocytochromes are first recognized via their C-terminal helices by the periplasmic CcmI-2 domain of the CcmFHI core complex (Figure 5, middle), and then

released from this domain upon transfer of heme *b* from CcmF to apocytochrome, and subsequently, the thioether bonds are formed. Once the thioether bonds are formed, cytochrome *c* folds into its final structure.

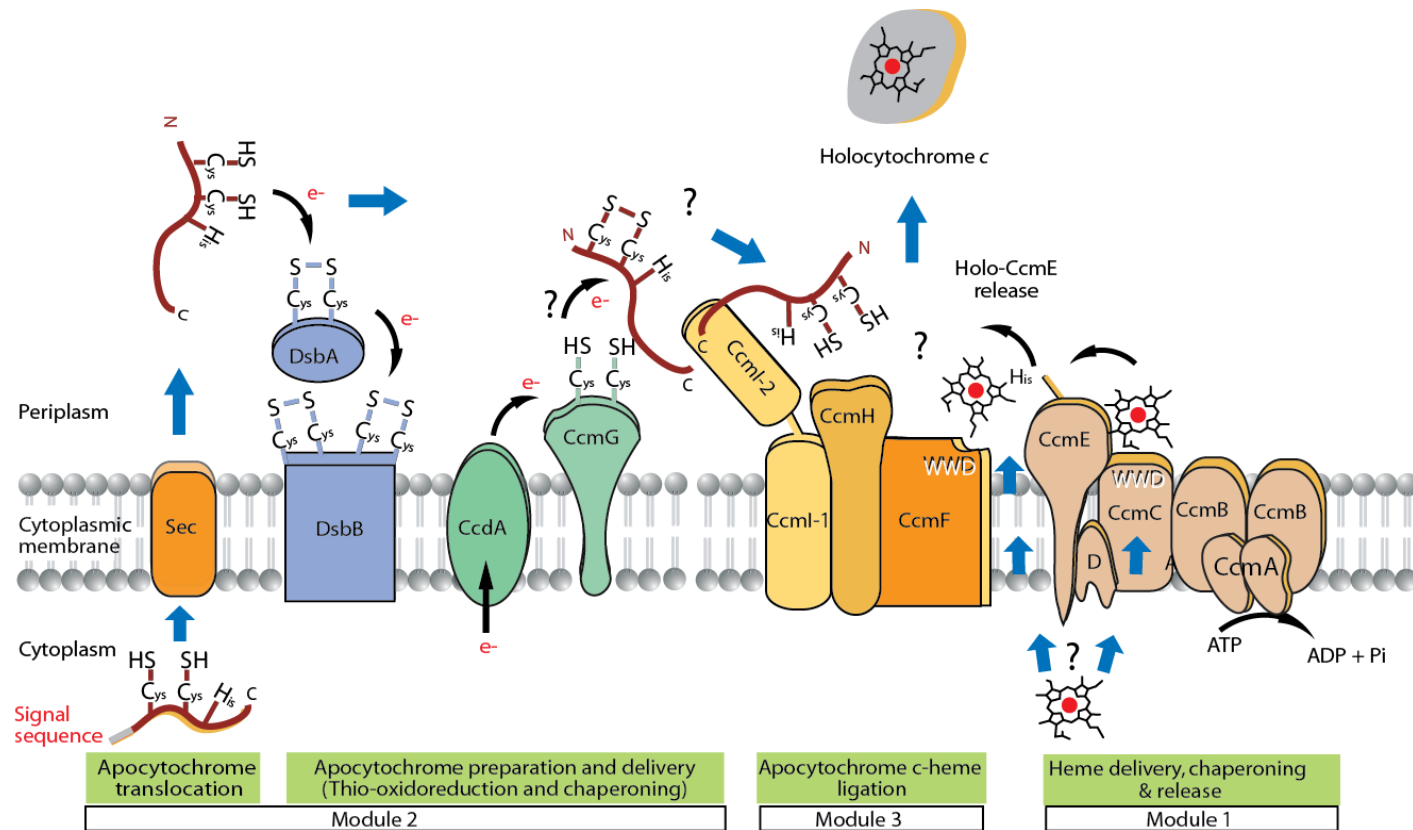


Figure 5. Cytochrome *c* maturation (CCM) system in *Rhodobacter capsulatus*

Figure 5. Cytochrome *c* maturation (CCM) system in *Rhodobacter capsulatus*: The cytochrome *c* maturation components can be divided into three modules. First module (right) is the transport of heme and its preparation for ligation to apocytochrome that involves CcmABCD proteins which forms a ABC-type transporter that has a role in the delivery of heme to the heme chaperone CcmE before the ligation. It is unknown whether heme is transported via CcmABCD complex or by another unknown protein. CcmA and CcmB are required for the release of heme-bound CcmE from CcmC and CcmD. CcmC and CcmD are involved in the heme attachment to CcmE. Second module (left) involves the apocytochrome thioredox and chaperoning processes. After the translocation of apocytochrome into the periplasm, the cysteine thiols are first oxidized by DsbA-DsbB, then reduced by CcdA, CcmG and/or CcmH. Third module (middle) consists of CcmHIF which is the heme ligation core complex. CcmI binds to C-terminal part of apocytochrome to deliver it to the core complex for the catalysis of the thioether bond formation between reduced apocytochrome and heme vinyl groups forming the mature cytochrome *c* (adapted from (Hunter et al. 2008)).

iii) Additional proteins involved in *cbb*₃-Cox biogenesis

Several additional proteins of not yet fully defined roles, including, DsbA (Deshmukh et al. 2003), TlpA, ORF277 might be involved *cbb*₃-Cox biogenesis, and described below.

DsbA: *R. capsulatus* mutants lacking the periplasmic thiol:disulfide oxidoreductase DsbA, primarily involved in oxidative protein folding pathway (Kadokura et al. 2003), overproduce the periplasmic protease DegP (Onder et al. 2008). They have pleiotropic phenotypes, including temperature sensitivity for growth (35° C), osmosensitivity, filamentation and decreased respiratory capabilities. Even at permissive growth temperature (25° C), they exhibit reduced *cbb*₃-Cox activity. Supplementation of the growth medium with redox active chemicals like cysteine/cystine or with Cu²⁺ restores both temperature sensitive growth and *cbb*₃-Cox defects of mutants lacking DsbA. Remarkably, DsbA knock out mutants revert frequently to regain their growth ability at 35° C without any need for redox active supplements, and concomitantly, they restore their *cbb*₃-Cox production. One group of the revertants acquires mutations in *degP* that decrease drastically the protease activity of DegP (Onder et al. 2008). These findings point to important links between the formation of disulfide bond, degradation of misfolded periplasmic proteins, and production of active *cbb*₃-Cox. However it is unknown whether exogenously supplied Cu²⁺ acts exclusively as a source of oxidant in the absence of DsbA, or whether it also eases acquisition of Cu as a missing cofactor for

*cbb*₃-Cox production, or both. Future studies might clarify these emerging links between major cellular processes and *cbb*₃-Cox biogenesis.

TlpA: A thioredoxin like protein called TlpA has been previously described to take a role in the biogenesis of *aa*₃-type Cox in *B. japonicum* (Loferer et al. 1993). TlpA is a membrane anchored protein shows a strong homology to the periplasmic thiol:disulfide oxidoreductases such as DsbA, DsbB, CcmG that catalyze the formation of disulfide bonds. *tlpA* mutants in *B. japonicum* has been shown to be devoid of active *aa*₃-Cox but the subunit I of this enzyme was still present in the membranes in lower amounts compared to the wild type (Loferer et al. 1993). Molecular mechanism of the function of TlpA remains unknown, but it is suggested that it has a role in keeping thiol groups of proteins involved in *aa*₃-Cox biogenesis reduced (Loferer et al. 1995).

ORF227: An ORF (called ORF277 previously in(Kulajta et al. 2006)) located upstream of *ccoNOQP* operon is previously detected in a mass spec analysis in a partially purified *cbb*₃-Cox implying a role in the assembly of this protein (Kulajta et al. 2006). The inactivation of ORF277 does not change the amounts of active *cbb*₃-Cox, but there might be a function related to protection against oxidative stress because of the homology of this protein to the universal stress proteins A that are linked to have a role in the resistance against oxidative stress (Kvint et al. 2003).

iv) **Role of membrane lipids for *cbb*₃-Cox biogenesis**

Membrane lipids, especially those that act as “lipo-chaperones”, are important determinants for membrane protein structure and activity (Bogdanov et al. 2008). These lipids bind to specific locations of proteins in stoichiometric amounts, and influence their folding, stability, steady-state amounts and activity (Dowhan et al. 2011). Phospholipids were shown to co-crystallize with heme-Cu: O₂ reductases from many different species (Qin et al. 2006; Shinzawa-Itoh et al. 2007; Tiefenbrunn et al. 2011), and the stability and organization of respiratory chain super complexes were significantly influenced by some phospholipids like cardiolipin (Paradies et al. 1997; Pfeiffer et al. 2003). A specific role of lipids on biogenesis and catalytic activity of *cbb*₃-Cox is also likely, as changes in membrane lipid composition seem to affect the amounts of *cbb*₃-Cox found in *R. capsulatus* membranes (Aygun-Sunar et al. 2006). Moreover, different lipid environments were also shown to affect carbonmonoxide binding to CcoP (Huang et al. 2010). *R. capsulatus* mutants that were defective in ornithine lipid (OL) biosynthesis lacked *cbb*₃-Cox (Aygun-Sunar et al. 2006). Studies of these mutants defined two genes, *olsA* and *olsB* coding for N-acyltransferase and O-acyltransferase, respectively, which are both required for OL biosynthesis (Aygun-Sunar et al. 2007; Aygun-Sunar et al. 2006). OL is a non-phosphorus membrane lipid that usually accounts only for a small fraction of the total lipids found in bacteria. In some species, OL are synthesized under phosphate limiting conditions, and are used as replacement for phosphate containing lipids (Benning et al. 1995; Geiger et al. 1999; Minnikin et al. 1974). In *R. capsulatus* OL biosynthesis is not regulated by phosphate availability, but absence of OL affects the steady-amounts of

a group of membrane proteins, including most of the *c*-type cytochromes. Mutants unable to synthesize OL have very low amounts of *cbb*₃-Cox and the electron carrier cytochromes *c*₂ and *c*_y. They contain a small amount of cytochrome *c*₁, and consequently, very low ubiquinol: cytochrome *c*₂ oxidoreductase (cytochrome *bc*₁) activity. Interestingly, these defects are both temperature- and growth medium-dependent. Especially on enriched media at regular growth temperature (35° C), mutants lacking OL are photosynthesis-incompetent and have no *cbb*₃-Cox. Pulse-chase studies conducted using cytochrome *c*_y indicated that absence of OL decreased drastically its cellular amount (Aygun-Sunar et al. 2006), suggesting that in the absence of OL, a group of proteins including *cbb*₃-Cox, may be more prone to misfolding and enhanced degradation.

***Rhodobacter capsulatus* as a model organism for studying *cbb*₃-Cox biogenesis**

Rhodobacter capsulatus is a facultative phototroph that belongs to the α -proteobacteria and is a purple nonsulfur bacterium. We choose to study the biogenesis and assembly of *cbb*₃-Cox in *R. capsulatus* because α -proteobacteria is the closest relatives of mitochondria. Therefore, they provide simpler models to study mitochondrial respiration. In addition, use of *R. capsulatus* for studying the *cbb*₃-Cox enzymes is advantageous because this organism contains only two terminal oxygen reductases: a quinol *bd* oxidase and a *cbb*₃-Cox (Gray et al. 1994). Thus, *cbb*₃-Cox is the only heme-copper oxidase present in this bacterium. The *cbb*₃-Cox activity can be directly detected by staining colonies with the NADI reagents (α -naphthol + dimethylphenylenediamine \rightarrow

indophenol blue + H₂O) (Keilin 1966). *R. capsulatus* strains that are deficient for all *c*-type cytochromes cannot grow photosynthetically (Ps⁻) and cannot catalyze the NADI reaction (NADI⁻) because of the absence of *cbb*₃-Cox (Zannoni et al. 1976). However the presence of a cytochrome *bd* oxidase is crucial because *R. capsulatus* mutants that do not have active *cbb*₃-Cox can still grow under respiratory conditions via this alternate terminal oxidase which is cytochrome *c* independent (Figure 6). Moreover, unlike the A family of Cox enzymes, it naturally lacks the Cu_A center, which makes the Cu_B center, the only copper atom in the enzyme. Besides, studying Cu-dependent maturation and assembly of the *cbb*₃-Cox might yield important drug targets, because the *cbb*₃-Cox are absent in humans while they are the only heme-copper terminal oxidases in some pathogenic bacteria such as *Vibrio cholera*, *Helicobacter pylori*, *Neisseria meningitides* and others (Pitcher et al. 2004). *R. capsulatus* also provides a convenient model for biogenesis studies because of the availability of well established genetic, biochemical, molecular and proteomic techniques. The genome of *R. capsulatus* has been sequenced and annotated by two different groups (Strnad et al. 2010) and is publicly available, providing a great tool for genetic and molecular studies. Construction of knock-out mutants of the desired genes are easily accomplished by intersposon mutagenesis via the use of virus like particles, called gene transfer agents (GTA) (Marrs 1974). Another useful feature of *R. capsulatus* is the intracytoplasmic membranes (ICM) formed by the invaginations of the cytoplasmic membrane (Golecki et al. 1980). These vesicles present a inside out configuration that allows us to study the cytochrome complexes that are localized in the inner cytoplasmic membrane.

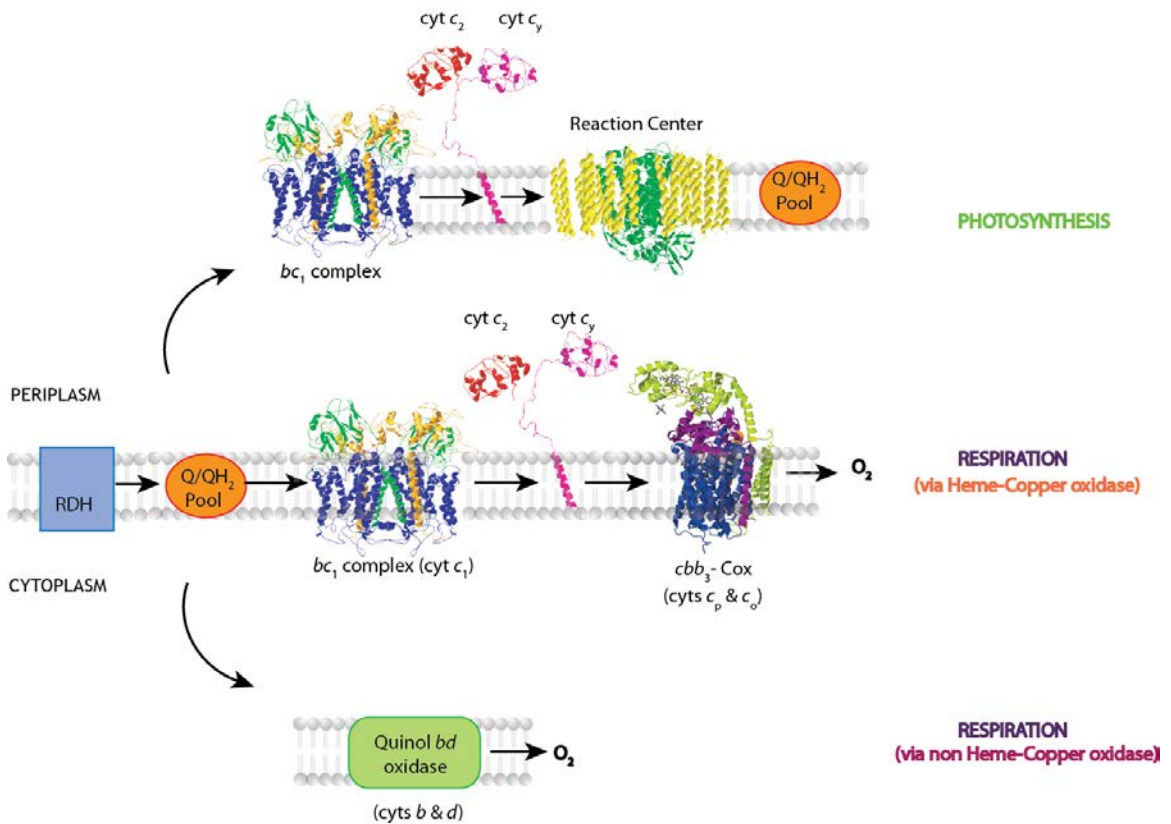


Figure 6: Photosynthetic and respiratory electron transport pathways of *R. capsulatus*.

In *Rhodospirillum rubrum*, the photosynthetic electron transport chain is anoxygenic and cyclic between the reaction center (RC), the cytochrome bc_1 and the quinones (Q Pool). Reducing equivalents are transferred from respiratory dehydrogenases (RDH) to Q Pool then to cytochrome bc_1 . Cytochrome bc_1 is then connected to RC by the cytochromes soluble cytochrome c_2 and membrane anchored cytochrome c_y . The respiratory electron transport chain is branched. One of the branches is mitochondrial like and is terminated by the cbb_3 -Cox which is a heme-copper oxidase and is connected to cytochrome bc_1 by the cytochromes c_2 and c_y . The second respiratory branch is *via bd*-type quinol oxidase which is not a heme-copper oxidase and does not involve any *c*-type cytochromes.

AIM AND SCOPE OF THIS STUDY

Metalloprotein maturation is a complex process that has been studied extensively by analyzing different model proteins and the cytochrome *c* oxidases (Cox) are one of such metalloproteins that have been studied for this purpose. Initially, most of the work was done on the biogenesis and assembly of the mitochondrial *aa*₃-Cox, and found to involve at least 30 proteins. In the last decade, the studies were initiated on the biogenesis of bacterial *cbb*₃-Cox and revealed many new components and steps of this process. However it is clear that many more components should be involved to produce an active *cbb*₃-Cox complex. This work was undertaken to identify and characterize new components and steps of the maturation of *cbb*₃-Cox in *R. capsulatus*.

First, a previously isolated *cbb*₃-Cox mutant was studied to find novel genes involved in the *cbb*₃-Cox maturation. This mutant had a Cu dependent *cbb*₃-Cox phenotype that suggested a defect in the Cu insertion into *cbb*₃-Cox's first subunit (Chapter 2). Complementation of this mutant identified CcoA, a novel component in *cbb*₃-Cox maturation. CcoA is a MFS type transporter that is involved in Cu acquisition to the cells and resulted in the *cbb*₃-Cox deficiency. *cbb*₃-Cox deficiency phenotype was bypassed by yet to be defined mechanism that rendered them sensitive to exogenous Cu (Chapter 4). Identification of these bypass suppressors together with characterization of CcoA function will provide new insights on Cu trafficking mechanism of *R. capsulatus*.

Second, identification of Cu acquisition pathways to *cbb*₃-Cox in the absence of CcoA transporter was attempted by searching for components required for producing active *cbb*₃-Cox in the presence of exogenous Cu. This approach led to the analysis of two mutants defective in cytochrome *c* maturation (CCM) process which was able to grow photosynthetically (Chapter 3). The first mutant was found to have a mutation in *ccmA* gene that led to the production of only some of the *c*-type cytochromes. The second mutant required a complete set of *ccmHIF* genes and could be partially bypassed by CcmE for cytochrome *c* biogenesis in *R. capsulatus*. Characterization of these mutants provides invaluable information about CCM system; however the alternative Cu acquisition pathways in the absence of CcoA still remains to be identified.

CHAPTER 2

CHARACTERIZATION OF A NOVEL TRANSPORTER REQUIRED FOR BIOGENESIS OF *cbb*₃-COX IN *RHODOBACTER CAPSULATUS*

INTRODUCTION

Cells require copper (Cu) as a cofactor of many metalloproteins, including cytochrome *c* oxidases (Cox) (Tsukihara et al. 1995), superoxide dismutases (Tainer et al. 1983) and multicopper oxidases (Roberts et al. 2002). Cu is an essential catalytic and structural cofactor of these important enzymes (Tsukihara et al. 1995), and its shortage causes severe human illnesses, like the Menkes and Wilson's or Alzheimer's diseases (Acevedo et al. 2011; Bull et al. 1993; Gaggelli et al. 2006; Hung et al. 2010; Petrukhin et al. 1994; Tanzi et al. 1993; Vulpe et al. 1993). Conversely, excessive amount of Cu is also toxic as it can activate O₂ to generate dangerous reactive radical species, harming cellular components (Halliwell et al. 1984; Macomber et al. 2009). Thus, cells need to control precisely Cu acquisition, trafficking and incorporation into the target proteins.

Cox biogenesis is a complex process because this membrane-integral enzyme contains various cofactors including heme groups and Cu atoms (Halliwell et al. 1984). Synthesis and maturation of individual subunits, their insertions into the membrane, and assembly of mature subunits into active enzymes are coordinated temporally and spatially. Regulated expression of the subunits and accurate insertion of the cofactors during biogenesis are necessary to ensure that free redox-active cofactors, or reactive assembly intermediates, do not harm the cells (Halliwell et al. 1984).

Currently, a number of components involved in mitochondrial Cox biogenesis are known, and several of them are linked to human diseases (Papadopoulou et al. 1999; Tiranti et al. 1998; Valnot et al. 2000). A smaller number of biogenesis components have been identified so far in the case of bacterial *cbb*₃-Cox, which is important for aerobic respiration, onset of photosynthesis, rhizobial symbiosis, and pathogenesis (Kulajta et al. 2006; Parkhill et al. 2000; Preisig et al. 1996; Stover et al. 2000; Tsukita et al. 1999). However, many of the components and steps governing biogenesis of an active *cbb*₃-Cox still remain undefined. In particular, those involved in forming the universally conserved heme-Cu_B binuclear center of Cox enzymes are unknown. In this work, we report molecular characterization of a *cbb*₃-Cox defective mutant that requires Cu²⁺ supplement to produce an active *cbb*₃-Cox. This mutant unveiled a novel transporter, CcoA, of the major facilitator superfamily (MFS) whose absence greatly diminishes the levels of intracellular Cu content and active *cbb*₃-Cox in *R. capsulatus*. To our knowledge, CcoA is the first example of a MFS type transporter required for efficient Cu acquisition and *cbb*₃-Cox production in bacteria.

MATERIALS AND METHODS

Strains, culture conditions, and phenotypes

The bacterial strains and plasmids used in this work are described in Appendix I. *R. capsulatus* strains were grown in enriched medium (MPYE) (Daldal et al. 1986) or in Siström's minimal medium A (MedA) (Siström 1960) supplemented when appropriate with antibiotics (at final concentrations of 10, 10, 70 and 2.5 µg/ml of spectinomycin (Spe), kanamycin (Kan), rifampicin (Rif) and tetracycline (Tet), respectively). Growth was at 35° C chemoheterotrophically (aerobic respiration) or photoheterotrophically (anaerobic photosynthesis) in anaerobic jars with H₂ + CO₂ generating gas packs from BBL Microbiology Systems (Cockeysville, MD). As needed, 5 µM CuSO₄ was added to MPYE to make MPYE+Cu, or 1.5 µM CuSO₄ was omitted from MedA to yield MedA-Cu media. Earlier ICP-MS analyses indicated that, in the absence of CuSO₄ supplement or chemical chelation of Cu both MPYE and MedA-Cu media contained approximately 150 nM of Cu²⁺ as “contaminants”, and bioavailable Cu was much lower in MPYE medium (Koch et al. 2000). *E. coli* strains were grown on Luria Bertani (LB) broth supplemented with appropriate antibiotics (at final concentrations of 100, 50, 50 and 12.5 µg/ml of ampicillin (Amp), Kan, Spe and Tet, respectively) as described previously (Jenney et al. 1993). NADI (*i. e.*, Cox activity) phenotypes of *R. capsulatus* colonies were revealed qualitatively using the NADI staining made by mixing 1:1 (v/v) ratio of 35 mM α-naphthol and 30 mM *N, N, N', N'*-dimethyl-*p*-phenylene diamine (DMPD) dissolved in ethanol and water, respectively (Marrs et al. 1973).

Isolation of Cu²⁺ dependent NAD^I mutants by transposon mutagenesis

The *E. coli* BW29427 strain (diaminopimelic acid (DAP) auxotroph, λ -pir⁺) carrying the transposon Tn5-RL27 (Kan^r-oriR6K) delivery vector pRL27 was conjugated into the wild type *R. capsulatus* strain MT1131 via triparental crosses using the helper plasmid pRK2013 on enriched medium MPYE supplemented with 100 μ M DAP. After 24 h incubation at 35°C, cells were spread on MPYE plates containing 5 μ g/ml kanamycin and no DAP. Kan^r colonies thus obtained were screened using the NAD^I staining, and mutants that were NAD^I on MPYE and NAD^I^{slow} on MedA (or NAD^I on MedA containing 15 μ M CuSO₄) were retained. Genomic DNA from 10 ml cultures of selected NAD^I mutants grown in MPYE was extracted using the DNeasy Blood&Tissue Kit (Qiagen Inc), and digested with either BamHI, BglII or BclI restriction enzymes whose recognition sites are absent in transposon Tn5-RL7, which carries the oriR6K origin of replication. Digested chromosomal DNA was circularized using T4 DNA ligase and transformed for Kan^r into the *E. coli* strain S17-1 (λ -pir⁺) to rescue plasmids that carried *R. capsulatus* chromosomal DNA flanking the transposon insertion sites. The primers TnmodRkan4-(5'-GAGCATTACGCTGACTTGAC-3') and OriR6Kseqprim1-(5'-GACACAGGAACACTTAACGGC-3') were used to define the DNA sequence at the insertion site of Tn5-RL7 using plasmid DNA isolated from Kan^r colonies.

Cu²⁺ sensitivity assays

Strains to be tested for Cu²⁺ sensitivity were grown up to the exponential phase (OD₆₃₀ of ~ 0.5) in enriched MPYE medium under respiratory or photosynthetic growth conditions. 1.7x10⁷ and 2.6x10⁷ cells (estimated using 1.0 OD₆₃₀ = 7.5 x 10⁸ *R. capsulatus* cells per ml) for respiratory and photosynthetic growth conditions, respectively, were added to 4 ml of same medium containing 0.7 % top agar and poured on top of 10 ml medium containing regular plates. Whatman 3MM paper discs (3 mm diameter), soaked with 8 µl per disc of desired concentrations of CuSO₄ solution, were placed on plate surfaces after solidification of top agar. Plates were incubated under desired growth conditions, scanned at the end of incubation period, and the size of growth incubation zones exhibited by different mutants were measured to estimate their response to CuSO₄ toxicity.

Molecular genetic techniques

Standard molecular biological techniques were performed according to Sambrook *et al.* (Sambrook *et al.* 2001), and all chromosomal insertion or insertion-deletion alleles were constructed by interposon mutagenesis using the Spe^r or Kan^r antibiotic cassette from pHP45ΩSpec or pHP45ΩKan, respectively (Prentki *et al.* 1984). The gene transfer agent (GTA) of *R. capsulatus*, which is a phage-like particle capable of transduction (Yen *et al.* 1979), was used to construct chromosomal knock out alleles of desired genes, as described earlier (Daldal *et al.* 1986). Starting with pSE2, a deletion-insertion allele of RCC02190 was obtained by replacing the 702 bp BstBI-ClaI fragment of with a Kan^r

cassette to yield pSE201, a deletion-insertion allele of RCC02191 was obtained by replacing the 247 bp BlnI-AsiSI fragment with a Spe^f cassette to yield pSE202, and a deletion-insertion mutation covering both RCC02190 and RCC02191 was obtained by replacing the 1058 bp ClaI-AsiSI fragment with a Spe^f cassette to yield pSE203 (Appendix I). Plasmid pSE204 was constructed by deleting the approximately 3300 bp HindIII-AsiSI fragment of pSE2. pSE3 and pBS_pSE3 was obtained by ligating into the XbaI and KpnI sites of pRK415 and pBluescript II-KS+ the 2812 bp region that contains 689 bp 5'- and 908 bp 3'- of *ccoA* (RCC02192), amplified using the GK1-Reverse 5'-ATTGGGTACCCCGCCCAAAGGGATTG-3' and GKI-Forward 5'-CCCGTCTAGACAGCCGCTCGCTCATC-3' primers containing 5'-XbaI and 3'-KpnI sites, respectively. At positions 997 and 1253 of the 2812 bp fragment of pBS_pSE3 the BglII and BamHI restriction enzyme sites were created by using QuikChange® Site-Directed Mutagenesis Kit, and the mutagenic primers GK1-QC-G997T-Sense (5'-TTGTTCGAGATCTACCCCGGTCAGGGCC-3') and GK1-QC-G997T-AntiSense (5'-GCCCTGACCGGGGTAGATCTCGAACAATTC-3') for BglII, and GK1-QC-Sense (5'-GCCACCAGGATCCCGGATCGCGCAAAAG-3') and GK1-QC-Antisense (5'-TGCGCGATCCGGGGATCCTGGTGGCG-3') for BamHI sites according to the manufacturer's instructions. A deletion-insertion allele of *ccoA* (RCC02192) was obtained by deleting and replacing with a Spe^R cassette this 256 bp BglII-BamHI fragment of pBS_pSE3 to yield pBS_pSE5, then XbaI-KpnI fragment was cloned into pRK415 to yield pSE5 and pSE6 was obtained by deleting the same fragment and ligating the ends filled in by T4 polymerase to yield pSE6 (Figure. 8). Plasmid pSE5 was

then used to obtain *ccoA* knock-out mutant SE8 by GTA cross to the wild type MT1131 strain.

R. capsulatus multicopper oxidase *cutO* (RCC02110) gene was PCR amplified from chromosomal DNA with the primers RCC02110F-(5'-AAG TCT AGA GCG GCGCGG TAG AAC GCG TCA ACG GTT T-3') and RCC02110R-(5'-AAT GGT ACC TGC AGC TTA CGC GCG AGC AAA TTC GGC-3') and cloned into pBluescript and pRK415 plasmids between their KpnI and XbaI sites, to yield pHY601 and pHY606, respectively. Plasmid pHY601 was then digested with EcoRI, and its 800 bp fragment was replaced with Kan^R cassette obtained by HindIII digestion of pHP45ΩKan, and filled in with T4 DNA polymerase, to yield pHY602. The $\Delta(\textit{cutO}::\textit{kan})$ allele thus constructed was transferred from pHY602 into pRK415 using the KpnI and XbaI sites to yield pHY603. The $\Delta\textit{cutO}$ mutant SE15 and $\Delta(\textit{cutO}\textit{-ccoA})$ double mutant SE16 were obtained by GTA crosses using the GTA overproducing strain Y262 that carries pHY602 as donor and MT1131 and SE8 as recipients, respectively.

RNA Isolation and RT-PCR

R. capsulatus cultures were grown semiaerobically in enriched medium until mid log phase (OD₆₃₀ of approx. 0.5) and total RNA was isolated from about 2×10^8 cells using the Qiagen RNAeasy Mini Kit, digested with DNase I for 25 min at room temperature, and ethanol precipitated. 50 ng of total RNA was used per RT-PCR reaction

with the QIAGEN one step RT-PCR kit, and the *ccoN* BHK20B 5'-CCAGTCGGGCAGCGCGGTAT-3' and N3-RT 5'-CGGCAACGGGATGCTGAACTTC-3' primers amplified a 652 bp long region internal to *ccoN* that corresponded to positions 299 and 972 of *ccoN*. The primers 16SrRNA-F 5'-ATATTCGGAGGAACACCAGTGGC-3' and 16SrRNA-R 5'-CAGAGTGCCCAACTGAATGATGG-3' were used as a control for amplification of a 450 bp region of 16S *rRNA* gene. In each case, RT-PCR controls were prepared by omitting the reverse transcriptase enzyme from the reaction, and the amplification products separated using 1% agarose gels, and their intensities compared by Image J (NIH).

Cell extracts preparation

Cells were grown in 10 ml of enriched MPYE medium by respiration and harvested at 4000 rpm for 10 min. Pellets were resuspended in 200 μ l of CellLytic B 2X Cell Lysis solution (Sigma Inc.) supplemented with 1 mM PMSF, 10 mM EDTA, 50 μ g lysozyme, 20 μ g DNase, and 10 mM MgCl₂, incubated at room temperature for 15 min and centrifuged at 14000 rpm for 10 min. Supernatants thus obtained were taken as whole cell extracts. Intracytoplasmic membrane vesicles (chromatophore membranes) were prepared in 50 mM MOPS (pH 7.0) containing 100 mM KCl and 1 mM PMSF as described earlier (Gray et al. 1994). Protein concentrations were determined using Bicinchoninic Acid assay according to the supplier's recommendation (Sigma Inc., procedure TPRO-562).

Enzyme activity measurements

Cytochrome *c* oxidase activity was measured by monitoring spectrophotometrically at 550 nm oxidation of reduced horse heart cytochrome *c* (Sigma, St. Louis, MO) in a stirred cuvette at 25⁰ C. Horse heart cytochrome *c* was reduced by incubation for 15 min at room temperature with 1 mM final concentration of fresh sodium dithionite (100 mM stock solution), which was then removed using a PD10 desalting column (GE Healthcare Life Sciences). *R. capsulatus* chromatophore membranes were detergent solubilized with 1 mg dodecyl β -D-maltoside per mg of membrane proteins (w/w) added to the assay buffer (10 mM Tris-HCl pH 7.0, 120 mM KCl about 25 μ M reduced cytochrome *c*). The enzymatic reaction was started and stopped by addition of solubilized membranes and 100 μ M KCN, respectively. Linear range of the assay was controlled by using different amounts of solubilized membranes, and KCN sensitive Cox activity was calculated as micromoles of cytochrome *c* oxidized per mg of membrane protein per min using an absorption coefficient ϵ_{550} of 20 as described earlier (Gray et al. 1994; Myllykallio et al. 1997). β -galactosidase activities of whole cell extracts prepared using 10 ml cultures of appropriate strains were measured spectrophotometrically at 420 nm using o-nitrophenylgalactoside (ONPG), as described earlier (Koch et al. 2000), and specific activity in nmoles of ONPG hydrolyzed per minute per mg of protein was determined using an absorption coefficient of ϵ_{420} of 21300 M⁻¹ cm⁻¹.

SDS-PAGE, immunoblotting and heme staining

For CcoN immunodetection, chromatophore membranes proteins (50 μ g) in 62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 25% (v/v) glycerol, and 0.01% (w/v) bromophenol blue, 5% β -mercaptoethanol were incubated at room temperature for 15 min prior to loading and were separated by 12% SDS-PAGE (Laemmli 1970). The gels were electroblotted onto Immobilon-P PVDF membranes (Millipore, Billerica, MA), and probed with *R. capsulatus* CcoN rabbit polyclonal antibodies (Koch et al. 1998). Alkaline phosphatase conjugated monoclonal anti-rabbit IgGs Clone RG-16 was used as the secondary antibody (Sigma-Aldrich, Saint Louis, MO) with BCIP/NBT as a substrate (Sigma-Aldrich, Saint Louis, MO) for the detection. For detection of the *c*-type cytochromes ~ 50 μ g of total membrane proteins were separated on 16.5% SDS-PAGE (Schagger et al. 1987), and the gels were stained for endogenous peroxidase activity of the *c*-type cytochromes by using 3,3',5,5'-tetramethylbenzidine (TMBZ) and H₂O₂ (Thomas et al. 1976).

Determination of cellular Cu content by ICP-DRC-MS

Cellular Cu content of various strains was determined using inductively coupled plasma-dynamic reaction cell-mass spectrometry (ICP-DRC-MS). In this technique, aliquots of sample digest are introduced into a radio frequency plasma where energy transfer processes cause desolvation, atomization, and ionization. The ions thus formed

are extracted from the plasma via a differentially pumped vacuum interface, and travel through a pressurized chamber (DRC) containing a specific reactive gas that preferentially reacts with interfering ions of the same target mass to charge ratios (m/z). A solid-state detector detects ions transmitted through the mass analyzer on the basis of their mass to charge ratio (m/z), and the resulting current is processed by a data handling system. For sample preparation, at least 1 hour prior to use, all containers, glassware and tubes were washed with 2 % nitric acid, and rinsed with metal-free MilliQ water to prevent metal contamination. Metal-free water and buffers were prepared by stirring 1 hour at room temperature with 5 g Chelex 100 per liter. For each strain, a one liter culture was grown by respiration in MPYE enriched medium to an OD_{630} of 0.8-0.9, cells were harvested by centrifugation, washed three times with a metal-free buffer of 20 mM Tris-HCl pH 8.0, and once with metal-free MilliQ water. Cell pellets were lyophilized until complete dryness, and shipped to Applied Speciation and Consulting, LLC, WA (www.appliedspeciation.com) for determination of total Cu, Zn, Mn, and Fe contents. 50 mg of lyophilized cells was digested completely with aliquots of concentrated HNO_3 and H_2O_2 at $95^\circ C$. The digests were diluted to a known final volume (50 ml) with metal-free reagent water and analyzed via ICP-DRC-MS according to a standard procedure of this company. The data was provided in μg of metal of interest per g of cells (ppm).

Chemicals

All chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Cu²⁺ supplement dependent *cbb*₃-Cox mutants

Previously, *R. capsulatus* mutants (*e.g.*, GK1, Appendix I) that exhibited very low or no *cbb*₃-Cox activity in a “growth medium-dependent” manner were isolated following mutagenesis with ethyl-methane sulfonate (Koch et al. 1998). These mutants had a completely NADI-minus (*i.e.*, no *cbb*₃-Cox activity) phenotype when grown on enriched (MPYE) medium, but exhibited a NADI^{slow} (*i.e.*, very low *cbb*₃-Cox activity) phenotype on minimal (MedA) medium (Figure. 7). Subsequently, additional similar mutants (*e.g.*, HY70, Appendix I) were also obtained after transposon mutagenesis (Materials and Methods). Two of these mutants (GK1 and HY70) were retained for further studies.

Testing of the chemical constituents of different growth media indicated that the growth medium-dependent NADI phenotype of GK1 was correlated with the Cu²⁺ content of the media used (Figure. 7). When Cu²⁺ was omitted from MedA (*i.e.*, “MedA-Cu”) medium, which normally contains 1.5 μM CuSO₄, GK1 became NADI-minus. Conversely, when MPYE, which normally has no Cu²⁺ supplement, contained 5 μM or more CuSO₄ (*i.e.*, “MPYE+Cu” medium) GK1 regained NADI^{slow} phenotype (Figure 7). Membranes of GK1 exhibited ~ 2% or 15% of the wild-type *cbb*₃-Cox activity (monitored as O₂ consumption activity in the presence of ascorbate and TMPD) when cells were grown on MPYE (*i.e.*, -Cu) or MedA (*i.e.*, +Cu) media, respectively (Koch et al. 1998). Addition of metal ions other than Cu²⁺, including Fe³⁺, Zn²⁺, Mn²⁺ or redox-active chemicals such as cysteine/cystine or oxidized/reduced glutathione did not affect

the NADI phenotypes of GK1, indicating that GK1 responded specifically to increased exogenous Cu^{2+} availability to exhibit *cbb₃-Cox* activity.

Genetic crosses using *R. capsulatus* structural (*ccoNOQP*) or assembly (*ccoGHIS*) genes of *cbb₃-Cox* (Koch et al. 1998; Koch et al. 2000), or other genes (*dsbAB*, *senC* and *olsAB*) known to affect its biogenesis (Aygün-Sunar et al. 2006; Koch et al. 2000; Onder et al. 2008) indicated that none of them could complement GK1 or HY70 for NADI⁺ phenotype in the absence of Cu^{2+} supplement. We therefore surmised that these mutants might reveal novel component(s) that affect Cu^{2+} dependent *cbb₃-Cox* biogenesis.

A new gene responsible for active *cbb₃-Cox* production.

The gene that was defective in GK1 was identified by complementation with conjugally transferable chromosomal libraries that were constructed using either EcoRI or HindIII restriction enzymes. These crosses yielded the plasmids pSE1 and pSE2 that complemented GK1 to NADI⁺ phenotype in the absence of Cu^{2+} supplement, and carried 8.0 kb EcoRI and 4.8 kb HindIII fragments, respectively. DNA sequence determinations of the end portions of these fragments, and their alignments with *R. capsulatus* reference genome (<http://www.ncbi.nlm.nih.gov>) identified the chromosomal region that complemented GK1. The EcoRI fragment contained six intact open reading frames (ORF), annotated as follows: protein of unknown function DUF88 (RCC02189); heavy metal translocating P-type ATPase (RCC02190); transcriptional regulator, MerR family (RCC02191); major facilitator superfamily (RCC02192); DNA-3-methyladenine

glycosylase II (RCC02193); and phospholipase/carboxylesterase family protein (RCC02194). The HindIII fragment, which was contained within the EcoRI fragment, carried only the RCC02190, RCC02191 and RCC02192 ORFs (Figure 8A). The plasmids derived from pSE2, namely pSE201, pSE202 or pSE203 containing a deletion in RCC02190, RCC02191 or both, respectively, and pSE204 containing only RCC02192 complemented GK1 to NAD^I⁺ in the absence of Cu²⁺ supplement (Figure 8A). Concurrently, the knock out mutants SE4, SE5 or SE6, with inactive copies of RCC02190, RCC02191, or both RCC02190-RCC02191, respectively, constructed by interposon mutagenesis (Materials and Methods), showed NAD^I⁺ phenotypes (Figure 8A). Plasmid pSE3 complemented both GK1 and HY70, whereas its derivatives, pSE5 and pSE6 with internal deletions on RCC02192 were unable to do so (Figure 8B) (Appendix I 1). These data showed that RCC02192 was defective in GK1 and HY70.

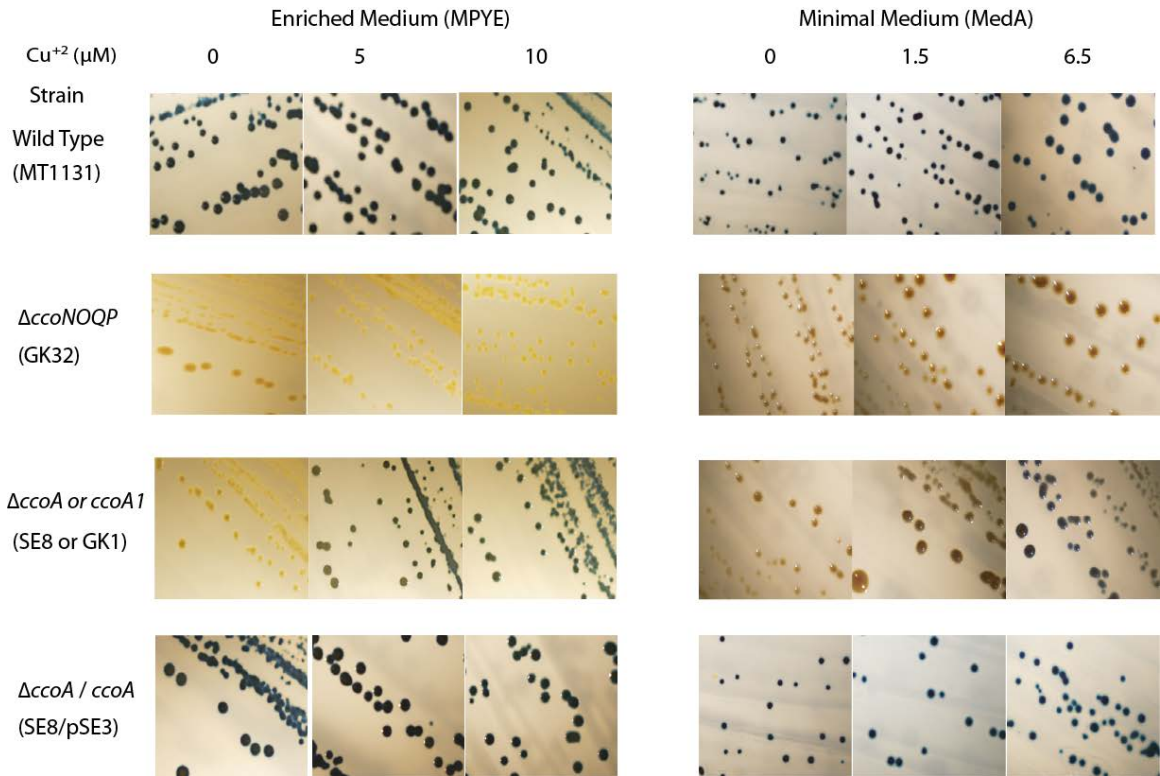


Figure 7: Growth medium-dependent NAD^I phenotypes of *R. capsulatus* mutants. Wild type (MT1131), *ΔccoNO* mutant (GK32), *ΔccoA* mutant (SE8) and the complemented mutant *ΔccoA/ccoA*⁺ (SE8/pSE3) strains grown at 35° C under respiratory conditions, on enriched MPYE or minimal MedA media supplemented with 0, 5 or 10 μM Cu²⁺, as indicated. Colonies that contain an active *cbb*₃-Cox (NAD^I⁺) turn blue immediately (< 0.5 min) when exposed to NAD^I stain described in Materials and Methods, and those that have no (NAD^I⁻) or low (NAD^I^{slow}) *cbb*₃-Cox activity remain green or become bluer upon longer (over 10 min) exposure times.

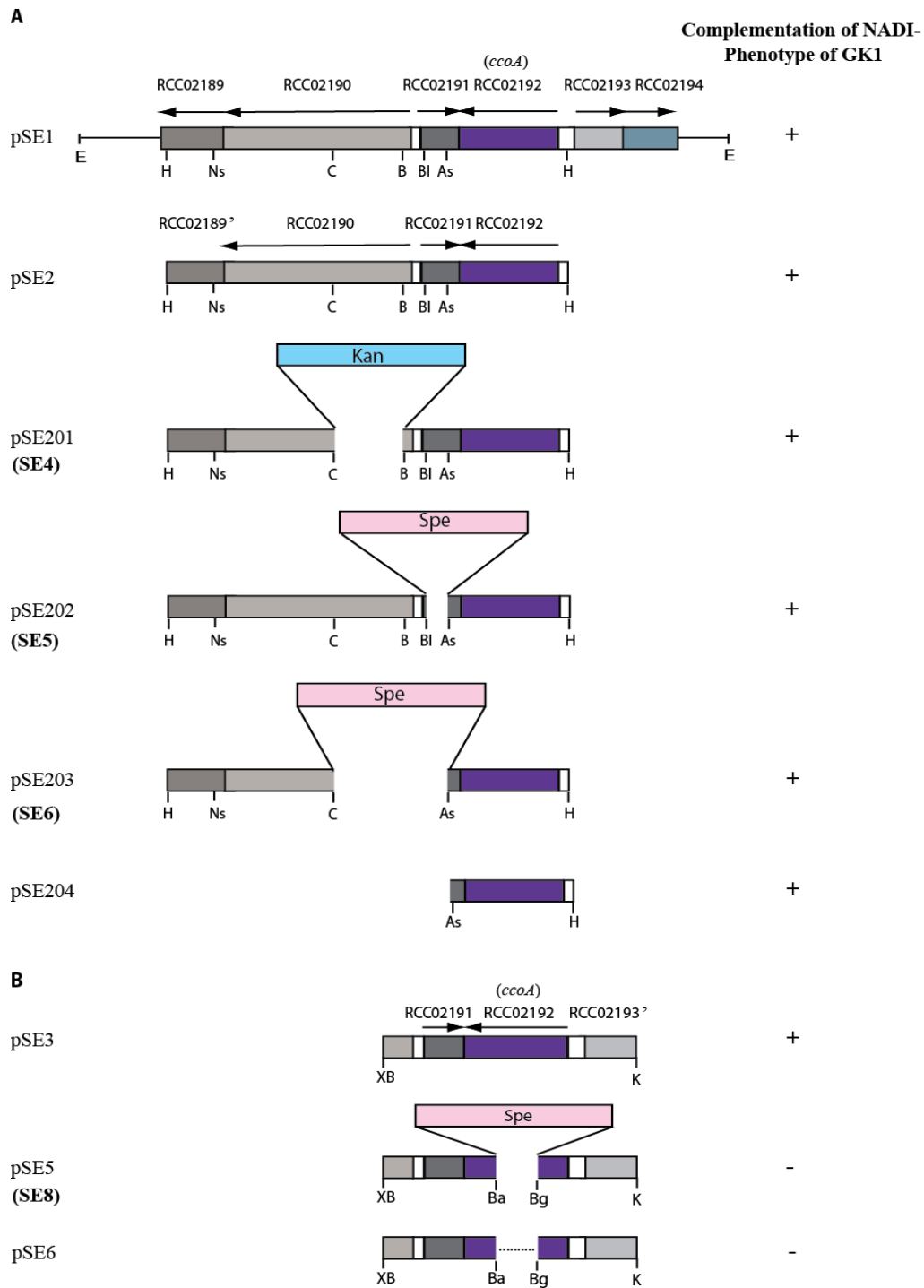


Figure 8: Restriction map of plasmids pSE1 and pSE2 and their derivatives used to complement GK1 and construct chromosomal knock out mutants.

Figure 8: Restriction map of plasmids pSE1 and pSE2 and their derivatives used to complement GK1 and construct chromosomal knock out mutants. Various plasmids pSE1, isolated or constructed as described in Materials and Methods are shown on the left and their ability to complement mutant GK1 on the right. When appropriate, *R. capsulatus* strains carrying the related chromosomal knock out alleles were also indicated under the plasmids. (A) pSE1, pSE2 and its derivatives are shown. See the text for annotations of the ORFs, RCC02189, RCC02190, RCC02191, RCC02192, RCC02193, RCC02194 that are carried by pSE1 and pSE2. (B) RCC02192 corresponds to *ccoA* that complements GK1, HY70 and SE8 and encodes a major facilitator superfamily (MFS) type transporter responsible for Cu²⁺ supplement dependent NADI phenotype and *ccb₃*-Cox production in these mutants. E, H, Ns, C, B, Bl, As, Ba, Bg, XB, K correspond to the restriction endonuclease sites for EcoRI, HindIII, NsiI, ClaI, BstBI, BlpI, AsiSI, BamHI, BglII, XbaI and KpnI enzymes, respectively. Superscript comma indicates partial gene.

A chromosomal knock out allele of RCC02192

A chromosomal deletion-insertion allele of RCC02192 was obtained using the gene transfer agent with pSE5 (Δ RCC02192:*spe*) as a donor (Materials and Methods) to yield the mutant SE8 (Figure 8B). Like GK1 and HY70, SE8 was NAD⁻ and NAD^{slow} on media lacking and containing Cu²⁺ supplement, respectively. Moreover, it was complemented to NAD⁺ phenotype in the absence of Cu²⁺ supplement by pSE3 but not by pRK-GK1 (Appendix I) carrying an identical chromosomal DNA fragment originated from GK1 (Figure 7). Thus, the defect in *ccb*₃-Cox activity seen in GK1 and HY70 was confined to RCC02192, which we subsequently named *ccoA* to recognize its role in *ccb*₃-Cox biogenesis. DNA sequencing of appropriate chromosomal regions encompassing *ccoA* defined the molecular bases of the mutation(s) in GK1 and HY70 (Materials and Methods). A single base pair change (C to T) at position 345 of *ccoA*, converting glycine 116 of CcoA to aspartate, and a six base pairs long insertion at position 257, resulting in an in-frame insertion of a threonine-alanine dipeptide between the positions 86 and 87 of CcoA, were found in GK1 and HY70, respectively.

CcoA belongs to the major facilitator superfamily of transporters.

In *R. capsulatus* reference genome *ccoA* (RCC02192) is annotated as a major facilitator superfamily (MFS) protein. Like the MFS type transporters (Pao et al. 1998), CcoA is an integral membrane protein with a predicted 405 amino acids long sequence forming 12 putative transmembrane helices split into two subdomains of six helices each, separated by a large cytoplasmic loop [see the “transporter classification database”

(www.tcdb.org)] (Saier et al. 2006; Saier et al. 2009) (Figure 9). These transporters contain two highly conserved DRXGRR motifs between their transmembrane helices two-three and eight-nine (AVYGRR and ARFGRE in CcoA) (Henderson et al. 1990). Remarkably, mutations that inactivated *ccoA* in GK1 and HY70 are located nearby the first motif (Figure 9), suggesting that this portion of CcoA is important for its function. An additional striking feature of CcoA is its richness in methionine residues, and the presence of several “Mets” motifs (M₃₀SM, M₇₀SSF_M, M₂₂₃ICGM and M₂₃₃NLVM) associated with Cu binding and transport in Ctr type Cu importers (Eisses et al. 2005; Puig et al. 2002). Moreover, mutating a conserved tyrosine residue located in the same transmembrane helix as a Met motif (YFLMLIFMT) was shown to decrease Cu transport by a Ctr type Cu importer (Eisses et al. 2005), and a similar sequence (Y230ALMNLVMT) is also present in the seventh helix of CcoA (Figure. 9).

Properties of a mutant lacking CcoA

In order to gain mechanistic insights on how the absence of CcoA decreased the activity of *cbb₃-Cox* in *R. capsulatus*, we compared the transcription levels of *ccoNOQP* gene cluster between a wild type (MT1131) and its Δ *ccoA* (SE8) derivative in the presence and absence of Cu²⁺ supplement. Total cellular RNA isolated from appropriate strains grown in enriched medium (MPYE) with or without Cu²⁺ supplement were subjected to RT-PCR using *ccoN* specific primers (Materials and Methods). Analyses of the amplification products (Figure 10A) indicated that the amount of *ccoN* mRNA was slightly lower in a mutant lacking *ccoA* than that in a wild type strain. In the presence of

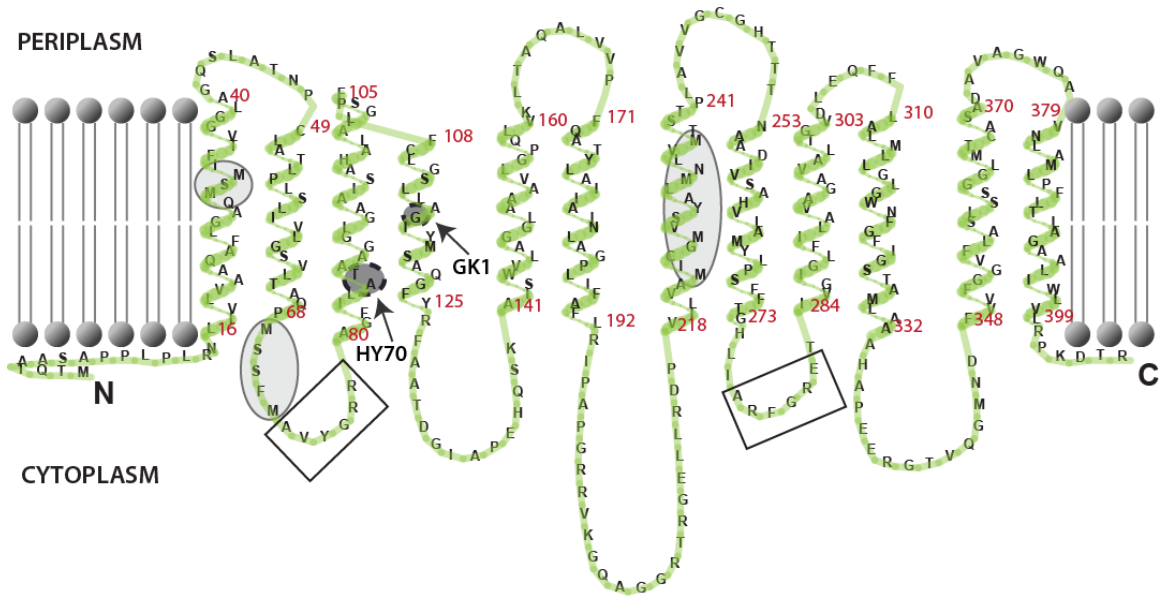


Figure 9: A topological model of CcoA (RCC02192) protein. CcoA protein is predicted to have 12 transmembrane helices, similar to MFS transporters. The topology model was drawn by using TransMembrane protein Re-Presentation in 2 Dimensions (TmRPres2D) program (Spyropoulos et al. 2004). The mutations present in GK1 and HY70 strains, the motifs similar to conserved motifs found in MFS transporters (rectangles), and the metal binding Mets motifs (ellipses) are indicated on the figure.

Cu²⁺ supplement these amounts decreased slightly and comparably in both strains, but absence of CcoA did not abolish the transcription of *ccoN*, and most likely that of *ccoNOQP* cluster, initiated from a promoter located immediately 5'-upstream of it (Koch et al. 1998).

Next, a transcriptional-translational *ccoN::lacZ* fusion construct (pXG1) that carried the 220 bp 5' of the ATG start codon and the first thirteen amino terminal codons of *ccoN* was conjugated into the wild type strain MT1131 and its Δ *ccoA* derivative SE8 (Koch et al. 1998). Absence of CcoA decreased roughly two fold the amounts of β -galactosidase activity produced in these strains grown in enriched medium (MPYE) without any Cu²⁺ supplement (Figure 10B). Upon 5 μ M Cu²⁺ supplementation this activity increased slightly and comparably in both strains. Thus, neither the transcription nor the translation initiation of *ccoN* in *R. capsulatus* was abolished by the absence of CcoA, and Cu²⁺ supplementation enhanced it only marginally.

The steady-state amounts of *ccb₃*-Cox subunits were examined in membranes of appropriate strains grown with or without Cu²⁺ supplement. The subunit I of *ccb₃*-Cox, CcoN, was monitored by immunoblot analyses using anti *R. capsulatus* CcoN polyclonal antibodies (Koch et al. 1998). The subunits II and III, CcoO and CcoP, were visualized using SDS-PAGE/tetramethylbenzidine (TMBZ) staining, which reveals specifically membrane bound *c*-type cytochromes (Materials and Methods). The amount of CcoN in SE8 lacking CcoA was much lower than that seen in the wild type strain MT1131, and addition of 5 μ M Cu²⁺ supplement increased this amount in both strains (Figure 11A).

Similarly, the amounts of CcoO and CcoP (cytochromes c_o and c_p , respectively) were lower in the absence of CcoA as compared with a wild type strain. Cu^{2+} supplementation increased these amounts (Figure 11B) even though no effect was seen with other *cbb*₃-Cox unrelated membrane bound *c*-type cytochromes (*e.g.*, cytochromes c_1 and c_y). Thus, in the absence of CcoA the steady-state amounts of the structural subunits of *cbb*₃-Cox in membranes were highly decreased, and Cu^{2+} supplementation palliated this defect(s) partially.

The *cbb*₃-Cox activity present in detergent dispersed membranes from cells lacking CcoA was determined using reduced horse heart cytochrome *c* (Materials and Methods). The total amount of *cbb*₃-Cox activity detected in SE8 lacking CcoA was, like GK1, ~ 5-10 % of that seen in the wild type strain MT1131 (Figure 11C). Upon addition of 5 μM Cu^{2+} supplement, *cbb*₃-Cox activity of SE8 increased to ~ 20 % of that of the wild type strain MT1131, which was unchanged under these conditions. As expected, upon complementation with a plasmid carrying *ccoA* (*e.g.*, SE8/pSE3) the NADI phenotypes, steady-state amounts and activities of *cbb*₃-Cox reached wild type levels (Figures 7 and 11). Therefore, absence of CcoA affected a step(s) that was subsequent to transcription and translation initiation of *ccoN* during biogenesis of *R. capsulatus cbb*₃-Cox under respiratory growth conditions. This defect(s) decreased drastically the steady-state amount and activity of *cbb*₃-Cox, and Cu^{2+} supplementation alleviated it partially.

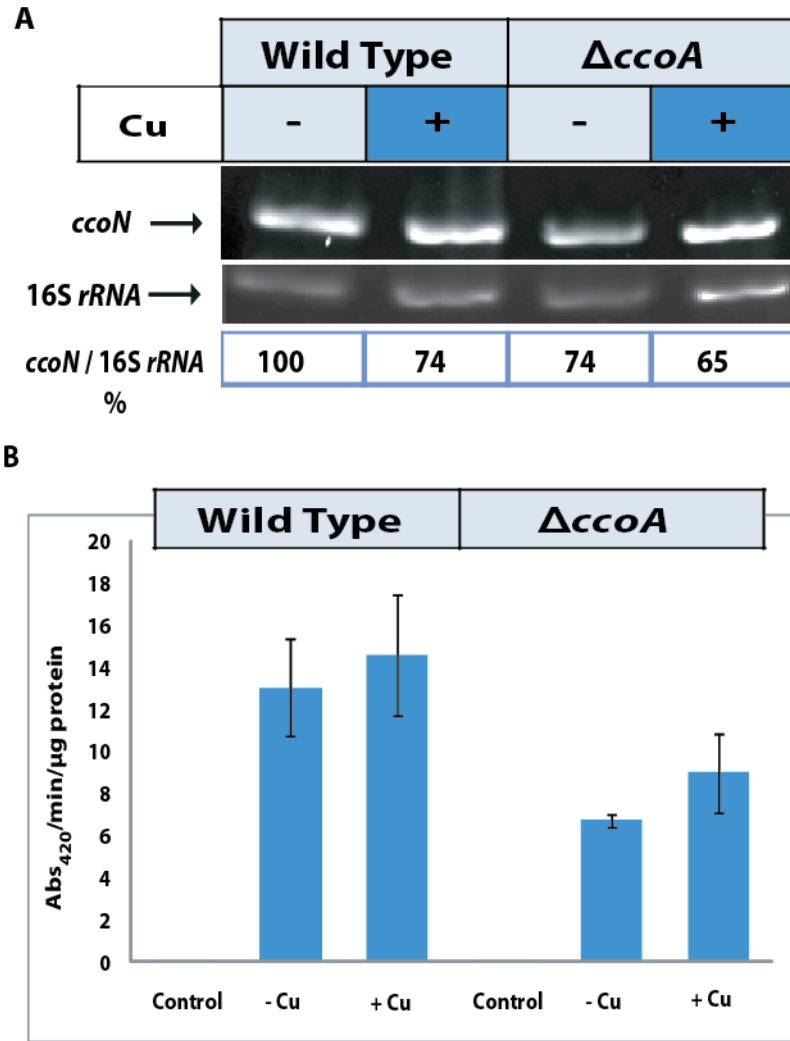


Figure 10: Effects of *ccoA* mutation on the expression of *ccoNOQP* structural genes of *cbb₃*-Cox.

Figure 10: Effects of *ccoA* mutation on the expression of *ccoNOQP* structural genes of *ccb₃-Cox*. (A) Top panel shows RT-PCR of total RNA from wild type (MT1131) and $\Delta ccoA$ mutant (SE8) strains grown in enriched media MPYE supplemented with 0 or 5 μM Cu^{2+} . Lower panel shows 16S *rRNA* expression that is used as an internal control, which is not affected by the *ccoA* mutation in these strains. Third panel shows the ratio of *ccoN*/16S *rRNA* expression by comparing the intensity of the bands. The intensity of bands was detected by ImageJ software (NIH). A control PCR by omitting reverse transcriptase enzyme was performed in each case to check the contamination (data not shown here). (B) β -galactosidase activities measured in the cell extracts prepared from the derivatives of the wild type (MT1131) and $\Delta ccoA$ mutant (SE8) strains carrying the *ccoN::lacZ* gene fusion grown on enriched media MPYE without Cu^{2+} (-Cu) or with 5 μM Cu^{2+} (+Cu) supplementation under semiaerobic conditions. Control reactions refer to the assays performed with the cell extracts of the same two strains (wild type and $\Delta ccoA$ mutant) carrying the pXCA601 plasmid that contains promoter-less *lacZ* that is used to construct the *ccoN* fusion. The assays were performed at least in duplicates and the activity is given in nmoles of ONPG hydrolyzed per minute per mg of protein.

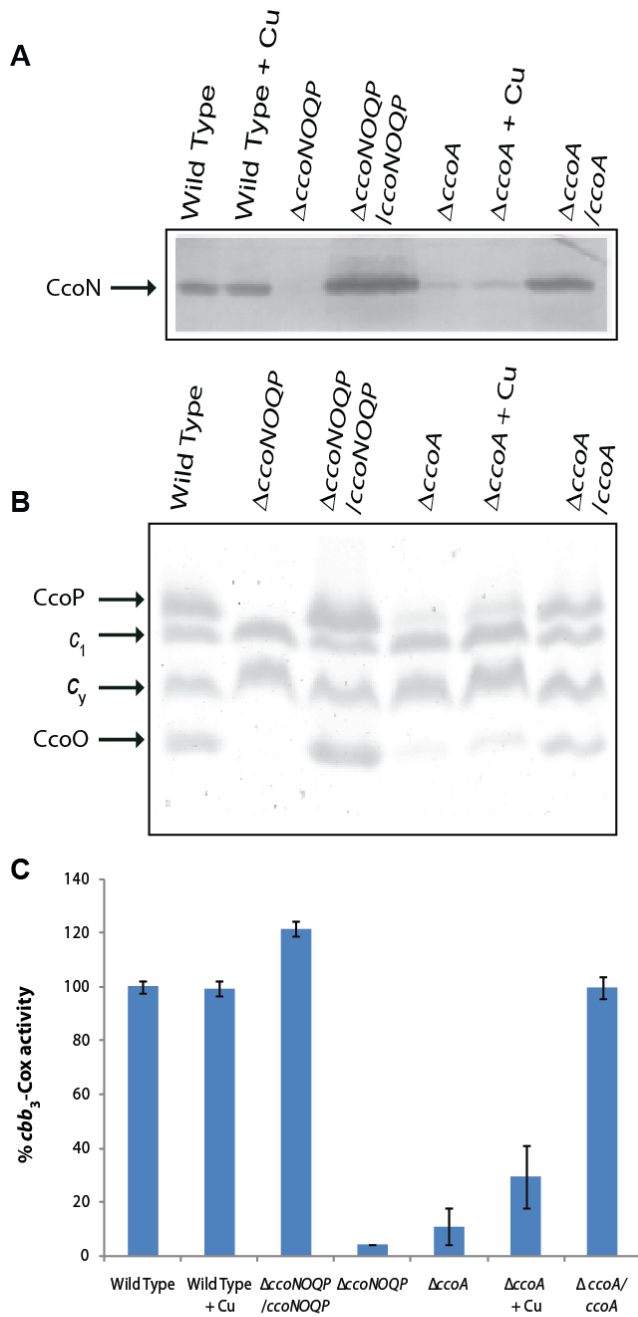


Figure 11: *cbb*₃-Cox subunit profiles and enzyme activity in membranes of various *R. capsulatus* strains

Figure 11: *cbb*₃-Cox subunit profiles and enzyme activity in membranes of various *R. capsulatus* strains. Chromatophore membranes from the following strains, wild type (MT1131), $\Delta ccoNO$ (GK32), $\Delta ccoNO/ccoNOQP$ (GK32/pOX15), $\Delta ccoA$ (SE8), $\Delta ccoA/ccoA$ (SE8/pSE3) grown at 35° C under respiratory conditions on enriched MPYE medium supplemented without or with 5 μ M Cu (designated as +Cu) were prepared as described in materials and Methods. **A)** Detection of the amounts of *cbb*₃-Cox subunit I, CcoN, by immunoblot analysis using anti-CcoN polyclonal antibodies. Approximately 50 μ g of chromatophore membranes were separated on 12% SDS-PAGE, and treated as described in Materials and Methods. **B)** Comparison of membrane associated cytochrome *c* profiles of various strains. Approximately 50 μ g chromatophore membranes prepared from appropriate strains grown as described above were separated using 16.5% SDS-PAGE, and the *c*-type cytochromes were visualized using TMBZ staining as described in Materials and Methods. CcoO and CcoP refer to subunits II and III of *cbb*₃-Cox, and *c*₁ and *c*_y correspond to the cytochrome *c*₁ subunit of cytochrome *bc*₁ complex, and the membrane attached electron carrier cytochrome *c*_y. **C)** *cbb*₃-Cox activities of various strains determined by monitoring at 550 nm the rate of oxidation of reduced horse heart cytochrome *c*. Detergent solubilized chromatophore membranes were prepared as described in Materials and Methods, and Cox activities were calculated as 1.5 μ M of cytochrome *c* oxidized Abs₅₅₀ per min per μ g proteins. The Cox activity exhibited by a wild type strain (MT1131) was taken as 100 % *cbb*₃-Cox activity to determine the relative amounts of Cox activities among the strains. A minimum of two independent duplicates were performed for each assay.

Absence of CcoA decreases total Cu content of *R. capsulatus* cells

Whether the absence of CcoA affected intracellular Cu content in appropriate mutants, cells grown in enriched medium with or without 5 μM Cu^{2+} supplement were analyzed by ICP-DRC-MS (Materials and Methods). In the absence of Cu^{2+} supplement, washed and lyophilized cells of SE8 lacking CcoA contained ~ 20% less Cu than its wild type parent MT1131, whereas the amounts of Fe, Mn, Zn found in these strains were unchanged (Table 2). Both wild type and CcoA-minus cells grown in the presence of 5 μM Cu^{2+} contained higher amounts of Cu, but the amount found in the absence of CcoA was again ~ 60% lower than that seen with wild type parent MT1131. Thus, absence of CcoA decreased significantly, but not abolished completely intracellular Cu accumulation in *R. capsulatus* cells under respiratory growth conditions.

Absence of CcoA does not affect the production of multicopper oxidase

In addition to *cbb₃-Cox*, *Rhodobacter* species also contain other Cu cofactor containing enzymes, like Zn-Cu superoxide reductase (Kho et al. 2004) or multicopper oxidase (laccase or CutO) (Wiethaus et al. 2006). Unlike *R. sphaeroides*, *R. capsulatus* does not contain a Zn-Cu superoxide dismutase, but it has the periplasmic enzyme CutO that confers resistance to Cu^{2+} . Mutants lacking CutO exhibit increased sensitivity against Cu^{2+} toxicity, and its protective effect is readily observed under anoxygenic photosynthetic growth conditions (Wiethaus et al. 2006).

Table 2: Metal contents of wild type (MT1131), Δ ccoA (SE8) and its revertants [Δ ccoA Rev1 and Rev2 (SE8R1, SE8R2)] determined by ICP-DRC-MS.

Strain	Cu addition during growth	(%)			
		Cu	Mn	Zn	Fe
Wild Type (MT1131)	-	100	100	100	100
	+	363	111	N/D	
Δ ccoA (SE8)	-	79	107	101	97
	+	146	118	N/D	
Δ ccoA Rev1 (SE8R1)	-	131	102	101	96
	+	490	137	N/D	
Δ ccoA Rev2 (SE8R2)	-	142	100	93	92
	+	433	129	N/D	

A mean value of 12 μ g Cu per g of lyophilized cells was determined and referred to as 100% for the wild type strain grown in the absence of Cu²⁺ in MPYE medium under respiratory growth conditions. For each strain, two sets of independently grown cells were analyzed, for each measurement, at least two repeats were done, and ~ 10-20% differences were observed between the measurements. For a given strain, the absolute amounts of metals determined varied from culture to culture in MPYE medium, but the trend of metal contents of different strains remained unchanged between the cultures. In each case, the mean value of all measurements was presented as a % of the value obtained with the wild type cells treated under the same conditions as described in Materials and Methods.

In order to test whether CutO enzyme was defective in the absence of CcoA, *R. capsulatus cutO* gene (RCC02110) was cloned, an insertion-deletion allele (*cutO::kan*) was constructed and introduced both into the wild type *R. capsulatus* strain MT1131 and its $\Delta ccoA$ derivative SE8 (Materials and Methods). Sensitivity to Cu^{2+} of a $\Delta cutO$ single (SE15) and a $\Delta ccoA cutO$ double mutant (SE16) (Appendix I) was determined by a plate growth inhibition assay. The sizes of growth inhibition zones surrounding filter disks soaked with various concentrations of Cu^{2+} were determined. As expected, a $\Delta cutO$ mutant (SE15) was sensitive to Cu^{2+} under photosynthetic growth conditions, as compared with a wild type strain (MT1131). Unlike the $\Delta cutO$ mutant, a $\Delta ccoA$ mutant (SE8) was not sensitive to Cu^{2+} like a wild type strain (MT1131) (Figure 12A) and unlike a $\Delta cutO ccoA$ double mutant (not shown). Similar but less pronounced Cu^{2+} sensitivity patterns were also observed when these strains were tested under respiratory growth conditions (data not shown). The data inferred that CutO was still functional to confer Cu^{2+} tolerance to a *R. capsulatus* mutant lacking CcoA, suggesting that its absence did not abolish CutO activity.

By-pass Suppressors of *ccoA* are hypersensitive to Cu^{2+}

During the complementation experiments using genomic libraries we noticed that GK1 reverted back to NADI⁺ phenotype at unusually high frequencies ($\sim 10^{-3}$ – 10^{-4}) when grown by respiration without Cu^{2+} supplement. DNA sequence analyses of the *ccoA* locus in several such revertants indicated that these revertants still retained the initial mutation (a C to T change at position 345 of *ccoA*) carried by GK1.

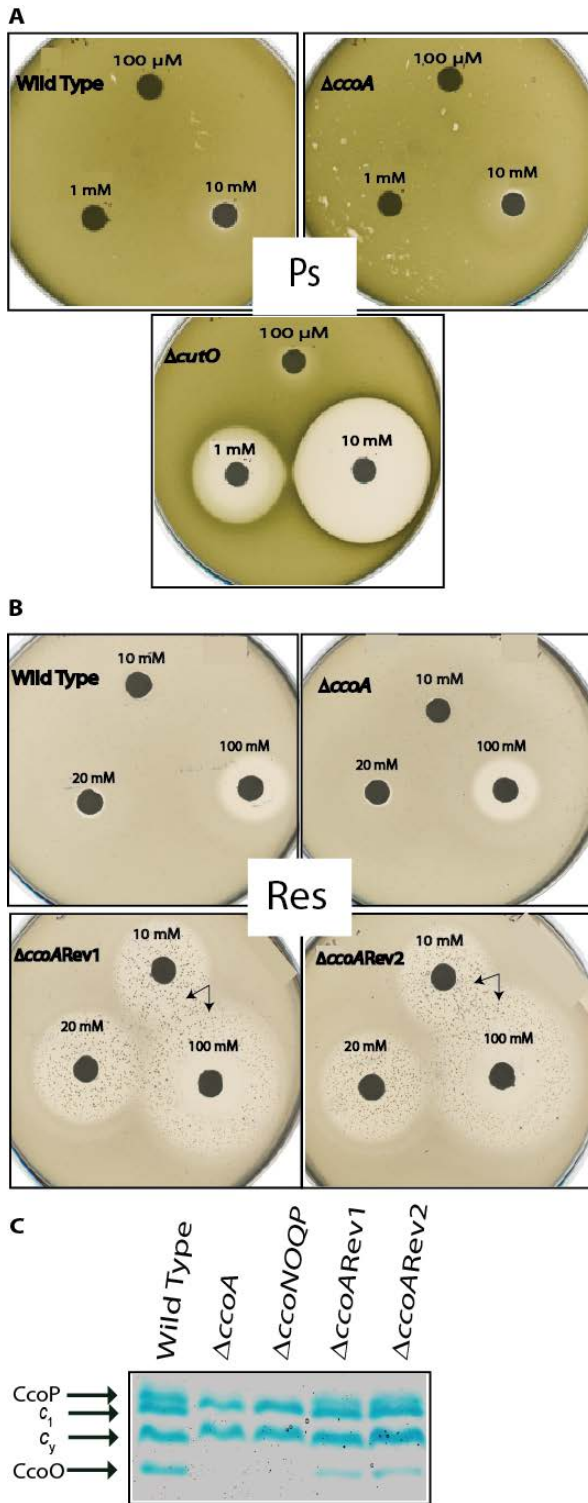


Figure 12: Cu^{2+} resistant or sensitive phenotypes of *R. capsulatus* strains lacking CcoA and its derivatives.

Figure 12: Cu²⁺ resistant or sensitive phenotypes of *R. capsulatus* strains lacking CcoA and its derivatives. **A)** Cu²⁺ sensitive or resistant phenotypes of *R. capsulatus* wild type (MT1131), $\Delta ccoA$ (SE8), and $\Delta cutO$ (SE15) strains grown on MPYE enriched medium under anoxygenic photosynthetic conditions as determined by plate growth assays. A given number of cells were incubated for two days with filter paper discs soaked in 100 μ M, 1 mM, 10 mM Cu²⁺ concentrations. Sensitivity of a given strain to a given Cu²⁺ amount presented on a filter paper disc was seen by the presence of a growth inhibition zone surrounding it. **B)** Cu²⁺ sensitive or resistant phenotypes *R. capsulatus* wild type (MT1131), $\Delta ccoA$ (SE8) and its by-pass suppressor derivative *ccoAREv1*(SE8R1) and *ccoAREv2* (SE8R2), grown on MPYE enriched medium under respiratory conditions as determined by plate growth assays as in A, except that 10 mM, 20 mM, 100 mM of Cu²⁺ concentrations were used to soak filter paper disks. **C)** Comparison of membrane associated cytochrome *c* profiles of various strains visualized as described previously in Figure 11.

Similar high reversion frequencies to NADI⁺ phenotype were also observed with SE8 that carried a deletion-insertion allele of *ccoA*, suggesting that the revertants restored the ability to produce *ccb₃*-Cox activity without any need for Cu²⁺ supplement by-passing the role of CcoA (Figure 12C). Unexpectedly, when tested for response to Cu²⁺ supplement, the *ccoA* suppressors (SE8R1 and SE8R2, Appendix I) showed extreme hypersensitivity to Cu²⁺ under both photosynthetic and respiratory growth conditions (Figure 12B). The *R. capsulatus* wild type strain MT1131 and its derivative SE8 lacking CcoA are tolerant up to ~ mM concentrations of Cu²⁺ for respiratory growth inhibition, but the *ccoA* suppressors SE8R1 and SE8R2 were sensitive to ~ μM amounts of Cu²⁺ supplementation. Indeed, these mutants were partially growth inhibited in minimal medium which contained 1.5 μM Cu²⁺, and completely growth inhibited by addition of ~ 25 μM Cu²⁺ supplement into the enriched medium. This hypersensitivity was specific to Cu²⁺ only, as no similar effect was seen when other metals, including Fe³⁺, Mn²⁺, Zn²⁺ or Ag⁺ and oxidants such as cystine or glutathione. We therefore concluded that SE8R1 and SE8R2 regained the ability to produce *ccb₃*-Cox at the expense of decreased tolerance to Cu²⁺ toxicity.

Total intracellular Cu contents of SE8R1 and SE8R2 cells grown in enriched medium with and without 5 μM Cu²⁺ supplement were also determined using ICP-DRC-MS analyses (Materials and Methods). In the absence of Cu²⁺ supplement total intracellular Cu content of these mutants were higher than their parent SE8 lacking CcoA, and similar or higher than that found in the wild type strain MT1131 (Table 2). In the

presence of Cu^{2+} supplement, all strains accumulated higher amounts of intracellular Cu. The levels found in SE8R1 and SE8R2 were much higher than those seen in the wild type strain MT1131 although the intracellular amounts of Mn used as an internal control were unchanged in all cases (Table 2). Thus, the suppressor mutation(s) by-passed the absence of CcoA by increasing specifically intracellular Cu accumulation at the expense of compromising cellular tolerance to this toxic metal. Molecular basis of this suppression remains to be identified.

DISCUSSION

The impetus behind this work was to understand how cells assemble catalytic metal cofactors into membrane-integral enzymes, like the heme Fe-Cu_B binuclear center of Cox, which is unknown. Using *R. capsulatus*, we initiated a genetic approach to investigate bacterial Cox biogenesis, and isolated various *cbb₃*-Cox defective mutants. In this work, we focused on mutants that produced an active *cbb₃*-Cox only upon exogenous Cu^{2+} supplementation. Studies of these mutants uncovered a novel gene, *ccoA*, which was distinct from *ccoGHIS* (Koch et al. 2000), *senC* (Swem et al. 2005), *olsAB* (Aygün-Sunar et al. 2006), *dsbA* and *degP* (Onder et al. 2008) known to affect this process. Mutants lacking CcoA were unable to produce normal amounts of *cbb₃*-Cox activity because the steady-state amounts of the subunits of this enzyme were drastically decreased in membranes. However, neither the transcription of the structural genes *ccoNOQP*, nor the translation initiation of the subunit I, CcoN, was abolished in the absence of CcoA, indicating that *cbb₃*-Cox assembly was defective.

A major finding was that *ccoA* gene encodes a multi span membrane protein, CcoA, of the MFS type transporters, which have not been implicated hitherto into *cbb₃*-Cox biogenesis in bacteria. Remarkably though, CcoA homologues with Mets motifs are present in most bacteria that contain *cbb₃*-Cox, except the ϵ -proteobacteria, suggesting that they are important for the production of this enzyme. How CcoA affects *cbb₃*-Cox assembly is intriguing. The MFS type secondary transporters use the electrochemical potential difference generated by ion or solute gradients, and transport a diverse range of substrates in and out of cytoplasm (Pao et al. 1998). Some MFS proteins have been implicated as importers or exporters of siderophores, including *E. coli* EntS (Furrer et al. 2002), *Erwinia chrysanthemi* YhcA (Franza et al. 2005), *Legionella pneumophila* LbtB (Allard et al. 2006), *Vibrio parahaemolyticus* PvsC (Tanabe et al. 2006), *Sinorhizobium meliloti* RhtX (Cuiv et al. 2004) and *Azotobacter vinelandii* CsbX (Page et al. 2003) that secrete enterobactin, achromobactin, legiobactin, vibrioferrin, rhizobactin and protochelin-like siderophores, respectively. *R. capsulatus* mutants lacking CcoA produce various *c*-type cytochromes that rely on efficient siderophore trafficking and Fe supply for heme production. Several lines of evidences suggest that CcoA is involved in cellular Cu acquisition. First, mutants lacking CcoA exhibit enhanced *cbb₃*-Cox activity upon increased exogenous Cu²⁺ supplementation (5 to 25 μ M addition into MPYE tested). This enhancement is specific to Cu²⁺ only as Zn²⁺, Mn²⁺ or Fe³⁺ addition has no similar effects. Mass spectrometry measurements indicated that only the total Cu content (and not the other metals) of cells lacking CcoA is lower than that of wild type cells under normal growth conditions. Upon Cu²⁺ supplementation, cellular Cu content increases in

mutants lacking CcoA although it never reaches wild type levels, whereas cellular contents of metals other than Cu, like Zn, Mn or Fe remain unaffected. These findings are consistent with CcoA being involved in a Cu influx rather than efflux pathway. We note that *R. capsulatus* mutants lacking CcoA still contain cellular Cu, indicating that it has other unrelated Cu acquisition pathway(s) that are unknown.

Second, mutants lacking CcoA are not more sensitive to Cu²⁺ supplement than a wild type *R. capsulatus* under various growth conditions, suggesting that CcoA is not involved in Cu detoxification, unlike for example the P1B-type Cu exporters (Gonzalez-Guerrero et al. 2010; Rensing et al. 2000; Solioz et al. 2010). Moreover, suppressor mutants that bypass the need for CcoA to recover *cbb*₃-Cox activity are extremely sensitive to very low amounts (~ 25 μM) of Cu²⁺ supplement in the medium. This sensitivity is specific to Cu²⁺ as these revertants exhibit normal tolerance towards Zn²⁺, Mn²⁺, Fe³⁺ and even Ag⁺, known to mimic Cu⁺ (Rensing et al. 2000; Winge et al. 1985). Mass spectrometry measurements indicated that in these suppressor mutants Cu content was similar to that seen with wild type cells, suggesting that the suppressors overcame the function of CcoA by enhancing Cu acquisition. Indeed, upon Cu²⁺ supplementation, the suppressor mutants accumulated intracellular Cu amounts (and not Mn for example) much higher than those seen with wild type cells to reach growth inhibitory levels. Based on the overall findings it is compelling to rationalize that the absence of CcoA induces intracellular Cu²⁺ shortage to decrease *cbb*₃-Cox production (Figure 13). Consequently, availability of increased exogenous Cu²⁺ supply, or occurrence of an additional

mutation(s), overcomes this shortage to yield normal amounts of *cbb*₃-Cox at the expense of compromised tolerance to Cu.

Interestingly, absence of CcoA does not affect the periplasmic Cu containing multicopper oxidase CutO in *R. capsulatus*. A possibility is that CcoA does not affect the periplasmic levels of Cu²⁺, but whether Cu is delivered to *cbb*₃-Cox or CutO from the cytoplasm or the periplasm is unknown. Another possibility is that CcoA might be highly specific for *cbb*₃-Cox. Investigation of bacterial species like *R. sphaeroides* or *B. japonicum* that have CcoA homologues and that also produce *aa*₃-type Cox might further elucidate its role in the biogenesis of other cytochrome *c* oxidases.

Bacteria utilize multiple transporters to achieve the movement of metals across membrane and maintain metal homeostasis without harmful cellular toxicity (Nies 2003). In the case of Cu, homeostatic pathways are multiple and complex (Banci et al. 2010; Banci et al. 2010). For example, energy dependent primary transporters of P1B subgroup of P-type ATPases are involved in Cu⁺ efflux from the cytoplasm to the periplasm across membrane. Of these, CopA1 type transporters have high efflux rates and are involved in Cu detoxification. Their expressions are induced by excess of Cu and their absence induces Cu sensitivity (Gonzalez-Guerrero et al. 2010; Kanamaru et al. 1994; Odermatt et al. 1993; Rensing et al. 2000). In contrast, CopA2 type transporters like *R. capsulatus* CcoI of the *ccoGHIS* cluster, have low efflux rates and no role on Cu toxicity, but are involved in *cbb*₃-Cox biogenesis (Gonzalez-Guerrero et al. 2010; Koch et al. 2000). RND (resistance-nodulation-cell division protein family)-type transporters are also involved in

the efflux of Cu ions from both the cytoplasm and the periplasm to extracellular milieu to detoxify cells (Kim et al. 2010). Clearly, while Cu efflux and detoxification pathways are elaborate, how Cu is imported into the bacterial cytoplasm is less well known (Banci et al. 2010; Solioz et al. 2010). Only a few proteins, including the *Enterococcus hirae* CopA (Odermatt et al. 1993), *Pseudomonas aeruginosa* HmtA (Lewinson et al. 2009), *Bacillus subtilis* YcnJ (Chillappagari et al. 2009), *P. syringiae* CopCD (Cha et al. 1993), the cyanobacterial P1B-type ATPases (CtaA located in the cytoplasmic and PacS in the thylakoid membranes) (Tottey et al. 2001), and two plant chloroplast P1B-type ATPases (PAA1/HMA6 of the inner membrane and PAA2/HMA8 of the thylakoids membrane) (Nouet et al. 2011) were implicated into Cu import. In eukaryotic microbes like yeast, mainly the Ctr type transporters located in the plasma membrane import Cu with very high affinity into the cytoplasm (Banci et al. 2010; Banci et al. 2010). However, no bacterial Ctr homologue was reported, and until very recently, no MFS type Cu transporter was also known. Beaudoin *et al.*, reported a novel forespore membrane Cu transporter, Mfc1, which is distinct from the Ctr type transporters (Beaudoin et al. 2011), and involved in meiotic and sporulating cells of *Schizosaccharomyces pombe* (Beaudoin et al. 2011). Mfc1 is a member of the MFS transporters, and it functions as a specific Cu importer during meiotic differentiation under Cu limiting conditions in *S. pombe*. Excitingly, the *S. pombe* Mfc1 is highly homologous to *R. capsulatus* CcoA, has a similar topology of 12 transmembrane helices with both amino and carboxyl terminal ends located in the cytoplasmic face of membrane, and contains other landmarks of MFS type transporters. Moreover, both Mfc1 and CcoA are methionine-rich and contain the Mets (MxM, MxxM and MxCxM) sequences involved in Cu binding. The Cu²⁺ importer

function of Mfc1 was established by direct transport assay using radioactive ^{64}Cu , and by its ability to complement a *S. cerevisiae* mutant lacking the known Cu importing Ctr type transporters. The pronounced similarity between CcoA and Mfc1 provides further support that CcoA might act as a Cu importer, although direct evidence remains to be obtained. If this is the case, then considering that cells lacking CcoA are not sensitive to silver, unlike the related P-type ATPase mutants in cyanobacteria (Kanamaru et al. 1994) or the Ctr family proteins known to transport Cu^+ and Ag^+ (Lee et al. 2001), CcoA might be a Cu^{2+} importer. Furthermore, as in some methane-oxidizing bacteria (methanotrophs) Cu is imported into the cytoplasm associated with siderophore-like molecules (called chalkophores or methanobactins) (Balasubramanian et al. 2008; Kim et al. 2004), whether CcoA-mediated Cu acquisition also involves additional compounds as those also seen in mitochondrial matrix (Cobine et al. 2004) needs further investigations.

In summary, our findings establish for the first time that the MFS type transporter CcoA is required for maintaining normal amounts of intracellular Cu content and *cbb*₃-Cox in *R. capsulatus*, and possibly in other bacterial species. Future work will hopefully further elucidate the links between CcoA, cellular Cu acquisition and *cbb*₃-Cox biogenesis.

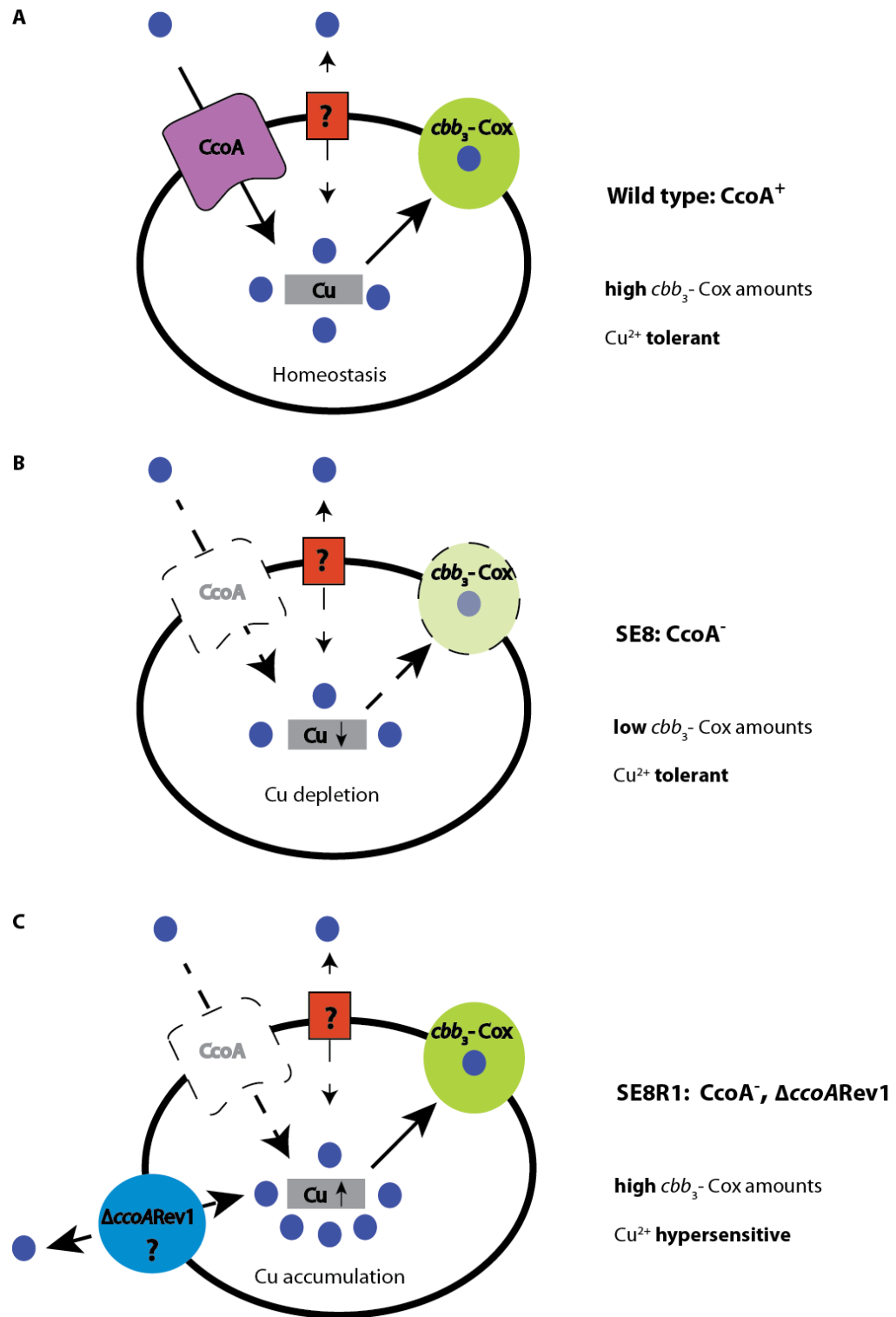


Figure 13: A hypothetical model on how CcoA affects cellular Cu content and production of cbb_3 -Cox in *R. capsulatus*

Figure 13: A hypothetical model on how CcoA affects cellular Cu content and production of *cbb*₃-Cox in *R. capsulatus*. **A)** A wild type strain (MT1131) requires CcoA to produce normal amounts of active *cbb*₃-Cox and to exhibit tolerance to Cu²⁺. **B)** In the absence of CcoA (SE8), *cbb*₃-Cox formation is abolished, intracellular Cu amounts are decreased, and cells are tolerant to Cu²⁺ stress. **C)** Absence of CcoA can be suppressed by a mutation of unknown mechanism, referred to $\Delta ccoA$ Rev (suppression of CcoA defect) that restores the production of normal amounts of active *cbb*₃-Cox, at the expense of rendering the cells sensitive to Cu²⁺.

CHAPTER 3

STUDIES ON THE CU-UNRESPONSIVE *CCOA* MUTANTS

INTRODUCTION

Cox of purple non-sulfur facultative phototrophic bacterium *Rhodobacter capsulatus* is a *cbb₃*-type Cox which has four subunits of which two being cytochrome *c* subunits, CcoO and CcoP (Gray et al. 1994). CcoO is a monoheme and CcoP is a diheme cytochrome *c* and both are thought to be matured by the cytochrome *c* maturation (CCM) system (Sanders et al. 2010). CCM involves up to 10 proteins that have different functions and can be grouped in three functional modules; 1) CcmABCDE-the transport and preparation of the heme molecule, 2) CcmG, CcdA, DsbA, DsbB-the thiol-oxidoreductase of apocytochrome 3) CcmHIF- the ligation complex. The roles are described extensively in Chapter 1 (Sanders et al. 2010).

In addition to cytochrome *c* subunits, the catalytic subunit CcoN is a well conserved 12 transmembrane protein that contains a low spin heme *b*, and a binuclear center composed of a high spin heme *b₃* and a Cu atom (Garcia-Horsman et al. 1994; Pereira et al. 2001). The insertion of these cofactors requires strict control of transport and trafficking of Cu and heme into this subunit. The fourth subunit CcoQ, is thought to be involved in the stability of *cbb₃*-Cox and does not contain any cofactors (Peters et al. 2008). Biogenesis and assembly of Cox depends on the proper regulation of the maturation of each one of these different subunits by different maturation pathways. Due to the complex structure of this enzyme, a multistep maturation and assembly pathway is required.

Previously we identified a novel protein, CcoA, which was required for making wild type levels of active *cbb₃-Cox* in *R. capsulatus*. CcoA is homologous to Major Facilitator Superfamily (MFS) type transporters. The mutants lacking CcoA exhibited a Cu responsive *cbb₃-Cox* phenotype which was NADI minus on the media without Cu and increased amounts of *cbb₃-Cox* upon supplementation with Cu (Chapter 2) (Figure 7). Also, Cu accumulation in $\Delta ccoA$ mutant was much lower than the wild type strain on both regular and Cu containing media indicating a defect in Cu acquisition to the cells. Therefore we hypothesized that in the absence of CcoA, Cu acquisition is provided by an alternative pathway(s). In order to get insights into this observation, we characterized three $\Delta ccoA$ mutants which were unresponsive to Cu supplementation. These mutants were defective in CCM gene *ccmA* and one of the *ccmHIF* genes, which rendered them unable to produce wild type levels of cytochrome *c* including the two subunits CcoO and CcoP of *cbb₃-Cox* complex. However in these mutants cytochrome *c* production was enough to sustain photosynthetic growth, although previous studies have shown that the insertion deletion mutants of these CCM genes lead to the absence of all *c*-type cytochromes and failure to grow under photosynthetic conditions suggesting that these mutants were leaky (Deshmukh et al. 2000). This study provides information about the threshold levels of the CCM functions required for production of different cytochrome *c*'s in *R. capsulatus*.

MATERIALS AND METHODS

Strains, culture conditions, and phenotypes.

The bacterial strains and plasmids used in this work are described in Appendix I. *R. capsulatus* and *E. coli* strains were grown as described in the materials and methods section of Chapter 2. NADI (*i. e.*, cytochrome *c* oxidase activity) phenotypes of *R. capsulatus* colonies were revealed qualitatively using the NADI staining made by mixing 1:1 (v/v) ratio of 35 mM α -naphthol and 30 mM *N, N, N', N'*- dimethyl-*p*-phenylene diamine (DMPD) dissolved in ethanol and water, respectively (Marrs et al. 1973).

EMS Mutagenesis

1.5 ml from two independent cultures of 10 ml overnight grown *R. capsulatus* strain SE8/pCW25 (Appendix I) was centrifuged at 10,000 rpm for 2 min. The pellet was resuspended in 1 ml of 100 mM KH_2PO_4 buffer and mutagenized by the addition of 30 μl of ethyl methanesulfonate (EMS) liquid (Sigma-Aldrich, Saint Louis, MO) by incubating the cells at 35°C for 30 minutes. After the incubation, cells were diluted to yield ~ 500 colonies per plate and were spreaded on enriched media MPYE plates containing 10 μM Cu^{2+} . Plates were incubated for two days under respiratory conditions and *cbb*₃-Cox minus colonies were retained after NADI staining. All NADI⁻ colonies were tested for their photosynthetic growth on enriched media MPYE at 35°C and NADI⁺, Ps⁺ colonies were kept and analyzed further. The strains were rescued from their plasmids (pCW25) (Appendix I) by growing them several times in the absence of the Tet antibiotic which is the antibiotic that pCW25 plasmid contains.

Molecular genetic techniques

Standard molecular biological techniques were performed according to Sambrook *et al.* (1989), and all chromosomal insertion or insertion-deletion alleles were constructed by interposon mutagenesis using the Kan^r cassette from pMA117 (Daldal *et al.* 1986) to make nonpolar insertions in the desired genes (Appendix I). The gene transfer agent (GTA) of *R. capsulatus*, which is a phage-like particle capable of transduction (Yen *et al.* 1979), was used to construct chromosomal knock out alleles of desired genes, as described earlier (Daldal *et al.* 1986). pSE10 plasmid contained XbaI and KpnI restriction sites flanking the BamHI restriction sites. Therefore pSE10 plasmid was used to construct pSE11 by the deletion of 4912 bp between XbaI-BstBI and pSE12 by the deletion of 2.7 kb between BstBI-KpnI. pSE14 was obtained by amplification of 334 bp 5' and 448 bp 3' of *ccmA* and *ccmB* from pSE11 plasmid by using the primers CcmAB-Fwd 5'- AGC GCT CTA GAC GAT CGA CGG CTA CGG ACC C -3' and CcmAB-Rev 5'- TTG GAG GGT ACT CCC ACC AGGTTC CCC ACAT -3' primers containing 5'-XbaI and 3'-KpnI and cloning into XbaI and KpnI sites of pBluescript. Insertion alleles of *ccmA* and *ccmB* was obtained by inserting Kan^r cassette at XhoI site and BstEII sites of pSE14 to obtain pSE15 and pSE16 respectively. Insertion-deletion allele of *ccmAB* was constructed by the replacement of 746 bp between XhoI-BstEII of pSE14 by Kan^r cassette and called pSE17. Later, XbaI-KpnI inserts of pSE15, pSE16, and pSE17 was cloned into the same sites of pRK415 obtain pSE21, pSE22, and pSE23 which were used to make the knock-out mutants of *ccmA*, *ccmB* and *ccmAccmB* double mutants which were named as SE17, SE18, SE19 in the case of $\Delta ccoA$ (SE8) background respectively,

and SE20, SE21, and SE22 in the case of wild type (MT1131) background respectively (Appendix I).

Heme staining

For detection of the *c*-type cytochromes ~ 50 µg of total membrane proteins were separated on 16.5% SDS-PAGE (Schagger et al. 1987), and the gels were stained for endogenous peroxidase activity of the *c*-type cytochromes by using 3,3',5,5'-tetramethylbenzidine (TMBZ) and H₂O₂ (Thomas et al. 1976).

Cu²⁺ sensitivity assays

Appropriate strains were tested for their sensitivity to Cu²⁺ as described in Materials and Methods section of Chapter 2.

Determination of cellular Cu content by ICP-DRC-MS.

Cellular Cu content of various strains was determined using inductively coupled plasma-dynamic reaction cell-mass spectrometry (ICP-DRC-MS) as described in Materials and Methods section of Chapter 2.

Chemicals

All chemicals were of reagent grade and were obtained from commercial sources.

RESULTS

Cu²⁺ supplementation of $\Delta ccoA$ mutants enhances the amounts of the *cbb₃*-Cox subunits

$\Delta ccoA$ mutant displays a NADI minus phenotype on media that does not contain biologically available Cu (enriched MPYE and minimal MedA-Cu) and a NADI^{slow} phenotype on media that contains Cu (MPYE+Cu and MedA). For example the *cbb₃*-Cox activity of $\Delta ccoA$ mutant when grown with 5 μ M Cu²⁺ reaches about 30% of the wild type activity (Chapter 2). Previously the transcription and translation initiation of *ccoNOQP* structural genes were measured in $\Delta ccoA$ mutants in the absence and presence of Cu when grown in enriched medium MPYE and was found to decrease to about 60% of the wild type. In contrast, the amounts of CcoN subunit were increased when the cells were grown with Cu supplement. To investigate how the amounts of the CcoO and CcoP subunits, which are *c*-type cytochromes, change with Cu addition, membrane associated *c*-type cytochromes were determined by growing $\Delta ccoA$ mutant (SE8) and wild type strain (MT1131) on enriched media MPYE with varying amounts of Cu supplementation (5, 10, 25 μ M) (Figure 14). As expected the amounts of CcoO and CcoP subunits were increased with the addition of Cu in $\Delta ccoA$ mutant, but did not reach the amounts seen in a wild type strain. On the other hand, the amounts of these subunits decreased slightly in the wild type strain after addition of Cu (Figure 14). Therefore it was clear that Cu addition showed a different effect in a $\Delta ccoA$ mutant than a wild type strain leading to the production of more *cbb₃*-Cox.

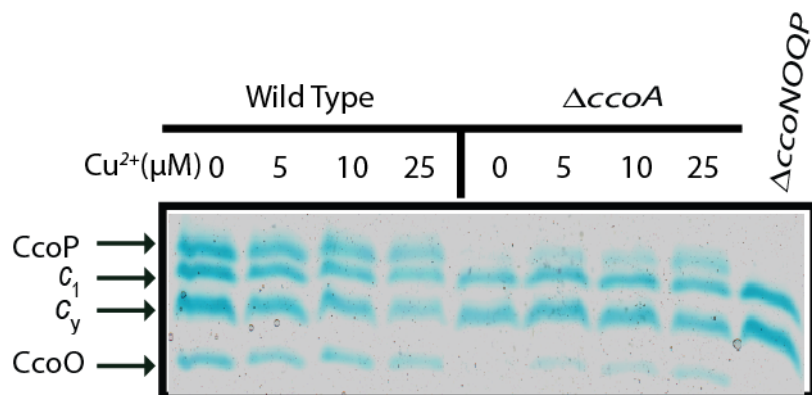


Figure 14: The amounts of *c*-type cytochrome subunits of *cbb*₃-Cox in respect to Cu²⁺ supplementation. Comparison of membrane associated cytochrome *c* profiles of wild type (MT1131) and $\Delta ccoA$ mutant (SE8) strains grown in enriched media MPYE with 0, 5, 10, or 25 μM Cu²⁺ supplementations under respiratory conditions at 35°C. Total 50 μg of chromatophore membrane proteins prepared from these strains grown as described above were separated using 16.5% SDS-PAGE, and *c*-type cytochromes were visualized by TMBZ staining, as described in Materials and Methods. CcoO and CcoP refer to subunit II and III of *cbb*₃-Cox, *c*₁ and *c*_y refer to cytochromes *c*₁ - subunit of the cytochrome *bc*₁ complex and cytochrome *c*_y - membrane attached electron carrier cytochrome *c*_y.

Isolation of “Cu²⁺- unresponsive” *ΔccoA* mutants

In order to obtain *ΔccoA* mutants that were “unresponsive to Cu²⁺ supplementation”, about 30,000 mutagenized colonies of *R. capsulatus ΔccoA* strain (SE8) containing an additional copy of *cbb₃-Cox* structural *ccoNOQP* and assembly *ccoGHIS* genes on a plasmid (pCW25) were screened after EMS mutagenesis on Cu containing media (MPYE + 5 μM Cu). The presence of a diploid set of *ccoNOQP* and *ccoGHIS* genes prevented the isolation of *cbb₃-Cox* minus mutants due to the inactivation of these genes. Three mutants (XJ3, XJ6, XJ11) that remained *cbb₃-Cox* minus in the presence of 5 μM Cu²⁺ were retained (Appendix I) and tested for photosynthetic growth as mutating the CCM genes would render them unable to grow photosynthetically due to the absence of *c*-type cytochromes (cytochrome *c₁* and at least one of the cytochromes *c₂* or *c_y* is needed for Ps growth). These mutant strains formed colonies smaller than a wild type strain after two days of photosynthetic growth, but reached similar colony sizes after an additional day of incubation under photosynthetic conditions. In order to see if the defect was due to the mutations in other known genes involved in *cbb₃-Cox* assembly, such as *olsAB* (Aygün-Sunar et al. 2006), *senC* (Swem et al. 2005) and *dsbA* (Onder et al. 2008), plasmids containing the wild type copies of these genes were transferred to the newly isolated mutants, and found not to complement the *cbb₃-Cox* minus phenotype on Cu containing media. These strains were cured from the plasmid that contained *cbb₃-Cox* structural and assembly genes (pCW25) by growing them in enriched media MPYE in the absence of antibiotic and yielded XJ3, XJ6 and XJ11 derivatives. These mutants were unable to perform respiration using *cbb₃-Cox*, independent of supplementation with Cu²⁺ on both minimal and enriched media, exhibited slow Ps growth and also were not

sensitive to other metals such as Mn^{2+} , Fe^{3+} , Zn^{2+} , Cd^{2+} , as well as to reducing and oxidizing agents, reduced/oxidized glutathione, cysteine, cystine, in respect to their *cbb3*-Cox phenotype.

Cu-unresponsive strains are not sensitive to Cu

To investigate whether Cu addition had any effect on the growth of these strains, Cu sensitivity or resistance were examined. These strains were grown in enriched media under respiratory and photosynthetic conditions and the sensitivity to Cu was determined by a plate growth inhibition assay as described in Chapter 2. After the growth for two days, the sizes of growth inhibition zones surrounding filter disks soaked with various concentrations of Cu^{2+} were determined. These strains did not show any difference in their sensitivity or resistance to Cu^{2+} than the wild type or *ccoA* mutant (data not shown).

Cu content of Cu- unresponsive mutant XJ3

In Chapter 2, we reported that ΔccoA mutants had a defect in the acquisition of Cu to the cells by measuring the total Cu content in the lyophilized whole cells by ICP-DRC-MS. ΔccoA mutant accumulated about 20% less than the wild type when grown in enriched media without Cu^{2+} , and 60% lower when grown with 5 μM Cu^{2+} .

Table 3: Respiratory, NADI, and photosynthetic phenotypes of isolated “Cu²⁺-unresponsive” *ΔccoA* mutant strains after EMS mutagenesis

Strains	MedA ¹ - Cu	MedA + Cu (1.5 μM)	MPYE ¹	MPYE + Cu (5 μM)
Wild Type (MT1131)	R ⁺⁺ , N ⁺⁺² , Ps ⁺⁺²	R ⁺ , N ⁺⁺ , Ps ⁺⁺	R ⁺ , N ⁺⁺ , Ps ⁺⁺	R ⁺ , N ⁺⁺ , Ps ⁺⁺
<i>ΔccoA</i> (SE8)	R ⁺ , N ⁻ , Ps ⁺⁺	R ⁺ , N ⁺ , Ps ⁺⁺	R ⁺ , N ⁻ , Ps ⁺⁺	R ⁺ , N ⁺ , Ps ⁺⁺
XJ3	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺
XJ6	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺
XJ11	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺	R ⁺ , N ⁻ , Ps ⁺

¹ MedA and MPYE refer to minimal and enriched growth medium, respectively.

² R and Ps, indicate respiratory and photosynthetic growth conditions, respectively. N refers to NADI reaction which indicates the *cbb₃*-Cox activity. The specifications ++, +, and – for NADI reaction refers to the speed of the reaction indicating the presence of *cbb₃*-Cox, for Ps growth, it refers to the absence or presence of growth under photosynthetic growth conditions and + and ++ refers to the size of the colonies.

We measured Cu content of the Cu²⁺ unresponsive *cbb₃-Cox* minus mutant XJ3 cells in order to determine whether it had similar or lower amounts of Cu compared to $\Delta ccoA$ mutant. XJ3 mutant Cu content was only 7% less than wild type Cu accumulation when grown in enriched media without Cu²⁺ supplement and 27% lower when grown with 5 μ M Cu²⁺ supplement (Table 4). The data indicated that XJ3 mutant Cu content was closer to wild type strain rather than $\Delta ccoA$ mutant (Table 4). These data suggested that XJ3 mutant had mutation(s) not in the alternative Cu²⁺ acquisition pathway in the absence of CcoA transporter which would result in even lower Cu²⁺ acquisition in the cells, but rather in some other gene(s) involved in *cbb₃-Cox* maturation and possibly in Cu export that would enhance accumulation when defective. Cu contents of two other Cu- unresponsive strains XJ6 and XJ11 will also be measured by the same method to determine if they show a difference in Cu acquisition.

Genetic complementation of XJ3 and XJ6 mutants with *R. capsulatus* genomic libraries

To locate the gene(s) defective in Cu-unresponsive mutants, a conjugally transferable *ccoA* minus revertant genomic library of *R. capsulatus* was used to complement their NADI phenotype on Cu containing media. A plasmid named as pSE10 was obtained from the genetic cross with XJ3 strain. This plasmid restored the NADI^{slow} phenotype of both XJ3 and XJ6 strains on Cu containing media but it did not complement the third strain XJ11.

Table 4: Metal contents of wild type (MT1131), $\Delta ccoA$ (SE8) and Cu-unresponsive XJ3 strain determined by ICP-DRC-MS.

Strain	Cu addition during growth	(%)	
		Cu	Mn
Wild Type (MT1131)	-	100	100
	+	363	111
$\Delta ccoA$ (SE8)	-	79	107
	+	146	118
XJ3	-	93	114
	+	266	123

The data suggested that XJ3 and XJ6 strains contained mutations different than XJ11. pSE10 contained a chromosomal 7.6 kb BamHI fragment. DNA sequences determined from the ends of the fragment were aligned to the *R. capsulatus* genome (<http://www.ncbi.nlm.nih.gov>) to locate the chromosomal region that complemented XJ3 and XJ6. The BamHI fragment contained eight intact and one partial open reading frames (ORF) annotated as follows: *mazG* family protein (RCC01779); peptidase, M20 family, amidohydrolase (RCC01780); protein translocase, YajC subunit (RCC01781); protein-export membrane protein *secD* (RCC01782); protein-export membrane protein *secF* (RCC01783); protein of unknown function DUF498 (RCC01784)); heme exporter protein A *ccmA* (RCC01785); heme exporter protein B *ccmB* (RCC01786); and partial heme

exporter protein C *ccmC* (RCC01787) (Figure 15). In addition, an existing plasmid (p2hel-404) (Appendix I) containing *ccmABCDG* genes when transferred, also complemented the NAD^I phenotype of XJ3 and XJ6 strains. In order to find the gene that contained the mutation, a BstBI site at 4912th bp of pSE10 was used to divide the BamHI insert of pSE10 into two fragments and plasmids pSE11 and pSE12 were constructed. pSE12 (4.9 kb) contained the fragment with four ORFs on the left side of BstBI site (*mazG* family protein (RCC01779), peptidase, M20 family, amidohydrolase (RCC01780), protein translocase, YajC subunit (RCC01781), protein-export membrane protein *secD* (RCC01782)). pSE11 (2.7 kb) contained three ORFs on the right side of BstBI site (protein of unknown function DUF498 (RCC01784)), *ccmA* (RCC01785), *ccmB* (RCC01786)). Both plasmids were transferred into XJ3 and XJ6 strains. pSE11 plasmid complemented the NAD^I phenotype of both strains same as the initial complementing plasmid pSE10 indicating that the mutation was in *ccmA* or *ccmB* genes.

Defining the mutation(s) in XJ3 and XJ6 strains

In order to pinpoint the mutation(s) in XJ3 and XJ6 mutants, three derivatives of pSE11 were constructed as described in Materials and Methods to yield the plasmids pSE21 [$\Delta(ccmA::Kan)$], pSE22 [$\Delta(ccmB::Kan)$], and pSE23 [$\Delta(ccmAB::Kan)$] by using a nonpolar kanamycin resistance cassette (Figure 15). Constructed plasmids, pSE21 (*ccmA*⁻, *ccmB*⁺), pSE22 (*ccmA*⁺, *ccmB*⁻), and pSE23 (*ccmA*⁻, *ccmB*⁻) were transferred into XJ3 and XJ6. Only the plasmid pSE22 was able to complement the NAD^I phenotype on media that contained Cu²⁺ for both strains

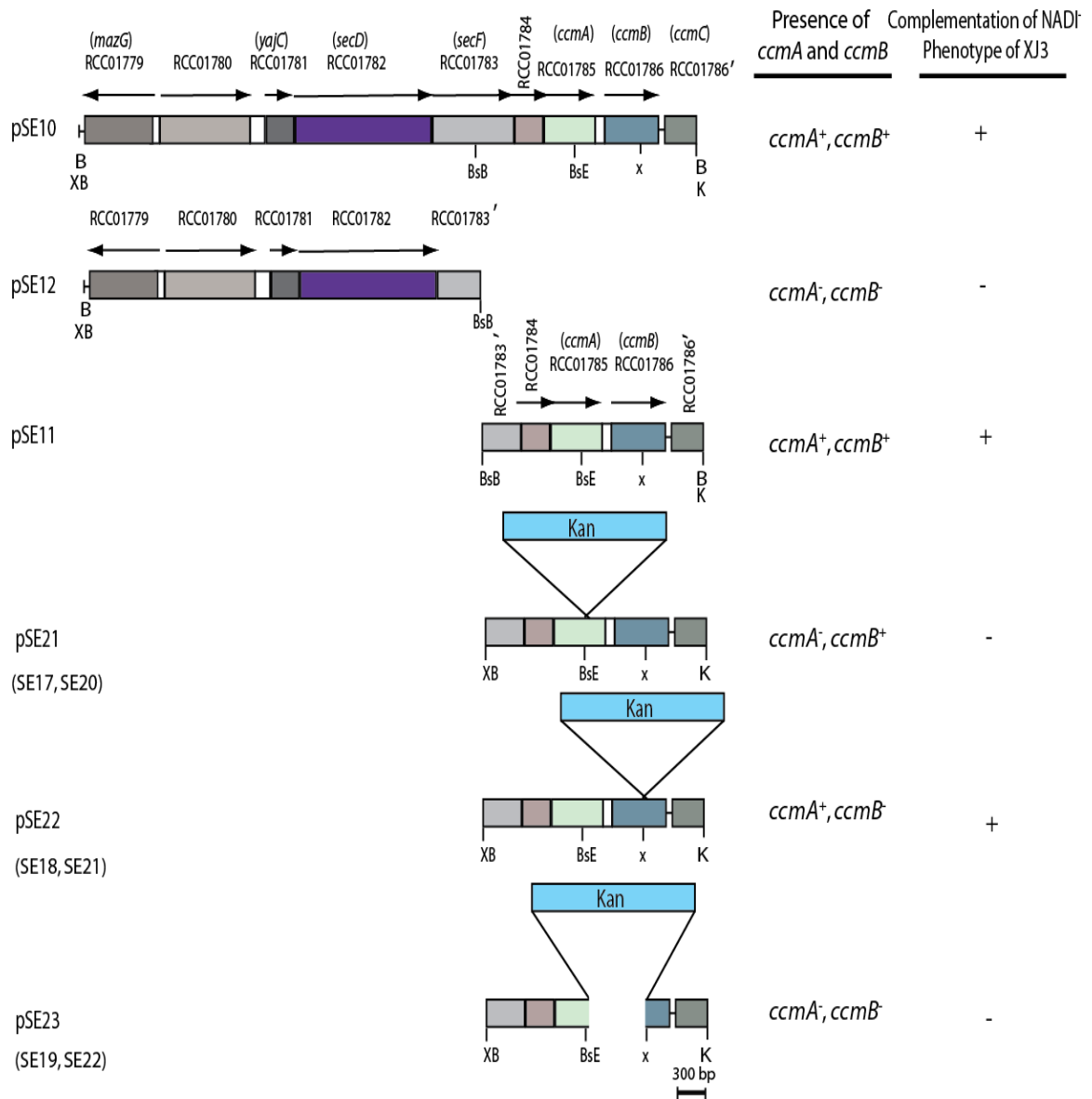


Figure 15: Restriction map of plasmids pSE10 and its derivatives used to complement XJ3, XJ6; chromosomal knock out mutants.

Figure 15: Restriction map of plasmids pSE10 and its derivatives used to complement XJ3, XJ6; chromosomal knock out mutants. Various plasmids, isolated or constructed as described in Materials and Methods are shown on the left and their ability to complement Cu independent $\Delta ccoA$ mutants (XJ3, XJ6) on the right. When appropriate, *R. capsulatus* strains carrying the related chromosomal knock out alleles were also indicated under the plasmids. pSE10 and its derivatives are shown. See the text for annotations of the ORFs, RCC01779, RCC01780, RCC01781, RCC01782, RCC01783, RCC01784, RCC01785, RCC01786 that are carried by pSE10. B, BsE, BsB, X, XB, K correspond to the restriction endonuclease sites for BamHI, BstEII BstBI, XhoI, XbaI and KpnI enzymes, respectively. Superscript comma indicates partial gene.

indicating that the mutation(s) was present in *ccmA* gene. To find the molecular basis of the mutation(s) present in these strains, *ccmA* gene was sequenced from the genomes of XJ3 and XJ6 strains. In both mutants, the same single basepair change (C to T) at position of 211 of *ccmA* that converted alanine 71 of CcmA to a valine residue was found. This alanine is located in a conserved region among different organisms that contained *ccmA* gene (Figure 16).

ccmABCD genes are required for *c*-type cytochrome maturation as described in Chapter 1, and predicted to form an ATP-binding cassette (ABC) containing transporter (Beckman et al. 1992; Goldman et al. 1998; Goldman et al. 1997; Kranz et al. 2009). The observation that XJ3 and XJ6 could grow photosynthetically in contrast to previously characterized Ps^- phenotype of *ccmA* and *ccmB* single mutants of *R. capsulatus* led us to construct insertion mutants of *ccmA* and/or *ccmB* in the background of *ccoA* insertion deletion. *ccmA* and *ccmB* genes form different subunits of an ABC type transporter, and predicted to work together, so we decided to construct mutants of both genes. Due to the presence of the mutation at the same location in both XJ3 and XJ6 strains, only XJ3 mutant was selected to continue with in our studies.

Construction and Characterization of *ccmA*, *ccmB* and *ccmAB* mutants

The plasmids pSE21, pSE22, and pSE23 were used to construct the nonpolar mutant strains of $\Delta(ccmA::Kan)$, $\Delta(ccmB::Kan)$ and $\Delta(ccmAB::Kan)$ genes in both wild type MT1131 and $\Delta ccoA$ (SE8) strain backgrounds via interposon mutagenesis (Materials

and Methods) to yield the strains SE17, SE18, SE19 (in the background of SE8) and SE20, SE21, SE22 (in the background of MT1131) (Appendix I).

These strains were unable to grow photosynthetically on both minimal and enriched media as observed before (Beckman et al. 1992), and excreted large amounts of coproporphyrins and protoporphyrins that was typical to some of the CCM mutants (e.g. *ccmE*, *ccmH*, *ccmF*) (Biel et al. 1990; Deshmukh et al. 2000; Richard-Fogal et al. 2010) which have been determined by NMR spectroscopy (Deshmukh et al. 2000). In addition they showed NAD⁻ and Ps⁻ phenotype on both enriched and minimal media indicating that as expected these mutants did not make any *c*-type cytochromes.

R. capsulatus ccmA, *ccmB*, and *ccmC* are located very close to each other in the genome, only separated by 119, and 84 bp respectively. To confirm that the insertions were not polar to the downstream genes, plasmids containing the wild type copy of mutated gene was introduced. In order to confirm that *ccmA* insertion was not polar to the *ccmB* gene downstream, pSE22 plasmid that only contained the wild type copy of *ccmA* was transferred to *ccmA* mutants (SE17 and SE20). Similarly, pSE21 plasmid was introduced into *ccmB* mutants (SE18 and SE21) to confirm that the insertion was not polar to *ccmC*. In both cases, the phenotypes of these strains were complemented to Ps⁺ confirming that the insertions were not polar and that *ccoA* mutation did not affect this phenotype.

```

Rcaps      MTL LAVDQLTVSRGGLAVLEGVSFSLAAGHALVLRGPNIGIKTTLLRRTLA 50
Ecoli      MGMLVRELLCERDERTLFSGLSFTLNAGEWVQITGSNGAGKTTLLRLLT 50
Bradyrhizobium MQL-SGRRVICVRGGREVFAGLDFEAVS GEAVAVVGRNGSGKTSLLRLIA 49
Rhodosphaeroides MDL-TVTNLACARGGVTVLERVSFRLSRGAALILRGPNIGIKTTLLRTVA 49
Agrobacterium MDL-TAENLGVRGGEDFIFMNISFKLSDGEALVLTGRNGSGKSTLLRTVA 49
          * : . : * . : : : * * : : * - * - * - * * * : :
          |
Rcaps      GLQPPLAGRVSMPP-----EGIAAAHADGLKATLSVRENLQFW 89
Ecoli      GLSRPDAGEVLWQGGPLHQVRDSYHQNLWIGHQPGIKTRLTALENLHFY 100
Bradyrhizobium GLLIPAGGTIALDGG--DA-ELTLPEQCHYLGHRDALKPALSAENLSFW 96
Rhodosphaeroides GLQPAVAGEISMPP-----EAVAYAAHADGLKATLTVAEENLAFW 88
Agrobacterium GLLRPEQGRVKIAGEGIDA-EMRPSEAFHYLGHARNAMKTELTVAEENLRFW 98
          ** . * : : : . * . : * . * * * * :
          |
Rcaps      AAIHAT-----DTVETALARMNLALEHRAAASLSAGQKRRRLGLARLLV 133
Ecoli      HRDGD-----AQCLEALAQAGLAGFEDI PVNQLSAGQRRVALARLWL 144
Bradyrhizobium ADFLGG---ER-LDAHESLATVGLDHATHLPAAFLSAGQRRRLSLARLLT 142
Rhodosphaeroides AAIYGT-----DRAARAIERMNLAAALADRQAQNL SAGQKRRRLGLARLLV 132
Agrobacterium KDFLGDFPGSTGVAIDEAAAIVGLAGITHLPFGYLSAGQRRRFAMAKLLV 148
          : . * . * * * * : * * : * * : * *
          |
Rcaps      TGRPVWVLDEPTVSLDAASVALFAEAVRAH LAAGGAALMATHIDLGLSEA 183
Ecoli      TRATLWILDEPFTALDVNGVDRLTQRMAQHTEQGGIVILTHQPLNVAES 194
Bradyrhizobium VRRPIWLLDEPTTALDVAGQDMFGGLMRDHLARGGLIAATHMALGIDSR 192
Rhodosphaeroides TGRPLWVLDEPTVSLDAASVALFGDVVRTHLAEGGAALMATHIDLGLAEA 182
Agrobacterium AWRPVWILDEPTAALDRAADAMFTDLVKSHLGKGGIVLAATHQPLGLEKA 198
          . . : * : * * * . : : : * * * : : * * * . : .
          |
Rcaps      RVLDLAPFKARPPEAGGHRGAFDHGFDGAF 214
Ecoli      KIRRISLTQT-----RAV 207
Bradyrhizobium -ELRIG-----VA 200
Rhodosphaeroides EVLDLAPYRAETPA--GTE-PADDPFAGVTA 210
Agrobacterium QELQMTGFAGVE-----TWA 213

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Figure 16: The amino acid homology between CcmA homologs from *R. capsulatus*, *E. coli*, *Bradyrhizobium japonicum*, *Rhodobacter sphaeroides*, and *Agrobacterium tumefaciens*. The mutated ala amino acid (changes to val) present in XJ3 and XJ6 strains is shown with arrow. The conserved structural motif of ABC transporters, Walker A motif (GPNIGIKTT), is boxed.

The phenotype related to the difference in the protoporphyrin secretion and photosynthetic growth between the missense mutant XJ3 and the insertion knock out mutants of *ccmA*, *ccmB* and *ccmAB* suggested that in XJ3 strain the maturation of *c*-type cytochromes was not completely abolished. Overall the phenotypes of *ccmA*, *ccmB* and *ccmAB* double mutants were the same in respect to the *c*-type cytochrome maturation and *cbb₃*-Cox production, indicating that both proteins are required to be able to have an active *cbb₃*-Cox and photosynthetic growth.

Characterization of cytochrome *c* profile of XJ3 strain

To understand the molecular basis of XJ3 Ps⁺ phenotype, the *c*-type cytochrome content was analyzed by using the chromatophores prepared from XJ3 strain grown in enriched media MPYE with 5 μM Cu²⁺ under respiratory or photosynthetic conditions and separated by SDS-PAGE/ stained with tetramethylbenzidine (TMBZ) (Materials and Methods). Interestingly only one faint band around 32 kDa was visible after the TMBZ staining in XJ3 strain. This band had a slightly larger size than the *cbb₃*-Cox subunit *c_p*, as well as the other cytochrome *c*'s detected by this method. The identity of this cytochrome remains to be defined. Other membrane bound cytochromes could not be detected. In addition we analyzed the cytochrome *c₂* content in soluble fractions, and were detected at very low levels in XJ3 as compared to wild type (Figure17). The data indicated that only very limited amounts of cytochromes were made in XJ3 in both presence and absence of Cu.

CcoA does not restore the *cbb*₃-Cox defective phenotype of XJ3

XJ3 mutant is a derivative of $\Delta ccoA$ mutant which is defective in making *cbb*₃-Cox. In order to check if the presence of *ccoA* gene changes the phenotype of XJ3 mutant, we introduced *ccoA* gene on a plasmid (pSE3) to XJ3 mutant and checked the presence of *cbb*₃-Cox by NADI staining the colonies. We found that *cbb*₃-Cox was still missing even though *ccoA* was now complemented (data not shown). This finding suggested that in the presence of *ccoA*, the *ccmA* missense mutation in XJ3 affected the production of cytochromes *c*_o and *c*_p more severely than those of cytochrome *c*₁ and *c*₂ to allow Ps growth and have a NADI phenotype.

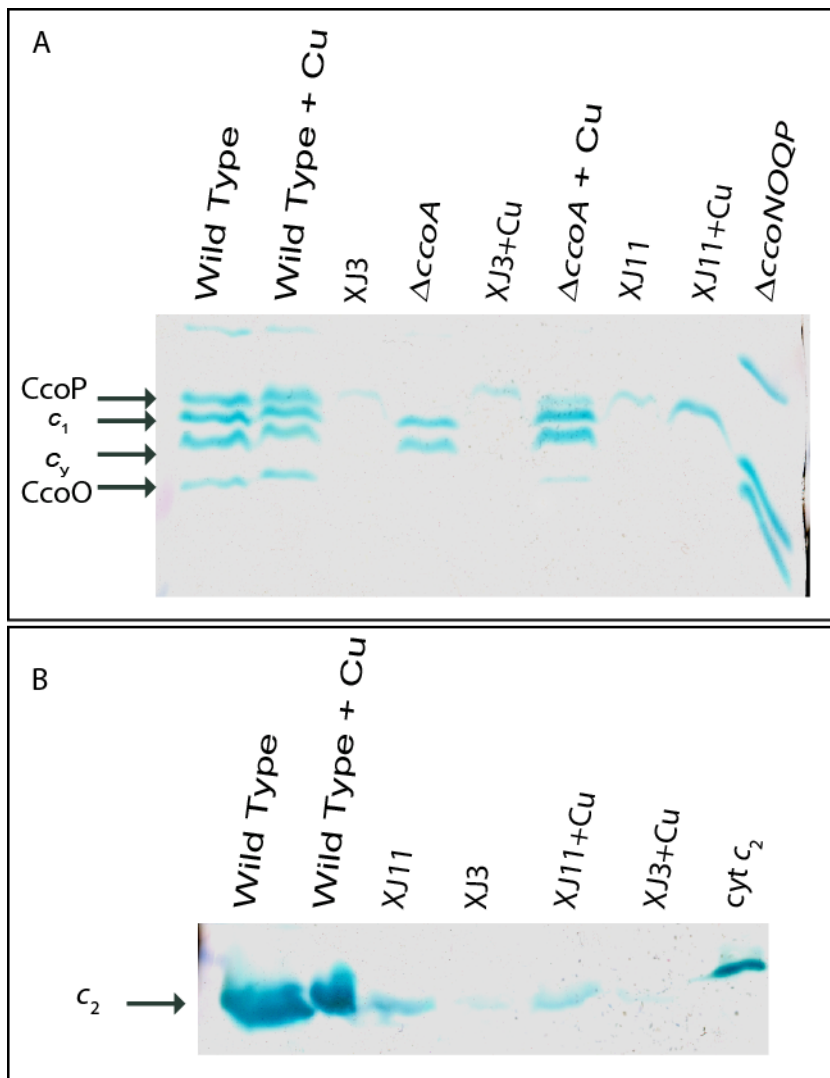


Figure 17: Cytochrome *c* profiles of Cu unresponsive mutants XJ3 and XJ11 strains under respiratory conditions.

Figure 17: Cytochrome *c* profiles of Cu unresponsive mutants XJ3 and XJ11 strains.

Membrane (panel A) and soluble (panel B) fractions from cultures grown under respiratory conditions on enriched medium (MPYE) in the absence or presence of 5 μ M Cu (designated as +Cu) were separated on SDS-PAGE (15% upper gel, 18% lower gel). Cytochrome *c* were detected on SDS-PAGE by TMBZ staining as described in Materials and Methods. Approximately 100 μ g chromatophore membranes or 150 μ g soluble fractions from Cu unresponsive strains XJ3 and XJ11 and about 50 μ g chromatophore membranes or 100 μ g soluble fractions from wild type (MT1131), $\Delta ccoA$ (SE8), and $\Delta ccoNO$ (GK32) were separated by this method. The cytochromes are indicated on the left. The descriptions of cytochromes are described in the figure legend of Figure 11.

Complementation of XJ11 strain with chromosomal libraries

The third strain XJ11 was not complemented by the previous plasmids, so another conjugally transferable BamHI genomic library of *R. capsulatus* was used to complement its NAD⁺ phenotype on Cu containing media. Two plasmids that contained a 2.3 kb BamHI chromosomal insert were able to complement NAD⁺ phenotype of XJ11 strain on enriched medium with Cu and named as pXJ01 and pXJ02. From the sequence analysis of both ends of the insertion and the alignment to the *R. capsulatus* genome revealed that both pXJ01 and pXJ02 were identical to a previously isolated plasmid in our lab pMD1 (Deshmukh et al. 2000). This plasmid contained three ORFs annotated as; *argC* (RCC0553), *ccmE* (RCC0554) and almost complete RCC0555 (only 2 bp missing) (protein of unknown function DUF847, a homolog of zliS) (Figure 18). To test whether the mutated gene in XJ11 was *ccmE*, pMD21 plasmid that contained a mutant form of it (a His123 to Ala mutation in the conserved heme binding site) was transferred to XJ11. pMD21 did not complement NAD⁺ phenotype of XJ11, suggesting that XJ11 mutant might be defective in CcmE function. However, pMD6 plasmid that contained an intact *ccmE* but lacked its upstream region containing *argC* did not complement XJ11 strain. This finding indicated that not only an intact *ccmE*, but also its upstream, a regulatory region that controls its expression was crucial for the complementation of XJ11 mutant.

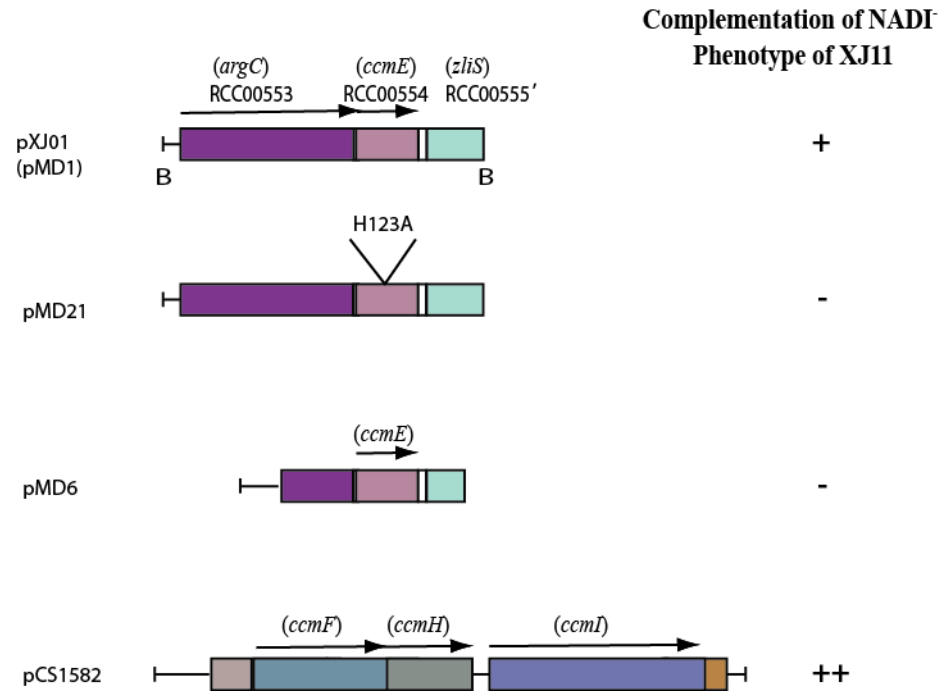


Figure 18: Map of the various plasmids and their derivatives complementing XJ11 strain. The ORFs and restriction sites present are located on the left, and their ability to complement XJ11 is shown on the right. ‘++’ means full complementation, ‘+’ shows partial complementation. B corresponds to the restriction endonuclease sites for BamHI. See the figure or the text for the annotations of RCC00553, RCC00554, RCC00555. Superscript comma indicates partial gene.

In order to locate the mutation on the genome of XJ11, the *ccmE* gene and its 800 bp upstream was sequenced and aligned to wild type genomic sequence. However, no mutations could be found in these regions. This led us to think that *ccmE* might be a multicopy suppressor of the mutation in XJ11. If this is so, then XJ11/pMD1 must overproduce CcmE, which remains to be seen.

***ccmE* is a multicopy suppressor of XJ11 phenotype**

Considering that *ccmE* might be a multicopy suppressor, we hypothesized that other Ccm genes could be responsible for the defect in XJ11 strain. As pSE10 which contained *ccmA* and *ccmB* was unable to complement XJ11, another plasmid with *ccmH*, *ccmI* and *ccmF* genes (pCS1582) was able to fully complement the *cbb₃-Cox* minus phenotype of XJ11 on Cu²⁺ containing media (Figure 18) suggesting that the molecular basis of XJ11 mutant might be related to *ccmHIF* genes. The crosses are being pursued to further define the defect in XJ11.

Characterization of *c*-type cytochrome content in XJ11 strain

Previously CcmH, CcmI and CcmF proteins have been shown to form the apocytochrome-heme ligation core complex, and mutants lacking either one of them have been found not to contain any *c*-type cytochromes. Again the Ps⁺ phenotype of XJ11 led us to determine its *c*-type cytochromes content by using SDS-PAGE and TMBZ staining. Just like XJ3, a light band visible around 32 kDa that was close to (but higher) the size of *c_p* cytochrome, was present in XJ11 strain. The identity of this band remains to be

defined by using antibodies against cytochromes c_1 and c_p . In addition it contained reduced amounts of soluble cytochrome c_2 and did not contain other membrane bound cytochromes (Figure 17). *ccmHIF* knockout mutants do not make any c -type cytochromes and excrete porphyrins to the media, so we predicted that this mutation did not abolish the function of these proteins completely but affected it enough so that it produced only very low amounts of c -type cytochromes which resulted much lower porphyrin secretion.

CcoA does not restore the *cbb*₃-Cox defective phenotype of XJ11

In order to check whether the presence of CcoA changed the c -type cytochromes and *cbb*₃-Cox deficiency phenotype of XJ11, we have introduced a wild type copy of *ccoA* gene on a plasmid (pSE3), and checked the *cbb*₃-Cox phenotype on enriched media MPYE that contains 5 μ M Cu²⁺ by NADI staining. The presence of CcoA did not change the NADI minus phenotype of XJ11, showing that the mutation in XJ11 alone was able to interrupt the formation of the wild type levels of active *cbb*₃-Cox most likely by decreasing the production of c -type cytochrome subunits (data not shown).

DISCUSSION

Rhodobacter capsulatus *cbb*₃-Cox biogenesis is a complex process due to the presence of two different maturation systems for different subunits that are the CcoO and CcoP by the CCM system and maturation of the catalytic subunit CcoN with the insertion of Cu and heme *b* cofactors (Gray et al. 1994). Previously we characterized a novel

transporter called CcoA involved in *cbb₃*-Cox biogenesis possibly in Cu acquisition required for the maturation of this enzyme (see Chapter 2). CcoA mutants were unable to make any of the subunits of *cbb₃*-Cox unless they were supplemented with exogenous Cu. We hypothesized that characterization of *ccoA* mutants that did not respond to Cu addition would reveal the alternative Cu acquisition pathway for the *cbb₃*-Cox in the absence of CcoA. We obtained two such mutants and characterized them to understand the molecular basis of their defect.

The first mutant, XJ3, was complemented by the *ccmA* gene. *ccmA* and *ccmB* are two CCM genes that are always located next to each other. CcmA encodes the ATP binding cassette domain that has two conserved motifs called Walker A and Walker B that were shown to be important for its function (Beckman et al. 1992). CcmA was shown to interact with the downstream gene *ccmB* in *E. coli* (Christensen et al. 2007; Goldman et al. 1997). CcmABCD proteins were suggested to function together as an ATP-binding cassette (ABC) transporter (Beckman et al. 1992). Other components of this complex CcmC and CcmD were shown to be copurified with CcmA supporting the formation of this multisubunit transporter complex (Goldman et al. 1997). The stoichiometry, exact composition and transport activities of CcmA, CcmB, CcmC, CcmD and CcmE remains to be determined.

ccmA null mutants are unable to grow photosynthetically. However XJ3 was able to grow photosynthetically indicating that at least two cytochromes; cytochrome *c*

subunit of *bc*₁ complex (cytochrome *c*₁) and either one or both of the cytochromes *c*, membrane attached cytochrome *c*_y or soluble cytochrome *c*₂, must be present. By TMBZ staining we were able to detect a band that did not correspond to any of the known cytochromes but had a size close to cytochrome *c*_p. In addition a small amount of soluble cytochrome *c*₂ was also detected. However our efforts to detect *c*₁ subunit and *c*_y was not successful. It could be that the amounts of these cytochromes are low due to the mutation and not detectable with the method used here. Although we could not detect all cytochromes in XJ3, it is clear that the photosynthetic growth and porphyrin secretion phenotypes of XJ3 strain are different than a *ccmA* deletion mutant. The identity of the observed cytochrome needs to be determined.

The *cbb*₃-Cox minus phenotype of XJ3 was not a result of the absence of CcoA as the introduction of a wild type copy of *ccoA* gene to XJ3 did not change its NADI phenotype. The two subunits, CcoO and CcoP of *cbb*₃-Cox were missing, however it remains to be seen if there is CcoN subunit production in this strain. It is not known how CcoN maturation is affected by the defects in CCM system. The CCM mutants do not have an active *cbb*₃-Cox, but if CcoN is made as wild type levels and degraded or it is not made at all remains to be seen (Pulse-chase experiments to detect the amounts of CcoN subunit in XJ3 mutant would be useful to distinguish between the two possibilities). Until now, CcmA has been only implicated in CCM system but it is not known if it has additional functions specific for *cbb*₃-Cox biogenesis. We do not know if this mutation in *ccmA* gene of XJ3 changes the levels of expression of *ccmA*. However we observed that

XJ3 do not secrete porphyrins as was observed in case of *ccmA* insertion mutants. It could be that CcmA levels are decreased but still enough to avoid excretion of porphyrins and also to make some cytochromes. It would be interesting to see cytochrome *c* profile of XJ3 strain containing the wild type copy of *ccoA* (pSE3).

The cargo of CcmAB complex (if there is one) is not clear. Initially these proteins were thought to transport heme, however there were several observations against this hypothesis. First, the heme chaperone CcmE could be loaded with heme in the absence of CcmAB and with overexpression of CcmC (Schulz et al. 1999). Second, the mature cytochrome *b* are produced in *ccmAB* mutants (Throne-Holst et al. 1997). It has also been suggested that a reductant could be their substrate (Christensen et al. 2007; Schulz et al. 1999) because heme needs to be kept reduced to be able to bind apocytochrome. However the supplementation of *ccmAB* mutants with exogenous reductants did not overcome the cytochrome *c* deficiency in this strain (Schulz et al. 1999). In addition neither heme nor reductants increased the rate of hydrolysis of ATP in the purified CcmAB complex and CcmA defect could not be suppressed by addition of exogenous heme (Christensen et al. 2007). It was proposed that the ability of this transporter to hydrolyze ATP was needed in order to release the heme bound CcmE from this complex for the ligation to apocytochrome (Christensen et al. 2007). Overall the nature of the substrate that CcmAB transport remains unknown but it is required for the holocytochrome *c* formation. In the absence of CcoA transporter, cells accumulate less Cu as we have discussed in Chapter 2. For that reason, we expected to find less Cu

accumulation in XJ3 due to the absence of CcoA and also due to the defects in alternative Cu acquisition pathway. However Cu content was very close to wild type levels rather than *ccoA* mutant. A possibility could be that CcmA and CcmB are involved in export of Cu from the cells in addition to their role in CCM. Cu transport assays or determination of total Cu content of *ccmA* knockout mutant and XJ3 mutant that contains a wild type copy of *ccoA* would be informative to determine if CcmA exports Cu. It has been observed that CCM genes also have implications in other biological processes (Cianciotto et al. 2005). A relation of CCM genes and Cu was previously observed in *Pseudomonas fluorescens* that was isolated from a Cu contaminated soil (Yang et al. 1996). In this organism, CcmI and CcmF were shown to be required for resistance to Cu by an unidentified mechanism.

Both heme and Cu are cofactors of CcoN, the catalytic subunit of Cox, therefore it is interesting to see if heme biosynthesis and/or trafficking changes by the availability of Cu. Previously it has been observed in *S. cerevisiae* that two Cu transporters Ctr1p and Ccc2p and a Cu chaperone Atx1p were required for trafficking Cu to the multicopper oxidase Fet3p which is responsible for iron (Fe) uptake (Dancis et al. 1994; Lin et al. 1997) indicating a link between Cu and Fe uptake. On the other hand, another study showed that heme *a* biosynthesis in *S. cerevisiae* that contains an *aa₃*-Cox, was not regulated by Cu at the level of heme *o* synthase and heme *a* synthase (Morrison et al. 2005) which are the enzymes responsible for converting heme *b* to heme *a*. How Cu affects heme (or iron) availability and if CcmABCD complex is involved in this process in *R. capsulatus* remains to be determined.

The second mutant XJ11 is complemented partially by the heme chaperone CcmE and fully by one of the ligation core complex components CcmHIF. It remains to be determined which one of these genes contain the mutation in XJ11.

Previously, similar relations to what we observed with XJ11 complementation (suppression of phenotype by overproduction of other CCM genes) in *R. capsulatus* were seen. The CcmI defect could be suppressed by overproduction of CcmF, and CcmH together or CcmI-1 domain on minimal media (Deshmukh et al. 2002) and on all media when CcmG or CcmI-2 or apocytochrome c_2 was overproduced in addition (Sanders et al. 2005). These findings suggested that the membrane-spanning domain of CcmI-1 is a part of the ligation complex with CcmH and CcmF, and the periplasmic domain could be an intersection domain between thiol-oxidoreduction processes catalyzed by CcmG and CcdA and the heme ligation complex. Definition of the mutation in XJ11 will help us to gain more information about the relationship between the heme-ligation complex CcmHIF and CcmE.

The complementation of XJ11 by *ccmE* gene was only achieved when the upstream region of *ccmE* that contained *argC* gene was present suggesting that this region could be responsible for controlling the levels of expression of *ccmE*. Previously it has been observed that insertion mutations located in *argC* gene are polar on *ccmE*. The *argC* mutants were unable to produce *c*-type cytochromes but excretion of porphyrins was absent in contrast to *ccmE* mutant (Deshmukh et al. 2000). This suggests that a small

amount of CcmE was still present in *argC* null mutant and was sufficient to prevent excretion of porphyrins but insufficient for the production of wild type levels of cytochromes. This observation appears to be similar to the phenotypes of XJ3 and XJ11 mutants, where the CCM proteins are functional enough to prevent excretion of porphyrins and support photosynthetic growth, but not enough for maintaining wild type levels of all *c*-type cytochromes especially in $\Delta ccoA$ background. The *argC* mutant reverted frequently to produce *c*-type cytochromes indicating an upstream transcriptional control of expression for CcmE gene. This was also consistent with the lack of complementation of XJ11 phenotype with a plasmid containing only *ccmE* gene without its upstream region. Therefore characterization of the *argC* revertants would reveal new information about the control of *ccmE* gene expression. In addition, the location of *ccmE* gene in the genome next to *argC* in *R. capsulatus* is unusual compared to other organisms. In other organisms such as *E. coli*, *ccmE* is located next to other CCM genes suggesting additional roles for CcmE in *R. capsulatus* (Thony-Meyer et al. 1995).

In *E. coli*, it has been shown that CcmE interacts with CcmF and CcmH suggesting that they form a heme-ligation complex (Ren et al. 2002). However whether this is also the case in *R. capsulatus* is not known. Our findings suggest that these proteins might have specific interactions that could be partly bypassed by overproduction of CcmE. It could be that if one of these genes is defective, the presence of more heme bound CcmE allows them to make more *c*-type cytochromes. As we observed from XJ11 cytochrome *c* profiles, this strain could make only cytochrome *c*₂ and an unidentified

cytochrome. The identity of this cytochrome remains to be seen to understand how this mutant grows under photosynthetic conditions. The location of the mutation would tell us more about the functions of these genes. In addition we did not observe porphyrin excretion in XJ11 strain as in case of CcmHIF and CcmE mutants. This also suggests that cytochrome *c* maturation is still present in this mutant. As for XJ3 strain, it would be interesting to see if XJ11 makes CcoN subunit of *cbb₃*-Cox to distinguish between CCM specific defects and *cbb₃*-Cox biogenesis. In addition, once the mutation is located, the levels of expression of these genes would be probed by using specific antibodies.

Here we analyzed two Cu-unresponsive CcoA mutants that were *cbb₃*-Cox minus even after Cu supplementation. The genetic analysis of these mutants led us to two CCM mutants that contained mutations in *cmaA* gene and one of the *cmaHIF* complex genes. The defect in CcmHIF complex was partially suppressed by the overexpression of CcmE. Additional studies with these mutants will provide us with valuable information about how the maturation of cofactor containing proteins such as *c*-type cytochromes or protein complexes such as *cbb₃*-Cox are achieved. In addition the alternative Cu acquisition pathway in the absence of CcoA still needs to be determined which will unveil important information on the Cu homeostasis in *R. capsulatus*.

CHAPTER 4
INVESTIGATION OF THE *CCOA* BYPASS MUTATION(S)

INTRODUCTION

R. capsulatus is a Gram negative facultative phototroph that contains a *cbb₃*-Cox as the only Cox (Gray et al. 1994). CcoA is a MFS type transporter involved in Cu acquisition and required for *cbb₃*-Cox maturation in *R. capsulatus* (Chapter 2). *ccoA* mutants are NADI but they restore ability to make *cbb₃*-Cox after supplementation with Cu. In the absence of CcoA, cells accumulate less Cu than the wild type cells as indicated by ICP-DRC-MS analysis. *ccoA* mutants frequently revert to NADI plus phenotype at high rates (10^{-3} - 10^{-4}) as described in Chapter 2. Interestingly, these revertants are highly sensitive to Cu supplementation, and the analysis of Cu content in two *ccoA* revertants (SE8R1 and SE8R2) shows that the accumulation of Cu is approximately 35% more than the wild type cells when grown on enriched media with or without supplementation with 5 μ M Cu. Thus, determination of the nature of this mutation should be informative about the role of CcoA and how Cu homeostasis is maintained.

In this chapter, we discuss approaches taken to determine the molecular nature of the *ccoA* bypass mutation(s). First we prepared genomic libraries derived from revertant or *ccoA* mutant and screened for complementation of *cbb₃*-Cox activity. Second, we have performed next generation sequencing analysis of *ccoA* mutant, its selected revertants and wild type to identify genomic loci that are linked to the observed phenotypes. Detailed studies of these revertants are not yet complete, but the preliminary results and exciting findings are presented. Understanding the molecular nature of these suppressor mutations will be invaluable to understand the Cu trafficking and related processes in *R. capsulatus*.

MATERIALS AND METHODS

Strains, culture conditions, and phenotypes.

The bacterial strains and plasmids used in this work are described in Appendix 1. *R. capsulatus* and *E. coli* strains were grown as described in materials and methods sections of Chapter 2.

Molecular genetic techniques

Standard molecular genetic techniques were performed as described (Sambrook, 2001). Genomic DNA was extracted from 10 ml cultures of selected *ccoA* revertants or *ccoA* mutant that were grown in MPYE medium by using the DNeasy Blood&Tissue Kit (Qiagen Inc). Quality and quantity of the isolated genomic DNA was analyzed by using nanodrop spectrophotometer and agarose gel electrophoresis prior to library preparation for next generation sequencing or for conjugal transfer. Genomic libraries for conjugal transfer were prepared by digesting the genomic DNA from the *ccoA* revertants (SE8R1 or SE8R2) or *ccoA* mutant (SE8) with specific restriction enzymes and then ligated to the pRK415 vector that was digested with the same enzymes.

Biochemical techniques

Intracytoplasmic membrane vesicles (chromatophore membranes) were prepared in 50 mM MOPS (pH 7.0) containing 100 mM KCl and 1 mM PMSF as described earlier (Gray et al. 1994). Protein concentrations were determined using Bicinchoninic Acid

assay according to the supplier's recommendation (Sigma Inc., procedure TPRO-562). Cytochrome *c* oxidase activity of colonies was detected by NADI staining as described previously in materials and methods section of Chapter 2. *C*-type cytochromes were detected by separating total membrane proteins on 16.5% SDS-PAGE (Schagger et al. 1987), and the gels were stained for endogenous peroxidase activity of the *c*-type cytochromes by using 3,3',5,5'-tetramethylbenzidine (TMBZ) and H₂O₂ (Thomas et al. 1976).

Cu²⁺ Sensitivity Assays

Cu²⁺ sensitivity of selected strains are tested as described in the materials and methods section of Chapter 2.

Next Generation Sequencing

The next generation sequencing of the wild type (MT1131), $\Delta ccoA$ (SE8), four $\Delta ccoA$ revertants (SE8R1, SE8R2, SE8R5, SE8R6) (Appendix I) was performed by Covance Genomics Laboratory LLC, Seattle, WA. Briefly, genomic DNA was sheared by using Covaris Adaptive Focused Acoustics system (Covaris, Woburn, MA). The genomic DNA libraries with insert size between 200-300 bp were prepared by using Illumina TrueSeq DNA sample prep kit for paired-end sequencing according to the manufacturer's instructions. The resulting genomic DNA libraries were sequenced by using Illumina HiSeq2000 sequencing machine to yield 12-15 million reads of length 100 bp. All reads were aligned to *R. capsulatus* SB 1003 reference genome (GenBank:

CP001312.1 and CP001313.1) by using bowtie (ref: PMID:19261174) alignment algorithm and Single Nucleotide Polymorphism (SNP) and indels were identified by using samtools (ref: PMID: 19505943).

RESULTS AND DISCUSSION

The phenotype of *ccoA* revertants

ccoA mutant is a Cu dependent *cbb₃*-Cox mutant as described in Chapter 2. This mutant does not make *cbb₃*-Cox on the media without Cu and is able to make *cbb₃*-Cox when supplemented with exogenous Cu. In addition, *ccoA* mutant reverts frequently (at the rate of 10^{-3} - 10^{-4}) to regain the ability to make *cbb₃*-Cox as indicated by their similar NADI phenotype to wild type independently of oxygen or Cu availability. However the reversion frequency is smaller when there is exogenous Cu in the media, or when they are grown under photosynthetic conditions.

Initially, we hypothesized that the expression of *cbb₃*-Cox structural genes *ccoNOQP* were increased by a mutation in the promoter region. Sequencing of the promoter region of the *ccoNOQP* operon from 4 different revertants (SE8R1-4) identified no mutations indicating that suppression mutations were located at some other loci in the genome.

As described in Chapter 2, these revertants show hypersensitivity to exogenous Cu supplementation (Figure 12) as well as several other interesting phenotypes. First, we

observed that they change their colony morphology when exposed to exogenous Cu (data not shown). Unlike wild type *R. capsulatus* colonies, the revertant colonies are not shiny or mucoid. Second, when the revertant strains are grown in enriched media with 5 μ M Cu, the color of the culture media displays a reddish color significantly different than the usual green color of *R. capsulatus* cultures.

Interestingly, during Cu sensitivity assays (Materials and methods of Chapter 2), revertant strains were noticed to regain Cu resistance when grown in Cu containing media pointing out that the Cu sensitive revertants also have a suppression mechanism to become resistant to Cu (Figure 19). Both Cu sensitive and resistant *ccoA* revertant colonies are NAD⁺. It would be interesting to probe the molecular nature of these mechanisms to understand the Cu acquisition in *R. capsulatus*. Therefore we investigated how the Cu sensitivity phenotype of *ccoA* revertants changes in the presence of the wild type *ccoA* gene. The Cu sensitivity phenotype of the revertants did not change upon introduction of the plasmid carrying the wild type copy of *ccoA* (pSE3) into the *ccoA* revertant strains (SE8R1 and SE8R2) indicating that CcoA did not decrease the Cu sensitivity consistent with its predicted importer function (Chapter 2) (data not shown).

Overall these observations and observed phenotypes suggest that the reversion mutation causes a change in mechanism of Cu acquisition by the cells.

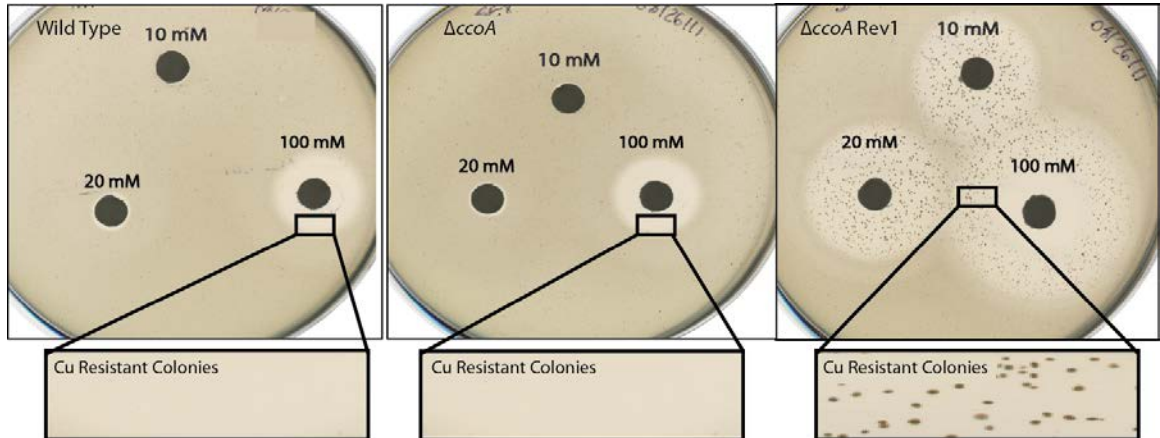


Figure 19: Cu^{2+} resistance or sensitivity phenotypes of *R. capsulatus* strains lacking CcoA and its derivatives. Cu^{2+} sensitive or resistant phenotypes of *R. capsulatus* wild type (MT1131), ΔccoA (SE8), and its revertant ΔccoA Rev1 (SE8R1) were determined by filter disk plate growth assays. Cells were grown on MPYE enriched medium under respiratory conditions and filter paper disks soaked in 10 mM, 20 mM, 100 mM of Cu^{2+} solutions were placed on plates. Sensitivity of a given strain to a given Cu^{2+} concentration was identified by the diameter of the growth inhibition zone surrounding the filter paper. The presence or absence of Cu^{2+} resistant colonies are indicated at higher magnification below each plate.

Genetic Complementation of *ccoA* revertants with genomic libraries

In order to identify the molecular nature of the bypass mutation(s), genomic libraries were constructed and transferred to appropriate strains and screened for NAD^I phenotype. As it is possible that either one of gain of function or loss of function mutations at a given locus may lead to observed phenotypes, two complementary approaches were undertaken.

1) Conjugal transfer of libraries prepared from $\Delta ccoA$ revertants: We constructed four genomic libraries from $\Delta ccoA$ revertants by using four different restriction enzymes and conjugally transferred them into $\Delta ccoA$ mutant background. Suppression of the NAD^I phenotype of a $\Delta ccoA$ mutant by revertant genomic library was investigated. If the reversion mutation is dominant over its wild type allele (i.e. gain of function mutation), then we expect that loci from the genomic library with the suppressor mutation should complement the NAD^I phenotype of $\Delta ccoA$ strain to NAD^I⁺ phenotype. Despite, several attempts we were unable to observe complementation of *ccb*₃-Cox minus phenotype suggesting that the suppressor mutation may not be a gain of function.

2) Conjugal Transfer of libraries prepared from $\Delta ccoA$ mutant: If the suppressor mutation is recessive over its wild type allele (i.e. loss of function mutation), a genomic library prepared from a *ccoA* mutant would reverse the NAD^I⁺ phenotype of the $\Delta ccoA$ revertants to NAD^I. Four different genomic libraries constructed using $\Delta ccoA$ mutant genomic DNA were transferred into two different revertants (SE8R1 and

SE8R2). As above, this complementation also did not identify any mutation suggesting that suppressor mutation may not be loss of a function mutation.

Despite all our efforts to obtain the gene(s) that suppress the *ccoA* defect, we were not able to identify it by library complementations. One of the remaining possibilities to explain these observations is the presence of the suppressor gene in a low copy plasmid leading to the expression of this protein in higher amounts than normally present in the cell. This would cause lethality due to Cu toxicity during the complementation experiments. Although the media used during the complementation experiments were without Cu supplement, Cu might be present in amounts toxic to these strains. Replicating these experiments using a medium completely depleted of Cu by using a Cu chelator bathocuproine in the media is necessary.

Another possibility is that the suppression might be accomplished by mutation(s) in multiple genes which would require the transfer of these genes all together during library transfers. This would be almost impossible by using genomic DNA libraries unless the desired genes are clustered together on a small DNA fragment and are in the same locus obtained by the restriction sites. However this possibility is not very likely due to the high frequency of the reversion events. It is also possible that the libraries we constructed may not contain the gene of interest because it might be located in an operon whose members cannot be transferred as whole operon because the restriction enzyme

cuts inside the operon or none of the restriction sites we used are present in the flanking regions.

Other approaches to determine the bypass mutation of revertants

While genetic studies need to be pursued, we also initiated other approaches to understand the suppression mechanism in *ccoA* mutant. First, we determined the whole genome sequences of four different *ccoA* revertant strains to the *ccoA* mutant and wild type strain by next generation sequencing analysis (NGS). *R. capsulatus*'s 3.8 MB genome has been sequenced from the wild type strain (SB1003) and available for public (<http://www.ncbi.nlm.nih.gov/pubmed/>). However in our experiments we use MT1131 strain, a derivative of the reference strain SB1003, which differs from SB1003 at least by a mutation that leads to deficiency of carotenoid biosynthesis. Surprisingly, our preliminary comparative analysis between MT1131 and SB1003 revealed that these strains also differ in other genomic locations since many SNPs were observed by NGS between these two strains. Elimination of the SNPs present in both MT1131 and SB1003 will yield those that are only present in the revertant strains. 7 strains were sequenced in this analysis in addition to the wild type (MT1131): Two different isolates from *ccoA* mutant (SE8), two different isolates from SE8R1, and 3 other *ccoA* revertant strains (SE8R2, SE8R5, SE8R6). For defining candidates responsible for the reversion, SNPs will be compared between each revertant to *ccoA* mutant and to wild type strain. Since we obtained high genome coverage (~300X) in our NGS experiment we expect to identify target genes even with single nucleotide changes that differ between mutants and

revertants. The presence of multiple replicates in our experimental design will allow not only account for experimental artifacts but also account for biological variability that can significantly affect our analysis.

We have observed differences between the two revertants (SE8R1 and SE8R2) in respect to the amounts of Cu accumulation and the NADI activity suggesting that there might be multiple different suppression mechanisms or different mutations in the same gene causing the suppression (Table 2). Therefore, inclusion of several mutant and revertant replicates will enable us to probe for different mechanisms as well. Once we identify the candidate genes, their knockout mutants in *ccoA* mutant background will be constructed to verify our predictions from computational analysis. We anticipate that if this reversion mutation is a gain of function mutation, the reversion will not be able to occur in the knockout and the colonies will stay NADI⁻. If the mutation is a loss of function mutation, the mutants will be NADI⁺.

When the wild type copy of *ccoA* gene (pSE3) was transferred to the revertants SE8R1 and SE8R2, the Cu hypersensitivity of these revertants were still present indicating that the presence of CcoA did not decrease Cu accumulation. Cu contents of these strains will be measured to provide more information about the molecular mechanism of Cu homeostasis.

We also initiated proteomic studies to compare the proteomes of the wild type, *ccoA* mutant and the *ccoA* revertant strains. We determined the proteome compositions of the whole cells of these three strains. Excitingly, we have observed that there are many changes and we are in the process of further analyzing these results. We believe that this analysis will be more powerful when combined with NGS analysis since we can use genome sequences from mutant and revertants as databases for peptide searches. Also we would like to extend this comparison by looking at the different parts of the cells by preparing periplasmic, membrane or cytosolic fractions of the cell.

In summary, understanding the molecular nature and the mechanism of suppression in *ccoA* revertants will be very informative about Cu trafficking and acquisition by *R. capsulatus*. We have already initiated, and are in the process of analyzing some of the exciting preliminary results. Once completed, this ensemble of genetic, molecular and computational analyses will help us to answer some of the intriguing questions such as i) How do these revertants become hypersensitive to Cu? ii) Does another transporter, which has much higher influx rate, replace CcoA or iii) Is it by inactivation of a Cu exporter that leads to Cu accumulation in the cells? Our data already hint at novel mechanisms for Cu trafficking and acquisition in *R. capsulatus* for Cox activity, future investigations of these questions may shed further light on the role of Cu for the assembly and biogenesis of Coxs in other organisms.

CHAPTER 5
SUMMARY AND FUTURE PERSPECTIVES

The biogenesis and maturation of Cox have been an exciting topic for many years mainly because their studies helped us to understand how multisubunit and cofactor bearing membrane proteins are assembled (Barrientos et al. 2009). This is a complicated process due to the fact that metals when exposed as soluble ligands can lead to the formation of reactive oxygen species or can bind to unspecific ligands resulting in nonfunctional proteins (Halliwell et al. 1984).

Studies on Cox maturation was initially focused on the biogenesis of mitochondrial *aa*₃-Cox which is composed of 13 subunits and requires at least 30 assembly components in yeast (Barrientos et al. 2009). In recent years, studies also focused on another class of Cox enzymes, *cbb*₃-type Cox, and the significant progress has been made toward understanding the assembly of this enzyme (Koch et al. 2000; Kulajta et al. 2006). In contrast to mitochondrial *aa*₃-Cox that contains two copper atoms and requires many assembly components, *cbb*₃-type Cox provides a simpler model with single Cu atom and far less number of assembly components to study cofactor insertion into multi-subunit membrane proteins (Koch et al. 2000; Kulajta et al. 2006; Peters et al. 2008). In addition, the availability of the first high resolution 3D structure of *cbb*₃-Cox from *Pseudomonas stutzeri* enables the structural and functional analysis of this enzyme in detail (Buschmann et al. 2010).

Rhodobacter capsulatus provides an excellent model organism to study this process because it can grow under respiratory conditions when the *cbb*₃-Cox is missing

due to the presence of the alternative *bd*-type oxidase that does not require any Cu cofactor (Gray et al. 1994). The second and third subunit of *cbb*₃-Cox are *c*-type cytochromes that are matured by cytochrome *c* maturation (CCM) system which have components different than *cbb*₃-Cox assembly genes and have been studied extensively in various organisms (Kranz et al. 2009; Sanders et al. 2010). The mutants of genes involved in CCM are unable to grow photosynthetically providing a tool to distinguish between the mutations in the CCM genes versus *cbb*₃-Cox biogenesis specific genes (Deshmukh et al. 2000).

Prior to this study, nine proteins were identified as being essential for *cbb*₃-Cox maturation in *R. capsulatus* (Aygün-Sunar et al. 2006; Deshmukh et al. 2003; Koch et al. 2000; Onder et al. 2008). In this study, we focused on determining the additional components and steps of this process especially related to Cu insertion into the first subunit of *cbb*₃-Cox. Our studies led to; i) characterization of a mutant that had a Cu dependent *cbb*₃-Cox phenotype ii) discovery of a novel component involved in this process (CcoA) iii) new insights about the Cu acquisition to the cells for insertion into the CcoN subunit of *cbb*₃-Cox and iv) identification of two Cu unresponsive derivatives of *ccoA* mutant (XJ3 and XJ11) that link CCM and Cox maturation.

What is the role of CcoA in *cbb*₃-Cox biogenesis?

In Chapter 2, we describe complementation analysis of a mutant that had Cu dependent *cbb*₃-Cox phenotype. Excitingly, this complementation revealed a novel

component of *cbb₃*-Cox biogenesis in *R. capsulatus* named CcoA. Amino acid sequence analysis showed that CcoA is highly homologous to MFS type transporters. Knockout of *ccoA* had lower Cu content compared to wild type indicating a defect in Cu acquisition. Interestingly, we frequently obtained bypass suppressors of *ccoA* mutant that were hypersensitive to Cu. In order to understand whether absence of *ccoA* affects presence of any of the *cbb₃*-Cox subunits we investigated the transcript and protein levels of the CcoN subunit. Even though the transcript levels of CcoN changed slightly in *ccoA* mutants, its protein product was missing. Whether the defect in the *cbb₃*-Cox biogenesis is due to low production of the subunits at the co-translational level or increased degradation of the subunits at the post translational level remains to be analyzed. It is important to know if the subunits are made at the wild type levels yet they are degraded due to absence of *ccoA*. Performing pulse-chase experiments in *ccoA* mutant to determine how CcoN subunit is expressed compared to wild type would be informative. CcoA is a novel component of *cbb₃*-Cox biogenesis that has never been identified before in any organisms. In addition, no MFS type transporters have been found to be involved in any type of Cox assembly to date. Here we show for the first time that CcoA is an MFS type transporter involved in *cbb₃*-Cox biogenesis in *R. capsulatus*. Our discovery opens up a whole new perspective for the assembly and biogenesis of multisubunit enzyme complexes. However, the molecular mechanism and functional role of CcoA for *cbb₃*-Cox remains to be seen.

The role of CcoA for CcoN maturation

There are a number of important questions waiting to be answered related to the maturation of CcoN subunit of *cbb*₃-Cox. For example, it is not known whether the heme and Cu cofactors are inserted into CcoN subunit co-translationally or post-translationally and if any order for insertion exists? In addition, whether Cu insertion happens in the cytoplasm or in the periplasm is not fully understood. Previously in *Schizosaccharomyces pombe*, a predicted copper chaperone Cox11 and ribosomes has been found to be associated supporting the co-translational insertion hypothesis. (Khalimonchuk et al. 2005). In contrast, post-translational insertion entails cofactor insertion into CcoN in the folded conformation which would require a channel for insertion and the elaborate cooperation of copper chaperones and transporters. Remarkably, in *R. capsulatus* a fully assembled *cbb*₃-Cox lacking both heme cofactors has been observed providing evidence that this subunit was stable without heme cofactors (Koch et al. 2000).

Activity of most proteins require various metal cofactors for proper functioning yet it is still not fully understood how cells achieve coordinated metal insertion into target apoproteins in a fast, highly specific and controlled manner. In the case of Cu, it was proposed that the different binding specificities of metallochaperones and the different affinities of cuproenzymes for these chaperone-Cu complexes provide specificity. (Banci et al. 2010; Waldron et al. 2009). Most Cu containing enzymes are located in the inner membrane of the bacteria or in the periplasm. It has been suggested that Cu should not be present freely in the cytoplasm and the insertion of Cu should be conducted in the

periplasm to avoid the production of ROS in the cells. However several evidences suggest that Cu can weakly bind to some small ligands or proteins such as metallotheins or chalkophores in the cytoplasm (Gold et al. 2008; Kim et al. 2004). For example, a cytosolic pool of Cu was detected in the yeast mitochondrial matrix (Cobine et al. 2004). In addition, cyanobacteria needs cytoplasmic Cu for insertion into Cox and plastocyanin enzymes located in the thylakoids (Cavet et al. 2003). Molybdenum cofactor biosynthesis in bacteria and archaea also requires the presence of Cu in the cytoplasm (Kuper et al. 2004).

In *R. capsulatus*, several components have been identified to achieve cofactor insertion into *cbb₃*-Cox (Koch et al. 2000). Based on our observations as described in Chapter 2, CcoA is predicted to import Cu⁺² into the cytoplasm (as suggested by the silver-insensitive phenotype of a CcoA mutant) which is then reduced by CcoG to Cu⁺ to provide the substrate for CcoI. The essential role of CcoI for *cbb₃*-Cox assembly suggests that it might transport Cu⁺ from the cytoplasm to the periplasm, which might then be inserted into CcoN (Figure 20). However it is surprising that CcoI mutant is not rescued by exogenous Cu addition, even though Cu is thought to be diffused freely across outer membrane into the periplasm (Koch et al. 2000). This could be explained by the possibility that either Cu⁺ transported by CcoI is inserted into CcoN during its transport across membrane, or that it is delivered upon transport to specific periplasmic Cu chaperones (perhaps SenC or PCu_AC), which might then deliver it to CcoN (Figure 20). This model would rationalize the cytoplasmic origin of Cu, and suggests that in the absence of CcoI, its loading onto the specific chaperone might be inefficient for the

maturation of CcoN. However more studies are needed to investigate the nature of this Cu insertion model.

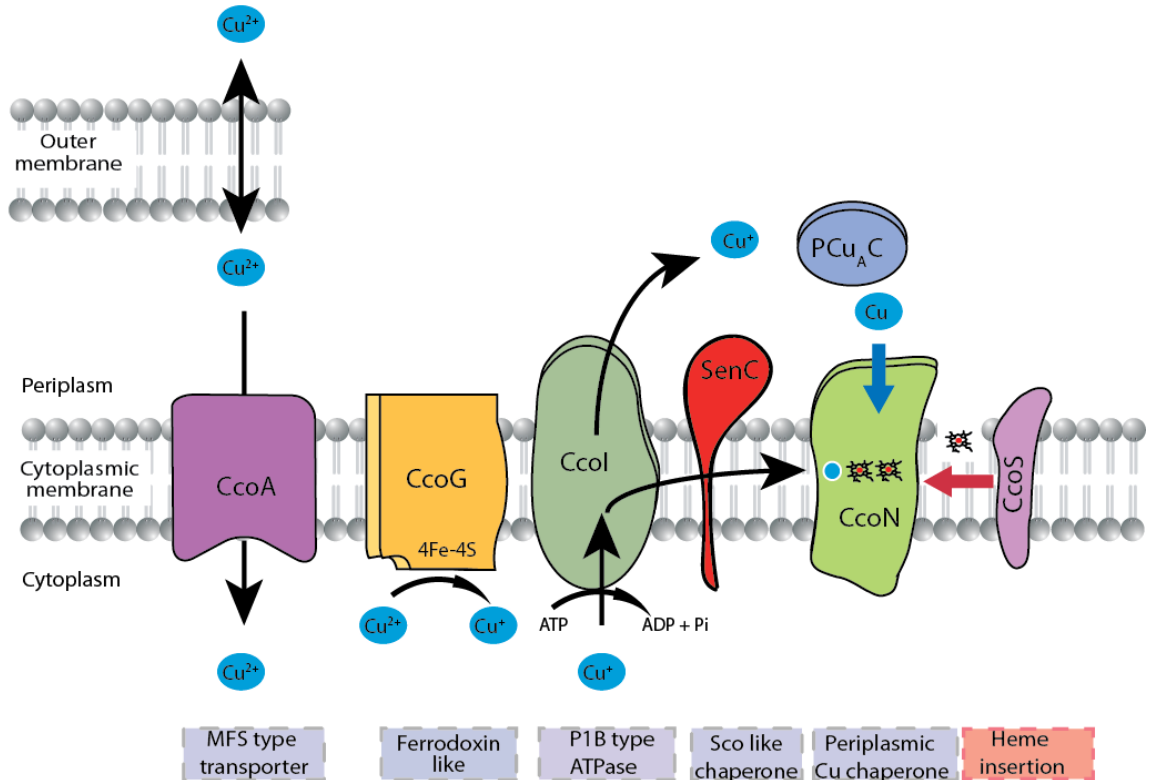


Figure 20: A hypothetical model of Cu and heme *b* insertion into the CcoN subunit of *cbb*₃-Cox in *Rhodobacter capsulatus*. Cu²⁺ is thought to diffuse across outer membrane into the periplasm, and then imported by CcoA into the cytoplasm. CcoG reduces Cu²⁺ to Cu⁺ before its transport by CcoI. SenC and PCu_AC are chaperones that are required for the biogenesis. They might deliver Cu from CcoI to CcoN as described in the text. CcoS could be involved in the heme insertion.

Recently *aa₃*-Cox enzyme from *Paracoccus denitrificans* was synthesized by cell-free protein synthesis method by addition of *E. coli* cell extract which contained u-shaped membranes, CuSO₄ solution, heme *a* and the coupled transcription/translation system with the template DNA that contained the structural genes (Katayama et al. 2010). Although these results demonstrate that the cell-free synthesis can be accomplished, in vivo synthesis of Cox demands faster and more controlled reactions. In addition in vitro reaction is not limited by the transport and availability of heme and CuSO₄ cofactors since the cell-free synthesis reaction includes both cofactors. However this system would be very useful to determine which components of biogenesis and assembly are strictly required for the assembly of Cox. It would be interesting to see which assembly proteins present in the cell extracts are used and how the amounts of synthesized protein change by adding or omitting known assembly factors from this mixture.

What is the role of CcoA in the biogenesis of other Cu containing enzymes?

In this study, we investigated the role of CcoA for the biogenesis of two Cu containing enzymes; *cbb₃*-Cox-a multisubunit membrane protein and multicopper oxidase (laccase)- a periplasmic soluble protein (Wiethaus et al. 2006). Although the absence of CcoA affected the *cbb₃*-Cox biogenesis severely, multicopper oxidase (CutO) activity for Cu detoxification was still present (Chapter 2). However these two proteins are localized to the different parts of the cell and in addition, CutO protein has TAT signal that would indicate that it is secreted to the periplasm after folding (Wiethaus et al. 2006). It would be remarkable to see if CcoA also affects the biogenesis of Cu containing enzymes other

than *cbb*₃-Cox biogenesis? *Rhodobacter sphaeroides* which is phylogenetically similar to *R. capsulatus* possesses a Cu/Zn containing superoxide dismutase (SOD). SOD is the only other Cu containing enzyme in mitochondria besides Cox and it was proposed to accept Cu from either inner membrane space or the cytosol (Banci et al. 2010). It is not obvious how bacterial SOD gets Cu, but if it is similar to mitochondria, one would expect Cu insertion to occur in periplasm. It would be interesting to see if SOD is also synthesized in *R. capsulatus* when transferred and if absence of CcoA has any effect on its synthesis and maturation. In addition, in *R. gelatinosus* CcoI homolog CtpA was shown to be also linked to the biogenesis of another Cu containing enzyme, nitrous oxide reductase (NosZ) (Hassani et al. 2010). It remains to be seen if similar link exists between NosZ, CcoI and CcoA in *R. capsulatus*. Finally, NADH(II) dehydrogenase that is proposed to contain Cu in its structure will be another candidate for our future studies.

CcoA homologs are present in most organisms that contain *cbb*₃-Cox and display high sequence homology (Figure 21). Investigating the functions of CcoA homologs in other *cbb*₃-Cox containing organisms such as *R. sphaeroides* or *B. japonicum* would be invaluable to understand its function at the mechanistic level. Since both of these organisms also possess other types of Cox enzymes (aa₃-Cox in *R. sphaeroides* (Garcia-Horsman et al. 1994), 4 Cox enzymes in *B. japonicum*, aa₃-Cox being the most predominant one (Thony-Meyer et al. 1994)), they provide suitable model systems to investigate the role of CcoA for the assembly of different Cox enzymes.

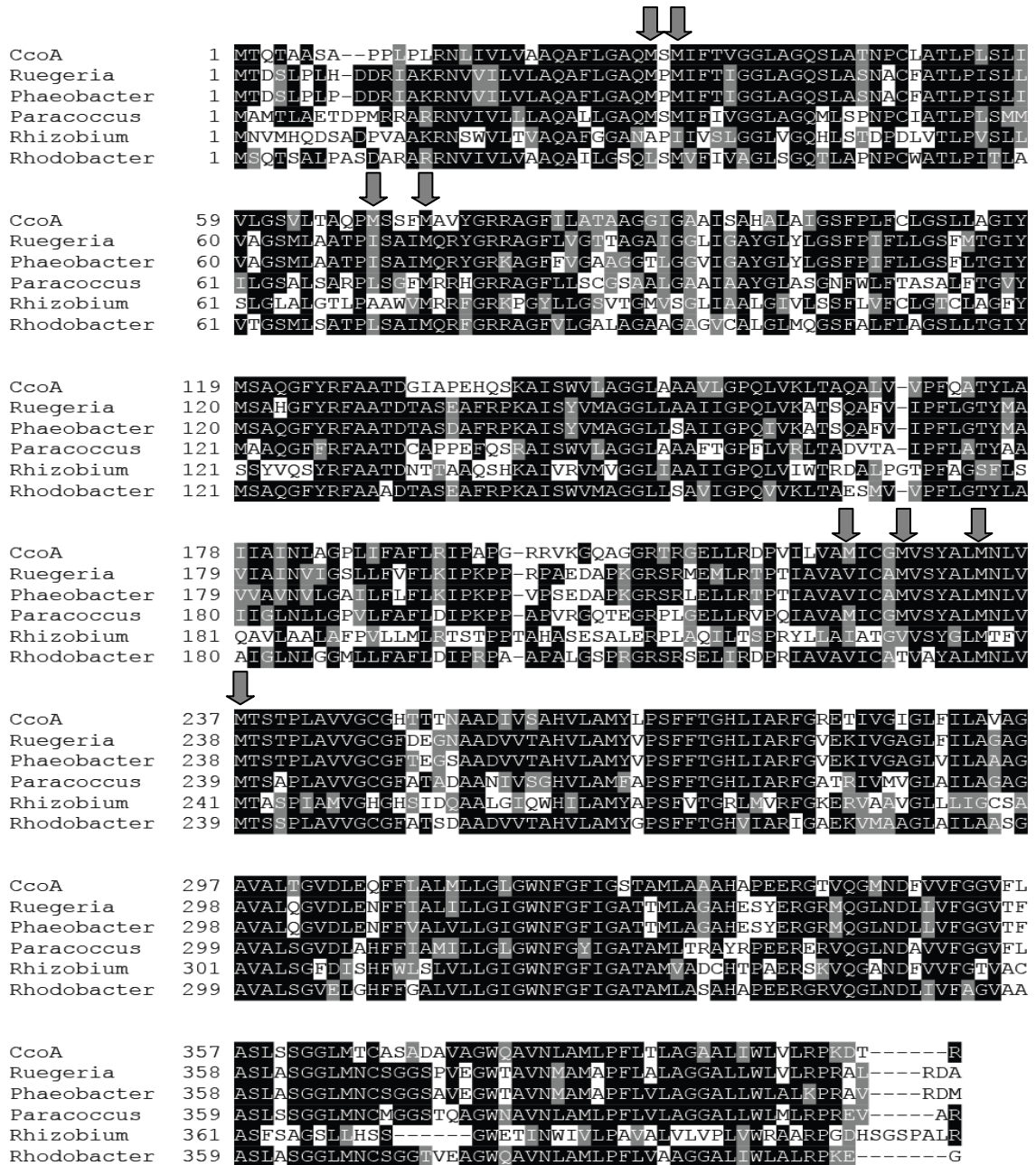


Figure 21: Multiple sequence alignment of amino acid sequences from five species reveals conservation of CooA.

Figure 21: Multiple sequence alignment of amino acid sequences from five species reveals conservation of CcoA. Amino acid sequences homologous to *R. capsulatus* CcoA (designated as CcoA) from *Ruegeria* species, *Phaeobacter gallaeciensis*, *Paracoccus denitrificans*, *Bradyrhizobium japonicum* and *Rhodobacter sphaeroides* were identified via BLAST searches and multiple sequence alignment was created by using ClustalW. Methionine residues of Met motifs (MX_XM) are shown under the arrows. Black and gray boxes correspond to identical and similar amino acid residues, respectively.

Furthermore, CcoA homologs in other well studied bacteria such as *E. coli* and yeast *S. cerevisiae* might be useful to explore if MFS type transporters constitute a conserved group of Cu transporters among bacteria and eukaryotes. Scanning the genome of these model organisms as well as others for the presence of MX_xM motif containing MFSs might help us to identify CcoA homologs. Once identified, plethora of information available in these model systems when combined with our findings might provide novel insights for Cu trafficking into multi-subunit proteins.

What is the cargo of CcoA?

The cargo of CcoA has not been determined yet. Although MFS type transporters are mostly known to transport sugars and drugs, our observations suggest that CcoA might be involved in metal transport, and more specifically in Cu transport (Pao et al. 1998). In *ccoA* mutants, Cu content is less than wild type levels as measured by ICP-DRC-MS. The bypass suppressors of *ccoA* mutant accumulate Cu and are hypersensitive to Cu. Remarkably, as we analyze our results, another MFS type transporter called Mfc1 was identified from *Schizosaccharomyces pombe* that was shown to transport Cu as its substrate (Beaudoin et al. 2011). Although Mfc1 was not involved in any Cox biogenesis, the authors were able to show that MFS type transporters could transport Cu. Based on the similarity between CcoA and Mfc1, and supported by our observations, we propose that CcoA is involved in Cu transport. The next challenge is to confirm this role by using radioactive Cu to measure Cu accumulation in wild type and CcoA deficient cells. Radioactive ⁶⁴Cu is available to measure accumulation of this metal in the cells. This

method has been frequently used to demonstrate the Cu transporting activities of P1B-type ATPases (such as for CopB from *Enterococcus hirae*, CopA1 and CopA2 from *Pseudomonas aeruginosa* (Gonzalez-Guerrero et al. 2010; Solioz et al. 2010)). The predicted direction of Cu transport by CcoA is towards the cytoplasm based on silver insensitivity phenotype. This radioactive Cu based assays might also assist us to support or refine this prediction to determine the direction of Cu transport.

It is also essential to test how CcoA expression changes inside the cell in response the Cu availability. One hypothesis is that if CcoA transports Cu, we expect to see difference in its expression in response to Cu. If it is a Cu importer, we would expect increased expression under the Cu limiting conditions and decreased expression in the presence of exogenous Cu. On the other hand, if it is an exporter, we would expect to see increased expression in response to increasing exogenous Cu amounts in the media.

Another possibility for the substrate for CcoA is a complex of Cu bound to other ligand molecules as seen with some metals (such as iron by siderophores). We discussed some of these molecules below. The methanobactins have been shown to be involved in Cu import to the cells (Balasubramanian et al. 2011). However the mechanism of the uptake or secretion of these molecules is not known. In addition, until now the presence of these molecules are only detected in methanotrophs (Balasubramanian et al. 2008). Recently the uptake of Cu-methanobactin complex has been demonstrated in a methanotroph species *Methylosinus trichosporium* and it was shown to occur via an

active transport dependent pathway by isotopic and fluorescent Cu labeling experiments (Balasubramanian et al. 2011). Since, it would be interesting to see if CcoA has similar role we have already initiated an assay to detect the methanobactins in *R. capsulatus* and further studies are in progress. This ligand bound to Cu could also be the cargo of CcoA because MFS type transporters can also transport larger molecules besides metals and there are examples of MFS proteins that can deliver siderophores, which are structurally similar to methanobactins (Franza et al. 2005; Furrer et al. 2002). Below are the known Cu binding ligands that have been shown to have a role in Cu acquisition. However the presence or absence of these molecules in *R. capsulatus* and their role in Cu acquisition remains to be identified.

Cu binding Ligands

Chalkophores: Methanotrophs requires very high amounts of Cu to regulate the production of methane monooxygenase enzymes (MMOs) to oxidize methane to methanol. One type of MMOs located in the cytoplasmic membrane, particulate MMO, is believed to have an active copper site in contrast to soluble MMO. Recently in methanotrophs a ligand that binds to Cu to meet the high Cu demands of these organisms have been detected and purified (Kim et al. 2004). The crystal structure of copper loaded ligand revealed a molecule with both amino acidic (Gly-Ser-Cys-Tyr) and non amino acidic functional groups (a pyrrolidine and two thionylimidazolate groups) that contained two cysteine residues forming a disulfide bond (Kim et al. 2004). These ligands were initially named chalkophores (chalko-Greek for Cu and sidero is Greek for Iron), then the name

was also accepted as methanobactin molecules (Balasubramanian et al. 2008). The biosynthesis pathway and the transporters involved in the trafficking of these molecules are yet to be determined. It is predicted that a nonribosomal peptide synthetase (NRPS) might be involved in the biosynthesis just like the case in siderophores (Lautru et al. 2004; Naismith et al. 2004). It would be exciting to identify similar molecules in organisms other than methanotrophs such as *R. capsulatus*. Luckily, there are assays available to screen these molecules for future investigations. (Semrau et al. 2010). Recently, the methanotroph *Methylosinus trichosporium* was shown to import both the Cu-Mb complex by the help of an active membrane transport process, whereas this transport could not be achieved in *E. coli* (Balasubramanian et al. 2011) and it remains to be seen if other organisms have similar mechanisms.

Metallothioneins: Metallothioneins are low molecular weight proteins containing large number of cysteine residues that are in the form of the CXXC or CXC motif. Although they are ubiquitously found in all kingdoms, only few have been identified in bacteria. MymT is a Cu binding metallothionein that was recently identified from *Mycobacterium tuberculosis* (MTb) (Festa et al. 2011; Gold et al. 2008). This molecule was shown to bind six Cu atoms and to be required for the survival of *M. tuberculosis* in the host. This observation supports the hypothesis that the immune system uses Cu as a defense mechanism to kill the pathogens which leads pathogenic bacteria such as MTb to develop elaborate systems to export excess Cu which will be discussed later in this chapter.

In addition to the predicted Cu transporter CcoA, *R. capsulatus* contains other type of transporters involved in Cu trafficking. One of the *cbb₃*-Cox assembly genes, CcoI protein in *R. capsulatus* is homologous to P1B- type ATPases, but it has never been shown biochemically to transport Cu (Koch et al. 2000). However its homolog from *P. aeruginosa* (named CopA2), has been demonstrated to export Cu (Raimunda et al. 2011). Interestingly this transport activity was at a slower rate than the other P1B-type ATPase present in this organism (CopA1) (Raimunda et al. 2011). This implied that Cu insertion into Cox needs more controlled trafficking of Cu in contrast to the activities of other P1B-type ATPases that function to quickly detoxify the cells from the extra Cu. Hence investigating and confirming the Cu transport role of CcoI in *R. capsulatus* is essential and will shed light on how CcoA and CcoI coordinate Cu trafficking for various Cu enzymes. Furthermore, determination of the Cu content in double mutants of CcoA, CcoI and CopA1 homolog by using ICP-MS might help us to understand epistasis, competition or hierarchy between these proteins for trafficking of Cu.

Copper transport in many organisms such as yeast has been shown to be achieved by multiple transporters that have different affinities for Cu (Banci et al. 2010; Banci et al. 2010). For example, in yeast, copper enters cells via Ctr1/3 transporters that are high affinity or Fet4/Smf1 low affinity transporters (Dancis et al. 1994; Hassett et al. 2000). Despite being involved in the same process these proteins belong to different family of transporters. Similarly, it is also possible that *R. capsulatus* employs multiple Cu transporters such as CcoA or CcoI to handle copper homeostasis under dynamically

changing environmental conditions. Presence of Cu in *ccoA* mutants indicate that additional pathways for acquisition of Cu exist and remain to be identified.

In *R. capsulatus* genome, CcoA is located next to a MerR type transcriptional activator and a P1B-type ATPase transporter both of which are associated with metal related functions. Previously in *Mycobacterium tuberculosis*, a Cu regulon was identified which was composed of a transcriptional activator (CsoR) and a P1B-type ATPase (Liu et al. 2007). Interestingly the homologs of CsoR in other organisms such as *Staphylococcus aureus* and other mycobacterial species were found to be always adjacent to a MFS type transporter (Liu et al. 2007). Cu sensitivity and *cbb₃*-Cox phenotype of the mutants of P1B-type ATPase (RCC02190) and MerR type transcriptional activator (RCC02191) located next to CcoA was not different than the wild type. However, we cannot completely rule out the possibility that they might have roles in Cu homeostasis. Further studies are needed especially to investigate their Cu related phenotypes and intracellular Cu levels in the absence of CcoA or CcoI when grown under Cu-depleted versus Cu-repleted conditions.

Copper homeostasis in health and disease

In addition to Cu being a cofactor for many proteins, there is a growing body of evidence that Cu has also roles in deadly diseases. First, Cu deficiencies and defects in Cu homeostasis are responsible for human diseases such as the Menkes and Wilson's in which Cu accumulation or deficiency leads to severe cellular abnormalities. Second, Cu

upregulation has been implicated in Alzheimer's disease (Adlard et al. 2006), one of the most common neurodegenerative disease and resistance of cancer cells to a cancer drug cisplatin (Leonhardt et al. 2009). Third, an important protein for the development of prion diseases such as scrapie in sheep, bovine spongiform encephalopathy in cattle, and Creutzfeldt-Jacob disease in humans called PrP^c was found to bind Cu and have role in Cu homeostasis. In the absence of PrP^c increased oxidative stress and sensitivity to Cu was observed (Brown et al. 1997; Kramer et al. 2001; Stockel et al. 1998; Viles et al. 1999). Even though our understanding of Cu homeostasis associated diseases has progressed tremendously in recent years, different mechanisms to maintain this homeostasis in response to various perturbations are not fully understood. In this study we have provided evidences for some of these novel mechanisms. Future investigations are vital to shed more light on mechanistic details of these processes.

Although changes in copper levels can cause deadly diseases in humans, there are experimental evidences that the immune system uses copper as a weapon to kill microbes during a bacterial infection. This has been shown initially by the observation that ATP7A transporter activity provided a bacteriocidal protection against *E. coli* in human macrophages by increasing the copper levels during the infection (White et al. 2009). Even though the mechanism was not clear it was proposed that the production of hydroxyl radicals induced by Cu via Fenton reaction could be the reason (White et al. 2009). This observation was also confirmed by studies that showed increased activity of Cu exporters in the infectious bacteria during infection such as *Pseudomonas aeruginosa* (Schwan et al. 2005), *Pseudomonas fluorescens* during plant colonization (Zhang et al.

2007). Recently a copper-responsive regulon was identified in *Mycobacterium tuberculosis* and the crucial role of Cu resistance mechanisms in this organism was suggested to be required for the survival in its host (Festa et al. 2011; Wolschendorf et al. 2011). Therefore, understanding Cu transport and homeostasis in model systems such as *R. capsulatus* provides invaluable information to extrapolate to pathogenic species for development of effective treatments for the deadly infectious diseases.

R. capsulatus is a simpler model system for studying Cu trafficking and acquisition in contrast to eukaryotic cells. However human cells and higher plants also provide other advantages to study the systemic regulation of Cu acquisition and distribution between different cellular compartments, different cell types and regulatory pathways. Using multicellular model systems help us to determine how cells decide and prioritize Cu delivery among different cellular compartments and components under Cu limiting conditions. In addition, we can tackle questions such as how these cells balance the Cu levels between different processes such as insertion into proteins as cofactors versus use in the immune system against infections

Is there an alternative Cu trafficking pathway in *R. capsulatus*?

In Chapter 3, we wanted to determine the alternative pathways of Cu acquisition in the absence of CcoA. However characterization of two Cu-unresponsive *ccoA* mutants (XJ3 and XJ11) revealed mutations in cytochrome *c* maturation (CCM) genes. Surprisingly these mutants were still able to make some *c*-type cytochromes that enabled

them to grow under photosynthetic conditions. XJ3 mutant was complemented by *ccmA* gene and XJ11 mutant was complemented fully by a plasmid carrying *ccmHIF* genes and partially by *ccmE* gene. Since CCM system requires reducing equivalents and heme translocation, further characterization of these mutants might uncover previously not identified links between Cu transport and CCM process in *R. capsulatus*. Especially that heme translocation is not fully understood, it is exciting to see complementation of Cu-unresponsive *ccoA* mutants by heme pathway related proteins. Obviously, future investigations are necessary to characterize these mutants.

One of the remaining questions is the presence of alternative Cu transport pathways that are still functional in the absence of CcoA and only physiologically relevant in the presence of exogenous Cu. In *R. capsulatus* there are 11 other MFSs in addition to CcoA, thus whether one of these could be the low affinity Cu transporter in the absence of CcoA remains to be tested. Our genome wide computational search for the presence of Mets motifs in these MFSs identified four candidates that contain several copies of the motif. Further characterization of these MFS proteins might help to identify alternative Cu transport pathways. However, analogous to yeast, this alternative pathway could contain different type of transporters than only MFS family. A possibility is the P1B-type ATPase family. In *R. capsulatus* there are 5 P1B-type ATPases. So far, we isolated mutants knocking out two of them (CcoI and RCC02190) and we are in the process of constructing the mutants for the remaining three genes. It will be interesting to knockout these transporters in a *ccoA* mutant background and check if the *ccb₃*-Cox defective phenotype can be suppressed in these double mutants by Cu addition.

Prior to this study, numerous other mutants were characterized as having a Cu dependent *cbb*₃-Cox activity such as the mutants of *dsbA* and *senC* (Buggy et al. 1995; Deshmukh et al. 2003). The role of the exogenous Cu supplementation in suppressing the observed phenotypes in these strains is not known. Defining this role is complicated by the fact that Cu might act as a cofactor of *cbb*₃-Cox but it also has an oxidative effect. If we can determine the alternative pathway of Cu acquisition in the absence of CcoA, we can knockout *dsbA* and *senC* in that background and investigate how these mutants respond to Cu supplementation. If their *cbb*₃-Cox phenotype responds to Cu supplementation, this would indicate an oxidative role for Cu in these strains.

Is there a link between cytochrome *c* maturation genes and Cu?

It has been observed that in some organisms mutations in cytochrome *c* maturation genes result in pleiotropic phenotypes that could not be explained by only cytochrome *c* deficiency (Cianciotto et al. 2005). This suggests that CCM genes might also have roles in other cellular processes. Remarkably, most of these phenotypes were associated with iron acquisition or reduced siderophore production (Cianciotto et al. 2005). In addition, at least in one case copper sensitivity was observed in *ccmF* and *ccmI* mutants of *Pseudomonas fluorescens* (Yang et al. 1996). Previously we have shown that *c*-type cytochromes CcoO and CcoP are expressed in CcoA mutants only when supplemented with exogenous Cu (Chapter 3). Although this could be related to the degradation of these subunits in the absence of CcoN caused by the Cu deficiency, we

cannot rule out the possibility that CcoA might also have a regulatory role for CCM expression. Exploring this previously unknown link between two important cellular processes will bring up exciting research possibilities.

In summary, *cbb₃*-Cox biogenesis and assembly has traditionally attracted much attention to understand the cofactor insertion into membrane proteins and multistep pathways of multisubunit protein complexes. In this study, by using *R. capsulatus* as model system we have tackled functional and structural aspects of Cu homeostasis, *cbb₃*-Cox biogenesis, and crosstalk between them by using novel molecular, genetic and biochemical tools. We have identified and characterized novel proteins related to Cu trafficking and *cbb₃*-Cox. We have also practiced biochemical analyses that can be applied to study other processes and model systems. Now we stand at the point that knowledge and tools produced from this study can be used to further explore exciting questions in this field. One of the most important questions of *cbb₃*-Cox biogenesis field will be the mechanism of the insertion of Cu and heme cofactors, including the trafficking of these cofactors in and out of the cell and assembly steps after the subunits are matured. Identifying new players in the maturation and assembly of this multisubunit membrane protein complex will be an active research field in the future and results presented in this study will be important landmarks.

APPENDIX I

Strain or plasmid	Description	Phenotype	Reference
<i>Strains</i>			
<i>E. coli</i>			
HB101	F ⁻ Δ(<i>gpt-proA</i>)62 <i>leuB6 supE44 ara-14 galK2 lacY 1</i> Δ (<i>mcrC-mrr rpsL20</i> (Str ^R) <i>xyl-5 mtl-1recA13</i>)	Str ^r	(Sambrook et al. 2001)
XL1-Blue	F'::Tn10 <i>proA⁺B⁺ lacIq</i> Δ(<i>lacZ</i>)M15/ <i>recA1 endA1</i> <i>gyrA96</i> (NaI ^R) <i>thi hsdR17</i> (<i>r_K⁻m_K⁺</i>) <i>supE44 relA1 lac</i>	Amp ^r	Stratagene
JM109	e14-(McrA-) <i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (<i>r_K-K+</i>) <i>supE44 relA1</i> Δ(<i>lac-proAB</i>) [F' <i>traD36 proAB</i> <i>lacIqZΔM15</i>]		Stratagene
BW29427	Donor for Tn5-RL27 delivery vector	DAP ^r	K.A. Datsenko and B.L. Wanner, unpublished
<i>R. capsulatus</i>			
MT1131	<i>crtD121</i> Rif ^r	Wild Type (NAD ^I)	(Scolnik et al.

Strain or plasmid	Description	Phenotype	Reference
			1980)
SB1003		Wild Type (NAD ⁺)	(Yen et al. 1976)
Y262		GTA overproducer	(Yen et al. 1979)
GK1	<i>ccoA1</i>	NAD ⁻ on MPYE NAD ^{slow} on MedA	(Koch et al. 1998)
SE8	$\Delta(ccoA(or RCC02192)::spe)$	Spe ^r , NAD ⁻ on MPYE NAD ^{slow} on MedA	This work
HY70	<i>ccoA70</i>	like GK1	This work
SE15	$\Delta(cutO::kan)$	Cu ^s	This work
SE16	$\Delta(cutO::kan) \Delta(ccoA::spe)$	Kan ^r , Spe ^r , Cu ^s	This work
SE4	$\Delta(RCC02190::kan)$	Kan ^r	This work
SE5	$\Delta(RCC02191::spe)$	Spe ^r	This work
SE6	$\Delta(RCC02190-RCC02191::spe)$	Spe ^r	This work
SE8R1-R5	by-pass suppressors of $\Delta(ccoA::spe)$	Spe ^r , NAD ⁺ , Cu ^s	This work

Strain or plasmid	Description	Phenotype	Reference
XJ3	<i>ccmA*</i> $\Delta(ccoA::spe)$	Spe ^r , NAD ^I on MPYE, MedA	This work
XJ6	<i>ccmA*</i> $\Delta(ccoA::spe)$	Spe ^r , NAD ^I on MPYE, MedA	This work
XJ11	<i>ccmHIF*</i> , $\Delta(ccoA::spe)$	Spe ^r , NAD ^I on MPYE, MedA	This work
SE17	$\Delta(ccmA::kan)$ $\Delta(ccoA::spe)$	Kan ^r , Spe ^r , NAD ^I , Ps ⁻	This work
SE18	$\Delta(ccmB::kan)$ $\Delta(ccoA::spe)$	Kan ^r , Spe ^r , NAD ^I , Ps ⁻	This work
SE19	$\Delta(ccmAccmB::kan)$ $\Delta(ccoA::spe)$	Kan ^r , Spe ^r , NAD ^I , Ps ⁻	This work
SE20	$\Delta(ccmA::kan)$	Kan ^r , NAD ^I , Ps ⁻	This work
SE21	$\Delta(ccmB::kan)$	Kan ^r , NAD ^I , Ps ⁻	This work
SE22	$\Delta(ccmAccmB::kan)$	Kan ^r , NAD ^I , Ps ⁻	This work

Strain or plasmid	Description	Phenotype	Reference
<i>Plasmids</i>			
pRK2013	Conjugation helper	Kan ^r ,	(Ditta et al. 1985)
pRK404	Broad-host-range vector	Tet ^r	(Ditta et al. 1985)
167 pRK415	Broad-host-range vector	Tet ^r	(Ditta et al. 1985)
pHP45Ω-Spc	Ω <i>spe</i> in pHP45 vector	Spe ^r	(Prentki et al. 1984)
pHP45Ω-Kan	Ω <i>kan</i> in pHP45 vector	Kan ^r	(Prentki et al. 1984)
pBluescript II KS+	Cloning vector	Amp ^r	Stratagene
pOX15	<i>ccoNOQP</i> in pRK404	Tet ^r	(Koch et al.

Strain or plasmid	Description	Phenotype	Reference
pCW25	<i>ccoNOQP</i> , <i>ccoGHIS</i> in pRK415	Tet ^r	1998) (Koch et al. 2000)
pSE1	8.0 kb chromosomal EcoRI fragment on pRK415	Tet ^r	This work
pSE2	4.8 kb chromosomal HindIII fragment on pRK415	Tet ^r	This work
pSE3	2.8 kb XbaI-KpnI fragment with <i>ccoA</i> on pRK415	Tet ^r	This work
pSE201	702 bp between ClaI-BstBI sites replaced by <i>kan</i>	Kan ^r , Tet ^r	This work
pSE202	247 bp between BlnI-AsiSI sites replaced by <i>spe</i>	Spe ^r , Tet ^r	This work
pSE203	1058 bp between ClaI-AsiSI sites replaced by <i>spe</i>	Spe ^r , Tet ^r	This work
pSE204	3358 bp between HindIII-AsiSI deleted.	Tet ^r	This work
pBS-pSE3	2.8 kb XbaI-KpnI fragment with <i>ccoA</i> on pBluescript II KS+	Amp ^r	This work
pBS-pSE5	256 bp between BamHI-BglII sites in <i>ccoA</i> replaced by <i>spe</i>	Amp ^r , Kan ^r	This work

Strain or plasmid	Description	Phenotype	Reference
pSE5	2.8 kb XbaI-KpnI fragment with $\Delta(ccoA::spe)$ in pRK415	Spe ^r , Tet ^r	This work
pSE6	256 bp between BamHI-BglII sites deleted	Tet ^r	This work
pRK-GK1	2.8 kb chromosomal PCR product containing <i>ccoA1</i> from GK1 in pRK415 between XbaI and KpnI sites	Tet ^r	This work
pXCA601	<i>lacZ</i> -based promoter cloning vector	Tet ^r	(Adams et al. 1989)
pXG1	<i>ccoN::lacZ</i>	Tet ^r	(Koch et al. 1998)
pHY601	<i>cutO</i> in pBluescript II KS+	Amp ^r	This work
pHY602	800 bp between EcoRI sites of <i>cutO</i> replaced by <i>kan</i>	Kan ^r , Amp ^r	This work
pHY603	KpnI-XbaI fragment with $\Delta(cutO::kan)$ in pRK415	Kan ^r , Tet ^r	This work
pHY606	<i>cutO</i> in pRK415	Tet ^r	This work
pRL27	Tn5-RL27 (Kan ^r - <i>oriR6</i> K) delivery vector	Kan ^r	(Larsen et al. 2002)

Strain or plasmid	Description	Phenotype	Reference
pMRC	<i>olsA</i> and <i>olsB</i> in pLAFR1	Tet ^r	(Aygün-Sunar et al. 2006)
pDsbA ^{WT}	<i>dsbA</i> in pCHB500	Tet ^r	(Deshmukh et al. 2003)
pDsbB ^{WT}	<i>dsbB</i> in pRK415	Tet ^r	(Deshmukh et al. 2003)
170 pSenCFlagC	<i>senC</i> in pBBR1mcs-2	Kan ^r	(Swem et al. 2005)
pMA117	Ω <i>kan</i> in pBluescript II KS+ vector	Kan ^r	(Daldal et al. 1986)
pSE10	7.6 kb chromosomal BamHI fragment on pRK415	Tet ^r	This work
pSE11	4912 bp between XbaI-BstBI deleted	Tet ^r	This work
pSE12	2714 bp between BstBI-KpnI deleted	Tet ^r	This work

Strain or plasmid	Description	Phenotype	Reference
pSE13	BstBI site filled in and ligated	Tet ^r	This work
pSE14	2389 bp XbaI and KpnI fragment that contains <i>ccmA</i> and <i>ccmB</i> cloned into XbaI-KpnI of pBluescript II KS+	Amp ^r	This work
pSE15	Kan ^r cassette inserted at the BstEII site of <i>ccmA</i>	Amp ^r Kan ^r	This work
pSE16	Kan ^r cassette inserted at the XhoI site of <i>ccmB</i>	Amp ^r Kan ^r	This work
pSE17	746 bp between BstEII and XhoI sites replaced by Kan ^r	Amp ^r Kan ^r	This work
pSE21	KpnI-XbaI fragment with $\Delta(ccmA::kan)$ in pRK415	Kan ^r , Tet ^r	This work
pSE22	KpnI-XbaI fragment with $\Delta(ccmB::kan)$ in pRK415	Kan ^r Tet ^r	This work
pSE23	KpnI-XbaI fragment with $\Delta(ccmAccmB::kan)$ in pRK415	Kan ^r , Tet ^r	This work
pXJ01, pXJ02	2.3 kb chromosomal BamHI fragment containing <i>ccmE</i> in pRK415	Tet ^r	This work
pMD1	2.3 kb chromosomal BamHI fragment containing <i>ccmE</i> in pRK404	Tet ^r	(Deshmukh et al. 2000)

Strain or plasmid	Description	Phenotype	Reference
pMD21	pMD1 with H123A mutation in <i>ccmE</i>	Tet ^r	(Deshmukh et al. 2000)
pMD6	PvuII fragment of pMD1 containing only <i>ccmE</i> in pRK415	Tet ^r	(Deshmukh et al. 2000)
pCS1582	Strep-CcmF ⁺ , CcmH ⁺ , CcmI-FLAG ⁺	Tet ^r	(Sanders et al. 2008)
172 p2hel-404	pRK404 derivative with <i>ccmABCDG</i>	Amp ^r Tet ^r	(Lang et al. 1996)

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