Copper starvation-inducible protein for cytochrome oxidase biogenesis in Bradyrhizobium japonicum

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COPPER STARVATION-INDUCIBLE PROTEIN FOR CYTOCHROME OXIDASE BIOGENESIS IN BRADYRHIZOBIUM JAPONICUM

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ETH ZURICH
for the degree of
Doctor of Sciences

presented by
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Table of contents

Summary 1

Riassunto 3

1 Introduction 5
   1.1 Copper acquisition and trafficking in bacteria 6
      1.1.1 Background 6
      1.1.2 Periplasmic copper uptake in Gram-negative bacteria 17
      1.1.3 Proteins for copper insertion into cuproenzymes 23
      1.1.4 Copper import and trafficking in the cytoplasm 34
      1.1.5 Concluding remarks 41
   1.2 Copper sorting in Bradyrhizobium japonicum 42
   1.3 Aim of this work 47

2 Copper starvation-inducible protein for cytochrome oxidase biogenesis in Bradyrhizobium japonicum 49
   2.1 Summary 51
   2.2 Introduction 52
   2.3 Experimental procedures 54
      2.3.1 Bacterial strains, media, and growth conditions 54
      2.3.2 Mutant constructions 54
      2.3.3 Complementation of ΔpcuABCDE 57
      2.3.4 Determination of Cu atoms in copper-free BVM 57
      2.3.5 RNA isolation and cDNA synthesis 58
      2.3.6 Quantitative real-time polymerase chain reaction (qRT-PCR) 58
      2.3.7 Primer extension 59
      2.3.8 Microarrays 59
      2.3.9 Plant growth 59
      2.3.10 Bacteroid isolation 59
      2.3.11 Nitrate and nitrite detection in the growth medium 60
      2.3.12 Determination of cytochrome c oxidase activity in membrane fractions 60
      2.3.13 Immunological techniques 60
      2.3.14 Purification of PcuC and its derivatives 63
      2.3.15 Test for Cu(I) binding to PcuC 64
      2.3.16 Bioinformatic analyses 64
   2.4 Results 65
      2.4.1 Comparative transcription profile of cells grown at different copper concentrations 65
      2.4.2 The pcuABCDE genes are transcribed as an operon 67
      2.4.3 Predicted proteins encoded by the pcuABCDE operon suggest a role in copper trafficking 68
      2.4.4 The ΔpcuABCDE strains have a pleiotropic phenotype 69
      2.4.5 pcuC is the only essential gene of the pcuABCDE operon for all tested functions 72
      2.4.6 B. japonicum possesses a second pcuC-like gene (blr7088) without an obvious function 73
      2.4.7 PcuC is a Cu(I)-binding PCuA-like protein 74
      2.4.8 PcuC and ScoI are required for the symbiotically essential cbb3-type cytochrome oxidase 76
   2.5 Discussion 77
3 In vivo effect of PcUC H79A and H113A/M115A substitutions

3.1 Summary 82
3.2 Introduction 82
3.3 Experimental procedures 83
  3.3.1 Bacterial strains and media 83
  3.3.2 Construction of the strains 83
  3.3.3 Phenotypization of the strains 84
3.4 Results 85
  3.4.1 H113 and M115 of PcUC are required in copper starvation 85
  3.4.2 Nitrogenase activity of pcuC[H113A/M115A] is strongly impaired 85
  3.4.3 The aa3 oxidase is defective in the pcuC[H113A/M115A] strain 86
3.5 Discussion 87

4 Future perspectives 89
4.1 A revised working model for cytochrome oxidase biogenesis 90
  4.1.1 The newly emerging role of Sco in proteobacteria 90
  4.1.2 PCuAC collaborates with Sco 91
4.2 Validating the model 92
4.3 Other possible copper trafficking proteins 93

References 95

Curriculum Vitae and Publications 117

Acknowledgments 119
Copper is both a trace and a toxic element. The Cu\(^{2+}/Cu^{+}\) couple potential is the highest among the biometals, making copper usage by life convenient for the extraction of electrons from molecules with already high redox potentials. For this chemical property copper enzymes are involved in electron transfer and redox reactions, for example in aerobic or anaerobic respiratory processes. For the same property, and because it is the most effective metal in binding biomolecules, copper is able to misplace other metal ions and/or catalyze the production of reactive oxygen species. In bacteria, this toxicity is a major menace both in the cytoplasm and in the periplasm. To deal with it bacteria compartmentalize copper enzymes outside the cytoplasm, where the most sensitive targets are located, use efficient efflux systems, and use metallochaperones to tightly control its transport to copper enzymes. In copper starvation conditions it is possible that import systems collaborate with metallochaperones in a more complex trafficking system.

Such copper chaperones have been described mainly for the biogenesis of heme-copper oxidases in mitochondria and in their prokaryotic relatives belonging to the \(\alpha\)-proteobacteria class like *Rhodobacter* and *Bradyrhizobium japonicum*. In the eukaryotic model, adopted also for prokaryotes, Sco1 transfers copper to (or reduces its cysteines of, or both) the Cu\(_A\) center of the cytochrome \(c\) oxidase, and Cox11 is responsible for the Cu\(_B\) center. Recently Sco has been linked to the biogenesis of Cu\(_B\) centers in prokaryotic \(aa_3\) and \(cbb_3\) oxidases (the latter containing only a Cu\(_B\) center) as well. Biogenesis of the \(cbb_3\) oxidase does not require Cox11-like proteins but instead is dependent on the integrity of predicted inner-membrane transporters. PCu\(_A\)C protein has been identified as a collaborator of Sco for copper transfer to its same targets (Cu\(_A\) and Cu\(_B\) centers of \(aa_3\) oxidase and Cu\(_B\) center of \(cbb_3\) oxidase).

The abundance of alternative terminal oxidases, most of them copper-dependent, is peculiar to the nitrogen fixing *B. japonicum*, and allows it to undergo different lifestyles such as free-living aerobiosis and anaerobiosis, and symbiosis (mainly with soybean). It has been shown that ScoI (Sco1-like) and CoxG (Cox11-like) collaborate for the biogenesis of the main aerobic \(aa_3\)-type cytochrome \(c\) oxidase in *B. japonicum*, while for the symbiotically-essential \(cbb_3\) oxidase the situation is less clear, but genes belonging to the \(fixGHIS\) operon, among them a predicted P\(_{1B}\)-type ATPase as product, are necessary.

This work aimed at identifying genes involved in copper import and trafficking in *B. japonicum*. For this reason, a transcriptomic survey of genes up-regulated in copper starvation and a search for copper chaperone homologues were carried out. Both approaches converged in the identification of an operon, *pcuABCDE*. This operon is the only genomic region whose expression is higher in copper-free medium compared to copper excess. It
encodes putative polypeptides related to copper trafficking, among them the PCuA homologue PcuC.

Strains with a resistance cassette replacing the operon were constructed and different phenotypes were evaluated. Importantly, the mutants were found to be defective in copper-starved growth, in anaerobic growth and in symbiosis. To check whether the phenotype is due to impaired biogenesis of the terminal oxidases, quantitative measurements of the $aa_3$ and $cbb_3$ oxidase activities were carried out, revealing decreased functionality for both of them. Interestingly, the defect of the $aa_3$ oxidase is restorable with copper supplementation. The $cbb_3$ oxidase misfunction is evident only when the activity is tested on bacteroid membranes, while in free-living anaerobic cultures (where the $cbb_3$ oxidase is also expressed) the defect is absent. The logical follow-up of the operon mutant characterization has been the creation of strains in which each single gene has been deleted, in order to assign to each ORF a weight in the different phenotypes observed for the whole operon mutant. Surprisingly, except for the anaerobic growth, for which seemingly all the genes have a partial role, the lack of $pcuC$ was found to be the only determinant of the $\Delta pcuABCDE$ strain phenotype.

Phenotypes of $\Delta scoI$ (previously characterized in this group) and $\Delta pcuC$ are strikingly similar. As for $\Delta pcuC$, the $cbb_3$ oxidase activity of $\Delta scoI$ is not impaired when membranes are isolated from anaerobically grown cells. The $cbb_3$ oxidase activity of bacteroid membranes was then measured in the $\Delta scoI$ strain as well, as this has not been tried formerly, and importantly revealed a strong defect, as for that observed in $\Delta pcuC$.

Furthermore, in-vitro studies confirmed the hypothesized Cu$^+$-binding nature of PcuC via conserved residues previously identified in the Thermus thermophilus homologue.

ScoI and PcuC are copper-binding proteins collaborating for copper insertion into the $cbb_3$ oxidase and into the $aa_3$ oxidase. These results are compatible with what has been concurrently observed in Pseudomonas aeruginosa PA01, Rhodobacter sphaeroides and Rhodobacter capsulatus, enforcing both a new view of the role of Sco, which is no longer restricted to the copper transfer/reduction of CuA centers, and its close link to PCuA. In this model it would be more likely that the soluble PCuA binds copper as it enters the periplasm and transfers it to the inner membrane-anchored Sco, which then sorts it to membrane bound chaperones/copper-enzyme targets. In order to assess the validity of this model further in vitro copper transfer studies are needed.
Il rame è sia un oligoelemento sia una sostanza tossica. Il potenziale della coppia Cu²⁺/Cu⁺ è il più alto tra i biometalli, rendendo il rame utile per l’estrazione di elettroni da molecole con un potenziale redox già elevato. Per questa caratteristica i cuproenzimi catalizzano principalmente trasferimenti di elettroni e reazioni redox, come ad esempio in svariati processi respiratori aerobi o anaerobi. Questa stessa caratteristica è però, assieme al fatto che il rame è il metallo che lega più efficientemente le biomolecole, anche il motivo della sua tossicità; infatti, esso può catalizzare la produzione di specie reattive dell’ossigeno e/o sostituire altri metalli. Nei batteri questa tossicità rappresenta una minaccia sia nel citoplasma che nel periplasma. Essi devono perciò evitare che atomi di rame rimangano liberi all’interno della cellula relegando i cuopronezimi fuori dal citoplasma, dove si trovano i bersagli più sensibili, utilizzando efficaci meccanismi di esporto e usando chaperones che scortano il metallo a destinazione. Quando il rame diventa limitante, è possibile che sistemi d’importo collaborino con chaperones in un più complesso network di smistamento.

Chaperones di questo tipo sono state descritte principalmente per la biogenesi di ossidasi terminali a eme-rame nei mitocondri e nei loro parenti procariotici più stretti appartenenti al gruppo degli α-proteobatteri, come *Rhodobacter* e *Bradyrhizobium japonicum*. Nel modello eucariotico, che è stato adottato anche per i procarioti, Sco1 trasferisce il rame nel centro Cuₐ della citocromo c ossidasi (o riduce le sue cisteine, o entrambe le cose), mentre Cox11 si occupa del centro Cuₙ. Più recentemente a Sco è stato attribuito un ruolo nel trasferimento del rame anche nel centro Cuₙ delle ossidasi *aa₃* e *cbb₃* (quest’ultima contenente solo un centro Cuₙ). La biogenesi dell’ossidasi *cbb₃* non dipende da proteine del tipo Cox11, ma piuttosto da putativi trasportatori della membrana citoplasmatica. PCuₐC inoltre sembra collaborare con Sco per la biogenesi dei suoi stessi bersagli.

L’abbondanza di ossidasi terminali, la maggior parte delle quali contengono rame, è una peculiarità dell’azotofissatore *B. japonicum*, e permette a questo batterio di adottare diversi stili di vita, quali replicazione in aerobiosi e anaerobiosi, o simbiosi, principalmente con piante di soia. È stato dimostrato che ScoI (omologo di Sco1) e CoxG (omologo di Cox11) sono necessari per la biogenesi dell’ossidasi *aa₃*, preponderante in aerobiosi, mentre non è chiaro come il rame sia inserito nell’ossidasi *cbb₃*, essenziale in simbiosi, anche se l’operone *fixGHIS*, contenente tra gli altri un gene per un’ATPasi di tipo P₌, è necessario.

Lo scopo iniziale di questo lavoro è consistito nell’identificazione di geni per l’importo e lo smistamento del rame in *B. japonicum*. A questo scopo sono state effettuate un’analisi trascrittomica dei geni la cui espressione è aumentata in assenza di rame, e una ricerca di geni omologhi a quelli codificanti chaperons note. Entrambi gli approcci hanno portato
all’identificazione dell’operone *pcuABCD*, i cui geni sono gli unici a subire una up-regolazione in terreni privi di rame rispetto a terreni con eccesso di rame. Le proteine teoricamente codificate da questi geni sono correlate con il trasporto del rame; tra di esse c’è PcuC, un’omologa di PCuAC.

Ceppi in cui l’operone è stato rimpiazzato da una cassetta di resistenza sono stati creati e analizzati per diversi fenotipi. La mancanza dell’operone causa un difetto di crescita in terreni privi di rame, in terreni anaerobici e in simbiosi. Successive analisi hanno dimostrato che la causa di questo fenotipo è la mancanza di attività delle ossidasi \( aa_3 \) e \( cbb_3 \). L’attività dell’ossidasi \( aa_3 \) è ripristinabile crescendo le cellule in eccesso di rame, mentre l’ossidasi \( cbb_3 \) risulta difettosa quando le membrane sono estratte da batteroidi, ma non quando sono estratte da cellule coltivate anaerobicamente (nelle quali la \( cbb_3 \) ossidasi è comunque espressa). Questo studio è stato approfondito creando ceppi mutanti per ciascun gene dell’operone e osservando nuovamente i fenotipi analizzati in precedenza, allo scopo di capire quale gene è responsabile per ciascun fenotipo. Sorprendentemente la mancanza di \( pcuC \) si è dimostrata l’unica causa per i tutti i fenotipi osservati nel ceppo \( \Delta pcuABCDE \) eccetto la crescita anaerobica, alla quale tutti i geni sembrano contribuire.

I ceppi \( \Delta scoI \) (caratterizzato precedentemente in questo gruppo) e \( \Delta pcuC \) condividono un fenotipo praticamente identico. Come in \( \Delta pcuC \), l’attività dell’ossidasi \( cbb_3 \) in \( \Delta scoI \) è paragonabile a quella del wild type quando le membrane sono estratte da colture aerobiche. Anche per \( \Delta scoI \) è stata quindi analizzata l’attività di \( cbb_3 \) in membrane estratte da batteroidi, dal momento in cui ciò non era stato tentato in passato. L’attività misurata è risultata fortemente ridotta, analogamente a quanto osservato in \( \Delta pcuC \).

Studi *in vitro* hanno confermato che PcuC è una chaperon del rame, essa lega infatti \( Cu^+ \) tramite i residui conservati precedentemente identificati nella corrispondente PCuAC di *Thermus thermophilus*.

Concludendo, ScoI e PcuC sono entrambe proteine che legano il rame, collaborando per la sua inserzione nelle due ossidasi. Questi risultati sono compatibili con quanto riportato per *Pseudomonas aeruginosa* PA01, *Rhodobacter sphaeroides* e *Rhodobacter capsulatus*, e rafforzano l’idea che la funzione di Sco non sia limitata al trasferimento del rame (o alla riduzione delle cisteine) del centro CuA, ma che piuttosto essa collabori con PCuAC per la biogenesi dei centri CuA e CuB. In un tale modello è logico immaginare che PCuAC, solubile, leghi il rame appena esso entra nel periplasma e lo trasferisca a Sco, che a sua volta lo smisterebbe alle chaperones e agli enzimi associati alla membrana. La validità di tale modello richiede comunque un’attenta valutazione tramite studi *in vitro* sul trasferimento del rame.
Chapter I

Introduction
Chapter I

1.1 Copper acquisition and trafficking in bacteria

Copper has been used by living beings since the oxygenation of the atmosphere billions of years ago. The uniquely high redox potential of the \( \text{Cu}^{2+}/\text{Cu}^+ \) couple allows copper enzymes to exploit as much as possible the energy embedded in the \( \text{O}_2 \) molecule, as well as catalyze electron transfer and oxidation of rather inert substrates, otherwise impossible. It is therefore not surprising that copper covers fundamental functions, on top of them respiration.

Copper is also the most efficient metal in binding biomolecules. For these two properties, copper is dangerous when not complexed since it can catalyze the generation of reactive oxygen species and occupy other metal sites. Bacteria deal with the copper double face in different ways. Firstly by using very efficient resistance systems, secondly by compartmentalizing copper-requiring proteins outside the cytoplasm, and thirdly by transporting copper tightly bound to biomolecules, namely metallochaperones, inside the cell. This last necessity becomes even more obvious for the biogenesis of copper proteins of the thylakoids, of the cytoplasmic membrane of Gram negatives, and for enzymes whose copper center does not build spontaneously. Less is known about copper uptake from the environment. Even though apparently copper is a widely available trace element, starvation situations requiring its import can still exist, and proved at least in the case of methane oxidizing bacteria.

This review summarizes the current knowledge of copper import from the environment to the cell, and its trafficking for the biogenesis of cuproenzymes in bacteria.

1.1.1 Background

Copper has not always been part of the life-essential elements on this planet. Life indeed evolved in an environment where copper was not accessible at all. The reducing atmosphere of the primordial Earth, with oceans filled with \( \text{H}_2\text{S} \) and completely devoid of \( \text{O}_2 \), forced copper to assume the oxidation state of +1, trapping it in extremely insoluble sulfides (Saito et al., 2003). The diverse prokaryotic forms of life populating the early seas were using mainly soluble ions such as \( \text{Fe}^{2+} \) and \( \text{Mn}^{2+} \) as redox-active metals. The \( \text{Fe}^{2+} \) ion redox ability fitted well with the bioenergetics, based on reactions taking place in the lower portion of the redox spectrum (\( \text{H}_2\text{S}/\text{S} \) and \( \text{H}_2/\text{H}^+ \) potentials are between 0.0 and –0.4 V) (Fraústo da Silva and Williams, 2011). Iron thus became an essential metal for the life evolved until then. Cyanobacteria started to exploit \( \text{Mn}^{2+} \) for photosynthesis around 2.7 billion years ago, releasing vast amounts of \( \text{O}_2 \) (Berkner and Marshall, 1965). This catastrophic event polluted slowly but irreversibly the atmosphere. The redox chemistry of the environment changed
completely as the redox spectrum upper limit reached 0.8 V (Rubino and Franz, 2012). Iron became mainly represented by the scarcely soluble $\text{Fe}^{3+}$, which precipitated, while soluble $\text{Cu}^{2+}$ was released from the sulfide deposits (Williams and Fraústo da Silva, 2006). Microorganisms adapted to this new situation in three ways. (i) Oxygen started to be used as a highly efficient electron acceptor. (ii) Since iron became indispensable they evolved chelates such as siderophores and storage proteins such as ferritin to overcome the unavailability of the metal (Rohrer et al., 1989). (iii) The newly available copper was introduced into proteins, and its ability to deal with the high oxygen redox potential was exploited by the cells (Crichton and Pierre, 2001; Saito et al., 2003). The $\text{Cu}^{2+}/\text{Cu}^{+}$ couple potential is in fact usually higher than $\text{Fe}^{3+}/\text{Fe}^{2+}$ in biomolecules, up to ~1 V in human ceruloplasmin (Machonkin et al., 1998; Crichton and Pierre, 2001). It is then no surprise that the main biological processes in which copper is involved are defense against oxidative stress and oxygen reduction in terminal oxidases of the electron transport chain. In the latter case copper allows organisms to have a heme-copper oxygen reductase, which is also a proton pump. Whether proton pumping occurs when copper is not involved is not entirely clear (bd type oxidases) (Poole, 1994; Pereira et al., 2008).

Therefore, copper brought an advantage to species which could handle oxygen. This observation is supported by the distribution of copper proteins across the tree of life. Most of the aerobic bacteria utilize copper proteins, while in most anaerobic species copper is dispensable. Archaea are mainly devoid of copper proteins. Eukaryotes evolved in an oxygenated environment; copper is thus indispensable for them (Ridge et al., 2008). During evolution copper proteins have been exploited for many functions that go beyond respiration and protection against oxidative stress damage. This is especially true in eukaryotes, where copper proteins are used to catalyze otherwise very difficult oxidative reactions, which can also involve radical mechanisms. As an example of this, one may reflect that the existence of vascular plants would not have been possible without this element; copper in fact is essential to lignin biosynthesis (Boerjan et al., 2003). In prokaryotes the number of functions a copper protein can assume are less than what is observed in eukaryotes, but protein families observed in eukaryotes are all present in bacteria as well.

**Bacterial copper enzymes**

In this review we refer to copper proteins, cuproproteins, copper enzymes or cuproenzymes when copper is part of the active site and is needed for the functionality of the protein. Cu-sensing regulators and exporters go beyond the scope of this review, and will not
be considered; copper acquisition and transfer proteins will be widely discussed in the following sections. All cuproproteins use copper ability of cycling between the +1 and +2 oxidation state in order to transfer electrons from a substrate, which is then oxidized, to another substrate, which is then reduced. The redox potential of a copper atom inside a protein is tuned for the specific function by varying the coordination sphere of the metal, and this gave rise during evolution to a conserved set of copper centers, where each type is involved in analogous functions. Copper properties make it potentially extremely useful to the bacterial cell, but because of its toxicity (see below) its occurrence in biomolecules is restricted to a limited number of protein families, which will be introduced in this section and which are listed in Table 1.1.

As already outlined, the most widespread usage of copper proteins is within the aerobic respiratory electron transport chain (Ridge et al., 2008). The heme-copper oxygen reductases are the most common terminal oxidases (García-Horsman et al., 1994). They conclude the electron transport chain by reducing O₂ and pumping protons outside the cytoplasm. In all the members of this family oxygen reduction is carried out in a binuclear
center constituted by a high-spin heme and a CuB center. The binuclear center is situated in the trans-membrane subunit I and is itself buried in the bilayer. It receives the electrons necessary for the O2 reduction from a low-spin heme in the same subunit (García-Horsman et al., 1994; Iwata et al., 1995; Abramson et al., 2000; Pereira et al., 2008; Sousa et al., 2012). Heme-copper oxygen reductases belonging to the A group (according to the classification of Pereira et al., 2001) possesses an additional bimetallic CuA center in the extracytoplasmic domain of subunit II where both copper atoms assume a mixed valence of +1.5. Its function is to receive electrons from the cytochrome c and sort them to the low-spin heme, which eventually transfers them to the high-spin heme-CuB center (Tsukihara et al., 1996). Belonging to this group is the mitochondria-like aa3-type cytochrome c oxidase (where a stands for a low-spin heme A and a3 stands for high-spin heme A in the binuclear center), which is widespread in Bacteria, especially in α-proteobacteria (Fig. 1.1). It is thought that mitochondria actually evolved from α-proteobacteria carrying this type of terminal oxidase (Andersson et al., 1998).

Table 1.1. Known families of bacterial cuproenzymes

<table>
<thead>
<tr>
<th>Family of cuproenzyme</th>
<th>Function</th>
<th>Occurrencea</th>
<th>Cu centers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme-copper oxygen reductase</td>
<td>Terminal oxidase of aerobic electron transport chain</td>
<td>91%</td>
<td>CuA, CuB</td>
</tr>
<tr>
<td>NADH dehydrogenase-2</td>
<td>Uncertain. Electron transport chain and/or oxidative stress protection.</td>
<td>34%</td>
<td>HMA domain (CxxC)</td>
</tr>
<tr>
<td>Cu/Zn superoxide dismutase</td>
<td>Oxidative stress protection</td>
<td>21%</td>
<td>4 His, tetragonal</td>
</tr>
<tr>
<td>Copper NO2− reductase</td>
<td>Reduction of NO2− to NO, denitification</td>
<td>10%</td>
<td>Type 1, type 2</td>
</tr>
<tr>
<td>N2O reductase</td>
<td>Reduction of N2O to N2, denitrification</td>
<td>n.a.</td>
<td>CuA, CuZ</td>
</tr>
<tr>
<td>Particulate methane monooxygenase</td>
<td>Oxidation of CH4 to CH3OH in methanotrophs</td>
<td>2%</td>
<td>Not clear</td>
</tr>
<tr>
<td>Blue copper protein (plastocyanin, azurin, pseudoazurin, amycianin, rusticyanin)</td>
<td>Electron shuttles for diverse electron transfer chains</td>
<td>15%</td>
<td>Type 1</td>
</tr>
<tr>
<td>Nitrosocyanin</td>
<td>Unknown</td>
<td>15%</td>
<td>Type 2</td>
</tr>
<tr>
<td>Copper amine oxidase</td>
<td>Oxidative deamination of primary amines</td>
<td>6%</td>
<td>Type 2</td>
</tr>
<tr>
<td>Laccase (multicopper oxidase)</td>
<td>Polyphenol oxidase; Mn2+ oxidase</td>
<td>1%</td>
<td>Type 1, trinuclear (type 2 + type 3)</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>Oxidation of p-monophenol and o-diphenols to quinones</td>
<td>1%</td>
<td>Type 3</td>
</tr>
</tbody>
</table>

a Percentage of sequenced bacterial genomes containing at least one member of the family, as determined by Ridge et al. (2008). n.a.: data not available.
Second most common bacterial copper-containing protein family is the copper dependent NADH dehydrogenase-2 (NDH-2) (Ridge et al., 2008). Its function as a NADH:ubiquinol oxidoreductase has been demonstrated in *Escherichia coli* (Jaworowski et al., 1981; Campbell and Young, 1983). Like the analogous mitochondrial complex I-like NADH dehydrogenase (NDH-1), also conserved in bacteria, it is a transmembrane protein which transfers electrons from NADH to the quinone pool, but it differs because of its inability to participate to the establishment of a proton gradient (Matsushita et al., 1987; Calhoun et al., 1993). NDH-2 is able to convey electrons from either NADH or quinoles to a Cu^{2+}, converting it to a Cu^{+} ion which then remains bound to a CxxC motif in a HMA domain (Rapisarda et al., 1999; Rapisarda et al., 2002). Since in *E. coli* the more efficient NDH-1 is also present, the quest for a complete understanding of NDH-2 physiological function is not considered closed yet. The production of Cu^{+} ions – more effective than Cu^{2+} in generating oxygen radicals (Silver and Phung le, 2005) – has been explained as a necessity because +1 is the copper oxidation state mainly required for other cellular functions (Fan and Rosen, 2002; Solioz and Stoyanov, 2003), but later quinones proved to be better players in this conversion (Volentini et al., 2011). NDH-2 mutants proved to be more susceptible to oxidative stress than their parental strains. This phenotype has been explained with a possible function of NDH-2 as a free-radical scavenger, since NDH-2 proved to be inactivated faster than other oxidases of the respiratory chain. In this latter interpretation a tightly-bound Cu^{+} is necessary to fish radicals which then react with the protein before inactivating other essential oxidases (Rodríguez-Montelongo et al., 2006). No data about a possible role for copper as cofactor in the ascertained activity of electron transfer from NADH to quinones is available; while in the second postulated function of protection from oxidative stress copper would be a substrate instead of a cofactor.

Another copper protein involved in oxidative stress protection is the Cu/Zn superoxide dismutase. It is a prerogative of Gram-negative bacteria, which export it to the periplasm via the Sec pathway (Steinman and Ely, 1990). Its function is to scavenge O_{2}^{-} produced in the periplasm or in the extracellular environment. While O_{2}^{-} produced during respiration is released in the cytoplasm and neutralized by copper-independent superoxide dismutases, Cu/Zn superoxide dismutase defends periplasmic targets from O_{2}^{-} produced exogenously (i.e. phagocytes for pathogenic bacteria) or endogenously during the stationary phase (Farrant et al., 1997; Gort et al., 1999). The active site of this protein is formed by a Cu^{2+} ion coordinated by four histidines (Pesce et al., 1997). Cu^{2+} is first reduced to Cu^{+} by O_{2}^{-} which...
is then converted to O$_2$ and released. The electron is then transferred to another O$_2^-$ together with two protons, reconstituting the Cu$^{2+}$ ion and releasing H$_2$O$_2$ (Fielden et al., 1974).

Apart from aerobic respiration, copper is also important for electron transport systems that use as final electron acceptor oxides of nitrogen, in the called denitrification process. In this kind of anaerobic respiration subsequent reduction steps converting nitrate to N$_2$ are coupled to proton pumping (Strohm et al., 2007). Conversion of NO$_2^-$ to NO is catalyzed by the nitrite reductase, which can be either a cytochrome $cd_1$ or a copper-containing enzyme, depending on the organism (Gamble et al., 1977). The **Cu-containing nitrite reductase** is a soluble periplasmic protein, exported via the Sec pathway (Glockner et al., 1993). It is active as a trimer in which each subunit contains type 1 and type 2 copper centers, coordinated by three histidines plus one cysteine and by three histidines, respectively (Adman et al., 1995). Electrons enter to type 1 centers usually from azurin/pseudoazurin or via cytochromes and are then transferred to the type 2 Cu center where reduction of NO$_2^-$ takes place (Zumft, 1997). The last step of denitrification in which N$_2$O is converted in N$_2$ is catalyzed by the multicopper enzyme **nitrous oxide reductase**. It is a homodimeric protein that is exported to the periplasm via the TAT pathway (Heikkilä et al., 2001). The copper content of this protein has recently been clarified thanks to the available structures. The N$_2$O reductase features a cytochrome oxidase-like Cu$_A$ center and a unique Cu$_Z$ center which is constituted by a [4Cu:2S] copper-sulphur cluster. N$_2$O binds between the two sites, where Cu$_Z$ activates N$_2$O while electrons are transferred by the Cu$_A$ center (Fig. 1.2; Pomowski et al., 2011). The electrons necessary for the conversion are transferred from $c$-type cytochromes or pseudoazurin (Zumft, 1997; Dell'Acqua et al., 2011).

![Figure 1.2.](image)

The ability of copper to activate O$_2$ is exploited by methanotrophic bacteria to convert an extremely inert compound such as methane to methanol, allowing its use as a carbon and electron source. The particulate **methane monooxygenase** (pMMO) is a transmembrane
protein complex located in the inner membrane of almost all methanotrophs. It is encoded by three genes, whose translation products arrange into a cylindrical $\alpha_3\beta_3\gamma_3$ trimeric structure. Even though a stoichiometry of 2-3 copper per protomer seems to be the most frequently observed, the number of copper ions bound to the protein is object of debate. To date, the reaction mechanism of oxygen activation has not been proven experimentally even though there is emerging evidence for oxygen binding at the dicopper center in the PmoB subunit (Hanson and Hanson, 1996; Ramakrishnan et al., 2007; Culpepper and Rosenzweig, 2012).

Blue copper proteins, or cupredoxins, are in a highly heterogeneous family of small single-domain proteins that share a typical common folding and a type 1 copper active site (De Rienzo et al., 2000). Their function of shuttling electrons from a donor to an acceptor, analogous to cytochrome $c$, is also conserved, but applied to a variety of physiological functions. Cyanobacterial plastocyanin is the most well studied member of this group. It participates in the light-dependent photosynthetic reactions in thylakoids by transferring electrons from the cytochrome $f$ to photosystem I (Diaz-Quintana et al., 2003). Bacterial members of this group include azurin and pseudoazurin, required for electron transfer during denitrification (De Rienzo et al., 2000), amicyanin, covering a similar role in some methylootrophic bacteria, and rusticyanin, involved in the electron transfer chain of the iron oxidizing bacterium *Thiobacillus ferrooxidans* (Baker, 1994; Yamanaka and Fukumori, 1995; Harvey et al., 1998). Nitrosocyanin, or red copper protein, a protein with a cupredoxin fold but with a type 2 copper center, has been isolated from *Nitrosomonas europaea*. The presence of the protein at molar levels comparable with those of proteins involved in the electron transfer pathway suggests a central metabolic role. Unlike the blue copper proteins, its low redox potential and the characteristic of the copper site do not seem to fit with an electron transfer activity, but rather with a catalytic function (Arciero et al., 2002).

Several bacterial proteins use the oxidative potential of copper in order to catalyze otherwise difficult oxidations. Copper-containing amine oxidases, multicopper oxidases and tyrosinases are three protein groups which accomplish this functionality. **Copper amine oxidases** couple the oxidative deamination of primary amines to their corresponding aldehydes to the reduction of $O_2$ to $H_2O_2$. The few studies on the bacterial members of this family, compared to the large number of studies on eukaryotic relatives, outlined a catabolic function on primary amines, used as source of C and N. The first documented substrate was 2-phenylethylamine in *E. coli* (Parrott et al., 1987). In following studies copper amine oxidase showed the same substrate preference together with other aromatic amines in *E. coli*, *Klebsiella oxytoca* and *Arthrobacter globiformis* (Hacisalihoglu et al., 1997; Klema and...
All the bacterial members are homodimeric and feature a Sec signal peptide or were isolated from periplasmic preparations. These proteins possess a 2,4,5-trihydroxyphenylalanine quinone cofactor (topaquinone or TPQ), which derives from the post-translational modification of a tyrosine residue in the enzyme precursor. A type 2 Cu\(^{2+}\) center forms the active site in close proximity to the TPQ cofactor. The peculiarity of these proteins is how the TPQ cofactor is synthetized. Only Cu\(^{2+}\) and O\(_2\) are in fact required for the conversion of the tyrosine in TPQ, in the following stoichiometry: ApoEnzyme + Cu\(_{2+}\) + 2 O\(_2\) \(\rightarrow\) HoloEnzyme + H\(_2\)O\(_2\) + H\(^+\) where ApoEnzyme is copper-free and with tyrosine, and HoloEnzyme is Cu\(^{2+}\)-bound and with TPQ. In a proposed mechanism copper catalyzes the tyrosine activation for the dioxygen molecule attack, and remains then in the protein as part of the active site. In the functional active site the amine is oxidized by the quinone cofactor, which accepts two electrons. The generated quinol form is converted back to quinone by reducing O\(_2\) to H\(_2\)O\(_2\) with the two electrons in excess. This last step is Cu\(^{2+}\) dependent, since the metal likely stabilizes the superoxide intermediate (Klema and Wilmot, 2012).

**Multicopper oxidases** are a large class of proteins characterized by the repetition (duplication and/or triplication) of a cupredoxin domain (Nakamura and Go, 2005). The aforementioned nitrite reductase also belongs to this family. In eukaryotes, in particular in plants and fungi, these proteins are represented by a large assortment of oxidases involved in secondary metabolism or in degradation (i.e. both biosynthesis and decomposition of lignin). The prokaryotic members so far characterized cover a much more limited number of functions, with the bulk of them being part of copper efflux systems. Multicopper oxidases which are instead proper cuproenzymes have distinguishable features. They have 4 copper atoms per monomer, organized in a type-1 center and an interdomain trinuclear center constituted by a type-2 copper and a binuclear type-3 copper (Nakamura and Go, 2005). Almost all the characterized proteins show a laccase-like activity (oxidation of phenolic compounds) with broad specificity but narrower than fungal laccases; four of them (CumA, MofA, CotA and MnxG) have a Mn\(^{2+}\) oxidase activity. Some of them (CotA, CumA, MofA and MnxG in *Bacillus subtilis*, *Pseudomonas putida*, *Leptothrix discophora* and *Bacillus* SG-1, respectively) are endospore coat proteins (Kepkay and Nealson, 1982; van Waasbergen et al., 1996; Francis and Tebo, 2001; Martins et al., 2002). Is not possible to exclude the existence of cytoplasmic members, even though the majority of laccases seems to be secreted (Ausec et al., 2011). The physiological function of the laccases could be the formation of melanin-like pigments for defensive functions against different stress factors such as oxidants, free radicals and UV radiation, but also lignin degradation (Hullo et al., 2001;
Ahmad et al., 2010), while the Mn$^{2+}$ oxidase activity function is not fully understood. PPO from *N. europaea* is a laccase with the unique ability of catalyzing nitrite reduction as well; it can in fact functionally replace a nitrite reductase defect (Dispirito et al., 1985). In general the catalytic mechanism consists of the transfer of four electrons from the substrate to O$_2$ and the release of H$_2$O. The oxidation is accomplished by the type 1 copper. The four electrons are then transferred to the trinuclear center where the copper atoms convey them to the oxygen molecule. The substrates are released as free radicals, undergoing spontaneous reactions like crosslinking, polymer degradation or aromatic ring cleavage (Claus, 2004). These laccase-like proteins seem to be widespread in bacteria based on two observations: (i) laccase activity has been demonstrated in some bacteria, and to date no copper-independent laccase activity is known; (ii) laccase genes are well conserved (Claus, 2004; Ausec et al., 2011).

**Tyrosinases** are similarly involved in phenol oxidation for melanin biogenesis, but they belong to a different family. Melanin biosynthesis may involve both tyrosinases and laccases (Mercado-Blanco et al., 1993; Castro-Sowinski et al., 2002). Tyrosinases feature a typical type 3 copper center, where the two ions are coordinated by six histidines. Molecular oxygen is usually placed between the two copper ions and it is used to catalyze two kinds of reaction: (i) *ortho*-hydroxylation of monophenols to *o*-diphenols and (ii) laccase-like oxidation (but with different mechanism) of *o*-diphenols to *o*-quinones, triggering spontaneous polymerization to melanin (Claus and Decker, 2006). Several types of tyrosinases have been identified, and their study is prompted by interest in their biotechnological application (Yang and Chen, 2009; Fairhead and Thöny-Meyer, 2012). Their cellular localization is not immediately recognizable from their sequences. In the best studied cases tyrosinases are secreted by interacting with a caddy protein which owns a TAT secretion leader sequence (Leu et. al., 1992; see section 1.1.3). The absence of such a TAT sequence probably causes the membrane localization of some tyrosinases (Yang and Chen, 2009). In other cases the leader sequence is present already in the structural enzyme, ensuring secretion (Fairhead and Thöny-Meyer, 2010). When both a leader sequence and a caddy protein are not identifiable, cytoplasmic localization cannot be excluded, though never proved so far. The physiological importance of these enzymes is not completely clear. Their melanogenic activity has been explained as useful for UV light protection, but given their wide substrate-specificity it may not be the only biologically relevant one (Saxena et al., 2002; Claus and Decker, 2006). In *Rhizobium etli* MelA tyrosinase is essential for symbiosis, and in *Streptomyces griseus* a tyrosinase is involved in a secondary metabolite biosynthesis (Suzuki et al., 2006; Piñero et al., 2007). In *Streptomyces* the co-occurrence of two different tyrosinase families led to a
proposed protection role against plant-produced phenolics for one family, and a toxic role by catalyzing the production of permeable quinones, which would confer competitiveness against non-resistant strains, for the other family (Yang and Chen, 2009).

**Handling of copper toxicity**

Ancient civilizations used copper as a salt for curing diseases or as a material to build vessels able to preserve liquids from decay, much before the concept of "microbe" was known at all (Dollwet and Sorenson, 1985). As often happens, the efficacy of traditional methods found scientific explanations in our times which legitimates them. What is clear today is that the very same reason that makes copper so useful in biology is actually a threat to life itself, microbes included. When in excess, copper inhibits bacterial growth. The mechanism of its toxicity is paradoxically not entirely clear, but two main hypotheses have been advanced. First, copper is able to generate reactive oxygen species (ROS) when in presence of hydrogen peroxide in the so-called Fenton reaction (Halliwell and Gutteridge, 1984):

\[
\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^- + \cdot\text{OH}
\]

This reaction can only happen in the presence of \(\text{H}_2\text{O}_2\), which is abundant wherever aerobic respiration takes place, so one possible toxicity mechanism is the degradation of biomolecules by the generated ROS. In this case \(\text{Cu}^+\) would be more effective than \(\text{Cu}^{2+}\) (Silver and Phung le, 2005). Second, copper is very effective in binding biomolecules in both oxidations states (Waldron and Robinson, 2009), making it a competitor of other metals or just a random binder, with a potential to inactivate the target molecule in both cases (Hartwig, 2001). Understanding which of the two mechanisms acts in specific cases has been challenging. Copper-generated ROS have been indicated as responsible for nucleic acid, protein, and lipid damage in animal cells and mitochondria (Rau et al., 2004; Sheline and Choi, 2004; Arciello et al., 2005; Krumschnabel et al., 2005). A closer look in bacteria revealed that during copper excess neither DNA damage nor a global oxidative stress response was triggered, while iron-sulfur clusters were demonstrated to be the primary targets of copper mediated toxicity. In this case is not possible to exclude a ROS-induced inactivation, but the observation of an analogous, if not worse, oxygen-independent toxicity points towards a metal-displacement mechanism (Macomber et al., 2007; Macomber and Imlay, 2009; Chillappagari et al., 2010).
Whatever the toxicity mechanism really is, cells have to deal with copper. There is no direct evidence of cytoplasmic localization of any copper protein, though it cannot be excluded for tyrosinases. Plastocyanins localize in thylakoids, where photosynthesis takes place, while other cuproenzymes are secreted via Sec or TAT pathways into the periplasm (in the case of Gram-negative bacteria) or in the extracellular environment. This observation indicates that copper toxicity is exerted mainly in the cytoplasm, or at least that the cell has an advantage in avoiding copper to be handled in this compartment. Coherently, in E. coli it was shown that the main targets of copper toxicity are indeed localized in the cytoplasm (Macomber and Imlay, 2009). Measurements of copper content in the yeast Saccharomyces cerevisiae revealed that almost no copper ion can be found free in this compartment (Rae et al., 1999). It is likely that in prokaryotes the situation does not differ much, since very efficient copper resistance systems, highly conserved among all bacteria, are well characterized (for a review, see Osman and Cavet, 2008) and since the copper sensor CueR has a zeptamolar (less than one atom per cell) sensitivity to free Cu$^+$ (Outten et al., 2000). Like CueR, all the other proteins involved in copper resistance bind Cu$^+$, the species found in the reducing environment of the cytoplasm (Solioz and Stoyanov, 2003).

The extent to which copper is toxic in the periplasm of Gram-negative bacteria is not clear. The localization of copper proteins leads one to believe that in this compartment the copper menace is a minor issue, but one must distinguish between free or chaperone-bound ions. Cells probably tolerate protein-bound copper better here than in the cytoplasm, since the total periplasmic copper content is significantly higher than that in the cytoplasm in E. coli, while in Pseudomonas syringae copper sequestration and accumulation in the periplasm has been inferred to as part of a resistance mechanism (Cha and Cooksey, 1991; Cooksey and Azad, 1992; Macomber et al., 2007). In E. coli a system for Cu$^+$ export outside the periplasm has been thoroughly characterized (Grass and Rensing, 2001b; Franke et al., 2003). The required level of free copper needed to activate this periplasmic resistance system is much higher than what is required to trigger the cytoplasmic export (Outten et al., 2001), pointing toward a better tolerance of the metal in this compartment. Moreover a periplasmic multicopper oxidase involved in converting Cu$^+$ to the less reactive Cu$^{2+}$ has been described (Grass and Rensing, 2001a). Since the periplasmic copper exporter is specific for Cu$^+$, it may be that free Cu$^{2+}$ is indeed less dangerous here, or maybe that the second species is better buffered by chaperones, or that efflux systems convert it to Cu$^+$ prior to export. Cu$^+$ was detected in the oxidizing periplasm of E. coli (Macomber et al., 2007), and it probably originates from cytoplasmic copper resistance, from Cu$^{2+}$ reduction by cysteines (kept in turn
reduced by DsbG; Depuydt et al., 2009), from the quinones pool, and from the NDH-2 (Rodriguez-Montelongo et al., 2006). An extracellular copper reductase activity was also detected in *Enterococcus hirae* (Wunderli-Ye and Solioz, 1999).

In summary, seemingly the evolution compartmentalized copper requiring proteins far from the cytoplasmic sensitive targets, minimizing the risk of major damages caused by fortuitous leakage from the chaperones. Copper is kept associated with biomolecules, more numerous in the periplasm than in the cytoplasm, because if let free, it may interfere with other metal molecules, including proteins involved in the homeostasis of other metal ions. A mutant of an abundant periplasmic Fe$^{3+}$-binding protein of *Synechocystis* PCC 6803 showed impaired copper-dependent processes, likely only because not enough Fe$^{3+}$ was buffered anymore, with a consequent displacement of copper from its specific metallochaperones (Waldron et al., 2007). This can be taken as an example of how important it is for a cell to ensure no metal is let free, even in the periplasm.

### 1.1.2 Periplasmic copper uptake in Gram-negative bacteria

As said before, the periplasm needs copper for a number of activities. Copper, available as soluble Cu$^{2+}$, is likely not limiting in the environment in most cases, since the description of periplasmic importers is rare in the literature. Thus, it is probable that active copper import into the periplasm is generally not required in most conditions faced by bacteria, and porins with promiscuous specificity could be instead used. However, given the array of possible conditions bacteria can survive, it cannot be excluded that in some cases active copper import is required, and in methanotrophic bacteria it has directly been proven. Copper import and sequestration in the periplasm has also been associated with resistance in *P. syringae*, but since the underlying mechanisms are to date not understood, this example will not be discussed further (Cha and Cooksey, 1991; Cooksey and Azad, 1992).

**Porins**

The outer membrane is considered to be a less discriminating barrier when compared to the inner membrane because of the presence of channels with no or broad specificity for small solutes, including metal ions. In the past outer membrane channels OmpB and OmpC have been identified in *E. coli* in screenings for copper resistant mutants, but they seem to also be involved in nutrient uptake (Lutkenhaus, 1977). Seemingly, copper passes through aqueous channels where the discrimination is solely based on size. Porins OmpC and OmpF from *E. coli* have also been linked to resistance to Ag$^+$, known to replace natural copper sites (Li et al., 1997). Moreover, when *Methylosinus trichosporium* OB3b was treated with the
porin inhibitor spermine, the organism showed an inability to accumulate copper when
presented as Cu\(^{2+}\) salt (Balasubramanian et al., 2011). The role of porins as unspecific
channels has been described in various occasions, and it is assumed that copper uses them as
an entrance to the periplasm, when it is not limiting (Nikaido, 2003).

**Pseudomonas NosA/OprC**

*Pseudomonas stutzeri* is a facultative anaerobe, which carries out denitrifying respiration
when oxygen is not available. Protein NosA was identified in a screening of an ICR-191
mutant library. It was missing in mutants able to grow anaerobically in the presence of
nitrate, but not in the presence of nitrous oxide, the first and the last intermediate of the
denitrification pathway, respectively. So generated mutant Δ*\text{nosA}\* still expresses the copper
dependent \text{N}_2\text{O} reductase (see "Background"), but when purified the protein is almost
completely copper free. Unexpectedly, when complemented, the strain could not restore the
\text{N}_2\text{O} reductase activity, indicating that the constructed mutant was defective in another
unidentified gene (Mokhele et al., 1987; Lee et al., 1991). To support this observation, an
insertional targeted inactivation of the *nosA* gene produced a functional \text{N}_2\text{O} reductase,
definitely ruling out the possibility that NosA is required for the enzyme biogenesis.
(Wunsch et al., 2003). Nevertheless despite these results the protein could still be linked with
copper import for the following reasons. (i) The amino-acid sequence revealed a porin-like
structure and shared similarity with outer membrane proteins belonging to the TonB-
dependent receptor family. (ii) The protein is a membrane channel, as experimentally proved
by measuring conductivity through artificial bilayers. (iii) NosA co-purified with copper. (iv)
\text{nosA} expression is down-regulated by copper. (v) The promoter region of \text{nosA} contains a
typical CopR binding region, where CopR from *Pseudomonas syringae* is a Cu-dependent
regulator involved in resistance. The gene is also repressed by oxygen, indicating that it may
be a copper importer in anaerobic copper starvation (Lee et al., 1989; Lee et al., 1991;
Wunsch et al., 2003). Similar observations were collected for the *Pseudomonas aeruginosa*
homologue, OprC, which showed analogous oxygen- and copper-dependent regulation,
channel forming properties and copper binding (Yoneyama and Nakae, 1996). Whether
copper is really imported and whether the import is linked to a TonB-ExbBD system has not
been directly demonstrated, but it is plausible that in some anaerobic and non-oxidizing
environments, copper is highly unsoluble, requiring to be complexed and actively introduced
for the cell to exploit it.
**Methanobactins**

Methanotrophic bacteria have a particular need for copper. Their metabolism is based on the oxidation of methane to methanol, which is then used as a carbon and energy source. This oxidation can be catalyzed by two types of methane monooxygenase. The most represented is the particulate methane monooxygenase (pMMO), a copper dependent inner membrane integral protein (see "Background" section), while in some organisms an iron-dependent soluble methane monooxygenase (sMMO) is alternatively expressed when copper becomes limiting. The underlying regulatory mechanisms required for the switch are not known yet. When pMMO is normally expressed, it accounts for ~20% of total cellular protein, making the demand of copper very high and favoring organisms with high ability to uptake copper in a competitive environment. Copper can indeed become limiting in the habitat of certain methanotrophs, such as subsurface environments, where it is found complexed by organic matter or bound to the surface of metal oxide soils (Semrau et al., 2010).

Mutants constitutively expressing sMMO, even in the presence of copper, were first isolated, and the reason for the missing switch mechanism was attributed to overproduction or missing uptake of a siderophore-like copper-complexing compound. In fact, copper, added as insoluble precipitates was solubilized in the medium of the mutants significantly more than in the wild type, which accumulated more copper in the cell (Phelps et al., 1992; Fitch et al., 1993). Subsequent purification of the pMMO from *Methylococcus capsulatus* Bath revealed the presence of associated soluble copper-containing small compound (hereafter methanobactin, Mb), localized in the extracellular or in the membrane fraction (Zahn and DiSpirito, 1996). Further studies showed that in *M. trichosporium* OB3b the Mb is present at low levels in the extracellular environment when copper is available (5 μM), but it accumulates when copper is limiting. In the mutant Mb accumulated independently of copper concentration. Furthermore in the wild type whole cell preparations or membrane fractions showed the presence of the copper-bound Mb, undetectable in the mutant. A model was then proposed where Mb is secreted constantly, but internalized only when it binds copper. The previously isolated mutants were unable to internalize Mb even when copper was bound, making them valuable strains for the identification of an importer. Apparently, these putative importer mutants are still able to grow normally and respire in low-copper medium, indicating that the Mb pathway is specific for the pMMO and its genetic regulation, while other needs for copper, as for the terminal oxidase, are satisfied in independent ways (DiSpirito et al., 1998; Téllez et al., 1998).
Methanobactin purification became standardized and the research in this field increased substantially, especially after the publication of the first crystal structure of the *M. trichosporium* OB3b isolate (Kim et al., 2004; for a review see Kenney and Rosenzweig, 2012; Fig. 1.3). Today we know that the *M. trichosporium* OB3b methanobactin is a modified peptide with a ~1200 Da mass, binding specifically one Cu$^+$ molecule via a novel S & N coordination, and it is also able to reduce Cu$^{2+}$ (Kim et al., 2004; Hakemian et al., 2005; Choi et al., 2006a; Choi et al., 2006b; Behling et al., 2008). Among the various determined biochemical details, the affinity for Cu$^+$ is remarkably high, $(6-7) \times 10^{20}$ M$^{-1}$, raising the issue of how methanobactin could actually release copper once bound (El Ghazouani et al., 2011). Multimerization was observed as a function of the Cu:Mb ratio, as when above 0.25 four Mb molecules bind one copper with high affinity, but decreasing binding constants have been measured for 0.5 and 1 ratios, where two and one Mb molecules bind one copper ion, respectively (Choi et al., 2006b). Methanobactin is able to solubilize insoluble sources of copper, such as Cu-doped Fe oxide or borosilicates (Knapp et al., 2007; Kulczycki et al., 2007). New X-ray and NMR structures have been also obtained, clarifying diverse biochemical aspects of the compound (Behling et al., 2008; El Ghazouani et al., 2011). Methanobactins have been identified and characterized in *M. capsulatus* Bath, in *Methylomicrobium album* BG8 and *Methylocystis* SB2; for the first two a several-fold lower affinity for copper has been determined, while for the latter species NMR studies have led to
a hypothetical structure. In all of them, differences from the *M. trichosporium* OB3b compound have been observed, indicating that the secretion of such compounds is widespread among the methanotrophs, but that their structure is rather variable, even though some key features, such as specific Cu$^+$ binding, seem to be conserved (Choi et al., 2008; Choi et al., 2010; Krentz et al., 2010; Yoon et al., 2011).

Copper import seems to be the main function of these molecules, but other roles have been demonstrated. Several studies on pMMO regulation agreed that copper is able to trigger the switch only if presented as a methanobactin complex (Knapp et al., 2007; El Ghazouani et al., 2011; Graham and Kim, 2011). The earlier observations on the sMMO constitutive mutants also agree with this, since the phenotype was most likely due to an impaired import of the Cu-Mb complex (DiSpirito et al., 1998; Téllez et al., 1998). Cu-Mb also proved to enhance the pMMO activity and protect against oxidative stress *in vitro*, but the *in vivo* relevance of these features have not been clarified. It cannot be excluded that Mb associate with the pMMO *in vivo* (Choi et al., 2003; Choi et al., 2005; Choi et al., 2008).

Despite the large amount of data collected some fundamental questions are not completely answered yet. Theoretically, the biosynthesis of these compounds could be accomplished either via nonribosomal peptide synthesis or via ribosomal synthesis, requiring in both cases a series of modifications. Since the genome of *M. trichosporium* OB3b became recently available (Stein et al., 2010), candidate genes for the putative precursor of the methanobactins have been identified, making the ribosomal synthesis pathway probable but not excluding nonribosomal option (Behling et al., 2008; Krentz et al., 2010). The latter is in fact known to be used for siderophore biosynthesis, and a putative nonribosomal peptide synthetase system is encoded by two methanotrophs with available genomes (Ward et al., 2004; Stein et al., 2010). Another open question is how these chalkophores (so called for analogy with the siderophores) are internalized by the cell's outer membrane. A recent isotope and fluorescent labeling experiment directly demonstrated that Cu-Mb is actually internalized in the cells. Methanobactin from *M. trichosporium* OB3b features a disulfide bond which is present in both the apo- and in the Cu-form, and is apparently not involved in the Cu$^{2+}$ reduction to Cu$^{+}$ (Fig. 1.3). This characteristic has been exploited by Balasubramanian et al. (2011), who labeled Cu-Mb complex with monobromobimane, a molecule which specifically binds thiol. Among a series of unexpected observations (see section 1.1.4), confocal microscopy revealed that methanobactins are also internalized when not bound to copper. When cells were treated with spermine, a porin inhibitor, unchelated Cu could not be internalized, but Cu-Mb still could. Conversely, when uncoupling agents as carbonyl cyanide, m-chlorophenylhydrazone
and methylamine were used, Cu-Mb entrance was blocked, while unchelated copper could enter. Such chemicals dissipate the proton-motive force across the inner membrane, and they have been reported to inhibit TonB-dependent receptor-mediated uptake of iron siderophores. That means that the most likely methanobactin mechanism of import is dependent on the TonB-ExbBD systems, which energizes an outer membrane receptor exploiting the proton-motive force, as for siderophores. Nevertheless the importer has not been identified yet (Balasubramanian et al., 2011).

In summary, methanobactins are undoubtedly one of the best studied vehicles of copper acquisition. They are needed to route copper from many sources, included insoluble ones, to the periplasm. They are internalized via an active importer. Once in the periplasm (i) they likely bring copper to the pMMO, (ii) they possibly contribute to its activity by association, (iii) and they trigger its expression. It is not clear whether all methanotrophs produce them or not, but the second option is more likely, since no methanobactins seem to be secreted in Methylocystis parvus OBBP (Yoon et al., 2011). It is possible that production of chalkophore is an advantage only when copper availability is restricted, such as in environments with low redox conditions in which copper is scarcely soluble.

**MopE/CorA protein from methanotrophs**

If methanobactin introduction is specific for the pMMO pathway and methanotrophs face conditions in which copper needs to be internalized, there should be an independent pathway to collect copper for other physiological copper-dependent processes. CorA protein was identified by SDS-PAGE as one out of three proteins up-regulated in the absence of copper (compared to 10 $\mu$M copper) in *M. album* BG8, and it co-localizes with the membrane fraction. The $\Delta$corA mutant shows defective growth independent of copper concentration (Berson and Lidstrom, 1997). MopE was identified among the five outer membrane associated protein of *M. capsulatus* Bath, sharing a weak homology with CorA (Fjellbirkeland et al., 1997). The protein was detectable both in the membrane fraction and in the spent medium; the secreted version of the protein lacks a short C-terminal sequence, which is likely cleaved (Fjellbirkeland et al., 2001). Western blot analyses showed that MopE can be found both in the surface-bound and in the excreted form independent of copper concentration. Importantly, its expression was inhibited at a copper concentration 4 $\mu$M, but still expressed at 1 $\mu$M CuSO$_4$, a concentration where sMMO activity was not detectable, because copper was still high enough to support pMMO expression. The observation that the protein levels are not linked to the type of oxygenase used, make the protein a candidate for
pMMO-unrelated copper import (Karlsen et al., 2003). Copper binding of the protein was confirmed by analyzing the metal content in purified MopE by inductively coupled plasma mass spectrometry. A ratio of 0.5-0.65 Cu atoms per molecule was measured. Copper was detected during the MopE crystal structure resolution as well. The structure revealed a novel protein fold and a previously unknown copper binding site, in which a kynurenine, an oxidized derivative of tryptophan, and two histidines form the metal coordination sphere (Fig. 1.4). Interestingly, when the protein was recombinantly expressed in *E. coli* this modification did not take place, and copper was not bound (Helland et al., 2008). To date, it is not known whether the protein transfers copper to outer membrane receptors.

**Figure 1.4.** Copper-binding site of the secreted copper chaperone MopE from *M. capsulatus* Bath containing kynurenine, the result of a modified tryptophan. The yellow sphere represents the copper atom, the red sphere either a water or a hydroxyl molecule (Helland et al., 2008).

### 1.1.3 Proteins for copper insertion into cuproenzymes

Copper, as seen above, is a cofactor for periplasmic enzymes, which usually bind it with high affinity in order to prevent its loss during redox cycling (Szilagyi and Solomon, 2002). Furthermore copper is the most effective transition metal in binding biomolecules (Waldron and Robinson, 2009). Given these two observations, if it was free in the periplasm, copper would not have trouble in finding the binding site of the cuproproteins. In most cases it is possible to create a functional holo-enzyme simply by adding copper *in vitro* (O'Halloran and Culotta, 2000). Of course the situation is not as simple *in vivo*. If periplasmic copper would be uncomplexed, it would displace other metal ions, compromising a number of other functionalities (Waldron and Robinson, 2009). This situation requires abundant copper binding proteins with the function to protect other metal sites, and it is indeed not rare that such proteins have been identified as copper resistance determinants (Cha and Cooksey, 1991; Franke et al., 2003). It is possible that soluble periplasmic cuproenzymes, due to their
high affinity, behave as such proteins, binding Cu\(^{2+}\) as soon as it enters the periplasm via porins, or Cu\(^+\) as soon as it is produced (see section 1.1.1). These kinds of cuproproteins theoretically would not need any chaperone in environmental conditions in which copper is not limiting, but whether this is the case it has not been experimentally confirmed yet. Proteins with the function of delivering copper to enzymes would anyway be required in the following eventualities: inner membrane-bound cuproenzymes, copper starvation conditions, and other situations in which the correct copper center formation needs assistance. In contrast, eukaryotic copper trafficking systems are more complex due to the presence of cellular compartments, and in multicellular organisms the metal must be delivered to the different tissues (O'Halloran and Culotta, 2000). Likely, it is not possible that the extreme copper starved conditions that bacteria would face in their natural niche can be reproduced experimentally. This explains the lack of information about copper insertion in cuproenzymes, simply because some phenotypes could not be detected. Consequently, many studies have focused on heme-copper oxygen reductases in \(\alpha\)-proteobacteria, where the similarity with the mitochondrial complex IV prompted the research (Cobine et al., 2006).

**Chaperones for heme-copper oxygen reductases**

For a summary of the known bacterial proteins for copper insertion into heme-copper oxygen reductases, see Table 1.2 at page 29. Some of the genes that today are known to take part in copper insertion into the prokaryotic cytochrome \(c\) oxidases were first identified in yeast. The current mitochondria model for copper insertion in the cytochrome \(c\) oxidase (CcO) includes proteins Cox17, Cox11 and Sco1. The first would work as a copper shuttle between the cytoplasm and the mitochondrial intermembrane space and deliver copper to the metallochaperones Sco1 and Cox11, both anchored to the inner mitochondrial membrane. Sco1 would then transfer copper to the Cu\(_A\) center located in subunit II of CcO, while Cox11 would be responsible for copper metallation of the Cu\(_B\) center (Fig. 1.5; Cobine et al., 2006). Homologues of Sco1 and Cox11, but not of Cox17, are conserved in bacteria. The evidence in favor of the proposed role of Sco1 and Cox11 in eukaryotes is reported here.

Absence of the gene Sco1 caused a defect in the cytochrome \(c\) oxidase assembly in Saccharomyces cerevisiae and an eventual proteolytic degradation of its subunits (Schulze and Rödel, 1988; Krummkeck and Rödel, 1990). Its overexpression, as well as the overexpression of its homologue Sco2, could restore functional CcO in a Cox17 mutant, but in the case of Sco2 addition of copper was needed. Because Cox17 mutation provokes a CcO defect restorable by copper excess, a role in the transfer of the metal to the CcO was
attributed to Sco1. The CcO defect of a Sco1 mutation was not similarly restorable, leading to speculation that Sco1 plays a downstream role relative to Cox17 in the copper trafficking to the oxidase. Sco2 mutant was not impaired in respiration, it was then considered not essential (Glerum et al., 1996b; Glerum et al., 1996a). In human, Sco1 and Sco2 mutations lead to decreased CcO activity and early death, supporting the hypothesized role of the protein and revealing the need for both the two homologues, unlike in yeast. Mutants of the two Sco have a distinguishable clinical presentation that is not due do tissue-specific expression (Shoubridge, 2001; Leary et al., 2007; Stiburek et al., 2009). A number of clues connect yeast Sco1 with the CuA center of subunit II: (i) proteolytic degradation of the subunit II is more pronounced than for other subunits in Sco1 mutants, (ii) Sco1 missense mutations led to the production of a cytochrome c oxidase missing the subunit 2, but with an unaltered CuB center, (iii) both CuA center and Sco1 bear a CxxxxC copper binding motif, (iv) and, more significantly, in yeast Sco1 and the subunit II co-precipitate. (Krummeck and Rödel, 1990; Dickinson et al., 2000; Lode et al., 2000). Unfortunately co-immunoprecipitation could not be reproduced in the same organism (Rigby et al., 2008). The scientific community has generally accepted the role of Sco1 as a chaperone for subunit 2. All the Sco proteins share a CxxxC motif and an essential histidine, which have been linked to copper binding. Human Sco1 and Sco2 missense mutations map in the vicinity of this domain, and mutational analyses support the fundamental role of this motif in the accumulation of functional CcO in yeast (Rentzsch et al., 1999; Leary et al., 2007). An experiment on purified yeast Sco1 demonstrated for the first time Cu⁺ binding via the two cysteines and the histidine (Nittis et al., 2001), as proposed previously. Later, human Sco1 and Sco2 and yeast Sco1 were revealed to be able to bind both Cu⁺ and Cu²⁺ species at the same binding site, but not to interconvert them. Cu²⁺ binding was observed less frequently and requires an aspartate residue involved also in the in vivo functionality of the protein. In all studies copper-bound protein was found as a purification product (Jaksch et al., 2001; Foltopoulou et al., 2004; Horng et al., 2005). Structures of human Sco1 and Sco2 and yeast Sco1 have been solved, revealing that the globular soluble domain possesses a thioredoxin-fold with exposed active-site cysteines. Given the ability of the two cysteines to form a disulfide bridge when oxidized, a possible dithiol:disulfide reductase function has been speculated. (Williams et al., 2005; Abajian and Rosenzweig, 2006; Banci et al., 2006; Banci et al., 2007). It is likely that both a copper-binding and oxidoreductase activity are coexisting in human Sco1. In this case Sco1 would reduce a hypothetic disulfide bond in the CuA center and transfer there the Cu⁺ ion at the same time (Banci et al., 2006). A similar situation has been shown for copper transfer
from Cox17 to Sco1, where Cox17 is also the reducing agent for Sco1 cysteines (Banci et al., 2008). This hypothesis implies that despite the reducing environment, catalyzed reduction is needed in the intermembrane mitochondrial space.

**Figure 1.5.** Structural representation of the hypothetical copper insertion in the subunit I (Cox1) and subunit II (Cox2) in mitochondria. Cox17 transfers copper to Sco1 and Cox11, which then insert it in the CuB and CuA centers, respectively. Cysteine residues involved in copper binding are depicted in red. Homologues of Sco1 and Cox11 are widespread in bacteria, and an analogous functions have been proposed. Picture from Cobine et al. (2006).

A bioinformatic search shows that Sco proteins are widespread in bacteria and even found in organisms lacking cytochrome oxidases, enlarging the possible range of functions of this family of proteins. All the characterized prokaryotic Sco proteins show conservation of the CxxxC motif, the histidine, but also a DxxxD motif, which includes the aspartate involved in the Cu$^{2+}$ binding identified in yeast (Arnesano et al., 2005). Bacterial Sco-like proteins have been linked to the biogenesis of CuA but also to the biogenesis of CuB centers in different types of heme-copper oxygen reductases. In *B. subtilis* the CuA/CuB-containing *caao* oxidase is defective in a Δ*ypmQ* (Sco-like) mutant, and copper excess could revert the phenotype. The CuB-only menaquinone oxidase activity was not affected. For this and for analogy with the prokaryotic homologue, the phenotype has been attributed to a lack of copper insertion in the CuA center (Mattatall et al., 2000). In *Rhodobacter capsulatus* no CuA centers are present, but still, a defect in respiration was found in a Δ*senC* (Sco-like) mutant. The only copper-
containing terminal oxidase is a \textit{cbb}_3 type, bearing a CuB center, whose low activity could be restored by adding copper in the \textit{ΔsenC} background (Buggy and Bauer, 1995; Swem et al., 2005). In \textit{Rhodobacter sphaeroides}, respiring via a CuB-only \textit{cbb}_3 oxidase and a Cu\textsubscript{A}/CuB-containing \textit{aa}_3 oxidase, \textit{ΔprrC} deletion (Sco-like) causes an impaired accumulation of both oxidases and a copper content reduction in both Cu\textsubscript{A} and CuB sites of the \textit{aa}_3 oxidase in low copper conditions (Thompson et al., 2012). In \textit{P. aeruginosa PA01 \textit{ΔsenC}} strains, a decrease in the \textit{cbb}_3 oxidase activity, replaced by an increased expression of a copper independent cyanide-insensitive quinol oxidase, has been observed (Frangipani and Haas, 2009). In \textit{Agrobacterium tumefaciens} mutation of \textit{sco} results in reduced cytochrome \textit{c} oxidase activity, restorable with copper repletion, but is not known to which extent the defect is due to the \textit{aa}_3 oxidase or to the \textit{cbb}_3 oxidase (Saenkham et al., 2009). In \textit{Bradyrhizobium japonicum}, the \textit{ΔscoI} mutant has a defective \textit{aa}_3 oxidase, but apparently functional \textit{cbb}_3 oxidase; the \textit{aa}_3 defect is restorable by copper excess (Bühler et al., 2010). For most of the bacterial examples listed above the mechanistic role of the Sco proteins is not clear. In many cases is not even clear for which copper center assembly they are needed. In case Sco acts directly for the biogenesis of the Cu\textsubscript{A} center, the doubt of a possible dithiol:disulfide oxioreductase activity is always present. The same cannot be inferred for the CuB center, since the copper coordination sphere does not include oxidizable cysteine pairs. What is certain is that the prokaryotic Cu\textsubscript{A} center needs to be reduced, since it is immersed in the oxidative periplasm, or in the extracellular space, but whether Sco proteins are always responsible is not known. Probably the specific function is species-dependent, making a definition of a generalized Sco function not possible. In \textit{B. subtilis}, YpmQ has been thoroughly characterized biochemically, showing a redox activity and a Cu\textsuperscript{2+} binding activity. There is no agreement about which of the two functions is the physiologically relevant, and a double function as for the eukaryotic homologue has also been postulated (Balatri et al., 2003; Ye et al., 2005; Cawthorn et al., 2009; Davidson and Hill, 2009; Siluvai et al., 2009; Siluvai et al., 2010; Bennett and Hill, 2011; Siluvai et al., 2011; Hill and Andrews, 2012; Lai et al., 2012). In \textit{Thermus thermophilus}, for which no phenotypic data are available, the action of Sco1 is clearly the reduction of the Cu\textsubscript{A} center of the \textit{ba}_3 oxidase \textit{in vitro} (Abriata et al., 2008). Interaction of a Sco protein and the CuB-containing subunit I of the \textit{R. capsulatus cbb}_3 oxidase has been directly detected, as well as the ability of SenC to bind copper. This important information supports the idea of a direct action of Sco proteins also for the CuB centers (Lohmeyer et al., 2012). On the other hand, \textit{R. sphaeroides} PrrC, whose action on both the copper centers has been proved \textit{in vivo}, revealed also a disulfide-reductase activity (Badrick et al., 2007). One
consideration is then possible. The two cysteines of the Sco-like proteins are suitable for both copper binding and disulfide reduction. In order to discriminate the physiological importance of the two functions, which may vary also among closely-related species, the necessity is becoming evident to combine quantitative characterization of the two possible activities with phenotypization. To my knowledge this has been done only in *B. subtilis* and in *B. japonicum*. In the first case the situation is still confused, in the latter it is possible to attribute a role to the Sco protein. The redox potential of *B. japonicum* ScoI was measured, and it does not fit with a reductase function for the Cu\(_A\) center. Copper binding was also observed. ScoI disulfide is reduced itself by another dithiol:disulfide oxidoreductase, namely TlpA, which is a good candidate for the reduction of the Cu\(_A\) center as well. There is sufficient evidence to state that in this organism the *in vivo* function is copper transfer rather than disulfide reduction (Loferer et al., 1993; Loferer and Hennecke, 1994; Bühler et al., 2010; Mohorko et al., 2012).

Similarly to Sco1, mutations of Cox11 lead to defects in assembly of the yeast CcO, with a major degradation observed for the subunit I. The protein localizes in the mitochondrial inner membrane. Its homologue was identified previously in the operon-like locus containing the genes for cytochrome *c* oxidase in *Paracoccus denitrificans*, (Raitio et al., 1987; Tzagoloff et al., 1990). In *R. sphaeroides*, isolation of the *aa*\(_3\) oxidase from Δ*cox11* mutants revealed the presence of an intact Cu\(_A\), but absence of the Cu\(_B\) center. Heme *a* and *a*\(_3\) were still present, while copper was totally missing (Hiser et al., 2000). When purified from yeast, the protein binds one equivalent of copper, and detailed analyses showed that a Cox11 dimer binds two copper atoms via a CxC motif. These residues, when mutated, confer yeast a Δ*cox11*-like phenotype (Carr et al., 2002). The homologue from *Sinorhizobium meliloti* showed conservation of the copper-binding site, and a copper-triggered dimerization. The quaternary structure adopts an immunoglobulin-like β-barrel fold (Banci et al., 2004). The Cu\(_B\) center is buried 13 Å below the membrane surface, requiring particular mechanism for its insertion. Cox11 possesses a conserved cysteine located at the interface between the periplasm and the inner membrane. Mutagenic analyses on *R. sphaeroides* Cox11 showed that this cysteine is not involved in dimerization or copper binding but it is essential for the assembly of the Cu\(_B\) center. It has been proposed that copper is transiently transferred to this residue prior to the insertion in the active site of the *R. sphaeroides* *aa*\(_3\) oxidase (Thompson et al., 2010). In *B. japonicum* the Cox11 homologue, CoxG, is involved in the biogenesis of the *aa*\(_3\) oxidase, but not of the *cbb*\(_3\) oxidase meaning that not all Cu\(_B\) centers are substrates of Cox11 (Bühler et al., 2010).
Table 1.2. Bacterial protein families for copper insertion into heme-copper oxygen reductases. All characterized bacterial proteins and exemplary eukaryotic members are reported.

<table>
<thead>
<tr>
<th>Family</th>
<th>Organism and Gene</th>
<th>Encoded protein features</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sco</td>
<td>Saccharomyces cerevisiae SCO1</td>
<td>Essential for cytochrome c oxidase assembly. Likely for the CuA center. Binds one Cu⁺ via CxxxC plus one His. Can bind also Cu²⁺ via an essential Asp.</td>
<td>Schulze and Rödel, 1988; Krummeck and Rödel, 1990; Rentzsch et al., 1999; Dickinson et al., 2000; Lode et al., 2000; Nittis et al., 2001</td>
</tr>
<tr>
<td></td>
<td>Saccharomyces cerevisiae SCO2</td>
<td>Not essential for cytochrome c oxidase activity.</td>
<td>Glerum et al., 1996a; Glerum et al., 1996b; Jakob et al., 2001</td>
</tr>
<tr>
<td>Homo sapiens SCO1</td>
<td>Essential for cytochrome c oxidase assembly. Binds one Cu⁺ via CxxxC plus one His. Can bind also Cu²⁺ via an essential Asp. Hypothesized concomitant Cu⁺ transfer and disulfide reduction of the Cu₄ center.</td>
<td>Shoubbridge, 2001; Horng et al., 2005; Williams et al., 2005; Abajian and Rosenzweig, 2006; Banci et al., 2006; Leary et al., 2007; Stiburek et al., 2009</td>
<td></td>
</tr>
<tr>
<td>Homo sapiens SCO2</td>
<td>Essential for cytochrome c oxidase assembly. Not overlapping with SCO1 function. Binds one Cu⁺ via CxxxC plus one His. Can bind also Cu²⁺ via an essential Asp. Binds Cu⁺ and Cu²⁺, and shows redox activity.</td>
<td>Shoubbridge, 2001; Leary et al., 2007; Foltopoulou et al., 2004; Horng et al., 2005; Banci et al., 2007</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis ypmQ</td>
<td>Essential for the Cu₄/Cu₈ containing cdd oxidase, not for the Cu₄-only menaquinone oxidase. Binds Cu⁺ and Cu²⁺ and shows redox activity.</td>
<td>Mattattall et al., 2000; Balanti et al., 2003; Ye et al., 2005; Cawthorn et al., 2009; Davidson and Hill, 2009; Silanikove et al., 2009</td>
<td></td>
</tr>
<tr>
<td>Rhodobacter capsulatus senC</td>
<td>Essential for Cu₄-only cbb₃ oxidase. Cross-links with Cu₄-containing subunit I.</td>
<td>Swern et al., 2005; Lohmeyer et al., 2012; Thompson et al., 2012; Badrick et al., 2007</td>
<td></td>
</tr>
<tr>
<td>Rhodobacter sphaeroides prrC</td>
<td>Involved in assembly of Cu₄ and Cu₈ centers in aa₃ oxidase and of Cu₄ center of cbb₃ oxidase. Disulfide-reductase activity.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PA01 senC</td>
<td>Essential for Cu₄-only cbb₃ oxidase.</td>
<td>Frangipani and Haas, 2009</td>
<td></td>
</tr>
<tr>
<td>Agrobacterium tumefaciens scoC</td>
<td>Essential for cytochrome c oxidase activity (aa₃ and cbb₃ oxidases combined activities).</td>
<td>Saenkham et al., 2009</td>
<td></td>
</tr>
<tr>
<td>Thermus thermophilus TTHA1942</td>
<td>Reduces cysteines of aa₃ oxidase Cua center, but does not transfer copper, in vitro.</td>
<td>Abriata et al., 2008</td>
<td></td>
</tr>
<tr>
<td>Streptomyces scoC</td>
<td>Needed for cytochrome c oxidase activity. Required for amine oxidase, tyrosinase and laccase activities.</td>
<td>Fujimoto et al. 2012</td>
<td></td>
</tr>
<tr>
<td>Bradyrhizobium japonicum scoC</td>
<td>Essential for aa₃ oxidase biogenesis. Binds Cu⁺² via CxxxC. Redox potential too high for disulfide reductase.</td>
<td>Bühler et al., 2010; Mohorko et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Cox11</td>
<td>Saccharomyces cerevisiae COX11</td>
<td>Essential for cytochrome c oxidase assembly. A dimer binds two Cu⁺ via CxC.</td>
<td>Tragolaroff et al., 1990; Carr et al., 2002</td>
</tr>
<tr>
<td></td>
<td>Paracoccus denitrificans ctaG</td>
<td>Part of the cytochrome c oxidase gene cluster. Essential for Cu₄ center assembly of the aa₃ oxidase.</td>
<td>Raitio et al., 2002; Hiser et al., 2006; Thompson et al., 2010; Abriata et al., 2004; Bühler et al., 2010</td>
</tr>
<tr>
<td>Rhodobacter sphaeroides cox11</td>
<td>Binds Cu⁺ via CxC. Dimerizes in presence of copper. Essential for the Cu₄/Cu₈ aa₃ oxidase biogenesis, but not for the Cu₄-only cbb₃ oxidase biogenesis.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sinorhizobium meliloti cox11</td>
<td>Essential for the Cu₄/Cu₈ aa₃ oxidase biogenesis.</td>
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<td></td>
</tr>
<tr>
<td>Bradyrhizobium japonicum coxG</td>
<td>Essential for Cu₄ oxidase assembly, likely for the Cu₄₅ center. Binds one Cu⁺ via CxxxC plus one His. Can bind also Cu²⁺ via an essential Asp. Hypothesized concomitant Cu⁺ transfer and disulfide reduction of the Cu₄ center.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCu₄C</td>
<td>Deinococcus radiodurans DR1885</td>
<td>Cu⁺⁺ binding via three M and one H. Cu⁺⁺ binding via two M and two H. Transfers two equivalents of Cu⁺⁺ to Cu₄ center of ba₃ oxidase in vitro.</td>
<td>Banci et al., 2005; Abriata et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Thermus thermophilus TTHA1943</td>
<td>Essential for Cu₄ oxidase assembly, likely for the Cu₄₅ center. Binds one Cu⁺ via CxxxC plus one His. Can bind also Cu²⁺ via an essential Asp. Hypothesized concomitant Cu⁺ transfer and disulfide reduction of the Cu₄ center.</td>
<td></td>
</tr>
<tr>
<td>Rhodobacter sphaeroides RSP, 2017</td>
<td>Involved in assembly of Cu₄ and Cu₈ centers in aa₃ oxidase and of Cu₄₅ center of cbb₃ oxidase.</td>
<td>Thompson et al., 2012</td>
<td></td>
</tr>
<tr>
<td>FixL/Cool</td>
<td>Bradyrhizobium japonicum fixL</td>
<td>Essential for cbb₃ oxidase.</td>
<td>Preising et al., 1996a; Koch et al., 2000; Ekici et al., 2012a</td>
</tr>
<tr>
<td>Rhodobacter capsulatus ccoL</td>
<td>Essential for cbb₃ oxidase.</td>
<td>Haas et al., 2007</td>
<td></td>
</tr>
<tr>
<td>Rubrivivax gelatinosa ctpA</td>
<td>Needed for N₂O reductase, Caa₃ and Cbb₃ oxidases.</td>
<td>Hassani et al., 2010</td>
<td></td>
</tr>
<tr>
<td>MFS</td>
<td>Rhodobacter capsulatus ccoA</td>
<td>Essential for cbb₃ oxidase. Involved in copper accumulation.</td>
<td>Ekici et al., 2012b</td>
</tr>
</tbody>
</table>
As reported above, Sco proteins have been linked to the biogenesis of CuB centers in both $aa_3$ and $cbb_3$ oxidases, but other mechanisms involved in $cbb_3$ biogenesis have been identified. In *B. japonicum*, mutation of the putative P$_{1B}$-type ATPase FixI leads to a defective $cbb_3$ oxidase. (Preisig et al., 1996a). Other two analogous situations have been described. *R. capsulatus* CcoI lack or mutation in its N-terminal metal binding domain causes a drastic decrease in the steady-state amounts of the $cbb_3$ subunits, not restorable with copper addition. Moreover the mutant is more resistant to Cu, indicating a possible import function as for other P$_{1B}$-type ATPases (see section 1.1.4) needed for the copper insertion (Koch et al., 2000; Ekici et al., 2012a). *Rubrivivax gelatinosa* CtpA also belongs to the same family, and its mutation led to a drastic reduction in both $cbb_3$ and $caa_3$ oxidase activity, but also in nitrous oxide reductase activity (Hassani et al., 2010). In all the three cases the gene for the ATPase localizes near the $cbb_3$ structural genes, and this situation seems to be well conserved. Unfortunately nothing more is known about a possible mechanism. Recently CcoA has been discovered as necessary for the $cbb_3$ biogenesis in *R. capsulatus*, adding to SenC and CcoI. It is a member of the major facilitator superfamily (MFS) and it was identified in a genetic screen for $cbb_3$ oxidase defective mutants restorable by Cu$^{2+}$ supplementation. The ΔccoA strain also features a decreased accumulation of copper, indicating that it may have a copper import function (Ekici et al., 2012b). It is possible that specific structural features of the $cbb_3$ oxidase complexes require copper to be imported prior to be transferred to their CuB centers, unlike $aa_3$ oxidases.

Another category of bacterial copper chaperones has been discovered. The PCu$_{1A}$C family members are encoded by conserved neighbours of some bacterial sco genes, predicted to be soluble periplasmic proteins; because of that a Cox17-like function was initially proposed. Structural analyses of the *Deinococcus radiodurans* homologue revealed that PCu$_{1A}$C binds to one Cu$^+$ via a novel MX$_{10}$MX$_{21}$HXM motif (Banci et al., 2005). In vitro studies on *T. thermophilus* PCu$_{1A}$C showed that in this organism Sco reduces the cysteines of the $ba_3$ oxidase Cu$_A$ center, while two PCu$_{1A}$C equivalents transfer both copper ions (*Tt*PCu$_{1A}$C structure is represented in Fig. 1.6). In this organism the Cu$^+$ binding site is constituted by two histidines and two methionines (HX$_{10}$MX$_{21}$HXM), as in the majority of the homologues (Abriata et al., 2008). *R. sphaeroides* strains where PCu$_{1A}$C was deleted showed a phenotype similar to that observed in ΔprrC strains of the same organism: reduced accumulation of both the $aa_3$ and $cbb_3$ oxidases and reduced copper content in both the copper centers of the $aa_3$ oxidase. The only difference between the two mutants was the seriousness of the defects, specifically they were higher in strains missing PrrC. Thus, the working model would be that
PCuAC transfers copper to the membrane-bound PrrC, which is then responsible for copper transfer to the Cu_A and Cu_B centers of both the oxidases (Thompson et al., 2012). The mechanism by which this is accomplished is to date unknown. The role of PcuC, the PCuAC homologue of \textit{B. japonicum}, will be discussed in chapter 2.

\textbf{Figure 1.6.} The cupredoxin-like structure of PCuAC from \textit{Thermus thermophilus} as determined by NMR (PDB ID: 2K6Z). Copper(I) is indicated by the orange sphere, methionine by blue sticks, histidines by red sticks.

\textbf{Copper transfer to the N}_2\textbf{O reductase}

The biogenesis of the N\textsubscript{2}O reductase, the enzyme responsible for reduction of N\textsubscript{2}O to N\textsubscript{2}, has been studied in depth (for a review see Zumft and Kroneck, 2007). In several denitrifying bacteria, the \textit{nosZ} enzyme gene is located clustered with other conserved genes in a \textit{nosRZDFYLX} organization, where \textit{nosX} is not always occurring. Moreover the gene encoding a Sco protein is always present in their genomes as well (Wunsch et al., 2003). Since the enzyme contains a Cu\textsubscript{A} and a [4Cu:2S] Cu\textsubscript{Z} center (Fig. 1.2), it has been a challenge to identify, if existing at all, which of these genes owns a copper metallochaperone activity. The maturation of the metal sites is likely happening in the periplasm in \textit{P. stutzeri}, since TAT export system mutants accumulate copper-free cytoplasmic N\textsubscript{2}O reductase which is correctly folded (Dreusch et al., 1997). The same situation is observed when N\textsubscript{2}O reductase is isolated from wild type strains grown in copper starvation (Wunsch et al., 2003). Gene \textit{nosR} is likely encoding a transcriptional regulator and/or an electron donor/acceptor, excluding it from the candidates (Wunsch and Zumft, 2005). Mutants of the genes \textit{nosX}, called \textit{aphE} in \textit{P. stutzeri}, show defective N\textsubscript{2}O respiration, but on the other hand \textit{P. denitrificans ΔnosX} mutants
produce a holo-enzyme, and many denitrifying organisms accomplish N$_2$O reduction in absence of this. The role of NosX in N$_2$O reduction is more likely in electron transfer to the enzyme, since it is likely flavine-cofactored (Chan et al., 1997; Saunders et al., 2000; Wunsch et al., 2003; Wunsch et al., 2005). NosDFY are similar to bacterial ABC transporter-type ATPases, inner membrane proteins involved in import and export of a wide range of compounds. Strains of $P$. stutzeri where any of the protein of this complex are inactivated lose their N$_2$O reductase activity, and the isolated enzyme is lacking the CuZ center (Riester et al., 1989; Zumft and Kroneck, 1996; Zumft, 1997; Rasmussen et al., 2000). It has been shown that only these three genes, together with NosZ, are required to form a functional enzyme when inserted in the non-denitrifying host $P$. putida. In the same experiment, the presence of nosRZ only caused the accumulation of an inactive N$_2$O reductase lacking the CuZ center, confirming the mutagenic analyses (Wunsch et al., 2003). The actual function of this putative transporter is not known and it is not predictable by sequence analysis. Only the ATPase activity has been demonstrated for the NosF subunit (Honisch and Zumft, 2003). Whether this system is needed for copper or for the sulfur required for the CuZ center is not known. NosL is a predicted lipoprotein of the outer membrane (Dreusch et al., 1996). NosL from Achromobacter cycloclastes owns a specific Cu$^+$-binding ability, possibly involving a cysteine, which is poorly conserved (McGuirl et al., 2001). Despite these features, a $\Delta$nosL $P$. stutzeri strain is not defective in N$_2$O reduction, and nosL is not needed to constitute a functional reductase in $P$. putida. Since the gene is a conserved member of the nos gene cluster it is possible that its function is replaceable by paralogues (Dreusch et al., 1996; Wunsch et al., 2003). Finally, the Sco-like protein ScoP from $P$. stutzeri has been considered a candidate for Cu$_A$ biogenesis of the N$_2$O reductase, for analogy to what was observed for the heme-copper oxygen reductases. Nevertheless its inactivation had no effect on the enzyme even in Cu-free medium. Interestingly, the gene locates in proximity of the $cbb_3$ oxidase gene cluster (Wunsch et al., 2003).

**Chaperone-dependent biogenesis and secretion of bacterial tyrosinases**

Tyrosinases have been identified in a variety of bacteria, but the best studied members are produced by the Gram-positive Streptomyces. They are encoded in a bicistronic operon bearing the melC1 and melC2 genes, where the first is a helper protein and the second is the structural gene. Early studies in Streptomyces antibioticus showed that $\Delta$melC1 strains accumulated an inactive apo-tyrosinase, whose activity was restored only partially by incubation with copper (Lee et al., 1988). A stable 1:1 MelC1-MelC2 complex could be
isolated via gel-filtration from strains grown in copper-deficient medium both in the intracellular and the extracellular fractions. Addition of copper caused the release of an active tyrosinase from the complex (Chen et al., 1992). MelC1 bears a TAT recognition sequence, which is not present in MelC2. When an arginine of the MelC1 TAT sequence was mutated, MelC2 was not exported, and it accumulated in the cell, whose extracts were deprived of tyrosinase activity even after copper incubation. Cell extract from another MelC1 point mutant which prevented MelC2 secretion, revealed that the activity was still present, whilst in the wild type, tyrosinase activity was equally distributed in cellular and extracellular extracts. Measurement of the tyrosinase activity requires cell lysis, meaning that free copper in the medium can reach components from which it is normally separated. Given these observations, it appears that MelC1 accomplishes a double action: activation of the protein for the correct placement of the metal and assistance for secretion. The mutated arginine has a role in the activation of the protein, while for the second point mutant only the export was impaired. It is not possible to say whether copper is inserted in the cytoplasm or extracellularly. Considering that the binary complex is needed for secretion and that copper dissociates the binary complex, the second option is most likely (Leu et al., 1992). A follow-up study on MelC1 substitutions identified two histidine residues as crucial for the activation of MelC2, and also showed that MelC1 causes conformational changes in MelC2 (Chen et al., 1993). Mutation of the copper binding sites in MelC2 rendered the complex unable to dissociate in the presence of copper, but it was still secreted. Likely, the correct insertion of copper in the binding site of MelC2 causes a conformational change in the tyrosinase which leads to the dissociation of the complex (Tsai and Lee, 1998).

Copper transfer to the Gram-negative Marinomonas mediterranea tyrosinase has also been analyzed. In this organism the structural gene encoding the enzyme PpoB1 is followed by ppoB2 in a co-transcribed unit. Cellular extracts from a ΔppoB2 strain grown in copper-deficient medium, but not in standard medium, are devoid of tyrosinase activity. A partial restoration of the wild type activity was possible by incubating ΔppoB2 extracts with copper. PpoB2 is a predicted transmembrane protein and bears CxxxC, MxxxMM and His-rich potential copper binding sites. Complementation by random integration of ΔppoB2 with wild type and substituted versions of ppoB2 revealed that all potential copper binding sites are necessary for PpoB2 activity (López-Serrano et al., 2004; López-Serrano et al., 2007). PpoB1 is found in the soluble fraction of M. mediterranea, but whether it is cytoplasmic or periplasmic it is not known (Fernández et al., 1999), even though no signal peptide is present.
The need for a helper protein for copper insertion has not been observed for all tyrosinases, and for many examples, unlike MelC2 from *Streptomyces*, it is possible to obtain a functional protein simply with the addition of copper *in vitro* (Fairhead and Thöny-Meyer, 2012). This does not exclude that *in vivo*, a dedicated copper insertion pathway is required.

### 1.1.4 Copper import and trafficking in the cytoplasm

To date, there is only one situation in which copper is clearly needed to cross the cytoplasm. This is the case of some photosynthesizing cyanobacteria, where both photosynthesis and respiration are carried out in the thylakoids (see below). Since plastocyanins are translocated in the thylakoids via the Sec pathway (Cavet et al., 2003), and cytochrome oxidases are folded in the thylakoid membrane with the help of other accessory proteins (Wang and Dalbey, 2011), copper needs to cross the cytoplasm from the periplasm to the thylakoid to be inserted in these cuproproteins, tightly bound to metallochaperones.

Copper importers have been characterized also in organisms apparently not requiring it in the cytoplasm, and they will be described in this section. The physiological relevance of the import remains often elusive but it may be necessary for the following situations: (i) for the biogenesis of not yet identified cytoplasmic copper enzymes, (ii) for the metallation of proteins which are then exported as folded via the TAT pathway, (iii) for the creation of particular copper sites in transmembrane proteins, as likely for *cbb*$_3$ oxidases, (iv) for accumulation in Gram-positive bacteria exposed to copper starvation, which then would store it tightly bound to dedicated biomolecules and release it for the biogenesis of cuproenzymes, (v) for copper transfer to sensor molecules which would then activate efflux mechanisms. Even though scenarios like the one reported in the last point have been hypothesized, they are difficult to interpret. One would in fact expect that soluble cytoplasmic regulators with high affinity sense copper as soon as it enters accidentally the cytoplasm as a consequence of high extracellular/periplasmic concentration. There would be no need to activate export if copper does not enter the cytoplasm passively, making then the utilization of an importer of little sense if the aim is to clear the cytoplasm of copper. On the other hand it is possible that importers are needed to channel copper ions directly to the sensor proteins minimizing the risk of having free copper passively entering the cytoplasm.

Many of the works here reported had to face technical challenges, such as the measurement of small amounts of copper entering the cytoplasm, or determining the direction of ion flow. As a consequence, the definition of copper importer is often missing conclusive
biochemical proof. Clarifying issues related to the function and the nature of copper import in bacteria is surely a challenging goal for future studies.

**P_{1B}-type ATPases and cognate copper chaperones**

Copper ATPases are the best studied transporters for this metal. The first description of a P-type ATPase as a copper transporter was in *E. hirae* (Odermatt et al., 1992) and since then the organism has become a paradigm for copper homeostasis. The search for homologues has led to the characterization of copper ATPases in all the kingdoms of life (Argüello et al., 2007), among them the human ATP7A and ATP7B, whose mutations lead to Menkes and Wilson diseases, respectively (Bull et al., 1993; Chelly et al., 1993; Mercer et al., 1993; Tanzi et al., 1993; Vulpe et al., 1993). All the copper ATPases belong to the P_{1B}-type group which is in turn a subgroup of the heavy metal transporter P-type ATPase, where P indicates a phosphorylation mechanism. They feature 6 to 8 transmembrane helices, where the three last C-terminal helices contain the proposed metal binding site for transport, and a series of cytoplasmic domains: an N-terminal soluble metal-binding domain, a large loop containing the ATP binding site and the phosphorylation domain, and loop containing the actuator domain (Solioz and Stoyanov, 2003; Argüello et al., 2007). The N-terminal metal-binding domain is not needed for the ATPase or transfer activities (Voskoboinik et al., 1999; Fan and Rosen, 2002; Mana-Capelli et al., 2003; Mandal and Argüello, 2003), and it is constituted of up to 2 (or 6 in eukaryotes) ferredoxin-like units, in many cases bearing a CXXC metal-binding motif (Argüello et al., 2007). Its interaction with a cognate protein which shares the same folding and metal-binding motif, with simultaneous transfer of copper, has been well documented in eukaryotes and bacteria (Larin et al., 1999; Wernimont et al., 2000; Multhaup et al., 2001; Tottey et al., 2002; Banci et al., 2003). The vast majority of characterized P_{1B}-type ATPases are exporters. In only three cases has import function been inferred: in *E. hirae* (Odermatt et al., 1994; Wunderli-Ye and Solioz, 2001), *Listeria monocytogenes* (Francis and Thomas, 1997a; Francis and Thomas, 1997b) and *Synechocystis* (Tottey et al., 2001). The catalytic mechanism for export has been studied in detail in other metal transporter ATPases. Copper exporter ATPases likely follow the same mechanism, as a number of biochemical studies corroborate this hypothesis. Since the orientation of the helices is ascertained, an import function would require a novel mechanistic explanation (Argüello et al., 2007).

The copper homeostatic machinery of the Gram-positive *E. hirae* includes the copper P_{1B}-type ATPase CopA. In the proposed working model this protein is defined as a copper importer because of the following observations: (i) strains show a defective grow in media in
which copper is complexed by the chelator 8-hydroxyquinoline (ii) ΔcopA strains are more resistant to Ag⁺ than in the wild type. It is known in fact that silver often binds to copper binding sites and its toxicity is much higher. Observations of ambiguous interpretation have been collected. CopA is located in the same operon of CopB, another P1B-type ATPase, which is on the contrary a copper efflux pump; the two proteins are then co-regulated. When copB is mutated copper accumulates in the cell during copper stress, but the same has not been observed for ΔcopA (Odermatt et al., 1994). Western blot analyses showed that the operon is up-regulated both in copper stress (20 mM CuSO₄) and in copper starvation mediated by the Cu²⁺ chelators o-phenanthroline, 8-hydroxyquinoline, and tetrathiomolybdate, but not by the Cu⁺ chelators BCA and BCS. Considering that copper is environmentally soluble as Cu²⁺ the latter observation is explainable by an internalization of copper in the +1 oxidation state, as a consequence of an extracellular Cu²⁺ reductase activity (Odermatt et al., 1993; Odermatt et al., 1994; Wunderli-Ye and Solioz, 1999). The cop operon is under the control of the repressor CopY, which is released from the DNA by Cu⁺ (Odermatt and Solioz, 1995; Strausak and Solioz, 1997; Cobine et al., 1999; Cobine et al., 2002). This mechanism does not account for the chelator-mediated up-regulation of the operon, requiring the existence of a separate regulation pathway. Mutants of the CopY-binding site in fact show a hyperinduction of the cop operon during copper stress but not when grown in presence of o-phenanthroline or tetrathiomolybdate Cu²⁺ chelators (Wunderli-Ye and Solioz, 1999). CopA has been purified and its ATPase activity was measured in vitro, as well as its inhibition by vanadate, typical of P-type ATPases. Inhibition was also observed in presence of Cu⁺ chelators, but not with Cu²⁺ chelators indicating that the pump is specific for Cu⁺ ions (Wunderli-Ye and Solioz, 2001). Like many other copper ATPases, CopA interacts with the cognate cytoplasmic chaperone CopZ. Both proteins own a CxxC motif, whose function in binding Cu⁺ has been demonstrated for CopZ, and it is extremely likely for the metal binding domain of CopA, given the characterized homologues. The interaction itself does not require copper, but the metal modulates it, by lowering the dissociation constant. A mutation of the CopA CxxC motif does not affect the interaction, but copper loses its ability to modulate it. The direction of the transport remains to be assessed in detail (Muthaup et al., 2001). Moreover CopZ is able to donate Cu⁺ to the repressor CopY in vitro. The transfer is likely one-directional because of the higher affinity of CopY for Cu⁺, and, also in this case, requires surface compatibility between the partners (Cobine et al., 1999; Cobine et al., 2002). Since ΔcopZ causes reduced expression of the cop operon (Odermatt and Solioz, 1995), it is likely that during copper stress CopZ transfers Cu⁺ to CopY which then de-
represses transcription of the operon at high copper levels. A scheme of the hypothesized model is reported in Fig. 1.7 B. To clarify the role of CopA as an importer, the direction of the transfer between CopA and CopZ, and the up-regulation of the operon in presence of copper chelators, further studies are needed.

![Figure 1.7](image)

**Figure 1.7.** Schematic representation of the working model for copper import and trafficking in *Synechocystis* PCC 6803 (A) and *E. hirae* (B). Copper is symbolized by brown circles, arrow indicates the direction of the transfer, yellow shapes indicate P$_{1B}$-type ATPases, blue shapes Atx-like molecules.

Up-regulation of a copper ATPase in presence of both copper stress and starvation has been observed in the Gram-positive *L. monocytogenes* as well. A divalent cation sensitive strain was identified in a transposon library of randomly integrated reporter genes. The fished promoter belonged to the *ctpA* gene, encoding a P-type ATPase with a high degree of similarity to characterized copper transporters. One insertion and one replacement Δ*ctpA* mutant were generated, showing that growth was inhibited by 5 μM 8-hydroxyquinoline, likely because of Cu$^{2+}$ chelation. RNA hybridization showed an up-regulation of the *ctpA* gene in the presence of 4 mM CuSO$_4$, 10 mM EGTA (a divalent ion chelator) or 5 μM 8-hydroxyquinoline, respectively, but not by Cd$^{2+}$, Hg$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ (Francis and Thomas, 1997a). HeLa and J774 cell lines were infected with the mutants without showing any particular phenotype, but when a mouse infection experiment was carried out, mutants were cleared more rapidly than the wild type from the liver but not from the spleen. Unfortunately control strains with random integration were not included in this study. One interpretation of these results would be that copper import and sequestration is required during infections,
since one possible defense of the host is decreasing the availability of the biometals in the serum. CtpA would then be an importer (Francis and Thomas, 1997b). Nevertheless, no evidences exclude that CtpA is instead an exporter; it is in fact known that hosts often use copper excess to clear a pathogen infections (Hodgkinson and Petris, 2012; Samanovic et al., 2012).

In many cyanobacteria thylakoids are the sites of both photosynthesis and the respiratory electron transport chain, even though is not clear whether respiration is functional in this compartment. *Synechococcus* PCC 7492 was the first cyanobacterium where copper ATPases, PacS and CtaA, were identified. The *pacS* transcript and product were detected when 5 or 10 μM CuSO₄ was added to the standard medium (0.3 μM CuSO₄) with Northern and Western blot analyses, respectively. Immunoblots also showed that PacS localizes to the thylakoid membrane but not to the inner and outer plasma membranes. A strain carrying a ΔpacS mutation showed an impaired growth in 10 μM CuSO₄-containing medium (Kanamaru et al., 1994). On the contrary, a ΔctaA mutant grew better than the parental strain in the presence of 10 μM CuSO₄ (Phung et al., 1994). *Synechocystis* PCC 6803 has been shown to possess respiratory systems as well as the photosystems I and II both in thylakoids and in the plasma membrane, but there is evidence that photosynthesis is only active in the thylakoids (Zak et al., 2001). As for other cyanobacteria and green algae, *Synechocystis* PCC 6803 adapts to copper deficiency by switching from copper containing plastocyanins (PetE) to cytochrome c₆ (PetJ) utilization as the photosynthetic electron carrier (Zhang et al., 1992). Homologues of the two *Synechococcus* PCC 7492 ATPases were found in this organism and named in the same way. Deletion of *pacS* in *Synechocystis* PCC 6803 led to a reduced tolerance to copper, with a complete block of growth in the presence of 1 μM CuSO₄, while no phenotype was observed in a ΔctaA mutant. Furthermore, ΔctaA strains accumulated less copper than the wild type, while ΔpacS behaved as the parental strain. In both mutants a switch toward the utilization of the copper-independent cytochrome c₆ at copper concentrations in which the wild type could still use plastocyanins as electron carriers was observed, as revealed from both spectroscopic and transcript analyses. This situation could be partially reverted by adding copper to the ΔctaA mutant. The addition of BCS to the medium of a strain carrying a ΔctaA ΔpetJ double mutation resulted in impaired growth when compared to a ΔpacS ΔpetJ mutant. Finally, total cytochrome c oxidase activity was lower in ATPases mutants than in the wild type in copper-free medium (Tottey et al., 2001). The working hypotheses is then that CtaA would work as a copper importer to the cytoplasm, while PacS would "export" the copper from the cytoplasm to the thylakoids. Thus, other
mechanisms are likely needed for copper routing to plastocyanins and cytochrome oxidase. The increased sensitivity to copper in ΔpacS strains could be explained with a smaller toxicity exerted by copper in the thylakoid compared to the cytoplasm. Also in this case a cognate copper chaperone has been identified. Named Atx1 after the characterized yeast ATX1 copper chaperone, the protein binds one copper atom via a CxxC motif in vitro. A bacterial two-hybrid assay showed interaction, dependent on the two cysteines, between Atx1 and the N-terminal metal binding domain of PacS, as for a many copper ATPases. The same assay showed also binding to CtaA, but not to a cobalt and a zinc ATPase. Copper was transferred from Atx1 to the N-terminal part of PacS in incubation experiments followed by chromatography, but the opposite direction of transfer was not tested. Strain carrying Δatx1 showed a reduced cytochrome c oxidase activity. When double mutants ΔpacS/Δatx1 and ΔctaA/Δatx1 were constructed, the atx1 deletion did not seem to further decrease the activity observed in ΔpacS, while it revealed to be synergic with the ctaA inactivation. In addition, the switch to the cytochrome c₆ resulted enhanced in the ΔctaA/Δatx1 double mutant compared to the ΔctaA single mutant. Atx1 is most likely a copper chaperone ferrying copper from the outer membrane importer CtaA to the thylakoid importer PacS and, given the synergistic effect of a ΔctaA Δatx1 double deletion, it is probably still able to gather copper without the importer (Fig. 1.7A; Tottey et al., 2002).

CopCD proteins

In P. syringae and E. coli, plasmid-borne copper resistance systems were already identified before the advent of the genomic era (Tetaz and Luke, 1983; Bender and Cooksey, 1987; Mellano and Cooksey, 1988a; Mellano and Cooksey, 1988b). In both organisms, plasmids contain homologous genes, copABCDRS in P. syringae and pcoABCDRS in E. coli. Despite the plasmids confer resistance in both species, the P. syringae copC and copD genes are able to cause copper hypersensitivity when inserted together in a strain missing the resistance plasmid, but not when they are singularly introduced. Since CopC is a periplasmic protein and CopD is a probable inner membrane protein, an import function was hypothesized (Cha and Cooksey, 1993). The import function should anyway be linked to copper detoxification, given that the two genes are required for a full resistance in P. syringae (Mellano and Cooksey, 1988b). Both CopC and PcoC revealed to be able to bind Cu⁺ and Cu²⁺ simultaneously at two different sites, specific to each ion. The peculiarity of these proteins is that the bound Cu⁺ is oxidized by O₂ to Cu²⁺ only if another CopC molecule with a free Cu²⁺ site is available. If this is the case, the newly formed Cu²⁺ is transferred there,
otherwise the Cu\(^{+}\)Cu\(^{2+}\)-CopC complex is stable in the oxidizing periplasm. In this way the protein buffers Cu\(^{2+}\) ions by balancing them with the Cu\(^{+}\) species (Arnesano et al., 2003; Koay et al., 2005; Zhang et al., 2006; Djoko et al., 2007). How these features affect on a larger scale copper resistance and/or acquisition has not been explained yet.

In many organisms a fusion of the \emph{copCD} genes occurs, but only one of them, \emph{B. subtilis} ycnJ, has been studied. While ORFs \emph{ycnK}, \emph{ycnJ} and \emph{ycnI} are all subsequent in the chromosome, \emph{ycnL} share the promoter region with \emph{ycnK}, but it is in the opposite orientation. Gene \emph{ycnJ} encodes an N-terminal CopC-like domain, where only the Cu\(^{2+}\) binding residues are conserved, and a transmembrane CopD-like C-terminal domain. The cysteine-rich YcnK product is a likely transcriptional regulator with an N-terminal DNA binding domain and a C-terminal domain with hypothesized sensor function, belonging to the Cu\(^{+}\)-binding NosL superfamily. Gene \emph{yncL} encodes a cysteine-rich putative reductase/disulphide isomerase. \(\Delta\text{ycnK}\) mutants showed enhanced growth in copper excess (1 mM CuCl) when compared to the wild type, while \(\Delta\text{ycnJ}\) mutants grow defectively in the presence of 0.5 mM BCS. All the 5 genes show up-regulation in microarray analyses when RNA was extracted from wild type cells grown in presence of 0.25 mM BCS compared to standard medium, and down regulation when RNA was isolated from cells growing in copper excess (0.5 mM CuCl) compared to standard medium. According to this, YcnJ would be an importer necessary during copper starvation. The role of YcnK as a \emph{ycnJ} repressor during copper availability was tested. Dot blot analyses showed an up-regulation of \emph{ycnJ} in a \(\Delta\text{ycnK}\) background in standard medium, but not as high as when the wild type strain faces copper starvation. To obtain such an up-regulation level, a double \(\Delta\text{ycnK}/\Delta\text{csoR}\) deletion was necessary, where in a \(\Delta\text{csoR}\) mutant copper resistance efflux sytems are constitutively derepressed. This led to the conclusion that CsoR directly participates in \emph{ycnJ} regulation. Inductively coupled plasma mass spectrometry was used to quantify copper in dry cells. In copper stress, a two-fold accumulation of copper was observed in \(\Delta\text{ycnK}\), while in \(\Delta\text{ycnJ}\) the amount did not differ from the wild type. Unexpectedly, in presence of BCS, the wild type accumulated more copper than in the standard medium, while in \(\Delta\text{ycnJ}\) the accumulation was the same in both cases. This observation fits with a model in which YcnK negatively regulates the importer YcnJ when copper is not limiting. When the YcnJ N-terminal periplasmic domain was purified and loaded on native PAGE gel, an oligomerization of the protein was detected only when Cu\(^{2+}\) was added (Chillappagari et al., 2009). In summary, a copper concentration-dependent regulation of \emph{ycnJ} mediated by \emph{ycnK} and \emph{csoR}, a growth defect during copper starvation in \(\Delta\text{ycnJ}\), and a Cu\(^{2+}\)-induced dimerization of YcnJ soluble domain were observed.
The role of YcnJ as an importer necessitates anyway deeper investigation since it cannot be excluded that the protein binds copper without importing it, possibly for the biogenesis of copper-requiring proteins located in the external side of the plasma membrane.

**Accumulation of copper-methanobactins in *Methylosinus trichosporium*OB3b cytoplasm**

Methanobactins have been already described in the 1.1.2 section. In addition to what was reported there, confocal microscopy on fluorescent-labeled Cu-Mb revealed a cytoplasmic localization. To rule out possible artifacts caused by the dye, the intrinsic fluorescence of the unlabeled Cu-Mb complex was measured, confirming what was observed previously (Balasubramanian et al., 2011). This interesting result deserves in-depth examinations, especially with regards to the cytoplasmic importer involved in the process.

**1.1.5 Concluding remarks**

By reviewing the world of the biological use of copper it is possible to outline some considerations. Copper is not exactly a trace element difficult to find. The demonstration of this is given by the easiness in which copper resistance systems have been characterized in contrast to copper import systems (Osman and Cavet, 2008). One may argue that the identification of a resistance trait presents less technical challenges and this is probably true, but the same cannot be said for other metals. The best example is copper’s counterpart, iron. The charm arisen by the comparison of the biological relevance of these two metals has been already rendered by Crichton and Pierre (2001). So far only import systems have been described for this metal, and almost nothing is known about iron resistance, even though, unlike copper, its involvement in the Fenton reaction which causes DNA damage has been proven (Mello Filho et al., 1984; Imlay et al., 1988; Cornelis et al., 2011). The opposite evolution of copper and iron in biology made iron insoluble, needing ingenious import systems and made copper soluble and apparently ubiquitous, requiring systems to get rid of it. It is also interesting to note how these two metals are used in the interface between pathogen and host. Hosts fight pathogens by restricting their access to iron, while copper excess is used to kill microbes (Haley and Skaar, 2012; Hodgkinson and Petris, 2012; Samanovic et al., 2012). In the middle is zinc. A number of prokaryotic systems for both its import and its export have been described (Blencowe and Morby, 2003). Copper is then seemingly more often an undesired guest, despite its usefulness. This view is likely familiar to those who have tried to experimentally reproduce copper starvation in a laboratory, forced to acid-treat glassware and use chelators to ensure its deprivation. It is probably true that in most cases its import is not needed at all. But some environments on the Earth still resemble the primordial
atmosphere, were copper is difficult to obtain. Re-population of these niches by descendants of organism which adapted to the oxygen life, and with that to the copper life, probably forced them to do what has been done for iron: to evolve small compounds that solubilize and import the metal. Methanobactins are undoubtedly the most evident example of how copper can be limiting, but whether this is really due to a reduced solubility of the metal is not known. Considering that bacteria populate any kind of environment, it is likely that the situation faced by certain methane oxidizing bacteria, may also be faced by other organisms.

Copper transfer is a related but different topic. Letting copper arrive to its destination without causing the damages it is able to, requires dedicated systems. In eukaryotes the destination is far from the source, so these systems are well known. In prokaryotes on the other hand source and destination are often close. It is therefore not surprising that much of what we know about copper trafficking comes from the insertion of the metal into a few functional enzymes. But if in certain cases of deprivation an importer may be needed, then it is logical to theorize that a linked sorting system is required to direct copper to important targets. The challenge is then to recreate these starvation conditions in a species that evolved such systems because of its life attitudes. Given the potential of today’s research and of bacteria in facing any kind of condition, the characterization of such systems may be only a question of time.

1.2 Copper sorting in *Bradyrhizobium japonicum*

*B. japonicum* is a Gram-negative α-proteobacterium from the soil, known for its ability to infect soybean's roots and undergo symbiosis, where it is able to fix atmospheric N\(_2\). It owns several genes encoding putative cuproenzymes. Among them only some have been so far characterized (Table 1.3). As a free-living organism it is extremely adaptable. In aerobic environments it respires oxygen, and it is also able use thiosulfate or H\(_2\) as electron donors while fixing CO\(_2\) (Lepo et al., 1980; Masuda et al., 2010). In the absence of oxygen it carries out denitrification, where the oxides of nitrogen are the final electron acceptors (Bedmar et al., 2005). During symbiosis the level of free oxygen is kept below 20 nM by the plant-secreted leghemoglobin, because of its toxicity against the nitrogenase complex (Hunt and Layzell, 1993). Despite this, *B. japonicum* consumes oxygen as an endosymbiont. This versatility is due to an array of different terminal oxidases, most of them copper dependent. In *B. japonicum* genome there are six gene clusters for heme-copper oxidases (plus two *bd*-type oxidases) and a set of genes for the complete denitrification pathway, including the
copper-cofactored NO$_2^-$ and N$_2$O reductases. In aerobic conditions the mitochondria-like $aa_3$-type cytochrome $c$ oxidase encoded by $coxBACF$ is the predominant oxidase, albeit other oxidases can replace it as aerobic growth is not impaired when these genes are mutated (Bott et al., 1990). In symbiosis the $cbb_3$ oxidase is essential for the production of the energy needed by the nitrogenase; its inactivation by mutation in the encoding $fixNOQP$ operon leads in fact to a drastic decrease in nitrogen fixation. The same effect is not revealed by mutating any other oxygen reductase (Preisig et al., 1996b). The $cbb_3$ oxidase differs from the $aa_3$ oxidase (described in section 1.1.1 and Fig.1.1) for the absence of a Cu$_A$ center in the subunit II, replaced by a monoheme cytochrome $c$. Its peculiarity is the presence of an additional diheme cytochrome $c$ in the subunit III, which is devoid of cofactors in other kinds of oxidases. Electrons from the cytochrome $bc_1$ are probably conveyed here, then transferred to the monoheme cytochrome $c$, and then onward to the oxygen reduction center (Fig. 1.8). Despite the availability of a crystal structure, it is not clear what the determinants for the high affinity for oxygen typical of this oxidase are (Preisig et al., 1996b; Buschmann et al., 2010).

Because of the abovementioned reasons, and because $B. japonicum$ is an $\alpha$-proteobacterium, relative of the mitochondrial ancestors, the biogenesis of the $aa_3$ and the $cbb_3$ cytochrome oxidases has often been a target of studies. $TlpA$ was the first protein described to be involved in $aa_3$ oxidase assembly. A screening for cytochrome $c$ oxidase-negative clones in a $Tn5$ mutant library of $B. japonicum$ identified the $tlpA$ gene. The reason for the defect was due to the lack of an active $aa_3$ oxidase. Its subunit I (CoxA) was still expressed, but barely detectable by immunoblots. $In vivo$ difference spectroscopy revealed the absence of the $aa_3$ cytochrome and the presence of $b$- and $c$-type cytochromes. The insertional mutant had a strong symbiotic defect, leading to a non-fixing phenotype. It was
then purposed that TlpA was involved in both the assembly of the \( aa_3 \) oxidase, and in an independent pathway essential for symbiosis. TlpA shares similarities with thioredoxins and protein disulfide isomerases but unlike other thioredoxins, TlpA features an uncleavable N-terminal signal sequence, which locates it to the periplasm, anchored to the inner membrane (Loferer et al., 1993). The protein forms two disulfides bridges via four cysteine residues, only one of them forming the redox active site. Measurement of TlpA activity revealed a redox potential of \(-0.256\) V, which is well comparable with other characterized thioredoxins (Loferer and Hennecke, 1994; Loferer et al., 1995; Mohorko et al., 2012). A crystal structure of its soluble domain has been solved (Capitani et al., 2001).

### Table 1.3. \textit{B. japonicum} cuproproteome. Homologues of known cuproproteins with conserved copper-binding sites are reported.

<table>
<thead>
<tr>
<th>Cuproenzyme family</th>
<th>Gene</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heme-copper oxygen reductase subunit I</td>
<td>bll1171 ((\text{coxA}))</td>
<td>( aa_3 ) oxidase subunit I, aerobic respiration</td>
<td>(Bott et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>bll3784 ((\text{coxN}))</td>
<td>Cytochrome ( c ) oxidase subunit I, possible role in low ( O_2 ) ( H_2 )-dependent chemolithotrophy</td>
<td>(Bott et al., 1992; Surpin and Maier, 1998)</td>
</tr>
<tr>
<td></td>
<td>bll0150 ((\text{cyoB}))</td>
<td>( ba_3 )-type quinol oxidase, unknown function</td>
<td></td>
</tr>
<tr>
<td></td>
<td>bll2715 ((\text{coxX}))</td>
<td>( bb_3 ) oxidase subunit I, possible minor role in symbiosis and in low ( O_2 ) ( H_2 )-dependent chemolithotrophy</td>
<td>(Surpin and Maier, 1998; Surpin and Maier, 1999)</td>
</tr>
<tr>
<td></td>
<td>bll4480</td>
<td>Cytochrome oxidase subunit I, unknown function</td>
<td>(Bühler et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>bll2673 ((\text{fixN}))</td>
<td>( cbb_3 ) oxidase subunit I, microaerobic respiration</td>
<td>(Preisig et al., 1993)</td>
</tr>
<tr>
<td>Cytochrome ( c ) oxidase ( Cu_{\text{A}_2} )-containing subunit II</td>
<td>bll1170 ((\text{coxB}))</td>
<td>( aa_3 ) oxidase subunit II, aerobic respiration</td>
<td>(Bott et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>bll3785 ((\text{coxM}))</td>
<td>Cytochrome ( c ) oxidase subunit II, possible role in low ( O_2 ) ( H_2 )-dependent chemolithotrophy</td>
<td>(Bott et al., 1992; Surpin and Maier, 1998)</td>
</tr>
<tr>
<td></td>
<td>bll4481</td>
<td>Cytochrome oxidase subunit II, unknown function</td>
<td>(Bühler et al., 2010)</td>
</tr>
<tr>
<td>Blue copper protein(^a)</td>
<td>bll2292 ((\text{petE}))</td>
<td>Unknown</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>bll3015</td>
<td>Unknown</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>bll2559</td>
<td>Unknown</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>bll1555</td>
<td>Unknown</td>
<td>–</td>
</tr>
<tr>
<td>Copper ( NO_2^- ) reductase</td>
<td>bll7089 ((\text{nirK}))</td>
<td>Denitrification</td>
<td>(Velasco et al., 2001)</td>
</tr>
<tr>
<td>( N_2O ) reductase</td>
<td>bll0315 ((\text{nosZ}))</td>
<td>Denitrification</td>
<td>(Velasco et al., 2004)</td>
</tr>
<tr>
<td>NADH dehydrogenase-2</td>
<td>bll3727</td>
<td>Unknown; highly similar to \textit{E. coli} NDH-2</td>
<td>–</td>
</tr>
<tr>
<td>Laccase (multicopper oxidase)(^a)</td>
<td>bll2293</td>
<td>Unknown</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>bll4946</td>
<td>Unknown</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^a\) Genes bll2209 encoding a blue copper protein and bll2210 encoding a multicopper oxidase have been omitted. They belong to the bll2208-bls2212 cluster likely involved in copper resistance.
ScoI was identified as homologue of the eukaryotic Sco1. Hence, its activity in the assembly of the \( aa_3 \) oxidase has been assessed. All the measured \( \Delta scoI \) phenotypes resemble what was observed for the \( \Delta tlpa \) strain: low immunodetectable levels of both the \( aa_3 \) oxidase subunits, absence of \( aa_3 \) cytochrome peak in difference spectroscopy experiments, absence of aerobic cytochrome \( c \) oxidase activity, and a symbiotic defect. Importantly, copper repletion (50 \( \mu M \)) was able to restore wild type-like levels for all the tested \( aa_3 \)-related phenotypes. To understand whether the symbiotic phenotype of \( \Delta scoI \) is due to a \( cbb_3 \) oxidase assembly defect, membrane proteins isolated from anaerobically grown \( \Delta scoI \) strain were tested for their ability to reduce cytochrome \( c \). In fact, genes \( fixNOQP \) encoding the \( cbb_3 \) oxidase are activated when the oxygen concentration falls under a certain threshold, thus, anaerobic cells produce functional \( cbb_3 \) oxidase even though they respire with nitrate (Nellen-Anthamatten et al., 1998). Under these conditions all cytochrome \( c \) oxidase activity is due to the \( cbb_3 \) oxidase, as observable in \( \Delta fixN \) negative controls. Apparently \( scoI \) is not responsible of \( cbb_3 \) oxidase assembly, since its mutant is as efficient as the wild type in reducing cytochrome \( c \). A purified soluble version of ScoI is able to bind \( Cu^{2+} \) \textit{in vitro} via its conserved cysteines, as a change in the absorption spectra is only observed when they are both present. This result did not exclude a possible role of ScoI as dithiol:disulfide oxidoreductase for the cysteines of the Cu\( \alpha \) center; its redox potential was then measured. The obtained value of \(-0.160 \) V excludes a role of ScoI as a reductase, since it is not negative enough when compared with characterized dithiol:disulfide oxidoreductases. A double role was then attributed to ScoI: (i) copper chaperone for the \( aa_3 \) oxidase Cu\( \alpha \), for analogy with the proposed role of the eukaryotic Sco (Fig. 1.5), and (ii) an unidentified function essential for symbiosis (Bühler et al., 2010; Mohorko et al., 2012).

Given the extremely similar phenotypes of the \( \Delta tlpa \) and the \( \Delta scoI \) strains, it was proposed that TlpA is the reductase for the cysteines of ScoI, which in turn need to be reduced. \textit{In vitro} experiments confirmed this hypothesis: the dynamic of the reaction between reduced TlpA and oxidized ScoI fit with a model by which ScoI is a specific substrate for TlpA (Mohorko et al., 2012). How the cysteines of the \( aa_3 \) oxidase Cu\( \alpha \) center are reduced is still not known, but ScoI, unlike what was shown in \textit{T. thermophilus} (Abriata et al., 2008), is not involved; TlpA is instead a good candidate for this function.

CoxG is a Cox11-like protein. Members of this protein family are involved in the biogenesis of the Cu\( \beta \) center of \( aa_3 \)-type cytochrome \( c \) oxidases (Cobine et al., 2006; section 1.1.3). \textit{B. japonicum} strains carrying a \( \Delta coxG \) deletion show the same phenotype as \( \Delta tlpa \) and \( \Delta scoI \) with regard to the assembly of the \( aa_3 \) oxidase, but, unlike them, they are efficient
in symbiosis. No cytochrome $c$ oxidase activity and no $aa_3$ peak in difference spectroscopy were revealed, and decreased amounts of both subunits I and II were observed by immunoblot experiments, when compared to the wild type. In contrast, subunits I and II of the $cbb_3$ oxidase were present and functional. CoxG is therefore likely transferring copper to the Cu$_B$ center of the $aa_3$ oxidase, but the same is not true for the Cu$_B$ center of the $cbb_3$ oxidase, which must utilize an independent copper incorporation system (Bühler et al., 2010).

A candidate for the biogenesis of the Cu$_B$ center of the $cbb_3$ oxidase is FixI, whose encoding gene lies in the $fixGHIS$ cluster, immediately downstream the $fixNOQP$ operon. This genetic situation is widespread in $cbb_3$ oxidase-containing bacteria. The $fixGHIS$ cluster presents the same expression pattern of $fixNOQP$ and the same kind of promoter. All the putatively encoded proteins are predicted to have transmembrane architecture, where FixG is an oxidoreductase and FixI a P$_{1B}$-type ATPase. Strains carrying deletions affecting the whole operon showed impaired symbiosis and, when they were grown anaerobically, had no cytochrome $c$ oxidase activity, and no $cbb_3$ oxidase subunits were detectable by immunoblot. It is not known to which extent each gene is responsible of the observed phenotype, but the homology of FixI with other copper-transporter proteins led authors to speculate a role of the ATPase in copper transfer to the Cu$_B$ center (Preisig et al., 1996a).

Figure 1.9. Model for copper insertion into the $aa_3$ and $cbb_3$ oxidases in $B. japonicum$. Copper is represented by brown circles, heme cofactors by pink lines over gray circles. Copper transfer is represented by blue arrows. Oxidase subunits are identified by roman numbering.

The collected information allowed hypothesizing a model, represented in Fig. 1.9. ScoI is reduced by TlpA, and is then able to bind copper for its insertion in the $aa_3$ oxidase Cu$_A$ center. The responsible enzyme for the reduction of the Cu$_A$ center has not been identified.
yet. CoxG likely transfers copper to the \( aa_3 \) oxidase Cu\(_B\) center. These proteins are seemingly not involved in the biogenesis of the \( cbb_3 \) oxidase Cu\(_B\) center, which may be assembled with the help of a copper transporter, FixI.

1.3 Aim of this work

This study aims at identifying genes involved in copper uptake and sorting to functional copper enzymes in \( B. \) japonicum in order to add information to the confused scenario of prokaryotic copper import and trafficking. Two approaches have been followed.

In the first approach, available microarray data (from the Ph.D. work of Dr. Doris Bühler and the M.Sc. work of Simona Huwiler) regarding the whole genome expression in cells grown in different amounts of copper have been analyzed. They allowed the identification of up-regulated genes in copper starvation which then became reliable candidates for copper import from the extracellular environment to the periplasm, and for its shuttling to the cuproenzymes when copper becomes limiting.

The second approach consisted of a bioinformatic search for conserved copper import and trafficking proteins in \( B. \) japonicum. This search excluded porins and the already characterized ScoI, CoxG and FixI, but included all the homologues of copper trafficking proteins reviewed in the introduction. Six homologues of copper P\(_{1B}\)-type ATPases are present. One of them is FixI, one is likely a copper exporter (bll0700), one is a potassium transporter (KdpB), and three of them have a weaker homology and they are probably not dealing with copper. A \( nosL \) homologue is present in the \( N_2O \) reductase operon; one \( ycnJ \) (\( copCD \) fusion) and two PCu\(_A\)C homologues are also encoded by the \( B. \) japonicum genome. Because of the difficult predictability of the role of P\(_{1B}\)-type ATPases homologues and because disruption of \( nosL \) failed to show detectable phenotype in \( Pseudomonas \) stutzeri (Wunsch et al., 2003), the \( ycnJ \) and PCu\(_A\)C homologues have been selected as candidates to test. The choice of the PCu\(_A\)C homologues as candidates is further supported by the conserved function in cytochrome oxidases biogenesis shown by this class of protein. Their characterization has then the potential to enrich the actual knowledge about copper transfer to cytochrome oxidases of \( B. \) japonicum.

Both the approaches converged in the identification of one candidate operon for copper import and sorting, \( pcuABCDE \), whose characterization is the main focus of this work.
Chapter II

Copper starvation-inducible protein for cytochrome oxidase biogenesis in *Bradyrhizobium japonicum*

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Chapter II

Contribution of the co-authors:

**Fabio Serventi:** planned and carried out the cloning procedures necessary to obtain the two ΔpcuABCDE (6611 and 6612) and the two Δblr7088 (6620 and 6621) marker replacement mutants. Constructed the two ΔΔpcuABCDE/blr7088 double mutants (6611-20 and 6611-21). Constructed the vector bearing the entire operon on pGEM-T Easy and planned the strategies to have in-frame deletions of pcuB, pcuC, pcuD, and pcuE. Partially (last steps carried out by Zeb Youard) participated to the construction of strains 6611-1650 (ΔpcuB) and 6611-6630 (ΔpcuE). Constructed strains 6632 (empty vector control in the wild type), 6611-32 (empty vector control in the operon mutant), 6611-33 (complementation of the operon mutant with the entire operon), and 6611-34 (ΔpcuC). Measured growth in aerobiosis, microaerobiosis and anaerobiosis, symbiotic efficiency, cytochrome c oxidase activity of strains 6611, 6612, 6620, 6621, 6611-20, 6611-21. Measured NO₃⁻ and NO₂⁻ during anaerobic growth of strains 6611, 6612, 6611-20, 6611-21. Measured symbiotic efficiency of strains 6611-1653 (ΔpcuA), 6611-1650 (ΔpcuB), 6611-34 (ΔpcuC), 6611-1654 (ΔpcuD), 6611-6630 (ΔpcuE). Isolated bacteroid membranes from 6611, 6611-34, 2574 (ΔscoI) and measured their cytochrome c oxidase activity. Did qRT-PCR on pcuD to verify the integrity of the transcript. Constructed the expression vector for PcuC periplasmic expression. Purified PcuC from both periplasmic and cytoplasmic expression systems and did all the in-vitro work. Designed and constructed expression vectors for substituted and truncated versions of PcuC. Supervised Miriam Richter. Wrote the manuscript.

**Zeb Youard:** constructed 6611-1653 (ΔpcuA) and 6611-1654 (ΔpcuD) strains starting from the vector bearing the entire operon on pGEM-T Easy. Completed construction of strains 6611-1650 (ΔpcuB) and 6611-6630 (ΔpcuE). Supervised Ronny Luchsinger.

**Valérie Murset:** did primer extension experiments and qRT-PCR on pcuA and pcuB to confirm microarray data.

**Simona Huwiler:** tested minimal medium and helped with the microarrays.

**Doris Bühler:** repeated and expanded microarrays.

**Miriam Richter:** analyzed truncated derivative of PcuC.

**Ronny Luchsinger:** tested aerobic and anaerobic growth and aerobic cytochrome c oxidase activity on strains 6611-1653 (ΔpcuA), 6611-1650 (ΔpcuB), 6611-34 (ΔpcuC), 6611-1654 (ΔpcuD), 6611-6630 (ΔpcuE).

**Hans-Martin Fischer:** co-supervised the work.

**Robert Brogioli:** determined Cu content in "Cu-free" medium.

**Martina Niederer:** constructed cytoplasmic expression plasmid for PcuC.

**Hauke Hennecke:** supervised the work and revised manuscript.
**Copper protein for cytochrome oxidase biogenesis**

**Background:** Copper trafficking in Gram-negative bacteria supplies cupro-enzymes via transporters and periplasmic chaperones.

**Results:** Symbiotic nitrogen fixation, denitrification, and copper-starved growth depend on a periplasmic, copper-binding protein named PcuC.

**Conclusion:** The pcuC mutant phenotypes are caused by defects in copper-containing respiratory enzymes.

**Significance:** Research on cytochrome oxidase biogenesis in α-proteobacteria, the extant relatives of mitochondria, helps to understand how mitochondria assemble the respiratory chain.

### 2.1 Summary

Microarray analysis of *B. japonicum* grown under copper limitation uncovered five genes named *pcuABCDE* which are co-transcribed and co-regulated as an operon. The predicted gene products are periplasmic proteins (PcuA, PcuC, PcuD), a TonB-dependent outer membrane receptor (PcuB), and a cytoplasmic membrane-integral protein (PcuE). Homologues of PcuC and PcuE had been discovered in other bacteria, namely PCuAC and YcnJ, where they play a role in cytochrome oxidase biogenesis and copper transport, respectively. Deletion of the *pcuABCDE* operon led to a pleiotropic phenotype including defects in the *aa3*-type cytochrome oxidase, symbiotic nitrogen fixation, and anoxic nitrate respiration. Complementation analyses revealed that, under our assay conditions, the tested functions depended only on the *pcuC* gene but not on *pcuA*, *pcuB*, *pcuD* or *pcuE*. The *B. japonicum* genome harbors a second *pcuC*-like gene (blr7088) which, however, did not functionally replace the mutated *pcuC*. The PcuC protein was overexpressed in *Escherichia coli*, purified to homogeneity, and shown to bind Cu(I) with high affinity in a 1:1 stoichiometry. The replacement of His79, Met90, His113, and Met115 by alanine perturbed copper binding. This corroborates the previously purported role of this protein as a periplasmic copper chaperone for the formation of the CuA center on the *aa3*-type cytochrome oxidase. In addition, we provide evidence that PcuC and the Cu chaperone ScoI are important for the symbiotically essential, CuA-free *cbb3*-type cytochrome oxidase specifically in endosymbiotic bacteroids of soybean root nodules, which could explain the symbiosis-defective phenotype of the *pcuC* and *scoI* mutants.
2.2 Introduction

This report deals with copper routing to membrane-bound heme-copper cytochrome oxidases in a Gram-negative bacterium. Based on the presence of different copper sites, two groups of heme-copper oxygen reductases must be distinguished (García-Horsman et al., 1994; Sousa et al., 2012): (i) those that carry only the high-spin heme-Cu_B center on subunit I, which is buried in the membrane part and forms the active site for O_2 reduction to H_2O (Abramson et al., 2000); and (ii) those that carry in addition a binuclear Cu-Cu center (Cu_A) on subunit II, which is exposed to the periplasm and serves as a recipient of the electrons delivered by periplasmic c-type cytochromes (Iwata et al., 1995; Tsukihara et al., 1996). Hence, members of the second group are cytochrome oxidases, represented prominently by the mitochondrial and bacterial aa_3-type oxygen reductases, whereas most of the bacterial quinol oxidases belong to the first group. The bacterial cbb_3-type cytochrome oxidase is in a class of its own (Ducluzeau et al., 2008). Although it lacks the Cu_A center, electrons are delivered to the heme-Cu_B site on subunit I (FixN or CcoN) via the c-type cytochromes FixO/CcoO and FixP/CcoP, which constitute subunits II and III of the enzyme complex (Buschmann et al., 2010). In contrast to mitochondria, which have only one energy-conserving respiratory chain with one terminal oxidase, bacteria often employ a branched respiratory chain terminating with disparate oxidases allowing them to efficiently adapt to a wide range of environmental oxygen concentrations (Poole and Cook, 2000; Han et al., 2011). The symbiotic nitrogen-fixing bacterium Bradyrhizobium japonicum, the organism investigated here, reflects this complexity most notably because it possesses up to eight different terminal oxidases. Two of these dominate, i.e., cytochrome aa_3 in oxically grown cells, and cytochrome cbb_3 in symbiosis (Bühler et al., 2010).

The question of how copper is delivered to, and assembled in, the Cu_A and Cu_B centers has been addressed not only in mitochondria but also in Gram-negative bacteria, primarily in members of the α-proteobacteria (e.g. Rhodobacter species, B. japonicum, Paracoccus denitrificans) because they are phylogenetically the closest extant relatives of mitochondria. Certain conserved functions seem to emerge for each of the following four biogenesis factors. (i) CoxG, also termed CtaG, is a homologue of the mitochondrial Cox11 protein (van der Oost et al., 1991; Hiser et al., 2000; Cobine et al., 2006; Bühler et al., 2010; Thompson et al., 2010). The protein is a membrane-anchored Cu(I) chaperone with the Cu-binding domain facing the periplasm or the mitochondrial intermembrane space. It plays a role in the assembly of the Cu_B center of cytochrome aa_3 (Hiser et al., 2000). For unknown reasons,
however, CoxG does not appear to be required for the symbiotically essential $cbb_3$-type oxidase in *B. japonicum*, even though this enzyme also carries a Cu$_B$ center (Bühler et al., 2010). (ii) FixI, also called CcoI or CtpA, might be the unknown factor for Cu$_B$ insertion into cytochrome $cbb_3$. If it is a Cu-translocating P$_{1B}$-type ATPase, as previously proposed (Hassani et al., 2010; Ekici et al., 2012a), this protein might work as an uptake system destined to insert Cu into subunit I. While Cu import has yet to be proven unequivocally, the dependency of cytochrome $cbb_3$ assembly on FixI (or its paralogs) is well documented (Preisig et al., 1996a; Koch et al., 2000; Hassani et al., 2010). (iii) ScoI, also called SenC or PrrC, is a homologue of the mitochondrial Cu-chaperone Sco1 (for a review, see Robinson and Winge, 2010). Like CoxG, the ScoI protein is anchored to the bacterial cytoplasmic membrane or the mitochondrial inner membrane where the copper-binding globular domain protrudes into the periplasm or the intermembrane space. Work with the *B. subtilis* (Cawthorn et al., 2009) and the mitochondrial Sco1 proteins has suggested a role in the metallation of the Cu$_A$ site on subunit II of the $aa_3$-type cytochrome oxidase (Robinson and Winge, 2010). Subsequent work with *B. japonicum* ScoI has adopted this view, supported by the fact that a *scoI* knock-out mutant was clearly defective in cytochrome $aa_3$, but not in cytochrome $cbb_3$ when the latter was tested in cells that had been grown micro-oxically or anoxically (Bühler et al., 2010). The symbiosis defect caused by the *B. japonicum scoI* mutant remained enigmatic (Arunothayanan et al., 2010; Bühler et al., 2010). Other reports have shown, however, that SenC and PrrC play a role in the formation of active cytochrome $cbb_3$ (Frangipani and Haas, 2009; Lohmeyer et al., 2012; Thompson et al., 2012). (iv) A more recently discovered Cu-chaperone is PCuAC which rivals Sco1 in its function to metallate the Cu$_A$ site. Abriata et al. (2008) designed *in vitro* experiments that documented the direct transfer of Cu(I) to the Cu$_A$ site of the *Thermus thermophilus* $ba_3$ oxidase. Sco1 functioned in this assay as a disulfide reductase to maintain the correct oxidation state of the subunit-II cysteine ligands. *In vivo* data with *Rhodobacter sphaeroides* confirmed that PCuAC and Sco1 co-participate in the assembly of a functional Cu$_A$ center in cytochrome $aa_3$ (Thompson et al., 2012). Intriguingly, the authors of this study noticed a role of PCuAC also in the formation of the Cu$_B$ center of cytochrome $cbb_3$. Homologous genes for PCuAC-like proteins had been recognized in the *B. japonicum* genome (Arunothayanan et al., 2010; Bühler et al., 2010), but detailed studies on their biochemical function had not been done.

The present work was initiated with the idea to find new genes for copper acquisition in *B. japonicum*. Incidentally, transcriptome analyses of Cu-starved cells uncovered an operon that also contained the gene for a PCuAC-like protein. What ensued was an extensive genetic and
biochemical investigation that proved its identity as a copper protein and provided evidence for its role in the biogenesis of both the \( \text{aa}_3\) - and \( \text{cbb}_3\) -type cytochrome oxidases.

2.3 Experimental procedures

2.3.1 Bacterial strains, media, and growth conditions

*Escherichia coli* was grown in Luria-Bertani (LB) medium (Miller, 1972) containing the following concentrations of antibiotics, if necessary: ampicillin, 200 μg/ml; kanamycin, 30 μg/ml; spectinomycin, 20 μg/ml; tetracycline, 10 μg/ml. *B. japonicum* was routinely cultivated in a peptone–salts–yeast extract (PSY) medium supplemented with 0.1% L-arabinose (Regensburger and Hennecke, 1983; Mesa et al., 2008). Buffered Vincent's minimal medium (BVM), here defined as vitamin-free modified Vincent's minimal medium (Vincent, 1970; Becker et al., 2004) supplemented with trace elements (Bishop et al., 1976), 10 mM MOPS (final pH adjusted to 6.8 with 2 M NH\(_3\)), and 0.3% L-arabinose, was alternatively used. This medium contains 20 mM CuSO\(_4\). Glassware was treated overnight with 0.1 M HCl and rinsed thoroughly with double distilled H\(_2\)O when used for experiments on copper limitation, and 10 μM bathocuproine disulfonate (BCS) and 1 mM ascorbate were added. Yeast extract–mannitol medium (YEM) (Daniel and Appleby, 1972) supplemented with 10 mM KNO\(_3\) was used for anoxic growth (nitrate respiration). Where appropriate, antibiotics were added to these final concentrations: kanamycin, 100 μg/ml; spectinomycin, 100 μg/ml; streptomycin, 50 μg/ml; tetracyclin, 50 μg/ml (solid media) or 25 μg/ml (liquid media). *E. coli* strains used in this work are listed in Table 2.1; *B. japonicum* strains are listed in Table 2.2.

2.3.2 Mutant constructions

Detailed information on plasmids and primers is given in Tables 2.1 and 2.3, respectively. For both the \( \Delta \text{pcuABCDE} \) and \( \Delta \text{blr7088} \) marker replacement mutants the upstream and downstream flanking regions of the target genomic sequences were amplified and cloned into pBluescript SK(+) (Stratagene, La Jolla, CA). The \textit{aphII} gene encoding kanamycin resistance or the Ω cassette encoding streptomycin resistance (\( \Delta \text{pcuABCDE} \) and \( \Delta \text{blr7088} \) mutants, respectively) was then inserted in both orientations between the upstream and downstream flanking regions. The DNA constructs were excised and inserted into the suicide plasmid pSUP202pol4 (Fischer et al., 1993) yielding plasmids pRJ6611, pRJ6612, pRJ6620 and pRJ6621. Mobilization of these plasmids into *B. japonicum* 110spc4 was carried out via *E. coli* S17-1 and followed by screening for double recombination events. The resulting strains
Copper protein for cytochrome oxidase biogenesis

6611 (ΔpcuABCDE, same orientation of the aphII gene) and 6612 (ΔpcuABCDE, opposite orientation) carry a deletion between positions 5,408,844 and 5,414,217 of the genome (Fig. 2.1). Strains 6620 (Δblr7088, same orientation of the Ω cassette) and 6621 (Δblr7088, opposite orientation) are deleted between positions 7,804,884 and 7,805,644. For the generation of double mutants 6611-20 (ΔpcuABCDE same orientation, Δblr7088 same orientation) and 6611-21 (ΔpcuABCDE same orientation, Δblr7088 opposite orientation) the plasmid pRJ6611 was mobilized into strains 6620 and 6621, respectively.

Table 2.1. E. coli strains and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (Φ80 lacZΔM15) hsdR17 recA1 gyrA96 thi-1 relA1</td>
<td>BRL, Gaithersburg, MD (Studier and Moffatt, 1986)</td>
</tr>
<tr>
<td>BL21 (DE3)</td>
<td>E. coli B F– dcm ompT hsdS(r38m80 M9) gal λ(DE3)</td>
<td>(Simon et al., 1983)</td>
</tr>
<tr>
<td>S17-1</td>
<td>SmR SpR hsdR (RP4-2 kan::Tn7 tet::Mu; integrated in the chromosome)</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript SK(+)</td>
<td>ApR cloning vector</td>
<td>Stratagene, La Jolla, CA</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>ApR cloning vector</td>
<td>Promega, Madison, WI</td>
</tr>
<tr>
<td>pEC415</td>
<td>ApR (pISC-2) ompA'·ccmEΔoΔH6 on a NdeI/XbaI fragment</td>
<td>(Schulz et al., 1998)</td>
</tr>
<tr>
<td>pBSL86</td>
<td>ApR KmR</td>
<td>(Alexeyev, 1995)</td>
</tr>
<tr>
<td>pBSL15-Ω</td>
<td>SmR SpR (pBSL15) Ω cassette on a 2.1-kb EcoRI fragment from pH45Ω</td>
<td>(Lindemann et al., 2010)</td>
</tr>
<tr>
<td>pSUP202pol4</td>
<td>TcR (pSUP202) part of the polylinker from pBluescript II KS(+) between EcoRI and PstI</td>
<td>(Fischer et al., 1993)</td>
</tr>
<tr>
<td>pKL1</td>
<td>ApR (pET-19b) TEV recognition site fused between H10 and NdeI cloning site</td>
<td>K. Locher laboratory collection (Kapust et al., 2001)</td>
</tr>
<tr>
<td>pRK793</td>
<td>ApR (pMal-C2) 'malE'-TEV recognition site-H10-TEV protease [S219V]-R3 expression vector</td>
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<tr>
<td>pRJ1650*</td>
<td>TcR (pSUP202pol4) pcuA[ΔB]CDE on a 5,894 SpeI/PstI fragment from pRJ6628 cloned into SpeI/SmaI-digested pSUP202pol4</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ1651*</td>
<td>TcR (pRJ6626) last 78 bp of pcuD and first 1040 bp of pcuE on a 1,121-bp HindIII/FspAI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ1652*</td>
<td>TcR (pRJ6626) overlapped-extension PCR-generated in frame deletion of pcuA on a 1,209-bp BamHI/SpeI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ1653*</td>
<td>TcR (pSUP202pol4) pcuA[ΔA]BCDE on a 6,782 SpeI/PstI fragment from pRJ1652 cloned into SpeI/SmaI-digested pSUP202pol4</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ1654*</td>
<td>TcR (pSUP202pol4) pcuABC[ΔD]E on a 6,497 SpeI/PstI fragment from pRJ1652 cloned into SpeI/SmaI-digested pSUP202pol4</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6336*</td>
<td>ApR (pGEM-T Easy linearized) pcuA promoter region on a 383-bp fragment</td>
<td>This work</td>
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<td>pRJ6603*</td>
<td>ApR [pBluescript SK(+)] 5′-flanking sequence of blr7088 on a 578-bp EcoRI/BamHI fragment</td>
<td>This work</td>
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<tr>
<td>pRJ6605*</td>
<td>ApR [pBluescript SK(+)] 5′-flanking sequence of pcuA on a 634-bp BamHI/EcoRI fragment</td>
<td>This work</td>
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<tr>
<td>pRJ6606*</td>
<td>ApR (pRJ6603) 3′-flanking sequence of blr7088 on a 637-bp BamHI/XbaI fragment</td>
<td>This work</td>
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<tr>
<td>pRJ6607*</td>
<td>ApR (pRJ6605) 3′-flanking sequence of pcuE on a 571-bp NotI/BamHI fragment</td>
<td>This work</td>
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<tr>
<td>Strain or plasmid</td>
<td>Relevant genotype or phenotype</td>
<td>Source or reference</td>
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<td>-------------------</td>
<td>--------------------------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>pRJ6608&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; (pRJ6607) ΔpcuABCDE::aphII, 1,242-bp BamHI fragment from pBSL86 (same orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6609&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Km&lt;sup&gt;+&lt;/sup&gt; (pRJ6607) ΔpcuABCDE::aphII, 1,242-bp BamHI fragment from pBSL86 (opposite orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6611&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Km&lt;sup&gt;-&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; (pSUP202pol4) 2,447-bp EcoRI/NorI fragment from pRJ6608</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6612&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Km&lt;sup&gt;-&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; (pSUP202pol4) 2,447-bp EcoRI/NorI fragment from pRJ6609</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6616&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; (pRJ6606) blr7088::Ω, 2,056-bp BamHI fragment from pBSL15-Ω (same orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6617&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Sp&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; (pRJ6606) blr7088::Ω, 2,056-bp BamHI fragment from pBSL15-Ω (opposite orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6618&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pEC415) Strept-tag II-TEV recognition site-pcuC&lt;sub&gt;str&lt;/sub&gt; on 535-bp SfiI/EcoRI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6620&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Sp&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; (pSUP202pol4) 3,271-bp EcoRI/BamHI fragment from pRJ6616</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6621&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Sp&lt;sup&gt;+&lt;/sup&gt; Sm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt; (pSUP202pol4) 3,271-bp EcoRI/BamHI fragment from pRJ6617</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6625&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pGEM-T Easy linearized) FseI/KpnI-bordered 3'-part of pcuABCDE on a 3,891-bp Taq polymerase-generated 5'-A overhang fragment (same orientation of pcuABCDE and bla)</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6626&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6625) 5'-part of pcuABCDE on a 2,440-bp KpnI/SpeI fragment</td>
<td>This work</td>
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<tr>
<td>pRJ6627&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6626) self-ligation of the 7,655-bp Spel fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6628&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6626) self-ligation of the 8,396-bp MluI/OciI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6629&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6626) last 36 bp of pcuC and first 110 bp of pcuD on a 139-bp AbsI/EcoRV fragment</td>
<td>This work</td>
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<tr>
<td>pRJ6630&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Tc&lt;sup&gt;-&lt;/sup&gt; (pSUP202pol4) pcuABCDE[ΔE] on a 5,243 SpeI/PsiI fragment from pRJ6627 cloned into Spel/Smal-digested pSUP202pol4</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6631&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6626) self-ligation of the 3,678-bp FseI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6632&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Tc&lt;sup&gt;-&lt;/sup&gt; (pSUP202pol4) 5'-flanking sequence of pcuA on a 1,266 SpeI/PsiI fragment from pRJ6631 cloned into SpeI/Smal-digested pSUP202pol4</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6633&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Tc&lt;sup&gt;-&lt;/sup&gt; (pSUP202pol4) pcuABCDE on a 6,857 SpeI/PsiI fragment from pRJ6626 cloned into SpeI/Smal-digested pSUP202pol4</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6634&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Tc&lt;sup&gt;-&lt;/sup&gt; (pSUP202pol4) pcuAB[ΔC]DE on a 6,599 SpeI/PsiI fragment from pRJ6629 cloned into SpeI/Smal-digested pSUP202pol4</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6635&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pKL1) pcuC&lt;sub&gt;str&lt;/sub&gt; on 455-bp NdeI/BamHI fragment</td>
<td>This work</td>
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<tr>
<td>pRJ6641&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6635) synthetic sequence encoding PcuC[ΔH79A] on a 370-bp NdeI/AjiI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6642&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6635) synthetic sequence encoding PcuC[ΔM90A] on a 370-bp NdeI/AjiI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6643&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6635) synthetic sequence encoding PcuC[ΔH113A] on a 370-bp NdeI/AjiI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6644&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6635) synthetic sequence encoding PcuC[ΔM115A] on a 370-bp NdeI/AjiI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6645&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6635) synthetic sequence encoding PcuC[ΔH79A/ΔM90A] on a 370-bp NdeI/AjiI fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pRJ6648&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; (pRJ6635) sequence encoding PcuC lacking the last 15 c-terminal amino acids on a 479-bp NcoI/BamHI fragment</td>
<td>This work</td>
</tr>
</tbody>
</table>

<sup>a</sup> Constructs for pcuABCDE marker replacement  
<sup>b</sup> Constructs for blr7088 marker replacement  
<sup>c</sup> Constructs for complementation of strain 6611  
<sup>d</sup> Used for primer extension ladder  
<sup>e</sup> Expression vectors
2.3.3 Complementation of ΔpcuABCDE

The genome sequence comprising the pcuABCDE operon and its upstream region necessary for a single cross-over event (corresponding to genome coordinates 5,408,585–5,414,850), with the addition of appropriate restriction sites, was cloned into vector pGEM-T Easy (Promega, Madison, WI), yielding plasmid pRJ6626. The restriction map of pRJ6626 allowed excision of operon fragments via simple digestions followed by self-ligation: FseI treatment deleted the entire operon leaving its 5’ region to give an empty-vector insertion for control (pRJ6631); SphI was used to excise pcuE (pRJ6627); MslI/OliI were used to in-frame delete pcuB (pRJ6628). When in-frame deletion of genes was not possible with the aforementioned strategy, alternative approaches were applied. Substitution of the naturally occurring HindIII/FspAI fragment with a shorter PCR-generated fragment, including the natural HindIII site on its 3’ end and an added FspAI site on its 5’ end, was carried out to obtain an in-frame pcuD deletion (pRJ1651). Analogously, natural AbsI/EcoRV restriction sites were exploited for the pcuC in-frame deletion (pRJ6629). An overlapped extension PCR (Higuchi et al., 1988) was used to obtain a fragment carrying a pcuA in-frame deletion which substituted the full-length gene via BamHI/SpeI restriction sites (pRJ1652). SpeI/PsiI fragments from pRJ6626, pRJ6631, pRJ6627, pRJ1651, pRJ6629, pRJ6628, and pRJ1652 were inserted into a SpeI/Smal-linearized pSUP202pol4 suicide plasmid yielding pRJ6633, pRJ6632, pRJ6630, pRJ1654, pRJ6634 (Fig. 2.1), pRJ1650, and pRJ1653, respectively. These plasmids were then mobilized into B. japonicum 6611 via E. coli S17-1, followed by screening for single recombination events (Fig. 2.1). Plasmid pRJ6632 (empty vector control) was mobilized into B. japonicum 110spc4 as well. All of the resulting strains are listed in Table 2.2.

2.3.4 Determination of Cu atoms in copper-free BVM

The concentration of Cu in CuSO₄-free BVM prepared with treated glassware was determined by graphite furnace atomic absorption spectroscopy (GF-AAS). The instrument used was an AAnalyst800 (PerkinElmer, Waltham, MA) equipped with a transversely heated graphite furnace and longitudinal Zeeman background correction system. A hollow cathode lamp was chosen as radiation source. A temperature program was applied to the furnace for solvent drying (110 °C for 30 sec followed by 130° for 30 sec under 250 ml/min Ar flow), matrix pyrolysis (1,200 °C for 20 sec under 250 ml/min Ar flow), and atomization (2,100 °C for 5 sec). Cu atoms were detected during the last step by measuring absorption at 324.8 nm. A three-point standard addition procedure was applied for the quantification of Cu in the
analyzed samples. 10-µl samples were mixed with a matrix-modifier solution (ca. 500 ng MgNO₃ and PdNO₃ per g of sample) and different amounts of a Cu standard solution (0, 0.9, 1.8, and 2.9 ng Cu per g with samples containing up to 3.5 ng; 0, 9, 18, 29 ng per g with samples containing more than 3.5 ng of Cu) in a final volume of 30 µl. Each measurement was repeated three times. All solutions were prepared in 0.5 % HNO₃. The instrumental limit of detection was 0.05 ng Cu per g of sample.

Table 2.2. *B. japonicum* strains used in this work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>110spc4</td>
<td>Sp⁺ wild type</td>
<td>(Regensburger and Hennecke, 1983)</td>
</tr>
<tr>
<td>COX132</td>
<td>Sp⁺ Km⁻ coxA::Tn5</td>
<td>(Bott et al., 1990)</td>
</tr>
<tr>
<td>3613</td>
<td>Sp⁺ Km⁻ fixN::Tn5</td>
<td>(Preisig et al., 1993)</td>
</tr>
<tr>
<td>2574</td>
<td>Sp⁺ Km⁻ ΔscoI::aphII (same orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>6611</td>
<td>Sp⁺ Sm⁻ Δblr7088::Ω (same orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>6620</td>
<td>Sp⁺ Sm⁻ ΔpcuABCDE::aphII (opposite orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>6621</td>
<td>Sp⁺ Sm⁻ ΔpcuABCDE::aphII (same orientation)</td>
<td>This work</td>
</tr>
<tr>
<td>6611-20</td>
<td>Sp⁺ Km⁻ Sm⁻ ΔpcuABCDE::aphII (same orientation), Δblr7088::Ω</td>
<td>This work</td>
</tr>
<tr>
<td>6611-21</td>
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<td>This work</td>
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<tr>
<td>6632</td>
<td>Sp⁺ Tc⁺ pSUP202pol4 chromosomally integrated in 110spc4</td>
<td>This work</td>
</tr>
<tr>
<td>6611-32</td>
<td>Sp⁺ Km⁻ Tc⁺ pSUP202pol4 chromosomally integrated in 6611</td>
<td>This work</td>
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<tr>
<td>6611-33</td>
<td>Sp⁺ Km⁻ Tc⁺ pcuABCDE on pSUP202pol4 chromosomally integrated in 6611</td>
<td>This work</td>
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<td>6611-34</td>
<td>Sp⁺ Km⁻ Tc⁺ pcuA[ΔC]DE on pSUP202pol4 chromosomally integrated in 6611</td>
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<td>6611-6630</td>
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<td>This work</td>
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<tr>
<td>6611-1654</td>
<td>Sp⁺ Km⁻ Tc⁺ pcuABC[ΔD]E on pSUP202pol4 chromosomally integrated in 6611</td>
<td>This work</td>
</tr>
<tr>
<td>6611-1650</td>
<td>Sp⁺ Km⁻ Tc⁺ pcuA[ΔB]CDE on pSUP202pol4 chromosomally integrated in 6611</td>
<td>This work</td>
</tr>
<tr>
<td>6611-1653</td>
<td>Sp⁺ Km⁻ Tc⁺ pcuA[ΔA]BCDE on pSUP202pol4 chromosomally integrated in 6611</td>
<td>This work</td>
</tr>
</tbody>
</table>

2.3.5 RNA isolation and cDNA synthesis

Cell harvesting, RNA extraction, and cDNA synthesis for microarray and qRT-PCR were performed as described (Hauser et al., 2007).

2.3.6 Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA from aerobically grown *B. japonicum* 110spc4, 6611-33, and 6611-34 was isolated in order to monitor the expression level of *pcuD* by qRT-PCR, similarly to what had been described previously (Lindemann et al., 2007). In order to analyze the influence of copper on the expression of genes *pcuA* and *pcuB*, RNA was extracted from *B. japonicum* 110spc4
grown in standard BVM or in copper-free BVM supplemented with 10 µM BCS and 1 mM ascorbic acid. Details on the primers used are listed in Table 2.3.

2.3.7 Primer extension

Mapping of the transcription start site of the pcuABCDEFG operon was carried out as previously described (Beck et al., 1997) using the primer named "primer_extension_1" (Table 2.3). RNA was isolated from B. japonicum 110spec grown aerobically in standard BVM or in copper-free BVM supplemented with 10 µM BCS and 1 mM ascorbic acid. The sequencing ladder was obtained by sequencing plasmid pRJ6336 (Table 2.1) with the same primer that was used for the extension reaction.

2.3.8 Microarrays

Transcriptomics experiments were carried out as previously described (Mesa et al., 2009) using a custom-designed Affymetrix GeneChip® (Hauser et al., 2007). The chip was hybridized with cDNA obtained from B. japonicum 110spec grown aerobically either in BVM containing 2 µM CuSO₄, in copper-free BVM (5 nM Cu), or in copper-free BVM supplemented with 10 µM BCS and 1 mM ascorbic acid. Three biological replicates were prepared for each condition. Data were analyzed using GeneSpring GX 7.3.1 software (Agilent, Santa Clara, CA). The data were filtered for probe sets which were called “present” or “marginal” in at least two out of three replicas, and a Student's t-test with a P-value threshold of 0.01 was applied. Differential expression in a comparison of two conditions was defined when the fold-change value was bigger than 2 or smaller than −2. Data sets generated in this work are deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo) with the record number GSE40437.

2.3.9 Plant growth

Sterilization of soybean seeds (Glycine max (L.) Merr. cv. Williams), cultivation of plants, and nitrogenase activity measurements were performed as previously described (Hahn and Hennecke, 1984; Göttfert et al., 1990a; Göttfert et al., 1990b; Bühler et al., 2010). Plants were evaluated for nodulation and nitrogen fixation 21 days after the infection with the appropriate B. japonicum strains.

2.3.10 Bacteroid isolation

Bacteroids were isolated from root nodules and separated from plant material as previously described (Sarma and Emerich, 2005; Delmotte et al., 2010).
2.3.11 Nitrate and nitrite detection in the growth medium

Adapted versions of described methods were used (Nicholas and Nason, 1957). Cells were removed from the growth medium by centrifugation. For nitrate detection, a 0.1-ml sample of 10-fold diluted supernatant was mixed with 0.1 ml 1% sulfamic acid and 0.8 ml of a 1:1 mixture of 98% H₂SO₄ and 85% H₃PO₄ (v/v). After 10-min incubation at room temperature, 0.1 ml of 0.12 % (w/v) 2,6-dimethyl phenol in 100% (v/v) acetic acid was added. Absorption at 334 nm was recorded after 90-min incubation at room temperature. A standard curve was recorded from 0 to 10 mM KNO₃ in YEM medium. For nitrite determination, a 0.4-ml sample of 80-fold diluted supernatant was mixed with 0.4 ml 1% (w/v) sulfanilamide in 7.4% (v/v) HCl and 0.4 ml 0.02% (w/v) N-(1-naphthyl)ethylenediamine. Absorption at 540 nm was measured after 30-min incubation at room temperature. A standard curve was taken from 0 to 10 mM NaNO₂ in YEM.

2.3.12 Determination of cytochrome c oxidase activity in membrane fractions

Preparation of the membrane fraction and determination of cytochrome c oxidase activity were carried out as described previously (Gerhus et al., 1990; Bühler et al., 2010) starting either from culture-grown cells or from purified bacteroids. Concentration of solubilized membrane proteins was determined with the Bradford method (Bradford, 1976), using a Bio-Rad assay (Hercules, CA) with bovine serum albumin as the standard.

2.3.13 Immunological techniques

Rabbit antibodies specific for CoxA and CoxB proteins were available from previous work (Loferer et al., 1993; Bühler et al., 2010). Western blot analyses were carried out on membrane proteins (35 µg/lane) separated by SDS-PAGE (Laemmli, 1970) and blotted as described previously (Loferer et al., 1993). Anti-rabbit IgG(H+L)–horseradish peroxidase conjugate (Bio-Rad) and peroxidase chemoluminescence detection kit (Thermo Fisher Scientific, Waltham, MA) were used to detect bands containing primary antibodies bound to CoxA and CoxB.
<table>
<thead>
<tr>
<th>Purpose&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Name</th>
<th>Position&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Oligonucleotide sequence (5’→3’)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>5′ extension purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pcuABCDE</strong> marker replacement</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3′ flanking sequence amplification</td>
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<td><strong>6611 (partial) complementation</strong></td>
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<td></td>
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<td>Pairs with 3′ end of UP amplicon for overlap extension</td>
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<td>Name</td>
<td>Position&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Oligonucleotide sequence (5'→3')&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5' extension purpose</td>
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<td>-----------------</td>
<td>------</td>
<td>----------------</td>
<td>-------------------------------------------------</td>
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<td>PcuC cytoplasmic overexpression</td>
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<td></td>
<td>bll4880_bamHI_rev</td>
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<tr>
<td>PcuC periplasmic overexpression</td>
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<td>CATATGCAGGCCTGGAGCCACCCGACGTCAAGGCCGGCGATCTCAGTCG</td>
<td>NdeI restriction site + C + Stul restriction site + Strep-tag II + TEV protease recognition site</td>
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<td>EcoRI restriction site</td>
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<td>on sigA</td>
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<td></td>
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<td>Primer_extension_1</td>
<td>5,414,135 d</td>
<td>CATTGGACACTGTCGAGATG</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Additional primers not listed here were used for confirmation PCR amplifications which have been carried out on the genome of newly constructed strains. In the marker replacement strains the presence of the inserted resistance gene, of the two flanking sequences, and the absence of the wild-type gene have been confirmed. In the complemented strains the presence of the integrated vector, the two flanking sequences, and the length of the in-frame deleted gene (if applicable) have been verified. Details about these primers are available from the authors upon request.

<sup>b</sup> In B. japonicum chromosome, except when indicated. d: direct strand; c: complementary strand.

<sup>c</sup> Unpairing 5' extension underlined.
2.3.14 Purification of PcuC and its derivatives

Details on the plasmids and primers are listed in Tables 2.1 and 2.3, respectively. For the periplasmic expression construct, the *B. japonicum* genomic sequence corresponding to the *pcuC* gene product (amino acids 25–173), excluding the predicted signal peptide, was amplified, adding a *Strep*-tag II sequence and a TEV protease recognition site (Parks et al., 1994) at the 5' end, and cloned into pEC415 (Schulz et al., 1998). This resulted in a plasmid for expression of PcuC that was additionally fused to the *E. coli* OmpA signal peptide. For cytoplasmic expression, DNA for the soluble part of PcuC was cloned into pKL1 to fuse it with a H10 tag followed by a TEV recognition site. Derivatives of PcuC in which H79, M90, H113, M115 and H113 plus M115 residues are substituted by alanine were obtained by exchanging the wild-type sequence of the cytoplasmic expression construct with synthetic fragments (custom-synthesized by Eurofins MGW Operon, Ebersberg, Germany) bearing the point mutations. A shortened version of *pcuC* missing the coding region for the 15-amino-acid, methionine-rich C terminus was created by PCR and inserting a stop codon at the 3' end. This was cloned into pKL1. Dense *E. coli* BL21 (DE3) pre-cultures containing the described expression vectors or the TEV protease expression vector pRK793 (Kapust et al., 2001) were used to inoculate 1-liter main cultures. Cells were grown at 37 °C until they reached an optical density (\(A_{600}\)) of 0.6; the expression was then induced by adding a final concentration of 0.1% (w/v) arabinose or 0.1 mM IPTG for the periplasmic PcuC expression and for the other expression constructs, respectively. Cultures were transferred to 30 °C, and after 2 h, cells were collected by centrifugation and disrupted by means of three passages at 9,000 p.s.i. through a French press. The periplasmically expressed protein was purified with a Strep-Tactin Sepharose column (IBA GmbH, Göttingen, Germany) according to the supplier's protocol. To obtain apo-PcuC, the purified protein was incubated with ~1000-fold molar excess of BCS, and the buffer was eventually exchanged with a PD-10 desalting column (GE Healthcare, Waukesha, WI). The poly-His tagged proteins were purified via Ni-NTA agarose columns (QIAGEN, Hilden, Germany). Cleavage of the tag was achieved by treatment with TEV protease, which was later removed with a Ni-NTA agarose column. Purity of the protein and the copper binding status were verified with electrospray ionization mass spectrometry (ESI-MS) measurements performed at the Functional Genomics Center Zurich.
2.3.15 Tests for Cu(I) binding to PcuC

PcuC from cytoplasmic expression was used for the experiment, which proved to be copper-free according to ESI-MS analysis. Alternatively apo-PcuC was obtained from periplasmic expression as described above. The titration buffer was obtained by filtering 100 mM HEPES pH 7, 10 mM NaCl through a Chelex 100 chelating ion exchange resin (Bio-Rad) into 0.1 M HCl-treated glassware in order to ensure a maximally possible absence of free copper. The protein elution buffer was exchanged with titration buffer using a PD-10 desalting column. The preparation of Cu(BCS)$_2^{3-}$ solution and the titration of apo-PcuC on the Cu(BCS)$_2^{3-}$ complex was carried out as described (Zhou et al., 2008), except that titration buffer was used. The experiment was performed both in anoxic and oxic conditions. In the first case, the solutions were prepared in an anaerobic glovebox (Coy Laboratory Products, Grass Lake, MI) with a maximal oxygen concentration of 80 p.p.m., and the UV-visible spectra were recorded on an Agilent diode array photometer. In the latter case, CuCl was dissolved directly into a BCS solution, and UV-visible spectra were recorded on a Hitachi U-3300 spectrophotometer (Hitachi, Tokyo, Japan).

2.3.16 Bioinformatics analyses

BLAST (http://blast.ncbi.nlm.nih.gov/) was used for homology searches. Multiple alignments were done with ClustalW (http://www.clustal.org/) and visualized with ESPript 2.2 (http://escript.ibcp.fr/ESPript/ESPript/). Presence and cleavability of signal peptide was predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP/). Extinction coefficient of purified proteins was calculated by ProtParam (http://web.expasy.org/protparam/). Homology modeling of the PcuC amino-acid sequence on the TtPCu$_4$C 3D structure (PDB ID: 2K6W) was done using SWISS-MODEL (http://swissmodel.expasy.org/).
2.4 Results

2.4.1 Comparative transcription profile of cells grown at different copper concentrations

Copper-limiting growth conditions were thought to cause an induction of genes possibly involved in copper uptake and sorting. This rationale in mind, we performed microarray analyses on *B. japonicum* cells grown in three variations of the BVM minimal medium. Variant 1 contained 2 μM CuSO₄ (copper excess). Variant 2 was prepared in HCl-treated glassware without any copper added (copper starvation). The residual copper concentration in this copper-starvation medium was analyzed by GF-AAS and determined to be 5 nM. Variant 3 (extreme copper limitation) was prepared like variant 2 but with the addition of 10 μM BCS and 1 mM ascorbic acid where BCS chelates Cu(I) selectively, and ascorbic acid reduces any Cu(II) to Cu(I). Changes in the transcription profiles were recorded by the pairwise comparison of cells grown in variant 2 vs. 1, and variant 3 vs. 2 (Tables 2.4 and 2.5, respectively). Only a small set of genes were differentially up- or down-regulated when copper-starved cells were compared with cells grown in copper excess (Table 2.4). Most notably, five genes located adjacent to each other on the *B. japonicum* genome displayed an increased expression: bll4882 to bll4878; (Table 2.4). For reasons that will become evident from subsequent research (*vide infra*), the five genes were named *pcuA*, *pcuB*, *pcuC*, *pcuD*, and *pcuE* (mnemonic of proteins for Cu trafficking). The genes with decreased expression are either of unknown function or – not surprisingly – play a role in copper resistance. Extreme copper limitation (variant 3 vs. 2) did not further enhance the expression of the five *pcu* genes. Instead, another cluster of adjacent genes was strongly up-regulated: bll0889 to bll0883.

### Table 2.4. Genes differentially expressed in copper-starvation conditions compared with copper-excess conditions in the *B. japonicum* wild type. Comparative microarray analyses were done with cells grown aerobically in copper starvation (BVM, 5 nM Cu) versus copper excess (BVM, 2 μM Cu). Protein-coding genes with either increased expression (fold-change >2) or with decreased expression (fold-change <–2) are listed.

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<thead>
<tr>
<th>ORF</th>
<th>Annotation</th>
<th>FC&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
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<td>TonB-dependent receptor (here named PcuB)</td>
<td>20.9</td>
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<tr>
<td>bll4882</td>
<td>PCu₅₇C-like protein (here named PcuA)</td>
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<tr>
<td>bll4880</td>
<td>hypothetical protein (here named PcuC)</td>
<td>5.4</td>
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<td>bll4797</td>
<td>hypothetical protein (here named PcuD)</td>
<td>5.3</td>
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<tr>
<td>bll4787</td>
<td>possible copper transport protein (here named PcuE)</td>
<td>4.6</td>
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<tr>
<td>bll4815</td>
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</tr>
<tr>
<td>bll4814</td>
<td>unknown protein</td>
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</tr>
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<td>bll5151</td>
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<td>hypothetical protein</td>
<td>–5.8</td>
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<tr>
<td>bll2209</td>
<td>copper tolerance protein, CopC-Like</td>
<td>–5.9</td>
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<sup>a</sup> Fold Change
Table 2.5. Genes differentially expressed in extreme copper limitation compared with copper-starvation conditions in the *B. japonicum* wild type. Comparative microarray analyses were done with cells grown aerobically in extreme copper limitation (BVM + 10 μM BCS + 1 mM ascorbic acid) versus cells grown in copper starvation (BVM, 5 nM Cu). Protein-coding genes with increased expression (fold-change >2) are listed in the left half of the table, whereas genes with decreased expression (fold-change < -2) are compiled on the right.

<table>
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<tr>
<th>ORF</th>
<th>Annotation</th>
<th>FC^a</th>
<th>ORF</th>
<th>Annotation</th>
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<td>bll1402</td>
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<td>bll0335</td>
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<td>bll2316</td>
<td>probable NADH-ubiquinone oxidoreductase, chain F</td>
<td>-2.1</td>
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<td>bll1149</td>
<td>5,10-methylene tetrahydrofolate reductase</td>
<td>3.5</td>
<td>bll0337</td>
<td>putative carbon monoxide dehydrogenase, medium chain</td>
<td>-2.3</td>
</tr>
<tr>
<td>bll0153</td>
<td>probable surfeit locus protein 1 (FixK2)</td>
<td>3.4</td>
<td>bll3876</td>
<td>aldehyde dehydrogenase</td>
<td>-2.4</td>
</tr>
<tr>
<td>bll2757</td>
<td>transcriptional regulatory protein</td>
<td>3.4</td>
<td>bll5219</td>
<td>small heat shock protein</td>
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</tr>
<tr>
<td>bll0150</td>
<td>putative quinol oxidase subunit I</td>
<td>3.3</td>
<td>bll0331</td>
<td>two-component response regulator</td>
<td>-2.6</td>
</tr>
<tr>
<td>bll0149</td>
<td>putative quinol oxidase subunit II</td>
<td>3.2</td>
<td>bll3149</td>
<td>putative oxalate:formate antiporter</td>
<td>-2.7</td>
</tr>
<tr>
<td>bll2221</td>
<td>adenylmethionine-8-aminooxyanolate aminotransferase</td>
<td>3.1</td>
<td>bll0330</td>
<td>two-component response regulator</td>
<td>-2.9</td>
</tr>
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<td>bll3148</td>
<td>unknown protein</td>
<td>-3.0</td>
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<tr>
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<td>hypothetical protein</td>
<td>-3.0</td>
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<td>-3.6</td>
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<tr>
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<td>2.7</td>
<td>bll333</td>
<td>unknown protein</td>
<td>-3.6</td>
</tr>
<tr>
<td>bll4303</td>
<td>putative amidase</td>
<td>2.6</td>
<td>bll3160</td>
<td>transcriptional regulatory protein, IclR</td>
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<td>bll2476</td>
<td>putative quinol oxidase subunit III</td>
<td>2.4</td>
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<td>2-oxoisovalerate dehydrogenase, beta subunit</td>
<td>-4.2</td>
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<td>bll0152</td>
<td>putative quinol oxidase subunit IV</td>
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<td>2-oxoisovalerate dehydrogenase, beta subunit</td>
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<td>bll4994</td>
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<td>2.3</td>
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<td>lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex E2</td>
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<td>bll4302</td>
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<td>2.2</td>
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<td>bll0332</td>
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<td>bll0332</td>
<td>unknown protein</td>
<td>-4.7</td>
</tr>
<tr>
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<td>bll0332</td>
<td>unknown protein</td>
<td>-4.7</td>
</tr>
<tr>
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<td>bll0332</td>
<td>unknown protein</td>
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<td>bll0332</td>
<td>unknown protein</td>
<td>-4.7</td>
</tr>
<tr>
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<td>bll0332</td>
<td>unknown protein</td>
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<td>bll0332</td>
<td>unknown protein</td>
<td>-4.7</td>
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<tr>
<td>bll1093</td>
<td>putative quinol oxidase subunit III</td>
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<td>bll0332</td>
<td>unknown protein</td>
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</tr>
<tr>
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<td>bll0332</td>
<td>unknown protein</td>
<td>-4.7</td>
</tr>
<tr>
<td>bll5476</td>
<td>putative quinol oxidase subunit III</td>
<td>2.1</td>
<td>bll0332</td>
<td>unknown protein</td>
<td>-4.7</td>
</tr>
</tbody>
</table>

^a Fold Change
which code for unidentified transport functions (Table 2.5). Incidentally, the list also includes the copper chaperone ScoI. As expected, the pairwise comparison of variant 3 vs. 1 (not shown) included again all of the differentially expressed genes listed in Table 2.4. Taken together, copper-limiting growth conditions have led to the de-repression of genes potentially involved in copper acquisition. In this work, we have focused on the investigation of the *pcuABCDE* gene cluster.

![Figure 2.1](image.png)

**Figure 2.1.** Map of the *pcuABCDE* gene cluster. Gene names and relevant homologies (if available) are given below the gene numbers. Genome coordinates are reported below the gene map. The top part of the scheme shows the transcription levels of *B. japonicum* wild-type cells grown copper excess BVM (2 μM CuSO₄, open circles) and in copper starvation BVM (5 nM Cu, closed circles) obtained from microarray analyses. The bottom part shows the genotype of the Δ*pcuABCDE::aphII* strain (same orientation) and the strategy used to partially complement it with an in-frame *pcuC*-deleted version of the operon (*pcuAB[ΔC]DE*), resulting in a Δ*pcuC* genotype. Analogous strategies were used to construct Δ*pcuA*, Δ*pcuB*, Δ*pcuD*, and Δ*pcuE* strains.

### 2.4.2 The *pcuABCDE* genes are transcribed as an operon

The short distances between the ORFs of the *pcuABCDE* cluster already suggested an operon organization in which the five genes would be co-regulated and co-transcribed. The tiling-like design of the oligonucleotides on the gene chip used for microarrays (Hauser et al., 2007) allowed us to confirm this inference because, consistently, transcript levels in copper-starved cells were higher throughout the entire *pcuABCDE* cluster as compared with copper-rich conditions (Fig. 2.1). Complemental information was brought about by applying qRT-PCR and primer-extension techniques. For these experiments, RNA was extracted from wild-
type cells grown in extreme copper limitation and from cells grown in standard BVM. When cDNA corresponding to \textit{pcuA} and \textit{pcuB} was produced thereof for qRT-PCR, the data reflected a respective up-regulation of 3.4±0.8- and 33.3±6.2-fold in copper limitation. The numbers compared well with the transcriptomics data. Likewise, a primer-extension product of the mRNA 5’ end was seen only in copper-limited cells (Fig. 2.2). The transcription start site was thus mapped at nucleotide position 5,414,300 in the \textit{B. japonicum} genome, which lies at a distance of 21 bp upstream of the \textit{pcuA} start codon. Sequence inspection of the associated promoter region did not allow predictions to be made on the possible mode of transcriptional regulation.

\textbf{Figure 2.2.} Transcription start site mapping of \textit{pcuA}. Total RNA from \textit{B. japonicum} wild type strain grown in standard BVM (0.02 µM CuSO₄, +) or in extreme copper limitation BVM (containing 10 µM BCS and 1 mM ascorbic acid, −). Extension products were obtained with the \([^{32}\text{P}]-\)labeled primer named "Primer_extension_1" and separated on a 6% denaturing polyacrylamide gel. The sequencing ladder was generated with plasmid pRJ6336 and the same primer. Part of the promoter region is shown on the right and the transcription start site (+1) is indicated (arrow).

\subsection*{2.4.3 Predicted proteins encoded by the \textit{pcuABCDE} operon suggest a role in copper trafficking}

The putative gene products can be grouped in two categories, soluble and membrane-bound proteins. PcuA, PcuC, and PcuD are predicted soluble, periplasmic proteins because all three have an N-terminal sequence for Sec-dependent secretion. PcuA is conserved only in the order Rhizobiales and does not contain a noteworthy amino-acid motif other than a CxxC sequence. PcuC is clearly homologous to the known copper chaperone PCuAC (for example, sharing 36% identity with the \textit{T. thermophilus} PCuAC protein; Fig. 2.3). PcuD is well conserved in bacteria, occasionally fused N-terminally with PcuC, but neither is its function known, nor does it contain a conspicuous amino-acid domain or motif. The PcuB and PcuE proteins are membrane integral proteins. PcuB is a member of the TonB-dependent receptor
Copper protein for cytochrome oxidase biogenesis

...protein family which implies a location in the outer membrane by virtue of the β-barrel structure. The high majority of the characterized members of this family are siderophore-iron or tetrapyrrole-metal importers (Krewulak and Vogel, 2011). It was tempting to speculate that PcuB might be a receptor for the uptake of a Cu-chelate complex through the B. japonicum outer membrane. PcuE is most likely a cytoplasmic membrane protein made up of an N-terminal CopC-like domain and a C-terminal CopD-like domain. The conserved CopC and CopD proteins have first been described in Pseudomonas syringae as part of the copper-resistance (export) system (Bender and Cooksey, 1986). By contrast, the B. subtilis YcnJ protein, which shares 27% identity with PcuE, is a CopCD-like fusion protein that was reported to have a copper-acquisition function (Chillappagari et al., 2009). In conclusion, the proteins encoded by the B. japonicum pcuABCDE operon appeared as strong candidates for playing a role in copper import and sorting.

2.4.4 The ΔpcuABCDE strains have a pleiotropic phenotype

Two deletion mutants were created by replacing the entire operon sequence with a kanamycin-resistance cassette in both orientations relative to the deleted genes, yielding strains 6611 (same orientation; Fig. 2.1) and 6612 (opposite orientation; Table 2.2). Aerobic growth was measured in different copper concentrations. The B. japonicum wild type and the two ΔpcuABCDE strains had an identical growth behavior in standard complete and minimal media or in copper-starvation medium under oxic conditions (data not shown). However, growth of the mutants was impaired in extreme copper limitation (Fig. 2.4A; here shown for strain 6611). Also, under conditions of anoxic nitrate respiration (YEM medium plus NO₃⁻, 0.2 μM CuSO₄), growth rate of the operon mutants was diminished when compared to the wild type (Fig. 2.5A). Concomitantly, a delayed consumption of nitrate and a transient accumulation of nitrite were observed in the mutants (Fig. 2.5B). This could be interpreted to mean that, in the mutants, the copper-dependent nitrite reductase (NirK; Velasco et al., 2001) is insufficiently supplied with the copper cofactor. In fact, attempts at limiting the copper concentration in such anoxic cultures led to a cessation of growth already with the wild type, making it impossible to further elaborate discriminative mutant phenotypes with respect to the denitrification pathway.
Figure 2.3. Multiple alignment of PCu₆C amino acidic sequences. The secondary structure of *Thermus thermophilus* PCu₆C extracted from its crystal structure (PDB ID: 2K6W) is shown above the alignment. The numbering refers to the *T. thermophilus* PCu₆C sequence. *B. japonicum* PcuC and *R. sphaeroides* 2.4.1 RSP_2017, *Rhodopseudomonas palustris* HaA2 RPB_2549, *Methylobacterium extorquens* PA1 Mext_1379, *Pseudomonas fluorescens* Pf0-1Pfl01_0598, *Vibrio cholerae* HC-02A1 VCHC02A1_2964, and *Nitrobacter hamburgensis* X14 Nham_2200 sequences are included in the alignment. Similarity and identity columns are shown as empty boxes and negative typing, respectively. Copper-binding residues of *Tt* PCu₆C are marked with triangles; the corresponding residues of PcuC are H79, M90, H113 and M115. The grey-shaded amino acids at the C-terminal of the PcuC sequence are those that were removed to test if they bind copper. The C-terminal 118-amino-acid domain of Blr7088 was omitted in the alignment.

Another important phenotype observed with the ΔpcuABCDE mutants was the complete lack of activity of the *aa₃*-type cytochrome oxidase, which is the predominant respiratory oxygen reductase in oxically grown *B. japonicum* wild type. The membrane protein fraction was isolated from oxically grown strains, and the ability to oxidize reduced horse heart cytochrome *c* was measured. As shown in Fig. 2.6, oxidase activity in wild type is due to cytochrome *aa₃*, since membrane proteins extracted from a *coxA* mutant (Tn5 insertion in *aa₃*-subunit-I gene) have almost no activity. Cytochrome *c* oxidase activity of both ΔpcuABCDE strains (here shown for 6611) is as low as that of the *coxA* mutant, indicating an involvement of the operon in cytochrome *aa₃* biogenesis. The wild-type phenotype could be restored by growing the cells in medium containing 50 μM CuSO₄. Western blot analyses of
all of these membrane extracts (Fig. 2.6, bottom part) revealed an absence of both the subunits I and II in the ΔpcuABCDE mutant, whereas they were detectable again in cells grown in copper excess. We tentatively concluded, therefore, that the pcuABCDE operon is involved in providing Cu for the aa3-type cytochrome c oxidase.

**Figure 2.4.** Decreased growth of the operon mutant ΔpcuABCDE under copper starvation is due to loss of pcuC. Strains were grown in BVM medium under extreme copper limitation (no copper added, 10 μM BCS and 1 mM ascorbic acid added). Aerobic growth was measured from a starting optical density of 0.02 until stationary phase, and values represent the average optical density of cultures measured in triplicate. A. Wild-type *B. japonicum* (filled square) and ΔpcuABCDE strain 6611 (open circle). B. Single-gene deletion strains ΔpcuE (open square), ΔpcuD (open triangle), ΔpcuC (filled circle), ΔpcuB (open diamond) and ΔpcuA (cross).

Finally, as we routinely test any *B. japonicum* mutant generated in our laboratory for its symbiotic efficiency, a 75% decrease of symbiotic nitrogen fixation activity (measured with the acetylene reduction test) was recorded in soybean root nodules induced with the ΔpcuABCDE strains (Table 2.6). The possible reason for this strong defect will be addressed further below (last Results section). Addition of excess copper to the plant growth medium did not help to abrogate the defect.

**Table 2.6.** Symbiotic properties of ΔpcuABCDE mutants inoculated on soybean. Mutants were tested in parallel with the wild type in three independent experiments. Shown here are data from one representative experiment.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Number of nodules</th>
<th>Nodule dry weight</th>
<th>Nitrogenase activity</th>
<th>Relative Fix activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>110spc4</td>
<td>wild-type</td>
<td>20.4 ± 4.0</td>
<td>2.4 ± 0.7</td>
<td>3.0 ± 0.7</td>
<td>100 ± 22.3</td>
</tr>
<tr>
<td>6611</td>
<td>ΔpcuABCDE-a</td>
<td>25.8 ± 7.8</td>
<td>1.3 ± 0.3</td>
<td>0.8 ± 0.5</td>
<td>25.9 ± 16.9</td>
</tr>
<tr>
<td>6612</td>
<td>ΔpcuABCDE-b</td>
<td>23.3 ± 5.1</td>
<td>1.4 ± 0.4</td>
<td>0.7 ± 0.4</td>
<td>24.4 ± 12.5</td>
</tr>
</tbody>
</table>

*a* Strain 6611 has insert in the same orientation (-a) and strain 6612 in the opposite orientation (-b)

*b* Nitrogenase activity is expressed as percentage of C2H4 formed/min/g

*c* Relative nitrogen fixation activity is expressed as a percentage of wild type
Figure 2.5. Anaerobic growth, NO_3^− consumption and transient NO_2^− accumulation in the ΔpcuABCDE mutant. The following strains were tested: wild type (closed squares), 6611 (ΔpcuABCDE insert in same orientation, open circles), and 6612 (ΔpcuABCDE insert in opposite orientation, open triangles). A. Growth curves in anaerobic YEM medium containing 10 mM KNO_3. B. Concentrations (mM) of NO_3^− (continuous lines) and NO_2^− (dashed lines) in the medium during growth. All the measurements were taken in triplicates.

Figure 2.6. Effect of the pcuABCDE deletion on aa_3-type cytochrome c oxidase activity. The relative cytochrome c oxidase activity of aerobically grown wild type (wt) and strains 6611 (ΔpcuABCDE) and COX132 (coxA::Tn5) is shown in the upper panel. Wild-type activity corresponds to 0.206 μmol horse heart cytochrome c oxidized/mg of membrane protein/min. 50 μM CuSO_4 was added to the PSY medium where indicated (+). Western Blot analysis for the detection of subunits I and II of the cytochrome aa_3 (CoxA and CoxB, respectively) was carried out on the same membrane proteins (bottom panels).

2.4.5 pcuC is the only essential gene of the pcuABCDE operon for all tested functions

Having established a pleiotropic phenotype associated with the operon deletion, it was necessary to find out how many and which of the five genes are to be made responsible. For this purpose, a comprehensive complementation strategy was applied in which either all five genes or five-minus-one were recombined back into the deletion mutant 6611. The latter
constructs consisted of operons in which a marker-less in-frame deletion was generated in each of the five genes, leaving the other four intact (see the ΔpcuC example in Fig. 2.1). This resulted in a set of five strains with single-gene deletions plus the controls (vector insertion, complete operon; Table 2.2). All strains were then tested for most of the phenotypes reported in the preceding section. Surprisingly, the ΔpcuC deletion proved to be the only responsible mutation for the decreased cytochrome c oxidase activity (Table 2.7), and this defect was restored to wild-type activity in cells grown with copper excess (Table 2.7). Deletions of pcuA, pcuB, pcuD, or pcuE did not cause a defect. Likewise, the ΔpcuC strain was the only one that exhibited an impaired aerobic growth in conditions of copper limitation (Fig. 2.4B), and it was the only one that had a decreased symbiotic nitrogen fixation activity (Table 2.7). The ΔpcuC phenotype was unlikely the result of polar effects of the deletion on the downstream operon genes, because the deletions of pcuE and pcuD themselves had no effect. Nevertheless, we ascertained by qRT-PCR that DNA downstream of the pcuC in-frame deletion was fully transcribed (data not shown).

**Table 2.7.** Complementation of ΔpcuABCDE (strain 6611) and effects of single-gene deletions on the phenotypes. Activities of the mutant strains are expressed as a percentage of wild-type activity.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genes added for complementation</th>
<th>Relative cytochrome c oxidase activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Relative Fix activity (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>50 μM Cu(SO)&lt;sub&gt;4&lt;/sub&gt;−</td>
<td>+</td>
</tr>
<tr>
<td>6611</td>
<td></td>
<td>16.8 ± 4.7</td>
<td>100.1 ± 12.8</td>
</tr>
<tr>
<td>6611-32</td>
<td>Only vector insert</td>
<td>14.4 ± 5.1</td>
<td>90.3 ± 9.8</td>
</tr>
<tr>
<td>6611-33</td>
<td>pcuABCDE&lt;sup&gt;+&lt;/sup&gt;</td>
<td>106.6 ± 10.5</td>
<td>nd</td>
</tr>
<tr>
<td>6611-6630</td>
<td>pcuABCD[ΔE]&lt;sup&gt;+&lt;/sup&gt;</td>
<td>113.4 ± 9.9</td>
<td>nd</td>
</tr>
<tr>
<td>6611-1654</td>
<td>pcuABC[ΔD]&lt;sup&gt;+&lt;/sup&gt;</td>
<td>120.5 ± 18.2</td>
<td>nd</td>
</tr>
<tr>
<td>6611-34</td>
<td>pcuAB[ΔC]&lt;sup&gt;+&lt;/sup&gt;D&lt;sup&gt;+&lt;/sup&gt;</td>
<td>24.8 ± 4.4</td>
<td>100.5 ± 10.1</td>
</tr>
<tr>
<td>6611-1650</td>
<td>pcu[ΔB]&lt;sup&gt;+&lt;/sup&gt;CDE&lt;sup&gt;+&lt;/sup&gt;</td>
<td>101.0 ± 17.0</td>
<td>nd</td>
</tr>
<tr>
<td>6611-1653</td>
<td>pcu(Δ)ABCDE&lt;sup&gt;+&lt;/sup&gt;</td>
<td>111.8 ± 19.5</td>
<td>nd</td>
</tr>
</tbody>
</table>

<sup>a</sup> The average wild-type cytochrome c oxidase activity (100%) is ~0.31 μmol horse heart cytochrome c oxidized/mg of membrane protein/min. The average wild-type Fix activity (100%) is 2.82 ± 0.4% C<sub>2</sub>H<sub>4</sub>/min/g. nd, not determined

2.4.6* B. japonicum* possesses a second pcuC-like gene (blr7088) without an obvious function

The predicted Blr7088 protein shares in its N-terminal half 40% identity with PcuC (cf. Fig. 2.3) whereas the C-terminal extension carries a weakly conserved membrane domain. The blr7088 gene is located next to the respiratory nitrite reductase gene (*nirK, blr7089*) which might implicate a function of the Blr7088 protein in the biogenesis of the Cu-
containing nitrite reductase. However, Δblr7088 strains (6620 and 6621, Table 2.2) were constructed and shown to be unaffected in anoxic growth with nitrate as the terminal electron acceptor. Also, these mutants had none of the phenotypes observed for the ΔpcuABCD strains, except for a slight delay in aerobic, Cu-limited growth. Furthermore, double-deletion strains (ΔpcuABCDE plus Δblr7088, Table 2.2) had phenotypes that were not more severe than those observed for the ΔpcuABCDE strains, except for perhaps an additive impairment in aerobic, Cu-limited growth (data not shown). Hence, the function of the Blr7088 protein remains elusive, and there was no indication that it might be able to functionally replace the PcuC protein in a pcuC mutant background. Therefore, research on blr7088 was discontinued.

![Figure 2.7](image)

**Figure 2.7.** PcuC binds one Cu(I) ion via H79, M90, H113 and M115. A. UV/visible spectra of incremental addition of apo-PcuC (wild type) to a 7.5 μM Cu(BCS)_2^3− solution. Spectra were taken when 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6 and 2.0 protein equivalents per copper atom were added. Arrows indicate the direction of the absorption change after the protein addition. B. Incremental addition of apo-PcuC[M90A] to a 7.5 μM Cu(BCS)_2^3− solution is shown as an example. An analog behavior was observed for the other substituted versions of PcuC (data not shown). C. Plot of A_{483} against the ratio of PcuC wild type (closed circles), PcuC[H79A] (open circles), PcuC[M90A] (squares), PcuC[H113A] (triangles), PcuC[M115A] (diamonds) and PcuC[H113A/M115A] (crosses) to total Cu(I). Saturation is observed only when PcuC (wild type) was used in the experiment.

### 2.4.7 PcuC is a Cu(I)-binding PCu₄C-like protein

Since pcuC turned out to be the only important gene of the pcuABCDE operon (under the conditions tested), we wished to obtain additional evidence for its role in copper metabolism. The PcuC protein was expressed in *E. coli* using both periplasmic and cytoplasmic overexpression systems (see Experimental Procedures), and the proteins were purified to apparent homogeneity from these compartments. ESI-MS analyses revealed that cytoplasmically produced PcuC was copper-free whereas periplasmically expressed PcuC contained one Cu ion bound to it. Cu could be removed from the latter by treatment with a >1,000-fold molar excess of BCS but not with EDTA (data not shown). Considering the high
specificity of the BCS chelate for Cu(I) (Smith and Wilkins, 1953; Zak, 1958), this pointed towards a preference of PcuC for Cu(I). Increasing amounts of Cu-free PcuC were then mixed as competitors with a constant amount of the Cu(BCS)$_2^{3-}$ complex, and the decrease of the characteristic 483-nm absorbance peak of the latter (Zhou et al., 2008) was recorded by UV/visible absorption spectroscopy until saturation occurred (Fig. 2.7A). The result showed that the affinity of PcuC for Cu(I) was higher than that of BCS. Applying the method elaborated by Zhou et al. (2008), we arrived at an estimated $K_D \leq 10^{-16}$ M. When the $A_{483}$ values were plotted against the PcuC/Cu(I) ratio, we observed disappearance of Cu(BCS)$_2^{3-}$ after the addition of one equivalent of PcuC, indicating a PcuC:Cu(I) stoichiometry of 1:1 (curve with black circles in panel C of Fig. 2.7). Similar data were obtained with a PcuC derivative in which the last 15, non-conserved amino acids had been deleted (data not shown). This carboxy-terminal extension is rich in possible Cu-binding amino acids (five Met, two His, one Glu, one Asp; Fig. 3). The unaltered activity of the truncated form precluded a role of this peptide region in copper binding.

**Figure 2.8.** Confirmation of molecular mass and homogeneity of PcuC and its derivatives. The graphs show ESI-MS spectra of purified proteins from cytoplasmic extraction after TEV protease treatment. The measured molecular masses, reported near the corresponding peaks, are compatible with the expected ones for every derivative. PcuC[H113A] and PcuC[M115A] show analogous results (omitted). The intensity axis is scaled to the highest peak.
Chapter II

The Cu-binding amino-acid ligands described for the homologous *T. thermophilus* PCuAC protein (Abriata et al., 2008) are conserved in PcuC (Fig. 2.3), and their similar spatial orientation was confirmed by modeling the PcuC primary structure onto the three-dimensional structure of *T. thermophilus* PCuAC (data not shown). Therefore, copper binding was also tested with purified mutant derivatives of PcuC in which each of the four presumed Cu(I)-binding residues had been substituted for alanine: H79A, M90A, H113A, M115A. Further, a double-replacement derivative was tested (PcuC[H113A/M115A]). Purity and identity of these proteins were documented by mass spectrometry (Fig. 2.8). All of the mutant proteins had an impaired capability of removing Cu(I) from the Cu(BCS)$_3^{3-}$ complex, as shown with PcuC[M90A] as an example (Fig. 2.7B). The plot in Fig. 2.7C illustrates that saturation occurred only with wild-type PcuC, whereas a moderate decrease in $A_{483}$ was observed for the substituted proteins; hence, they bound Cu(I) more weakly than wild-type PcuC. The experiments supported the notion that PcuC binds one Cu(I) ion with high affinity via H79, M90, H113, and M115.

2.4.8 PcuC and ScoI are required for the symbiotically essential *cbb*$_3$-type cytochrome oxidase

As described above, deletion of the *pcuC* gene caused a defect in symbiotic nitrogen fixation (Tables 2.6 and 2.7). Having shown that the PcuC protein is a periplasmic Cu(I) protein, we asked whether or not it is involved in the biogenesis of the symbiotically essential *cbb*$_3$-type oxidase. To assess its functionality, cells were first grown anoxically with nitrate as the terminal electron acceptor. Under these conditions, virtually all of the cytochrome *c* oxidase activity present in wild-type membranes stems from cytochrome *cbb*$_3$ but not from cytochrome *aa*$_3$ (Fig. 2.9A, compare *coxA* mutant with *fixN* mutant). The activity measured with membranes extracted from anoxically grown Δ*pcuABCDE* cells was as high as that from the wild type. It thus appeared as if PcuC was not important for the formation of cytochrome *cbb*$_3$. Prompted by recent findings of Thompson et al. (2012), who reported that PCuAC was a biogenesis factor for cytochrome *cbb*$_3$ in *R. sphaeroides*, we tested membrane proteins that had been extracted from endosymbiotic cells. For this purpose, root nodule bacteroids of the wild type and of the deletion mutants 6611 (Δ*pcuABCDE*) and 6611-34 (Δ*pcuC*) were separated from plant material via sucrose gradient centrifugation prior to membrane isolation. Bacteroids of *coxA* and *fixN* mutants served as positive and negative controls, respectively. The *cbb*$_3$-type oxidase activity was strongly impaired in membranes of *pcuC* mutant bacteroids (Fig. 2.9B). In contrast to the result of Fig. 2.9A, this now suggested a role of PcuC
in cytochrome \( cbb_3 \) biogenesis and provided a possible explanation for the symbiotic nitrogen fixation defect of \( pcuC \) mutants.

In previous work (Bühler et al., 2010), we found that a \( B. japonicum \) \( scoI \) mutant had very similar phenotypes as described now for the \( pcuC \) mutant, such as the absence of an effect on \( cbb_3 \)-type oxidase activity in cells grown anoxically. The unexpected condition-dependent difference (free-living, anoxic cells vs. symbiotic bacteroids) led us to investigate the \( cbb_3 \) oxidase activity of the \( scoI \) mutant also in symbiosis. Indeed, oxidase activity was strongly impaired in membranes of \( scoI \) mutant bacteroids (Fig. 2.9). Hence, the ScoI protein is important for the formation of cytochrome \( cbb_3 \) specifically in symbiosis, just like PcuC.

![Figure 2.9](image-url)

**Figure 2.9.** PcuC and ScoI are involved in \( cbb_3 \) oxidase biogenesis in bacteroids. A. Relative cytochrome \( c \) oxidase activity of anaerobically grown wild type (wt), and strains 6611 (\( \Delta pcuABCDE \)), COX132 (\( coxA::Tn5 \)), and 3613 (\( fixN::Tn5 \)). Wild-type activity corresponds to 0.891 \( \mu \)mol horse heart cytochrome \( c \) oxidized/mg of membrane protein/min. B. Relative cytochrome \( c \) oxidase activity of bacteroid membrane proteins from wild type (wt) and strains 6611 (\( \Delta pcuABCDE \)), 6611-34 (\( \Delta pcuC \)), 2575 (\( \Delta scoI \)), COX132 (\( coxA::Tn5 \)), and 3613 (\( fixN::Tn5 \)). Wild-type activity corresponds to 1.15 \( \mu \)mol horse heart cytochrome \( c \) oxidized/mg of membrane protein/min.

### 2.5 Discussion

Our expectation to discover de-repressed genes in \( B. japonicum \) cells grown in copper-limited conditions was fulfilled. Which and how many of these are in fact important for copper acquisition is a matter of ongoing and future research. Extreme copper limitation led to the expression of seven clustered genes (bll0889–bll0883), preceded by a predicted transcription activator (bll0890), which code for two putative cytoplasmic membrane
complexes of the ABC transporter family and a major-facilitator-superfamily (MFS) transport protein (bll0889). Interestingly, Ekici et al. (2012b) have reported an MFS-type transporter (CcoA) that is specifically required for cytochrome $cbb_3$ biogenesis in $R. capsulatus$. Although $B. japonicum$ has several $ccoA$ homologues, the bll0889 gene product might play a similar role because of its low-copper inducibility. Undoubtedly, this cluster of $B. japonicum$ genes is worthy of further investigations.

For the present work, we decided to explore the $pcuABCDE$ operon as it attracted attention for the following reasons: (i) it is already induced by more moderate copper-starvation conditions (5 nM residual Cu in so-called “Cu-free” medium); (ii) it looked as if the five genes are organized and co-regulated as an operon, for which we then obtained experimental evidence; and (iii) the encoded proteins promised to constitute a novel relay system for copper acquisition in a Gram-negative bacterium consisting of a TonB-ExbBD-dependent outer-membrane receptor (PcuB), one or two periplasmic copper chaperones (PcuC, and perhaps PcuA), and a Cu-specific cytoplasmic membrane transporter (PcuE). This idea of a co-operating import system was further strengthened by the pleiotropic phenotype of a $pcuABCDE$ deletion mutant which suffered defects in Cu-limited growth, symbiotic nitrogen fixation, and anoxic denitrification. In a first approximation these phenotypes were supposed to be caused by a critical shortage of Cu for the synthesis of Cu-containing enzymes such as the $aa_3$- and $cbb_3$-type cytochrome oxidases, and the nitrite and nitrous-oxide reductases. After a functional gene-by-gene analysis, we demonstrated that the missing $pcuC$ gene alone could be made responsible for all of the defects. Why the $pcuA$, $pcuB$, $pcuD$ and $pcuE$ genes are functionally inconspicuous remains a mystery at present. Given the substantial induction of expression and clear co-regulation together with $pcuC$ under copper starvation, the precise conditions in which cells will need the other four genes have yet to be identified. The minimal copper concentrations one can reach in laboratory media might still be too high to mimic the copper-limited niches that bacteria may encounter in natural environments. What speaks in favor of this argument is the ease with which the $pcuC$ defect could be abrogated by the simple supplementation of media with more copper, albeit at a different threshold level to achieve phenotypic compensation.

Further investigations focused on $pcuC$ and its product. The results have ascertained the cupro-protein nature of PcuC. The reduced form of copper ($Cu^+$) was found to be bound to purified PcuC in a 1:1 ratio, and the estimated affinity ($K_D \leq 10^{-16}$ M) compares well with data obtained for other copper chaperones (Banci et al., 2010). Deduced from the structure of a PcuC-homologous protein (PCuAC; Abriata et al., 2008), the two histidines (H) and two
methionines (M) for copper binding lie in an unusual protein motif \((H{-}X_{10}{-}M{-}X_{22}{-}HXM)\) in which not only the four ligands but also the fairly large spacing is evolutionarily well conserved. We confirmed here by mutational analysis that all four liganding amino acids are needed for efficient Cu binding to PcuC. Furthermore, we made sure by truncation that the 15 carboxy-terminal amino acids, which are rich in M and H, are dispensable. This truncated version might become useful in crystallization studies because efforts to crystallize the full-length PcuC protein have been unsuccessful. In vivo, the PcuC protein was shown here to be involved in the biogenesis of the \(aa_3\)-type cytochrome oxidase. This was anticipated since Abriata et al. (2008) had demonstrated the transfer of Cu(I) from \(T.\ thermophilus\) PCuAC to the the Cu\(_A\) site of the \(ba_3\)-type oxygen reductase \textit{in vitro}. Less expected was the role that PcuC appears to play in the formation of the \(cbb_3\)-type oxygen reductase because this oxidase lacks the Cu\(_A\) center. Two independent studies (Thompson et al., 2012, and this work) have now arrived at this new function. With an expedient combination of genetic and biochemical approaches, Thompson et al. (2012) have recently shown for \(R.\ sphaeroides\) that PCuAC works both for the metallation of the Cu\(_A\) site of its \(aa_3\)-type oxidase and for the metallation of the Cu\(_B\) site of its \(cbb_3\)-type oxidase. Furthermore, these authors argued that PCuAC might even be involved to some extent in the formation of the Cu\(_B\) center in the \(aa_3\)-type oxidase in spite of the presence of Cox11 (see Introduction) which is still the major player in this process (Thompson et al., 2010). Our own data with \(B.\ japonicum\) was less straightforward because the dependencies of cytochrome \(cbb_3\) formation on PcuC differed fundamentally in anoxic, culture-grown cells and in symbiotic bacteroids, where a \(pcuC\) mutant had no effect in the former but a strong defect in the latter. Formally, we cannot rule out that a PcuC-compensating protein is synthesized in free-living conditions but not in symbiosis. In any case, we showed that the PcuC-homologous Blr7088 protein was not involved. We rather suspect that the copper concentrations play a decisive role. In order to achieve optimal growth of \(B.\ japonicum\) under denitrifying conditions (even with the wild type), the copper concentration should not fall below 0.2 \(\mu M\), which might already be high enough to bypass the need for PcuC in the assembly of active cytochrome \(cbb_3\). In symbiosis, however, it is possible that such a functional compensation does not work if the copper concentration that is available for bacteroids in root nodules is very low. Unfortunately, it is practically difficult, if not impossible, to assess and distinguish Cu contents in separate compartments such as the cytosol of colonized plant cells in nodules and the symbiosome (\textit{i.e.}, bacteroids plus peribacteroid membrane-surrounded space). Our attempt to abrogate the symbiotic defect by
increasing the copper content in the plant growth medium has not been successful. The bottom line of our investigations was that PcuC is important for cytochrome \( cbb_3 \) formation specifically in endosymbiotic bacteroids. This fact alone is sufficient to explain the low nitrogen fixation activity in \( pcuC \) mutant-infected soybean nodules because this bacteroid process depends on micro-oxic respiration via the high-affinity \( cbb_3 \)-type oxidase (Preisig et al., 1996b). Along the same line, we now showed that the \( B. \) japonicum \( scoI \) mutant had a similar condition-dependent phenotype as the \( pcuC \) mutants, i.e., the \( scoI \) mutation negatively affected cytochrome \( cbb_3 \) activity in bacteroids (Fig 2.9B) but had no effect on this oxidase in cells grown under anoxic, denitrifying conditions (Bühler et al., 2010). Therefore, we must revise a previous interpretation according to which ScoI was not believed to be an important biogenesis factor for cytochrome \( cbb_3 \) in \( B. \) japonicum (Bühler et al., 2010). Other recent studies have provided good evidence for a role of ScoI-like proteins (SenC) in cytochrome \( cbb_3 \) formation in \( P. \) aeruginosa (Frangipani and Haas, 2009), \( R. \) sphaeroides (Thompson et al., 2012), and \( R. \) capsulatus (Lohmeyer et al., 2012).

In conclusion, a unifying concept seems to emerge at least for members of the \( \alpha \)-proteobacteria according to which two proteins (ScoI-like and PcuC-like) are required collectively for the biogenesis of two types of cytochrome oxidases (\( aa_3 \) and \( cbb_3 \)). To which extent each of the two biogenesis factors is involved in the metallation of the \( \text{Cu}_A \) and \( \text{Cu}_B \) sites, and how they cooperate with each other, needs to be explored. Lack of ScoI (Bühler et al., 2010) or PcuC (cf. Fig. 2.6) leads to an apparent destabilization and/or degradation not only of subunit II but also of subunit I of cytochrome \( aa_3 \). Therefore, one cannot readily attribute an exclusive function to the chaperones in either \( \text{Cu}_A \) or \( \text{Cu}_B \) formation. Intuitively, one would like to propose that the soluble, periplasmic PcuC protein disseminates Cu to the membrane-bound ScoI protein and perhaps to other membrane-bound recipients (e.g., CoxG/Cox11, FixI/CcoI, and CcoA) as has been suggested in parts also by others (Arunothayan et al., 2010; Ekici et al., 2012a; Lohmeyer et al., 2012; Thompson et al., 2012). However, more biochemical work such as establishing a copper transfer assay with purified components \textit{in vitro} will be needed as proof.
Chapter III

In vivo effect of PcuC H79A and H113A/M115A substitutions
3.1 Summary

PcuC is a *B. japonicum* periplasmic protein able to bind Cu\(^+\) *in vitro* via the conserved residues H\(_{79}\), M\(_{90}\), H\(_{113}\), and M\(_{115}\). Its deletion causes defective copper-limited growth and misassembly of the *aas* and *cbb*\(_3\)-type cytochrome oxidases, both cuproenzymes. These data support the view of PcuC as a copper-transferring protein.

In this study, M90A and H113A/M115A substitutions in the copper-binding site have been integrated in the genome of *B. japonicum* in order to investigate whether the copper-binding ability of PcuC is essential for its functionality.

The phenotypization of the two strains revealed that *pcuC[H113A/M115A]* phenotype is similar to that of Δ*pcuC*, whereas the *pcuC[M90A]* phenotype is almost overlapping that of the wild type. A copper-independent function for PcuC is unlikely, but further studies are required to understand whether the difference in phenotype between the two substituted versions of PcuC is simply due to a different affinity for copper.

3.2 Introduction

In the previous chapter it has been shown that *pcuC[H113A/M115A]* phenotype is similar to that of Δ*pcuC*, whereas the *pcuC[M90A]* phenotype is almost overlapping that of the wild type. A copper-independent function for PcuC is unlikely, but further studies are required to understand whether the difference in phenotype between the two substituted versions of PcuC is simply due to a different affinity for copper.
assessment of the effect that mutations in the copper-binding residues have on the copper-binding capability of a PCuAC protein was reported. The mutation of any of the residues belonging to the motif causes a drop in the affinity for Cu⁺. Our interest is to evaluate the effect of the same substitutions in vivo, in order to understand whether the role of PcuC is dependent entirely on its ability to bind copper. In this chapter, the phenotype of two B. japonicum strains containing two different PcuC amino-acid substitutions will be described and discussed.

3.3 Experimental procedures

3.3.1 Bacterial strains and media

Growth media and antibiotic concentrations used for E. coli and B. japonicum cultures have been already described in section 2.3.1. B. japonicum strains used in this work are listed in Table 3.1.

3.3.2 Construction of the strains

Strains, plasmids and primers used are listed in Table 3.1. In order to create substituted versions of pcuC in the genome of B. japonicum the entire pcuABCDE operon bearing the desired substitutions in pcuC had to be inserted in the ∆pcuABCDE strain 6611, analogously to what carried out to obtain single gene mutants of the operon (see section 2.3.3). In the restriction map of the plasmid pRJ6626, bearing the entire operon on pGEM-T Easy, EcoRV and FspAI were identified as the only suitable unique restriction sites for the excision of the pcuC region containing the amino acids to substitute. In the expression vectors pRJ6642 and pRJ6645, carrying soluble versions of pcuC[M90A] and pcuC[H113A/M115A] (see section 2.3.14), the FspAI site is not present since it naturally occurs inside pcuD. These two vectors were then used as PCR template in which the reverse primer (80subst_rv) contains a 95-bp extension identical to the genomic region comprised between the pcuC stop codon and the FspAI restriction site. The resulting amplicons and pRJ6626 were digested with EcoRV and FspAI; the wild-type fragment of pRJ6626 was then replaced with the [M90A] and [H113A/M115A] fragments, yielding plasmids pRJ6649 and pRJ6650, respectively. The two substituted versions of the operon have been then transferred in the pSUP202pol4 suicide plasmid. The so-constructed pRJ6651 and pRJ6652 plasmids were mobilized into B. japonicum 6611 via E. coli S17-1, and followed by screening for single recombination events, originating strains 6611-51 (pcuC[M90A]) and 6611-53 (pcuC[H113A/M115A]).
### Chapter III

#### Table 3.1. *B. japonicum* strains, plasmids, and primers used in this work

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<td>(Fischer et al., 1993)</td>
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<td>Chapter 2</td>
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### 3.3.3 Phenotypization of the strains

Sterilization of soybean seeds (*Glycine max* (L.) Merr. cv. Williams), cultivation of plants, and nitrogenase activity measurements 21 days after infection were performed as previously described (Hahn and Hennecke, 1984; Göttfert et al., 1990a; Göttfert et al., 1990b; Bühler et al., 2010).
Preparation of the membrane fraction and determination of cytochrome c oxidase activity were carried out as described (Gerhus et al., 1990; Bühler et al., 2010).

3.4 Results

3.4.1 H113 and M115 of PcuC are required in copper starvation

The substituted strains have been evaluated for their capability of growing in different media. Growth in aerobic rich or minimal medium with the standard copper concentration of 0.02 µM did not differ between wild type and the amino-acid substituted strains 6611-51 and 6611-52 (data not shown), as observed for the operon mutant. Given the defect in copper starvation observed for ΔpcuC it was interesting to evaluate if the amino-acid substitutions would provoke a similar growth delay. Interestingly, the 6611-51 strain bearing pcuC[H113A/M115A] shows a limited grow comparable with that of the ΔpcuC strain, while pcuC[M90A] seems to grow with an efficiency which lies in the middle between the wild type and ΔpcuC (Fig. 3.1).

![Figure 3.1. Effect of the aminoacid substitutions in copper depleted growth. The following strains were tested: wild type (filled squares), 6611 (ΔpcuABCDE, open circles), 6611-33 (6611 complemented with the operon, open squares), 6611-51 (pcuC[M90A], filled diamonds), and 6611-52 (pcuC[H113A/M115A], filled triangles). Values represent the average optical density of cultures measured in triplicate.]

3.4.2 Nitrogenase activity of pcuC[H113A/M115A] is strongly impaired

One of the most interesting phenotypes observed for the ΔpcuC strain is the defect in nitrogen fixation, which is due to the lack in functional cbb3-type cytochrome oxidase. When plants were infected with pcuC[M90A] and pcuC[H113A/M115A], a decrease in the nitrogenase activity was observed only in the doubly-substituted strain, while the pcuC[M90A] phenotype resembled the wild type one (Fig. 3.2). The reduced functionality though does not seem as evident as when the entire pcuC gene is missing (cf. Table 2.7).
3.4.3 The \(aa_3\) oxidase is defective in the \(pcuC[H113A/M115A]\) strain

Membrane proteins have been isolated from aerobically grown strains 110spec4 (wild type), COX132 (\(Δ\)coxA), 3613 (\(Δ\)fixN), 6611-34 (\(Δ\)pcuC), 6611-51 (\(pcuC[M90A]\)), and 6611-52 (\(pcuC[H113A/M115A]\)), and their ability to oxidize reduced cytochrome \(c\) was evaluated. As shown in sections 2.4.4 and 2.4.5 \(pcuC\) is essential for the \(aa_3\) oxidase activity. Similar to what was observed in the nitrogenase activity measurements, only the double H113A/M115A substitution causes a decrease in cytochrome \(c\) oxidase activity, whereas the M90A substitution has no negative effect on the oxidase biogenesis (Fig. 3.3).
3.5 Discussion

In this work, we wanted to assess whether the copper-binding properties of PcuC are essential for its in-vivo functionality. To do so, two strains bearing two different amino-acid substitutions, which in vitro caused a decreased copper-binding affinity, were constructed.

The substitution of two out of four copper binding residues (pcuC[H113A/M115A]) resulted in a phenotype which resembles the ΔpcuC deletion concerning growth in copper limiting medium, symbiosis, and aa₃ oxidase activity. This led to the interpretation that copper-binding is necessary for PcuC functionality.

What was observed in the pcuC[M90A] strain is not as straightforward. This substitution clearly disrupted high-affinity copper binding in the in-vitro BCS competition assay described in section 2.4.7, but it did not seem to cause a defect in the biogenesis of the aa₃ and cbb₃ oxidases, while in the copper-starved growth it had an apparent weak influence. One could speculate that the affinity of pcuC for Cu⁺ decreases significantly below the BCS one when Met₉₀ is replaced by an alanine, but not as much as when His₁₁₃ and Met₁₁₅ are both replaced. According to this interpretation the BCS competition assay would not allow discrimination of differences in copper affinities when they are significantly lower than the BCS affinity for Cu⁺. The residual affinity of pcuC[M90A] for copper would still be enough to bind the metal and transfer it to the targets, but not to bind it as tightly as the wild-type version when it becomes limiting, explaining the small defect in copper-limited growth.

A function of pcuC that is independent of metal binding is not likely, but it cannot be excluded completely because of what was observed for the pcuC[M90A] strain. To prove whether the small defect observed in the pcuC[M90A] strain is indeed due to the retaining of a higher copper affinity than the one pcuC[H113A/M115A] has, an independent method for the extrapolation of the copper-binding dissociation constant may be necessary. Moreover, creation of other strains with different combinations of substitutions could in future help for a better understanding of the role of the different copper-coordinating residues.
Future perspectives
4.1 A revised working model for cytochrome oxidase biogenesis

The model presented in Fig. 1.9 (see page 46) has to be updated as a consequence of the results presented in this work, in particular regarding two main aspects: the updated role of ScoI and the addition of a new player, PcuC.

4.1.1 The newly emerging role of Sco in proteobacteria

At the time of ScoI characterization it was assumed a generalized role of Sco as protein involved in the biogenesis of the CuA center of cytochrome oxidases. The defect in cytochrome c oxidase activity observed in ΔscoI mutants has been therefore attributed to a lack of copper insertion into the CuA center, considering its ability to bind copper in vitro. This idea was enforced by the apparent lack of activity of ScoI toward the biogenesis of the CuA-free \(cbb_3\) oxidase. The symbiotic defect which characterizes strains lacking scoI remained unexplained (Bühler et al., 2010). The reasons why Sco proteins have been associated with the CuA center, and not to CuB, have been listed in the section 1.1.3. There is indeed lack of strong evidence for this link both in eukaryotes and in prokaryotes, and in no case could it be excluded that Sco is involved in the assembly of the CuB center as well, even though Cox11-like proteins have been more clearly related to its biogenesis (Hiser et al., 2000). The situation is furthermore complicated by the ambiguous biochemical activity of Sco, which apparently can be either a copper chaperone, a dithiol:disulfide oxidoreductase, or both, probably depending on the organism.

Recently, a role of Sco proteins in the biogenesis of CuB centers has been inferred in three different proteobacteria: \(P.\ aeruginosa\) PA01, \(R.\ capsulatus\) and \(R.\ sphaeroides\) (Swem et al., 2005; Frangipani and Haas, 2009; Lohmeyer et al., 2012; Thompson et al., 2012). In all of them the assembly of the \(cbb_3\) oxidase depends on the presence of a Sco-like protein, and in the third it seems that even the CuB center of the \(aa_3\) oxidase is defective when the sco-like gene is deleted. A direct interaction between the CuB center and Sco has been detected in \(R.\ capsulatus\). These studies prompted us to verify if indeed the \(cbb_3\) oxidase is active in ΔscoI bacteroids. It was extremely interesting to observe that also in \(B.\ japonicum\) the \(cbb_3\) oxidase is indeed impaired, even though the defect is evident only when membrane proteins are isolated directly from the bacteroids.

This result enforces a view in which, at least in proteobacteria, Sco function is not restricted to the CuA center biogenesis, but is extended to the CuB center of \(cbb_3\). This also means that in these organisms, differently to the distant-related \(T.\ thermophillus\) (Abriata et al., 2008), Sco would be a copper chaperone, rather than a dithiol:disulfide oxidoreductase,
since there is no cysteine to be reduced in the CuB center. Moreover the redox potential of *B. japonicum* ScoI is not low enough to allow the reductase activity (Mohorko et al., 2012). The CuA center cysteines, embedded in the oxidizing periplasm, would then require other reductases. *B. japonicum* TlpA is responsible for the reduction of the ScoI cysteines, so it might also work for the CuA center.

### 4.1.2 PCuAC collaborates with Sco

The second main revision to the previous model consists of the addition of PcuC, the PCuAC-like protein. The close link between Sco and PCuAC has been key to the identification of PCuAC itself, since its encoding gene is a conserved *sco* neighbor (Banci et al., 2005). In *T. thermophilus* it has been shown that they collaborate for the biogenesis of the CuA center *in vitro*, where Sco in this case is a dithiol:disulfide oxidoreductase and PCuAC directly inserts copper into the CuA center of the *ba*3 oxidase (Abriata et al., 2008). In *R. sphaeroides*, both the proteins are involved in copper delivery to the CuB center of the *cbb*3 oxidase and the CuA and CuB centers of the *aa*3 oxidase. Their mutants share strikingly similar behaviour, excluding that the effect on several phenotypes of *sco* deletion is stronger than that of deleting PCuAC (Thompson et al., 2012).

An analogous scenario has been described in this work on *B. japonicum*. Also in this case Sco and PCuAC collaborate for the biogenesis of the *cbb*3 and *aa*3 oxidases, even though it is not possible to understand which copper center of the second is targeted. Moreover we know that ScoI and PcuC are both copper-binding proteins. The other copper chaperones for the two oxidases identified in *B. japonicum* probably act downstream of this protein pair, since they have an oxidase-specific phenotype: Δ*coxG* results in an inactive *aa*3 but in an active *cbb*3 oxidase, while Δ*fixI* behaves in the opposite way (Preisig et al., 1996a; Bühler et al., 2010). Furthermore, we speculate that PcuC transfers copper to ScoI, which then delivers it directly or indirectly to the terminal oxidases, according to the following observations. First, copper has to reach ScoI and the other membrane-bound chaperones, requiring a soluble periplasmic chaperone, which would be PcuC. Second, in *R. sphaeroides* Sco is probably downstream of PCuAC, since Sco deletion causes greater defects in copper insertion (Thompson et al., 2012). Third, in *R. capsulatus*, crosslink experiments placed Sco at the end of the copper delivery pathway, since it interacts with one of the final cuproenzymes, the *cbb*3 oxidase (Lohmeyer et al., 2012). The model depicted in Fig. 4.1 summarizes these considerations.
4.2 Validating the model

Our proposed model for copper trafficking to the terminal oxidases is highly hypothetical and requires fundamental questions to be solved.

Some information is missing about the actual copper binding nature of the copper chaperones. It is surely of interest to understand what is the affinity for both Cu$^+$ and Cu$^{2+}$ of the chaperones ScoI, PcuC and CoxG, in a similar way to what has been done in this work for the Cu$^+$-PcuC interaction. Since this requires the protein to be purified, such experiments are probably less applicable to transmembrane proteins as the ones encoded in the fixGHIS operon. Eventually the copper transfer pathway should be followed in the most direct possible way in order to clarify the topology of the model. Different combinations of apo- and holo-PcuC, -ScoI$_{sol}$ and -CoxG$_{sol}$ could be mixed together and copper transfer followed by NMR, for example (as what was done by Abriata et al., 2008). Alternatively, the final solution can be analyzed by ESI-MS or by separating the protein mix with a size-exclusion chromatography and assess their copper content by spectrometry, when possible, or by inductively coupled plasma mass spectrometry (ICP-MS). In this experiment the Cu$_A$ center of the $aa_3$ oxidase could be included, but would require the establishment of a purification protocol.

In order to identify in-vivo interactions, fishing experiment could be carried out, using a His-tagged query protein as bait. The crosslink with the interactor can be achieved using
formaldehyde (as for *R. capsulatus*, Lohmeyer et al., 2012), the tagged protein can be then purified exploiting the tag and run on an SDS-PAGE, where bands can be analyzed by immunoblotting and/or with mass spectrometry. This technique is likely applicable also to membrane proteins, as it was done by Lohmeyer et al. (2012), and is probably one of the few suitable methods for the investigation of the biogenesis of the CuB centers.

The way FixI, FixG, FixH and FixS work for the correct functionality of the *cbb*$_3$ oxidase is not clear. The homology between FixI and copper P$_{1B}$-type ATPases links the *fixGHIS* operon with copper transfer. The need of a P$_{1B}$-type ATPase for the biogenesis of the *cbb*$_3$ oxidase has been observed also in *R. gelatinosus* and *R. capsulatus* (Koch et al., 2000; Hassani et al., 2010). They belong to a class of Cu-P$_{1B}$-type ATPases featuring high affinity for substrate and a slow export rate, as reported for the *P. aeruginosa* and *Synechocystis* PCC 6803 members (González-Guerrero et al., 2010; Raimunda et al., 2011). How copper export to the periplasm would result in the biogenesis of the CuB center is not understood. Probably a crosslinking experiment would help identify possible interacting partners.

Finally, it is of interest to identify whether TlpA is responsible for the reduction of the Cu$_A$-center cysteines, in addition to those of ScoI. A soluble version of subunit II (CoxB) needs to be purified, and an experimental setup similar to that described by Mohorko et al. (2012) could be applied. The typical transient formation of a heterodisulfide bridge between dithiol:disulfide oxidoreductases and their targets could be exploited to trap *in vivo* specific targets of TlpA. This method requires the expression in *B. japonicum* of a tagged TlpA in which one of the two cysteines is mutated in order to avoid the resolution of the transient disulfide bridge, and a subsequent tag-specific purification, similarly to what proposed above. Such an experiment could confirm TlpA activity on ScoI *in vivo*, and reveal other possible TlpA targets, determining whether the *aa*$_3$ oxidase subunit II is also included.

### 4.3 Other possible copper trafficking proteins

The transcriptomic analyses carried out in the work here presented revealed other genes possibly acting in copper import and trafficking (see section 2.4.1 and Tables 2.4 and 2.5). The *pcuABCDE* operon has been selected for this study because of its inducibility in copper starvation and for the interesting predicted features of the encoded proteins. Even though PcuC is the only gene with a clear function in the tested conditions, it is difficult to imagine that the other genes have no role in copper trafficking. Anaerobic growth is the only tested phenotype which was revealed to be due to all of the genes of the operon. Its study was
complicated by the impossibility to grow wild type *B. japonicum* anaerobically in copper starvation. It is very likely that the *pcuABCDE* proteins are indeed involved in the biogenesis of cuproenzymes for the anaerobic respiration, including to date unidentified azurins or pseudoazurins (to be searched among the four hypothetical blue copper proteins listed in Table 1.3, page 44). Anaerobic growth curves from the single gene mutants in decreasing copper concentration may in future help in finding threshold conditions under which these genes become essential for denitrification. A more general copper import and sorting function could have been masked by functional redundancy, or because even in the extreme copper limitation medium the available copper was still enough to make these genes unnecessary. Moreover it is possible that the target of these proteins is an uncharacterized cuproenzyme whose corresponding phenotype has not been characterized. To verify whether an import function is actually encoded in the *pcuABCDE* operon, measurement of cellular copper amount via ICP-MS in the wild type and in the operon mutant may be an option.

Another remarkably interesting gene cluster has been revealed by the microarray analysis when comparing copper starvation conditions with extreme copper limitation (Table 2.5). It consists of seven genes, from bll0889 to bll0883, encoding two putative ABC transporter complexes and an MFS (Major facilitator superfamily) transport protein. Moreover a putative regulator, bll0890, was found just upstream the first gene of the cluster. Recently, an MFS-type transporter has been associated with copper transport, since it is required for the biogenesis of the *cbb₃* oxidase of *R. capsulatus* (Ekici et al., 2012b). The characterization of this gene cluster in *B. japonicum* is surely another challenging follow-up of the project presented here.
References


Baker, E.N. (1994) *Copper proteins with type 1 sites*, Wiley Interscience, Chichester, UK


kinetic, and thermodynamic properties of Cu(I) and Cu(II) binding by methanobactin from *Methylosinus trichosporium* OB3b. *Biochemistry* **45**, 1442-1453


Hartwig, A. (2001) Zinc finger proteins as potential targets for toxic metal ions: differential effects on structure and function. Antioxid. Redox Signal. 3, 625-634


Li, X.Z., Nikaido, H., and Williams, K.E. (1997) Silver-resistant mutants of Escherichia coli display active efflux of Ag⁺ and are deficient in porins. J. Bacteriol. 179, 6127-6132


bacterial laccase that occurs as a structural component of the Bacillus subtilis endospore coat. J. Biol. Chem. 277, 18849-18859
FixLJ-dependent regulatory cascade for control of genes inducible by low oxygen levels. *J. Bacteriol.* **180**, 5251-5255


References


Ye, Q., Imriskova-Sosova, I., Hill, B.C., and Jia, Z. (2005) Identification of a disulfide switch in BsSco, a member of the Sco family of cytochrome c oxidase assembly proteins. *Biochemistry* 44, 2934-2942

Yoneyama, H., and Nakae, T. (1996) Protein C (OprC) of the outer membrane of *Pseudomonas aeruginosa* is a copper-regulated channel protein. *Microbiology* 142, 2137-2144


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