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PERIPLASMIC PROTEINS INVOLVED IN THE BIOGENENSIS OF CYTOCHROME OXIDASES IN *BRADYRHIZOBIUM JAPONICUM*

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DORIS BÜHLER

Master of Science, University of Berne

Born August 16, 1981

Citizen of Aeschi b. Spiez

accepted on the recommendation of

Prof. Dr. Hauke Hennecke

Prof. Dr. Hans-Martin Fischer

Prof. Dr. Rudolf Glockshuber

Dr. Guido Capitani

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Summary

Bradyrhizobium japonicum, the nitrogen-fixing root-nodule symbiont of soybean, has four quinol oxidases, two aa_3-type cytochrome *c* oxidases, one novel type of cytochrome *c* oxidase, and one cbb_3-type cytochrome *c* oxidase. The latter is specifically required for microoxic respiration in symbiosis. In contrast to cytochrome aa_3 which has a copper A center (Cu_A) in subunit II and a copper B center (Cu_B) in subunit I, the cytochrome cbb_3 contains only Cu_B in subunit I, whereas subunit II is a monoheme cytochrome *c*.

The present thesis aimed at unraveling the biogenesis of these two cytochrome oxidases. It turned out that they rely on disparate pathways for the maturation of their copper sites. ScoI (Blr1131) and CoxG (Blr1174) are periplasmic, membrane-anchored proteins that are widely conserved through the kingdoms of life and that had been proposed to be required for the biogenesis of the Cu_A and Cu_B centers, respectively.

A Δ*coxG* deletion mutant of *B. japonicum* could undergo a fully functional symbiosis with soybean but was strongly impaired in free-living respiration. Thus, we concluded that this factor is involved in biogenesis of the aa_3-type cytochrome oxidase but is dispensable for formation of the symbiotically essential cbb_3-type cytochrome oxidase.

The phenotype of a Δ*scoI* mutant was more pleiotropic than the phenotype of a Δ*coxG* mutant. In addition to a similar respiratory defect, a Δ*scoI* mutant was also impaired in symbiosis. However, the activity of the cbb_3 cytochrome oxidase was not affected in a Δ*scoI* mutant. Accordingly, we concluded that the symbiotic defect of the latter is not due to a contribution of ScoI in the biogenesis of the high-affinity cbb_3 oxidase. All the other proteins containing a predicted Cu_A site, namely CoxM (subunit II of an alternative aa_3 cytochrome oxidase), Bll4481 (a predicted subunit II of an unknown cytochrome oxidase), and NosZ, an N_2O reductase, were not required for an effective symbiosis of *B. japonicum* with soybean. Therefore, they were not considered to be symbiosis-relevant targets of ScoI. In order to obtain a list of possible target proteins of ScoI, we performed a bioinformatics approach. The genome of *B. japonicum* was screened for proteins encoding a periplasmic, simplified Cu_A binding site, namely a CXXXC-motif. We combined this search with results from proteome and transcriptome analyses that had been performed with *B. japonicum* bacteroids obtained from soybean nodules. Only candidates that were expressed in symbiosis were retained. This resulted in a list of 34 putative target proteins. Among these, we found a family of five highly conserved proteins that were present only in *B. japonicum* and its closest relatives. Their function is unknown, and one member of this family, namely Blr1130, is encoded in the immediate vicinity of *scoI* on the *B. japonicum* chromosome.

ScoI, like its homologs, contributed to the biogenesis of the aa_3 cytochrome oxidase. Two possible mechanisms, or a combination of both, had been proposed for Sco-like proteins to achieve their function: a copper-chaperone activity, or a disulfide-reductase activity. To clarify by which of those mechanisms ScoI contributed to the biogenesis of the aa_3 cytochrome oxidase, biochemical analyses were performed. ScoI was shown to bind Cu(II) *in vitro*, and the conserved cysteine residues 74 and 78, which belong to the Sco-motif (CXXXC), were essential for binding. This is in contrast to the conserved histidine residue 162 which was found to be dispensable for copper binding. ScoI was shown to be inactive in an insulin reduction assay; moreover, its redox potential was measured to be approximately –155 mV. These biochemical findings challenge the disulfide-reductase hypothesis and point towards a copper-chaperone activity. This is corroborated by the fact that the respiratory defect of a Δ*scoI* mutant could be complemented by copper addition to the growth medium. The redox potential of ScoI makes it a possible target for TlpA. TlpA (Bll1380) is a periplasmic, membrane-anchored *B. japonicum* protein having a redox potential of –213 mV and possessing dithiol-disulfide oxidoreductase activity. A Δ*tlpA* mutant displays a strikingly similar pleiotropic phenotype as a Δ*scoI* mutant. We therefore hypothesized that TlpA keeps the active-site cysteines of ScoI reduced prior to copper binding.

Due to its toxic effects, free copper is present at a very low concentration in cells. The hypothesis that copper reaches the periplasm only passively has been contested in a few organisms lately. Thus, we were curious to learn more about adaptation mechanisms of *B. japonicum* towards high copper concentrations and towards copper starvation. A transcriptome analysis of *B. japonicum* grown under different copper concentrations was performed. The regulon obtained from this analysis helped identify candidate proteins involved in copper export and import. Interestingly, *scoI* was among the genes upregulated under copper starvation. This supported again our conclusion that ScoI is most probably involved in copper distribution rather than in a disulfide-reductase activity.

Résumé

Bradyrhizobium japonicum est une bactérie capable de vivre en symbiose avec le soja. Dans ce cas, elle induit le développement de nodosités situées sur les racines de son hôte, où elle peut ensuite fixer l'azote et fournir celui-ci à son hôte. *B. japonicum* possède quatre quinole oxydases, deux cytochromes oxydases du type aa_3, une cytochrome *c* oxydase d'un type inconnu et une cytochrome oxydase du type cbb_3. Celle-ci sert à la respiration de la bactérie lorsqu'elle vit en symbiose. Contrairement à l'oxydase du type aa_3 qui possède un site cuivre du type A (Cu_A) localisé dans la sous-unité II et un site cuivre du type B (Cu_B) dans la sous-unité I, la cytochrome oxidase du type cbb_3 ne possède que le Cu_B. La sous-unité II est remplacée par un cytochrome *c*.

Le but de cette thèse était d'élucider la biogenèse de ces deux cytochromes oxydases (aa_3, cbb_3). Il s'est avéré que toutes les deux utilisent des voies différentes afin d'assurer la maturation de leurs site cuivre. ScoI (Blr1131) et CoxG (Blr1174), deux protéines périplasmiques largement conservées à travers les règnes, sont considérées comme des facteurs requis pour la maturation de respectivement Cu_A et de Cu_B.

Une souche mutée dans le gène *coxG* (Δ*coxG*) est parfaitement capable d'établir une symbiose fonctionnelle avec son hôte, alors que sa respiration « free-living » est quasi nulle. Ceci nous a permis de conclure que CoxG est bien requis pour la maturation de la cytochrome oxydase aa_3, alors qu'elle n'est pas nécessaire pour la maturation de la cytochrome oxydase cbb_3.

Une souche mutée dans le gène *scoI* (Δ*scoI*) possède un phénotype plus pléiotropique que Δ*coxG*. En plus de la déficience respiratoire similaire dans les deux mutants, Δ*scoI* rencontre de sérieuses difficultés lorsqu'il s'agit d'établir la symbiose avec le soja. Cependant, l'activité de l'oxydase cbb_3 n'étant pas défectueuse dans la souche Δ*scoI,* la déficience en symbiose de cette souche ne peut être due à une contribution de ScoI dans la biogenèse de l'oxydase cbb_3.

Toutes les protéines pour lesquelles un site Cu_A a été prédit, c'est à dire CoxM (une sous-unité II d'une cytochrome oxydase aa_3) Bll4481 (une sous-unité II d'une cytochrome oxydase de type inconnu) et NosZ (une réductase à oxyde nitreux) ne sont pas requises pour la mise en place d'une symbiose fonctionnelle entre *B. japonicum* et le soja. C'est pourquoi, elles ne peuvent êtres des cibles de ScoI essentielles pour la symbiose. Nous avons effectué une recherche bioinformatique afin d'obtenir une liste de candidats potentiels qui pourraient être dépendants de ScoI pour leur biogenèse. Les protéines exprimant un site Cu_A simplifié, c'est-à-dire ayant le motif CXXXC dans le périplasme, ont été cherchées dans le génome de *B. japonicum*. Des données obtenues dans des analyses du protéome et du transcriptome de bactéroides de *B. japonicum* en symbiose avec le soja ont été combinées à cette recherche. Ainsi, seuls les candidats dont l'expression a été démontrée en symbiose (au niveau transcriptionnel ou translationnel) ont été retenus. Une liste de 34 cibles possibles de ScoI

a ainsi été obtenue. Parmi celles-ci, une famille de cinq gènes hautement conservés et que l'on ne trouve que dans le génome de *B. japonicum* et dans quelques bactéries très proches phylogénétiquement a été détectées. La fonction de ces protéines est inconnue, mais relevons encore que l'un des membres de cette famille, le gène *blr1130*, se situe juste en amont du gène *scoI* sur le chromosome de *B. japonicum*.

ScoI, tout comme ses homologues, contribue à la biogenèse de la cytochrome oxydase du type aa_3. Deux mécanismes, ou une combinaison des deux, pourraient être à la base de la fonction des protéines Sco. Les protéines Sco pourraient, en effet, servir de chaperonne distribuant le cuivre ou de disulfide-réductase. Afin de clarifier par lequel de ces mécanismes ScoI agit sur la biogenèse de la cytochrome oxydase du type aa_3, des analyse biochimiques ont été effectuées. Nous avons ainsi démontré que ScoI est capable de lier du Cu(II) *in vitro* et que les cystéines conservées C74 et C78 sont requises pour cette interaction. Ces cystéines font partie du motif Sco (CXXXC) et, de plus, elles sont essentielles pour le fonctionnement de ScoI. Par contre, un autre acide aminé conservé de ScoI, l'histidine 162, n'est quant à lui pas requis pour lier le cuivre. De plus, nous avons démontré que la ScoI purifiée était incapable de réduire l'insuline et que le potentiel d'oxydo-réduction de ScoI est d'environ –155 mV. Les propriétés biochimiques de ScoI remettent en cause une fonction de disulfide-réductase et suggèrent plutôt une fonction de chaperonne responsable de la distribution du cuivre.

En raison de ses effets toxiques, la concentration de cuivre en solution est généralement très basse dans toute cellule. De plus, l'hypothèse que l'import du cuivre dans le périplasme des bactéries se fait passivement a été fortement contestée récemment. De ce fait, il nous a paru intéressant de savoir comment *B. japonicum* réagit en présence de concentration élevées de cuivre ainsi qu'en l'absence (virtuelle) de cuivre. Le transcriptome de *B. japonicum*, cultivé avec des concentrations de cuivre variables, a été analysé. Nous avons ainsi identifié des protéines susceptibles d'assumer un rôle dans la tolérance face à ses diverses conditions. Parmi ces protéines, certaines participent probablement à l'import, et d'autres à l'export du cuivre. ScoI, la chaperonne qui a été l'objet principal de cette thèse, était parmi les protéines surexprimées dans des conditions de manque de cuivre. Ce fait confirme une fonction de distributrice de cuivre, telle qu'elle l'a déjà été suggérée par les propriétés biochimiques de la protéine, et sous-entend qu'une fonction de disulfide-réductase est plutôt improbable pour ScoI.

CHAPTER 1

Introduction: Biogenesis of cytochrome oxidases in Bacteria

Cytochrome oxidases are terminal enzymes of the respiratory chain. Their function is to catalyze the reduction of dioxygen to water while the gained redox energy is used for proton translocation across the mitochondrial inner membrane or the bacterial cytoplasmic membrane (Wikström, 1977; Richter & Ludwig, 2003; Hosler *et al.*, 2006; Belevich & Verkhovsky, 2008). Per catalytic cycle, 8 protons are taken from the mitochondrial matrix, respectively from the cytoplasm, four are pumped across the membrane and four are used for the reduction of O_2 to two molecules of water (Wikström, 2004). The study of cytochrome oxidases began in 1925 when Keilin recognized the importance of these "pigments" in respiration and their ubiquity among species (Keilin, 1925). Since then, they have captivated generations of scientists. Cytochrome oxidases are present in Eukarya, Archaea and Bacteria, thus it is assumed that they are of monophyletic origin. It has been proposed that their evolutionary forerunners are the nitric oxide reductases which also belong to the heme-copper superfamily (van der Oost *et al.*, 1994; Saraste, 1994; Castresana *et al.*, 1994; Zumft, 1997; Hendriks *et al.*, 1998, Giuffre *et al.*, 1999, Zumft, 2005b). Cytochrome oxidases may vary in terms of subunit composition, heme-types and electron donor. However, the central subunit I always consists of 12 transmembrane helices and contains 6-fold histidine-coordinated low-spin heme (A or B) and a high-spin heme (A_3, O_3 or B_3) associated with a copper center (Cu_B). The latter is the common denominator of all cytochrome oxidases (García-Horsman *et al.*, 1994; Castresana *et al.*, 1994; Trumpower & Gennis, 1994; Poole & Cook, 2000; Pereira *et al.*, 2001). As they are composed of several subunits, require cofactors, and in the case of eukaryotes are encoded partly by the mitochondrial genome and partly by the nuclear genome, their assembly is a complex process which requires a plethora of assisting factors. Recently, the factors involved in cytochrome *c* oxidase biogenesis in Eukarya were reviewed (Carr & Winge, 2003; Khalimonchuk & Rödel, 2005; Herrmann & Funes, 2005; Fontanesi *et al.*, 2008; Stiburek & Zeman, 2010). It turns out that to date, more than 30 assisting factors were identified to be necessary for efficient cytochrome oxidase biogenesis in eukaryotes (Tzagoloff & Dieckmann, 1990; Fontanesi *et al*., 2008). While for some of them the bacterial counterparts were discovered, genes encoding others appear to be absent in prokaryotes. In this review, we give a survey on the current knowledge in the field of cytochrome oxidase biogenesis in Bacteria.

1.1 Different cytochrome oxidases in Bacteria and their subunit composition

In eukaryotes, cytochrome oxidases (complex IV of the respiratory chain) are highly conserved and always follow the same architecture, at least in the three mitochondrially encoded core subunits. On the contrary, in Bacteria, a great diversity of different oxidases is found. Most aerobic bacteria possess

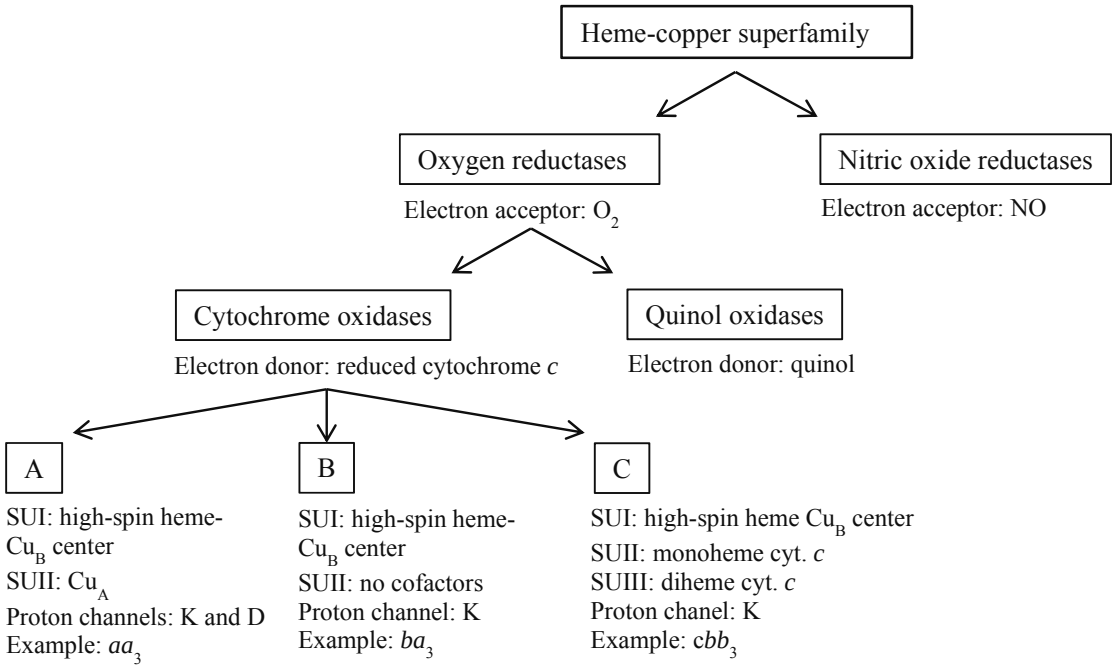
several terminal oxidases, leading to branched respiratory chains which allow adaptation to different growth conditions. This reflects the versatility of Bacteria with regard to lifestyle and metabolism (Anthony, 1988, Richardson, 2000). All eukaryotes, except a few strictly anaerobic unicellular ones like *Trichomonas vaginalis* (Ellis *et al*., 1992*)*, have cytochrome oxidases, but some prominent members of the bacterial world lack them entirely and rely exclusively either on quinol oxidases, or on anaerobic oxidases using nitrite, fumarate, DMSO or also much more exotic electron acceptors like the radionuclide uranium (Lovley & Phillips, 1992), and finally, some bacteria do not respire at all because they fully cover their energy needs with fermentation. An example is the strictly anaerobic *Clostridium* sp. which does not possess cytochromes or an electron transport chain and which produces its ATP via substrate-level phosphorylation (Nölling *et al.*, 2001; Shimizu *et al.*, 2002). Among the aerobic bacteria that are devoid of cytochrome oxidases we find important human pathogens like *Salmonella typhi*, *Staphylococcus aureus*, and *Yersinia pestis*, and also *Escherichia coli*; however, they have quinol oxidases (Kranz *et al*., 2002).

The commonly used classification of cytochrome oxidases proposes three groups named A, B, and C (FIG. 1.1). To date, the A group counts the biggest number of described members, but this may be biased because it includes all the mitochondria-like aa_3 cytochrome oxidase. However, designating the oxidases by their heme content is treacherous, as there are aa_3 oxidases that do not belong to the A-group (Pereira *et al*., 2001). The aa_3 oxidase encoded by the *soxABCD* genes that is found in the Archaeon *Sulfolobus acidocaldarius* illustrates how confusing heme names can be. This aa_3 oxidase results from a fusion of a "traditional" aa_3 cytochrome oxidase and a quinol oxidase and, contrary to the general rule, it does not oxidize cytochrome *c* but quinols (Anemüller & Schäfer, 1990; Anemüller *et al.*, 1992; Lübben *et al.*, 1992; Lübben *et al.*, 1994a; Lübben *et al.*, 1994b).

The core of the A-type cytochrome oxidases comprises a subunit I containing a copper B center (Cu_B) (see later in FIG. 1.3.B) associated with a heme A_3 forming the high-spin heme-copper center where the reduction of dioxygen to water takes place. Another heme, the low-spin heme A, is also non-covalently attached to subunit I. Its function is to shuttle electrons from the entry point (in aa_3 oxidases this task is fulfilled by the copper A center ($Cu_{A)}$) to the high-spin active center which is buried in the hydrophobic core of the enzyme. Subunit II harbors the aforementioned Cu_A (see later in FIG. 1.3.A). Subunit III is devoid of redox-active cofactors and can be removed from the complex without drastic alteration in catalytic activity. Its function rather seems to be related to stabilization of the multimeric structure of the oxidase (Haltia *et al.*, 1989). Eukaryotic mitochondria exclusively use the aa_3 cytochrome oxidase, and in Bacteria it is very widespread, especially in the α-proteobacteria, the supposed ancestors of mitochondria (Margulis, 1970). However, it appears that not all α-proteobacteria have an A-type cytochrome oxidase. *Rhodobacter capsulatus*, for example, lacks this type. On the other hand, the distribution of aa_3 oxidases in Bacteria is not restricted to α-proteobacteria: the Gram-positive *Bacillus subtilis*, for example, has a caa_3-type cytochrome oxidase,

whereas the *c* stands for a cytochrome *c* domain that is fused with the oxidase (Saraste *et al.*, 1991; van der Oost *et al.*, 1991b; Lauraeus & Wikström, 1993; Andrews *et al.*, 2005). Cytochrome oxidases of the aa_3-type have also been found in the respiratory chains of many aerobic Archaea.

FIG. 1.1. Classification of the heme-copper superfamily. The criteria that are used for the classification are indicated below the boxes. SU stands for subunit.

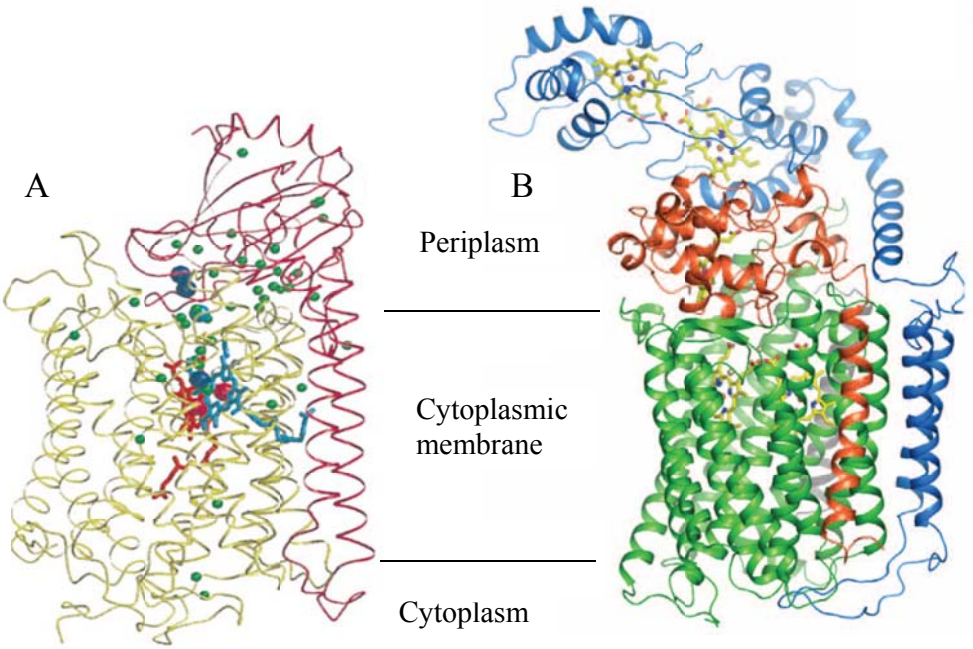


The C-group of cytochrome oxidases is also widespread among Bacteria, as a genomic survey showed. They are found in almost every phylum and, thus, this group is also proposed to be evolutionarily ancient (Ducluzeau *et al.*, 2008). In addition to the phylogenetic findings, the fact that most of them are high-affinity oxidases suggests that they might have been present already in early evolutionary times when the atmosphere had not yet reached the present day O_2-level (Castresana *et al.*, 1995). The only bacterial phyla in which the C-group cytochrome oxidase has not been found, so far, are the Thermotogales, the Deinococcales, and the Firmicutes. Moreover, the C-group cytochrome oxidases are completely absent in Archaea (Ducluzeau *et al.*, 2008). The *fixNOQP* encoded high-affinity oxidase of *Bradyrhizobium japonicum* was the first member of the C-group of cytochrome oxidases that has been described and characterized (Preisig *et al.*, 1993; Preisig *et al.*, 1996b). It plays a central role in the legume-root nodule symbiotic lifestyle of this bacterium. It has also been suggested that the cbb_3-type oxidases assume a central role in pathogenesis since it is necessary for

Vibrio cholerae and *P. aeruginosa* to efficiently colonize the intestinal and the respiratory tracts of their hosts (Myllykallio & Liebl, 2000). Whereas subunit I of C-type cytochrome oxidases (FixN/CcoN) is homologous to the subunit I of the A-group cytochrome oxidases, the subunits II (FixO, CcoO) and III (FixP, CcoP) are not homologous to the A-group cytochrome oxidase subunits II and III. These subunits are mostly replaced by membrane anchored mono- and di-heme cytochromes in the C-group members.

The structures of two representatives of the aa_3-type oxidases, namely the one from bovine mitochondria (Tsukihara *et al*., 1995; Tsukihara *et al*., 1996) and *Paracoccus denitrificans* (Iwata *et al*., 1995 and Ostermeier *et al.*, 1997) have been solved more than a decade ago (FIG. 1.2.A). Only very recently the structure of the *Pseudomonas stutzeri* cbb_3–type cytochrome oxidase was described (FIG. 1.2.B). These structural data provide evidence that although O_2-binding and reduction is conserved among these two families, there are substantial differences regarding e^- shuttling and proton pumping (Buschmann *et al.*, 2010).

FIG. 1.2. Ribbon representations of structures of two of the four subunits of the aa_3-type cytochrome oxidase from *P. denitrificans* (A) and of the cbb_3-type cytochrome oxidase *from P. stutzeri* (B). Figures are taken from Ostermeier *et al.* 1997 and Buschmann *et al*., 2010, respectively. Subunits I are represented in green, subunits II in red, and in B subunit III is represented in blue. A. Heme A is represented in red, heme A_3 in blue, the copper atoms are represented in blue and the green globes represent water molecules. B. All hemes are represented in yellow and the heme B_3-associated copper atom (Cu_B) was omitted. Localization and orientation of the subunits in the membrane is schematically indicated between the two oxidases.



As for the A- and C-group oxidases, the features of subunit I are also conserved in B-group oxidases; however, the subunits II and III are devoid of cofactors (Pereira *et al.*, 2001). The founding member of the B-group of cytochrome oxidases is the ba_3 oxidase of *Thermus thermophilus* (Zimmermann *et al.*, 1988). This oxidase was crystallized and the resolved structure revealed some peculiarities with regard to the hydrophobic channels that are thought to represent diffusion pathways for O_2 (Luna *et al.*, 2008). Like the A-group oxidases, B-group members are widespread among Bacteria and Archaea. Additionally to subunit composition, another criterion differentiates the oxidases from the A-group with the two others. It appears that while the A-group oxidases have two proton channels, called K and D, the others lack the D channel (Hemp & Gennis, 2008) (FIG. 1.1).

An expanded classification of the cytochrome oxidases has been proposed by Hemp and Gennis (Hemp & Gennis, 2008). This classification is the result of an extensive metagenomic analysis that particularly focused on the highly diverse, and poorly explored, archaeal cytochrome oxidases. Five new groups (D, E, F, G and H) were established that would, according to the former classification (Pereira *et al.*, 2001), belong to the B-group of oxidases.

1.2 Cofactors associated with cytochrome oxidases

The universal cofactors associated with cytochrome oxidase are heme moities and copper atoms. The heme molecule is a heterocyclic organic ring with an iron ion in its center. Its synthesis and function has gathered a lot of attention from the scientific community since more than half a century. However, the interest in the copper cofactor is more recent. Most of the cofactors of the cytochrome oxidase are found in subunit I, namely the low-spin heme, and the binuclear center formed by a high-spin heme and the Cu_B. In the case of groups A and C of cytochrome oxidases, subunit II also carries a cofactor, namely a Cu_A center, or a heme, respectively, but in B group cytochrome oxidases, subunit II is devoid of any cofactor. In addition to heme and copper, zinc has also been reported to be associated with cytochrome oxidase. Zn binds to subunit IV in yeast and plays a stabilizing role (Coyne *et al.*, 2007). Mg(II) or Mn(II) has been reported to associate with cytochrome oxidases in Eukarya, but also in Bacteria like *Rhodobacter sphaeroides* (Hosler *et al.*, 1995). This ion is non-redox active and localized at the interface of subunits I and II where it shares a ligand with the Cu_A center (Florens *et al.*, 2001). Its function is not clear yet; however, it seems that this ion is involved in the water exiting from the reduction site (Schmidt *et al.*, 2003). As Zn does not play a role in prokaryotic cytochrome oxidases, and since data on the Mg(II)/Mn(II) cofactor is very scarce, the present review will not consider these any further. Although the amount of available data for heme biogenesis and insertion exceeds by far the knowledge on the copper cofactor insertion, the focus of this review is put on the

latter as the topic of heme biosynthesis and insertion has been thoroughly reviewed before (Thöny-Meyer, 1997; O'Brian & Thöny-Meyer, 2002; Frankenberg *et al.*, 2003; Hamza, 2006; Richard-Fogal *et al.*, 2009).

1.3 Assembly of subunits

In Eukarya, assembly and biogenesis of the oxidase complex has gained significant interest because several hereditary diseases are due to mutations in cytochrome oxidase or factors involved in its biogenesis (Barrientos *et al.*, 2009, Fernandez-Vizarra *et al.*, 2009). The overall assembly process is described as a four-step process which starts from an S1 intermediate, composed of subunit I inserted in the mitochondrial inner membrane. Then, after progressing through intermediate S2, composed of three to four subunits, and intermediate S3, the holocomplex (S4) is assembled. The final step is the dimerization of the holocomplex (Lenaz & Genova, 2010). At this stage, the biogenesis of the cytochrome oxidase can be considered as finished, but very often, supercomplexes, also called respirasomes, are formed. Respirasomes are found in mitochondria (Boumans *et al.*, 1998, Eubel *et al.*, 2004) and in Bacteria (Schägger, 2002; Stroh *et al.*, 2004) and they designate the association of complex I (NADH dehydrogenase), complex III (cytochrome bc_1 complex) and complex IV (cytochrome oxidase). The advantage of supercomplex formation is twofold: the stability of the included complexes (I, III and IV) is enhanced and catalysis is optimized since the diffusion distances for substrates are shortened. In *P. denitrificans*, complex I content is strongly decreased when complex III and IV are deleted, suggesting that stability of complex I depends on the formation of the respirasome (Stroh *et al.*, 2004). Similar effects occur at the level of the cytochrome oxidase subunits, where one can observe that some subunits are mandatory for stabilization of the other subunits whereas others are not. In the *B. japonicum* cbb_3 cytochrome oxidase, the presence of subunit I (FixN) and of subunit II (FixO) is essential for assembly of the oxidase. This means that in the absence of FixN or FixO, the third essential subunit FixP is probably unstable or gets degraded (Zufferey *et al.*, 1996). A similar effect can be seen in the *P. denitrificans* aa_3 cytochrome oxidase in which subunit I (CtaD) is destabilized in the absence of subunit II (CtaC) (Steinrücke *et al.*, 1991). In *R. capsulatus*, interactions between CcoQ and CcoP (Peters *et al.*, 2008) on one hand, and between the CcoNOQ inactive complex and CcoH and CcoS (Kulajta *et al.*, 2006) on the other hand, are crucial for biogenesis. The latter interaction seems to be essential for recruitment of SUIII (CcoP) and the genes coding for those factors belong to the *ccoGHIS* operon, also called *fixGHIS*. The *ccoGHIS/ fixGHIS* operon generally maps immediately downstream of the operon encoding the structural proteins of the cbb_3 oxidase (Ducluzeau *et al.*, 2008). It was first described in the context of an involvement of the encoded proteins in symbiosis in different rhizobia, namely *Rhizbium meliloti* (Kahn *et al.*, 1989), *Azorhizobium caulinodans* (Mandon *et al.*, 1993) and *B. japonicum* (Preisig *et al.*, 1996a). A deletion

of the *fixGHIS* operon leads to a defect as prominent as if the structural genes where deleted, *i.e.* defective nitrogen fixation (Preisig *et al.*, 1996a). Although the exact functions of the four gene products are still not very well understood, it is suggested that CcoH (FixH) and CcoS (FixS) are involved in the recruitment of the subunits and their stabilization during assembly of the oxidase complex (Kulajta *et al.*, 2006). CcoG, an iron-sulfur cluster-containing protein that possesses a putative ferredoxin domain, and CcoI, a homolog of P-type ATPases, might be involved in copper cofactor delivery (Preisig *et al*., 1996a; Koch *et al.*, 2000) (further discussed in paragraph 1.6.1).

The respiratory chain of eukaryotes is located in the inner mitochondrial membrane within the mitochondrion, and is separated from the cytoplasm by an additional membrane, the outer mitochondrial membrane. This can be considered as an additional level of complexity compared to Bacteria. Therefore, it is not surprising that most of the factors involved in the insertion of the subunits into the inner mitochondrial membrane in Eukarya are missing in Bacteria. One example of such a factor is Mss2 which was identified in yeast, but is absent in Bacteria. It participates in the translocation of the C-terminal tail of subunit II (Broadley *et al.*, 2001). On the contrary, the eukaryotic protein Oxa1, also involved in subunit insertion into the mitochondrial membrane, has a bacterial homolog, named YidC. In *E. coli*, the insertion of the subunit II (CyoA) of the bo_3 oxidase into the cytoplasmic membrane is assisted by the YidC translocase (van der Laan *et al.*, 2003). A String-analysis (http://string-db.org/) shows that YidC is largely conserved among Bacteria, and it can therefore be postulated to play a similar role for membrane insertion of subunits of oxidase of different types, but so far, this has not been shown.

1.4 Heme biosynthesis

Like chlorophylls, hemes belong to the tetrapyrroles which are the most abundant pigments found in nature (Heinemann *et al.*, 2008). As the name indicates, tetrapyrroles consist of four pyrrole rings and they have the ability to chelate divalent cations, Fe(II) in the case of heme and Mg(II) in the case of chlorophyll. Hemes are essential components of the energy-conserving electron transport chains of almost all organisms. They are also involved in other central cellular function like oxidative stress management and they are a cofactor for the enzyme catalase (Heinemann *et al.*, 2010). Although most of the organisms require heme, not all of them possess the enzymes to synthetize it, as for example *Haemophilus influenzae*, which relies on its host's heme (White & Granick, 1963). However, the genes for heme biosynthesis appear to be conserved to a large extent (Panek & O'Brian, 2002). Heme biosynthesis has been extensively studied and has been reviewed before (Thöny-Meyer, 1997; O'Brian & Thöny-Meyer, 2002; Frankenberg *et al.*, 2003).

In the recent years, structural and biochemical data of the enzymes catalyzing the steps of heme biosynthesis has accumulated, leading to a very thorough understanding of the mechanisms of this multistep process (Heinemann *et al.*, 2008; Layer *et al.*, 2010). For every step of heme biosynthesis, the respective enzyme has been crystallized and the structure resolved. Details can be found in (Layer *et al*., 2010) and references therein. The reaction sequence will not be described in detail here, as this has been done before, and only the main steps will be highlighted shortly.

Heme biosynthesis is divided into three parts. As for all tetrapyrroles, it starts with the formation of 5-aminolevulinic acid (ALA) which represents the only source of carbon and nitrogen that can be exploited to generate protoheme IX. Two possible pathways lead to the synthesis of ALA: the Schemin pathway and the C5 pathway. The originality of the C5 pathway, which is mainly used by plants, Archaea and most bacteria such as *B. subtilis*, *P. aeruginosa* or *T. thermophilus* (Beale, 1990), is that it utilizes tRNA-bound glutamate that gets converted into glutamate semialdehyde (by the enzyme HemA, glutamyl tRNA reductase) which in turn gets converted into ALA by HemL (glutamate semialdehyde aminomutase). In the Shemin pathway (Shemin & Russell, 1953; Neuberger & Scott, 1953; Kikuchi *et al.*, 1958a; Kikuchi *et al.*, 1958b; Gibson *et al.*, 1958) ALA is synthetized via a condensation of glycine and succinyl-CoA by HemA (aminolevulinate synthase). This pathway is utilized by Eukarya and many proteobacteria (Ferreira & Gong, 1995). The next step in the heme biosynthesis is the formation of the first cyclic tetrapyrrole uroporphyrinogen III. Three catalyzed reactions are necessary for this: HemB (porphobilinogen synthase) converts ALA into porphobilinogen, which in turn gets converted to pre-uroporphyrinogen by HemC (porphobilinogen deaminase), which gets converted to uroporphyrinogen III by HemD (uroporphyrinogen synthase). Uroporphyrinogen is an important branching point in prokaryotes as the molecule can be either further processed to heme D1, vitamin B12, and siroheme, or, as in Eukarya, be converted to protoheme IX by the subsequent action of HemE (uroporporphyrinogen III decarboxylase), HemF (O_2-dependent coporporphyrinogen oxidase) or HemN (O_2-independent coporporphyrinogen oxidase), HemG (O_2-independent protoporporphyrinogen oxidase) or HemY (O_2-dependent protoporporphyrinogen oxidase), and HemH (ferrochelatase) which inserts the Fe(II) ion. Protoheme IX is also called heme B. It serves as a precursor to synthetize the other hemes C, O and A. CtaB (also called Cox10, CyoE, heme *o* synthase or protoheme IX farnesyltransferase) and CtaA (heme A synthase, heme O monooxygenase) catalyze the reactions that lead to hemes O and A (Mogi *et al.*, 1994). Although the field has been extensively studied, resulting in a huge amount of biochemical and structural data available for almost every step of heme biosynthesis, some questions remain open. One concerns the archaeal heme biosynthetic pathways. Many archaea are known to utilize heme but they generally lack the biosynthetic enzymes downstream of uroporphyrinogen (Panek & O'Brian, 2002). Other knowledge gaps concern many Gram-negatives like the well-studied *P. aeruginosa*,

Helicobacter pylori or *Agrobacterium tumefaciens* which appear to lack both known types of protoporporphyrinogen oxidases (HemG and HemY) (Panek & O'Brian, 2002).

1.5 Heme insertion

Heme C insertion into cytochrome was extensively studied. It turns out that heme C is covalently bound to the unfolded apoprotein via two cysteines generally belonging to a CXXCH motif, and the formation of this covalent bond is catalyzed by heme lyases in mitochondria (system III), whereas in bacteria, it relies either on system I which is represented by the genes *ccmABCDEFGHI* (Thöny-Meyer, 1997; Sanders *et al.*, 2010) in δ-proteobacteria or the homologous *cyc*/*hel*/*ccl* genes in α-proteobacteria, or on system II. The less well characterized system II is present in Gram-positive bacteria, some Gram-negative bacteria, and in thylakoids of plants or algae. It is often involved in heme insertion in proteins that require more than one heme. It comprises four proteins: CcsX, CcsB, CcsA, and CcsC (also called ResA, ResB, ResC and CcdA, respectively) (Stevens *et al.*, 2004; Ferguson *et al.*, 2008; Kranz *et al.*, 2009). This detailed knowledge is in contrast to insertion of other types of hemes (A, B, and O-type) which is less well studied.

Contrary to the heme C moieties, the A-, B- and O-type hemes are non-covalently linked to their apoprotein via histidine ligands and therefore lyases are not needed. For a long time it was unclear whether protein factors are involved in the process or whether the heme moieties can spontaneously reach their locations in the folded protein (Thöny-Meyer, 1997). However, in the recent years, the significance of Surf proteins in this process became apparent (Smith *et al.*, 2005; Bundschuh *et al.*, 2008). Surf1 in human (Tiranti *et al.*, 1998) and the yeast homolog Shy1 (Mashkevich *et al.*, 1997, Nijtmans *et al.*, 2001) were recognized to be relevant for cytochrome oxidase biogenesis, although they are not part of the complex. In human, the topic has earned a lot of interest as defects in Surf1 are the most frequent cause for Leigh's disease (Tiranti *et al.*, 1998). The exact mode of action of Surf proteins is not yet understood, but, since residual amounts of about 10% of correctly assembled cytochrome oxidases remain in the membranes, a chaperoning-role, either in assembly or in stabilization, is suggested. In bacteria, *surf* genes are also found, often even in more than one copy in the genome. Two cases are best studied in the bacterial world, namely the *P. denitrificans* Surf1c and Surf1q (Bundschuh *et al*., 2008) and the *R. sphaeroides* Surf1p (Smith *et al*., 2005). *P. denitrificans* Surf1c and Surf1q are both capable to bind heme A in a 1:1 stoichiometry, and the first probably serves the biogenesis of an aa_3-type cytochrome oxidase whereas the second one serves the biogenesis of a quinol oxidase (Bundschuh *et al*., 2008; Bundschuh *et al.*, 2009). In *R. sphaeroides*, the content of assembled aa_3 cytochrome oxidase was assessed in a Δ*surf1p* mutant, and it turned out that the

majority was lacking heme A_3 (Smith *et al.*, 2005). In *B. japonicum*, two copies of genes encoding Surf proteins were identified, namely *blr0153* which turns out to be encoded in a putative operon encoding a quinol oxidase and *blr1177* which is within the immediate vicinity of the operon encoding the aa_3 cytochrome oxidase (*coxBAC*) (Göttfert *et al.*, 2004). This distribution of the genes resembles very much the aforementioned findings in *P. denitrificans*, and it seems likely that this resemblance is also reflected at the functional level. A String analysis (http://string-db.org/) shows that while *surf* genes are widespread in proteobacteria, they are missing in Gram-positives and also in some Gram-negatives. *Salmonella*, *Pseudomonas*, *Yersinia* lack *surf* genes. *T. thermophilus* is an interesting case as it also lacks a *surf* gene, but very recently an alternative protein, that might fulfill the function of Surf was proposed: *cbaX* is cotranscribed with the ba_3 oxidase locus and is essential for proper assembly of the oxidase (Werner *et al.*, 2010). However, the presence of the *cbaX* gene is restricted to the *Thermaceae* group (Werner *et al.*, 2010) and thus, it is very likely that other mechanisms for heme insertion are yet to be unraveled. Equivalently to Surf proteins which bind heme A, the product of *ccmE*, one of the genes of the *ccmABCDEFG* operon (Thöny-Meyer & Künzler, 1997), was also shown to bind heme *c*, but covalently (Stevens *et al.*, 2005). It was shown that from there, heme C gets transferred to its cognate sites, which means that CcmE acts as a heme chaperone for cytochrome *c* biogenesis (Schulz *et al.*, 1998).

1.6 Copper cofactors

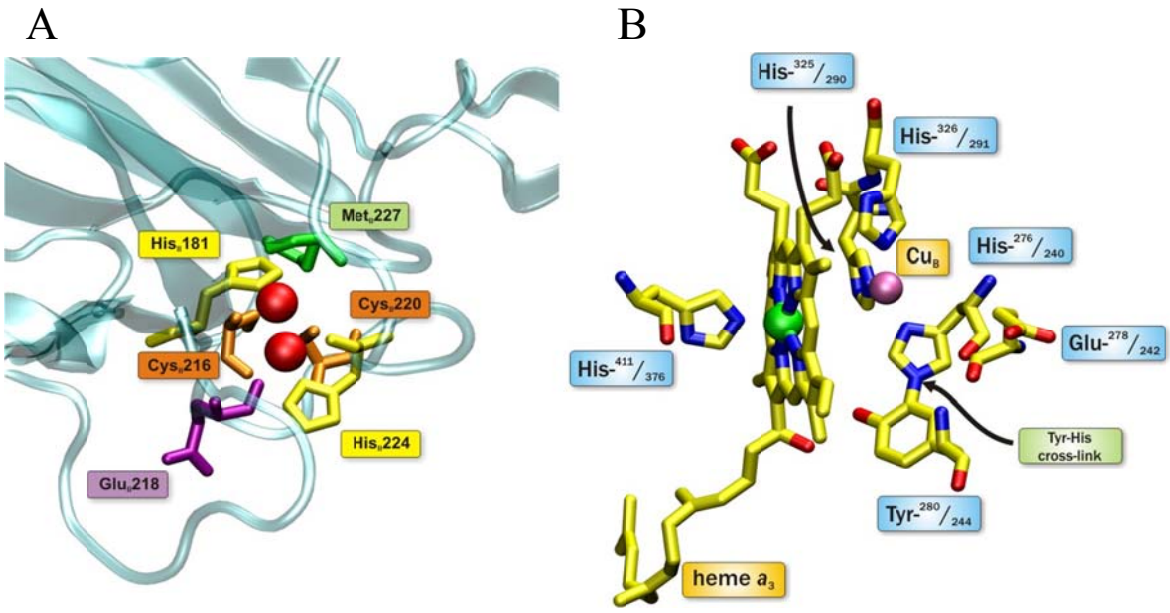
Given its toxic effects, the concentration of free copper in cells has to be very low (Rae *et al.*, 1999). It was estimated that 10^4 copper ions are present in an *E. coli* cell, but virtually all of it is protein-bound (Finney & O'Halloran, 2003). The need for copper distributing systems within cells is evident (O'Halloran & Culotta, 2000; Harrison *et al.*, 2000; Robinson & Winge, 2010), and as all cytochrome oxidases harbor at least one copper site, their requirement for Cu-chaperones and possibly also copper-importing systems (see chapter 6) for proper biogenesis is obvious.

1.6.1 Cu_B center

The Cu_B center (FIG. 1.3.A) is common to all cytochrome oxidases. In nitric oxide reductases, the other group also belonging to the heme-copper oxidases, Cu_B is often replaced by an iron-center. In association with a heme moiety, the Cu_B center forms the active site where the reduction of O_2 to H_2O or of NO to N_2O is catalyzed. Cox11p was first discovered in a screen for respiration-deficient mutants in yeast (Tzagoloff *et al.*, 1990). The eukaryotic Cox11 and its bacterial homologs Cox11/CtaG/CoxG were shown to be essential for biogenesis of cytochrome oxidase, most probably by copper insertion into Cu_B of subunit I, in a series of organisms, namely *Saccharomyces cerevisiae*

(Cobine *et al.*, 2006; Horng *et al.*, 2004, Banting & Glerum, 2006), *R. sphaeroides* (Cao *et al.*, 1992; Thompson *et al.*, 2010), *P. denitrificans* (Greiner *et al.*, 2008), and *B. japonicum* (Bühler *et al.*, 2010). Members of the Cox11/CtaG/CoxG family are, with the exception of *P. syringae*, encoded near the genes coding for the structural subunits of cytochrome oxidase (Arnesano *et al.*, 2005). In proteobacteria, other proteins also involved in biogenesis of the oxidase, namely CtaB and Surf1 (see paragraph 1.5) are often also found within the same operon.

FIG. 1.3. Copper centers of cytochrome oxidases are depicted. A. The bimetallic Cu_A center is shown in which the copper atoms are complexed by residues of the H–X_{34}–CXEXCXXXHXXM consensus sequence. B. The Cu_B center is shown in which the single copper atom is liganded by 4 histidine residues and in close association with the heme moiety which is also depicted. (Figures are taken from Ilya Belevich, http://www.biocenter.helsinki.fi/bi/hbg/research_CcO.html).



The encoded proteins are membrane-anchored and are found in all eukaryotes (with complete genome sequence) and in many proteobacteria; however, this protein family is absent from other bacterial phyla. This means that some aa_3-type cytochrome oxidase-possessing bacteria, like *T. thermophilus* or *B. subtilis* lack a CtaG/CoxG encoding gene, suggesting that they rely on alternative mechanisms. The solution structure of *Sinorhizobium meliloti* Cox11 has been solved, and Cox11 appeared to adopt a β-immunoglobulin-like fold (Banci *et al.*, 2004). As shown in FIG. 1.4, Cox11 is thought to form dimers in which the complexed copper atoms would be at the interface of the two monomers (Carr *et al.*, 2002; Banci *et al.*, 2004). In yeast Cox11p and in *R. sphaeroides* Cox11, cysteine-mediated

copper binding was demonstrated (Carr *et al.*, 2002; Thompson *et al.*, 2010). Direct transfer of copper from Cox11/CtaG/CoxG has never been shown so far. Therefore, the mechanism by which Cox11/CtaG/CoxG contributes to biogenesis of cytochrome oxidase is not completely clear yet. However, as Cu_B was shown to be missing in a *R. capsulatus* Δ*cox11* mutant whereas Cu_A and hemes were detectable (Hiser *et al.*, 2000), the hypothesis that Cox11 specifically delivers copper to Cu_B is strongly supported and generally accepted in the field. Hence, the research community strongly supports a copper-chaperone activity. Interestingly, a co-immunoprecipitation experiment performed in yeast showed that Cox11 interacts, at least transiently, with ShyI, a protein involved in heme insertion that is also present in many Bacteria (generally called Surf in Bacteria). This suggests that, in addition to the generally assumed role in formation of Cu_B (Cobine *et al.*, 2006), Cox11 might play a stabilizing role for other steps of the oxidase assembly process (Khalimonchuk *et al.*, 2010). The residues involved in Cox11 function have been studied in several organisms and a fully conserved CXC motif, most probably involved in copper liganding, has emerged from those studies (Carr *et al.*, 2002).

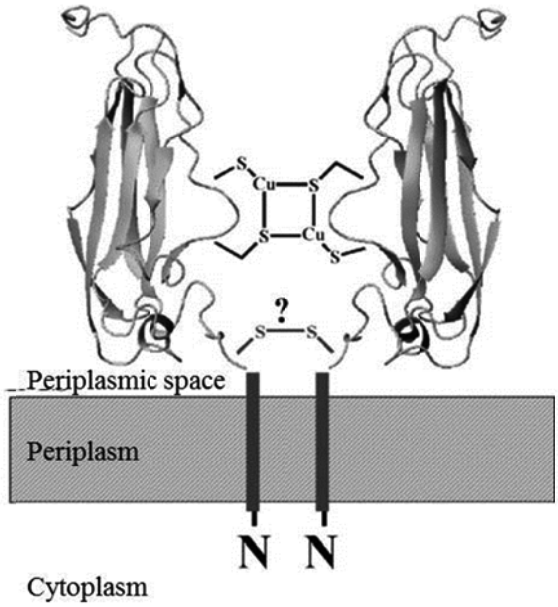


FIG. 1.4. Model for the dimeric form of *S. meliloti* Cox11 is depicted. The two monomers possess a β- immunoglobulin-like fold, consisting of 10 β-sheets which arrange in β–barrel-like manner. The copper atoms that are possibly complexed at the interface of the two monomers are schematically represented. The proposed disulfide bond that is thought to stabilize the dimer (via Cys9) is indicated with an interrogation mark. Figure was taken from Banci *et al.*, 2004.

Rhodobacter appears to be an interesting genus with respect to respiratory chains and biogenesis factors. Although *R. capsulatus* and *R. sphaeroides* are closely related purple bacteria that both harbor a highly versatile metabolism and that can live photosynthetically, they differ substantially with respect to their respiratory chains. Whereas *R. capsulatus* lacks an aa_3-type cytochrome oxidase, *R.*

sphaeroides possesses one. The maturation of this oxidase depends on Cox11 in *R. sphaeroides* (Thompson *et al*., 2010), whereas *R. capsulatus* lacks a gene for Cox11, implying that the biogenesis of the Cu_B center of the *cbb*$_3$ cytochrome oxidase of *R. capsulatus* is accomplished with another system. This suggests that if indeed Cox11 is the cuprochaperone delivering copper to the Cu_B center of *aa*$_3$-type cytochrome oxidases it is not required for the biogenensis of the Cu_B center of *cbb*$_3$-type cytochrome oxidases. This correlates with a finding in *B. japonicum* which relies on CoxG for biogenesis of the low-affinity *aa*$_3$ cytochrome oxidase that the bacteria use under free-living conditions but does not require CoxG for biogenesis of the symbiotic *cbb*$_3$ cytochrome oxidase (Bühler *et al.*, 2010). As discussed above (paragraph 1.3), the gene products of the *ccoGHIS* (*fixGHIS*) operon are crucial for biogenesis of the *cbb*$_3$ cytochrome oxidase, and two of the encoded proteins are supposed to be involved in the Cu_B center biogenesis of the oxidase, namely CcoG and CcoI. In *Rubrivivax gelatinosus*, a photosynthetic β-proteobacterium, experimental evidence was recently given that, in addition to the *cbb*$_3$ cytochrome oxidase, two additional cuproenzymes, NosZ and the *caa*$_3$-type cytochrome oxidase, also depend on the activitiy of CtaP (CcoI/FixI). This finding strongly supports the hypothesis that indeed CtaP/CcoI/FixI work as copper-importing P-type ATPases (Hassani *et al.*, 2010). *R. capsulatus* appears to be an exceptional case with regard to maturation of its *cbb*$_3$ oxidase. It also depends on the products of the *ccoI*, *ccoH* and *ccoS* genes, which, in that case, are not transcribed as an operon (Koch *et al*., 2000). In addition, and this seems to be a unique case so far, biogenesis of the *cbb*$_3$ oxidase in this organism depends on SenC, a Sco-like protein. In general Sco-like proteins are dedicated to *aa*$_3$ cytochrome oxidase biogenesis (Buggy & Bauer, 1995) (further discussed in paragraph 1.6.2). As the respiratory defect of a Δ*senC* mutant can be complemented by addition of copper into the medium, it is thought that SenC delivers copper into the Cu_B center of the *cbb*$_3$ oxidase (Swem *et al.*, 2005).

As mentioned earlier, genes encoding proteins from the Cox11/CtaG/CoxG-family are absent in Gram-positives; but, incongruently, a gene encoding a protein required for *caa*$_3$ cytochrome oxidase maturation in *B. subtilis* was also called *ctaG* although it is not homologous to the *cox11*/*ctaG*/*coxG* family. *B. subtilis* CtaG lacks the typical CXC motif and has 7 transmembrane domains. Its function specifically affects the *caa*$_3$ cytochrome oxidase and has no implication for the formation of the *aa*$_3$ quinol oxidase; its mode of action is so far unknown. The gene is widespread among Gram-positives and often associated with the operon encoding the structural subunits of cytochrome oxidases (Bengtsson *et al.*, 2004).

1.6.2 Cu_A center

The Cu_A center (FIG. 1.3.A) is different from the Cu_B (FIG. 1.3.B) center in multiple ways. The two centers differ in function, location, architecture and distribution. The Cu_A center is the entry point for

the e^- that is coming from cytochrome *c* and thus it is located on the peripheral domain of subunit II, exposed towards the periplasm and accessible to the solvent. Copper binding is mediated by a highly conserved motif: $HX_{34}CXECX_3HX_2M$. On the contrary, Cu_B is liganded via four histidine residues, and it is deeply buried and isolated from the solvent in order to offer a very controlled environment for O_2 reduction (Iwata *et al.*, 1995; Pereira *et al.*, 2001). Given their distinct locations, it is not surprising that different systems deliver the copper atoms to these two disparate copper centers. Furthermore, Cu_B is a common feature of all heme-copper oxidases, whereas Cu_A is only found in the A-class of cytochrome oxidases (see paragraph 1.2). In addition, it should be noted that, contrary to the Cu_B site, the existence of Cu_A centers is not confined to heme-copper oxidases. NosZ, a central enzyme in denitrification, also possesses Cu_A centers. The biogenesis of the NosZ Cu_A centers is well understood and appears to depend on a different machinery than the Cu_A centers of cytochrome oxidase. NosA, a Cu porin, and NosH, a Cu-binding chaperone are the key players of this process (Zumft, 2005a).

Sco, which stand for synthesis of cytochrome oxidase, was first discovered in a screen for respiration-deficient yeast strains (Schulze & Rödel, 1988). The hypothesis that this membrane-anchored periplasmic protein is involved in the biogenesis of the Cu_A site emerged a few years later (Glerum *et al.*, 1996). This hypothesis was supported by the demonstrated interaction of Sco with the Cu_A-carrying subunit II in yeast (Lode *et al.*, 2000). A systematic ortholog search showed that *sco* genes can be present in several copies within one genome: up to five in *Pseudomonas putida* or *Pseudomonas fluorescens*, for example (Arnesano *et al.*, 2005), whereas yeast (Glerum *et al*., 1996) and human (Leary *et al.*, 2004) code for two *sco* copies whose products have overlapping but not identical functions. The genes for Sco are widespread and even found in organisms that lack cytochrome oxidases, suggesting that Sco can also assume other functions than biogenesis of cytochrome oxidases. Their genetic neighborhoods reflect this variability: they can be found near genes encoding cytochrome oxidase, nitrite reductase, or multicopper oxidases (Arnesano *et al.*, 2005).

Structures of Sco proteins have been resolved for human Sco1 (Balatri *et al.*, 2003), yeast Sco1 (Abajian & Rosenzweig, 2006) and *B. subtilis* BsSco (Ye *et al.*, 2005) (FIG. 1.5). All solved Sco-protein structures have in common a thioredoxin-fold (FIG. 1.5) with exposed active-site cysteines that appear to be crucial for function. Involvement in cytochrome oxidase biogenesis was reported in several organisms: yeast Sco1 (Nittis *et al.*, 2001), human Sco1 and Sco2 (Balatri *et al*., 2003), *R. sphaeroides* PrrC (Swem *et al.*, 2005), *T. thermophilus* Sco1 (Abriata *et al.*, 2008), *B. subtilis* BsSco (Cawthorn *et al.*, 2009), *Agrobacterium tumefaciens* Sco (Saenkham *et al.*, 2009), and *B. japonicum* ScoI (Bühler *et al.*, 2010). However, these different studies do not converge towards a uniform mode of action by which Sco-proteins contribute to cytochrome oxidase maturation. As copper-binding via

essential and highly conserved cysteine residues was demonstrated for several Sco-proteins, namely human Sco1 and Sco2 (Horng *et al.*, 2005), *B. subtilis* (Mattatall *et al.*, 2000), *B. japonicum* (Bühler *et al.*, 2010), copper chaperone activity has been proposed in those cases. On the other hand, the same conserved cysteines that belong to the so-called Sco-motif have been reported to act as disulfide-reductases in human Sco2 (Leary *et al.*, 2009), in *R. sphaeroides* PrrC (Badrick *et al.*, 2007) , in *B. subtilis* BsSco (Imriskova-Sosova *et al.*, 2005) and in *T. thermophilus* Sco (Abriata *et al.*, 2008). This activity was shown to be essential for biogenesis of the Cu_A center. An *in vitro* experiment showed that *T. thermophilus* Sco reduces the disulfide bridge that formes between the two cysteines of the Cu_A binding motif ($HX_{34}CXECX_3HX_2M$). These cysteines must be reduced in order to be capable of copper binding. Upon reduction, another factor, PCu_AC inserts the copper ions into Cu_A (Abriata *et al.*, 2008). As genes encoding PCu_AC-like proteins are widespread among Bacteria, it is to be expected that in the near future they will be described to fullfill similar functions in other bacteria (see chapter 6).

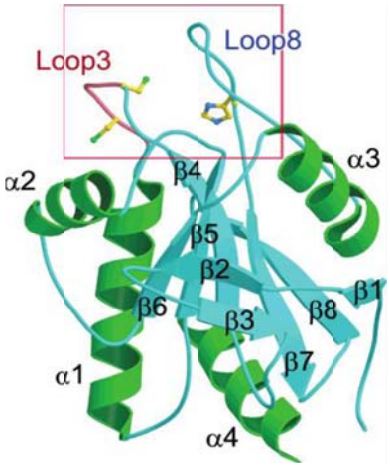


FIG. 1.5. Structure of the soluble domain of *B. subtilis* BsSco, that was determined from a crystal at 1.7 Å resolution. (Figure taken from Ye *et al.* (2005)). The typical elements of a thioredoxin fold can be seen: namely central β-sheets (blue) that are flanked by α-helices (green). The pink box highlights the conserved Cys45, Cys 49 and the His135 residues that were shown, in other Sco-protein, to mediate copper binding.

Coming back to Sco-proteins, as some of them seem to be capable of both copper-chaperone activity and redox activity, the concept of a framework that reconciles these two activities was recently proposed (Banci *et al.*, 2007a). According to this concept, Sco proteins may have the capability to fullfill copper-chaperoning functions, redox activities, or both.

As already mentioned, two cysteines, separated by three amino acids, form the so-called Sco-motif (FIG. 1.5 and FIG. 1.6). In addition to these two residues, a histidine, located approximately 86 amino acids distal to the second cysteine is also strictly conserved (FIG. 1.6). Its role is not completely elucidated, but it was shown to be essential for Sco function in some cases (Siluvai *et al.*, 2009, Saenkham *et al.*, 2009) and its participation in copper liganding was demonstrated several times

(Nittis *et al*., 2001), although it is dispensable for copper binding in other cases (Siluvai *et al.*, 2009) (see also chapter 4).

It appears that Sco proteins harbor another conserved motif namely DXXXD (Arnesano *et al.*, 2005) (FIG. 1.6). The function of these residues is poorly understood, except in yeast Sco1 where one of those aspartic acids, namely the N-terminal one of the two, is essential for Sco1 function. Indeed, a Δ*sco1* strain transformed with a plasmid expressing a Sco1D182A was unable to complement its respiratory defect (Horng *et al.*, 2005). Aspartic acid is a charged residue; moreover the known structure of yeast Sco1 (Abajian & Rosenzweig, 2006) shows that the DXXXD is exposed to the surface and located in the vicinity of the conserved cysteines of the CXXXC motif. Therefore, it is well possible that these conserved aspartic acid residues play a role in the interaction of Sco-proteins with the target protein(s).

FIG. 1.6. Alignment of *S. cerevisiae* Sco1, *B. japonicum* ScoI and *B. subtilis* BsSco. The predicted transmembrane domains are underlined, the conserved two cysteines and the histidine that are involved in copper liganding are bold and underlined, and the aspartic acids of the DXXXD motif are bold. Another aspartate (that is only partly conserved) was shown to be essential for copper binding in yeast and human (Horng *et al.*, 2005), is also highlighted in bold. Conserved residues in all three proteins are shaded.

```
S.c.Sco1        MLKLSRSANLRLVQLPAARLSGNGAKLLTQRGFFTVTRLWQSNGKKPLSRVPVGGTPIKD 60
B.j.ScoI        ------------------------------------------------------------
B.s.Sco         ------------------------------------------------------------

S.c.Sco1        NGKVREGSIEFSTGKAIALFLAVGGALSYFFNREKRRLETQKEAEANRGYGKPSLGGPFH 120
B.j.ScoI        -------------MSSATRPLVIATAFAASLIVGLLIMFWAMGGVGKVAQPAAIGGPFQ 46
B.s.Sco         ---------------------- MKVIKGLTAGLIFLFLCACGGQQIKDPLNYEVEPFT 36

S.c.Sco1        LEDMYGNEFTEKNLLGKFSIIYFGFSNCPDICPDELDKLGLWLNTLSSKYGITLQPLFIT 180
B.j.ScoI        LTDQNGKAVTDKSLKGKPTLIFFGYTHCPDVCPTSLFEISEVLRAMG-KDADKVNAIFIS 105
B.s.Sco         FQNQDGKNVSLESLKGEVWLADFIFTNCETICPPMTAHMTDLQKKLK-AENIDVRIISFS 95

S.c.Sco1        CDPARDSPAVLKEYLSDFHPSILGLTGTFDEVKNACKKYRVYFSTPPNVKPGQDYLVDHS 240
B.j.ScoI        VDPERDTPATMKNYLSSFDPHLEGLSGDPAEIAKVITSYRVYAKKVP--TKDGDYTMDHT 163
B.s.Sco         VDPENDKPKQLKKFAANYPLSFDNWDFLTGYSQSEIEEFALKSFKAIVKKPEGEDQVIHQ 155

S.c.Sco1        IFFYLMDPEGQFVDALGRNYDEKTGVDKIVEHVKSYVPAEQRAKQKEAWYSFLFK 295
B.j.ScoI        ALIYLMDRDGRFVSPFN----LKRTPEEAAADLKRYL----------------- 196
B.s.Sco         SSFYLVGPDGKVLKDYNG--VENTPYDDIISDVKSASTLK-------------- 193
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1.7 Concluding remarks

The biogenesis of the A-group of cytochrome oxidases is much better understood than the biogenesis of the B- and C-groups. It can be expected that there are many unknown factors and mechanisms which are awaiting to be discovered and which should lead to a better understanding of biogenesis of cytochrome oxidase, a very complex process.

Many open questions are still to be answered concerning the copper cofactor, two of them have priority in our eyes and they concern copper import and copper delivery. How is copper imported into cells? Three possible tracks may lead to an answer. First, methanobactin, a non-ribosomally synthetized peptide that is the copper-equivalent of iron-complexing siderophores was shown to work as a copper importer (Balasubramanian & Rosenzweig, 2008). Secondly, there is a copper-importing machinery that was described to be dedicated for nitrous oxide reductase but which might also be relevant for other copper-requiring systems (Zumft, 2005a). Third, there are copper-specific P-type ATPases which could also fulfill the task (Solioz & Stoyanov, 2003). Moreover it is unclear whether Bacteria require shuttling intermediate chaperones, equivalent to the Cox17 protein in Eukarya (Cobine *et al*., 2006), specifically providing the cuprochaperones with their metal.

Mysteries surround also the delivery of copper cofactors to their cognate target proteins. It was shown that the thermodynamic force that is provided by the respective copper affinities of copper chaperones and copper-requiring target proteins is often not sufficient to drive efficient metal transfer from the chaperone to the target protein (Huffman & O'Halloran, 2000). Thus, it is suggested that the metallochaperones exert a kinetic control on this transfer (O'Halloran & Culotta, 2000). Studies on the role of conserved residues in the chaperones, like histidine 239 in yeast Sco (Abajian & Rosenzweig, 2006) (see also chapter 4), and improved *in vitro* assays for copper transfer (Greiner *et al.*, 2008) might shed some light on this enigma in the future.

CHAPTER 2

Disparate pathways for the biogenesis of cytochrome oxidases in *Bradyrhizobium japonicum*

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2.1 Abstract

This work addresses the biogenesis of heme-copper terminal oxidases in *Bradyrhizobium japonicum*, the nitrogen-fixing root-nodule symbiont of soybean. *B. japonicum* has four quinol oxidases and four cytochrome oxidases. The latter include the aa_3- and cbb_3-type oxidases. While both have a Cu_B center in subunit I, the subunit II proteins differ in having either a Cu_A center (in aa_3) or a covalently bound heme C (in cbb_3). Two biogenesis factors were genetically studied here, the periplasmically exposed CoxG and ScoI proteins, which are the respective paralogs of the mitochondrial copper-trafficking chaperones Cox11 and Sco1 for the formation of the Cu_B center in subunit I and the Cu_A center in subunit II of cytochrome aa_3. We could demonstrate copper binding to ScoI *in vitro*, a process for which the thiols of cysteine residues 74 and 78 in the ScoI polypeptide were shown to be essential. Knock-out mutations in the *B. japonicum coxG* and *scoI* genes led to loss of cytochrome aa_3 assembly and activity in the cytoplasmic membrane, whereas the cbb_3-type cytochrome oxidase apparently remained unaffected. This suggests that subunit I of the cbb_3-type oxidase obtains its copper cofactor via a different pathway than cytochrome aa_3. In contrast to the *coxG* mutation, the *scoI* mutation caused a decreased symbiotic nitrogen fixation activity. We hypothesize that a periplasmic *B. japonicum* protein other than any of the identified Cu_A proteins depends on ScoI and is required for an effective symbiosis.

2.2 Introduction

The common denominator in respiratory heme-copper oxidases is a membrane-integral subunit I that carries as cofactors a low-spin heme and a unique high-spin heme-copper binuclear center (Cu_B site) where reduction of O_2 to H_2O takes place (García-Horsman *et al.*, 1994).

There is diversity regarding the number and cofactor content of the other subunits, which relates to the substrates used as electron donor (Trumpower & Gennis, 1994; Poole & Cook, 2000). Reduced *c*-type cytochromes donate electrons to cytochrome oxidases, whereas reduced quinones deliver electrons to quinol oxidases. The latter possess a cofactor-free subunit II, whereas cytochrome oxidases have cofactors bound to subunit II. In most cases this is a binuclear Cu–Cu center (Cu_A site) that is liganded by six highly conserved amino acids (Pereira *et al.*, 2001, Iwata *et al.*, 1995). The subclass of cbb_3-type oxidases is exceptional as its members have a subunit II (CcoO or FixO) that is a monoheme *c*-type cytochrome instead of the Cu_A-containing protein (Ducluzeau *et al.*, 2008; Pereira *et al.*, 2001). Subunit III in cbb_3-type oxidases, which is a diheme cytochrome *c*, is thought to relay the electrons from the cytochrome bc_1 complex via CcoO to the redox centers of subunit I (Zufferey *et al.*, 1996; Kulajta *et al.*, 2006). Subunit III of all other heme-copper oxidases is cofactor-free, just like the non-conserved small subunit IV (Castresana *et al.*, 1994; García-Horsman *et al.*, 1994; Iwata *et al.*, 1995;

Pereira *et al.*, 2001). With few exceptions, the four-subunit composition is typical for prokaryotic heme-copper oxidases, whereas the eukaryotic, *i.e.* mitochondrial, counterparts are much more complex (Tsukihara *et al.*, 1996).

Knowledge about subunit and cofactor composition is pivotal for an understanding of the biogenesis of heme-copper cytochrome oxidases. How do subunits assemble in the membrane, and how are the cofactors (hemes, Cu_A, Cu_B) inserted? Furthermore, the topology of subunits and redox centers has to be considered in the context of cofactor delivery which is routed either from the cytoplasm or from the outer side of the membrane. While the low-spin heme and high-spin heme-Cu_B are embedded in the membrane-integral segment of subunit I, the Cu_A center on subunit II lies peripheral to the membrane (Tsukihara *et al.*, 1996; Iwata *et al.*, 1995), which is equivalent to the inter-membrane compartment in mitochondria and the periplasmic space in Gram-negative bacteria.

Given the complexity of eukaryotic cytochrome oxidase, with possibly more than 30 factors involved in its formation (Carr & Winge, 2003; Khalimonchuk & Rödel, 2005), the use of the comparatively simpler bacterial aa_3-type oxidases facilitates biogenesis studies. Members of the alpha-proteobacteria (e.g., *Paracoccus denitrificans*, *Bradyrhizobium japonicum*, *Rhodobacter* sp.) are attractive model organisms for this purpose because they appear to be the closest extant relatives of a mitochondrial ancestor (Castresana et al., 1994). In fact, a fairly small number of chaperoning proteins have so far been identified as being instrumental in the maturation of bacterial aa_3-type cytochrome oxidases: Surf1 for Heme A insertion into subunit I (Bundschuh *et al.*, 2008; Bundschuh *et al.*, 2009); CtaG (or CoxG), a homolog of mitochondrial Cox11 (Cobine *et al.*, 2006), for copper trafficking to the Cu_B site in subunit I (van der Oost *et al.*, 1991; Cao *et al.*, 1992; Greiner *et al.*, 2008); Sco1 (also called SenC or PrrC), for copper delivery to the Cu_A site in subunit II (Nittis *et al.*, 2001; Balatri *et al.*, 2003; Swem *et al.*, 2005; Abajian & Rosenzweig, 2006; Cawthorn *et al.*, 2009); and TlpA, a protein dithiol:disulfide oxidoreductase with an unknown role in cytochrome aa_3 formation in *B. japonicum* (Loferer *et al.*, 1993; Capitani *et al.*, 2001). A specialized case appears to be that of PCu_AC, which is involved in generating the Cu_A site of the *Thermus thermophilus* ba_3-type oxidase (Abriata *et al.*, 2008). All of these proteins are membrane-bound, and the active domains of Sco1 and PCu_AC face the periplasm, which is consistent with their role in Cu_A assembly on subunit II. Concerning assembly, the cbb_3-type oxidase is again in a class of its own. Since subunits II and III are *c*-type cytochromes, their synthesis requires the complete set of maturation proteins for the covalent attachment of heme (Thöny-Meyer, 2002; Zufferey *et al.*, 1997). Additional assembly factors are needed whose biochemical functions have not been elucidated (Preisig *et al.*, 1996a; Koch *et al.*, 2000). Although the cbb_3- and aa_3-type cytochrome oxidases have in common a conserved subunit I, it was not clear whether they depend on similar assembly factors for that subunit. Part of the work reported here addresses this issue.

B. japonicum, a facultatively symbiotic, nitrogen-fixing bacterium investigated in our laboratory, has eight terminal oxidases, of which two are *bd*-type oxidases (Jünemann, 1997), and six are heme-copper oxidases, the latter being further divided into two quinol oxidases and four cytochrome oxidases (TABLE 2.1).

TABLE 2.1. Terminal respiratory oxidases in *B. japonicum*

Gene number[a]	Gene name	Subunit (SU)	Cytochrome type	Cu center	Pheno-type[b]	FC Bact/Air[c]	References
Heme-copper cytochrome oxidases							
blr1170	*coxB*	SU II		Cu_A	Fix^+	–	(Bott *et al.*, 1990)
blr1171	*coxA*	SU I	aa_3	Cu_B		-2.1	
blr1173	*coxF*	SU IV				-2.4	
blr1175	*coxC*	SU III				–	
bll3785	*coxM*	SU II		Cu_A	Fix^+	5.1	(Bott *et al.*, 1992)
bll3784	*coxN*	SU I	aa_3-like	Cu_B		3.1	
bll3783	*coxO*	SU IIIA				–	
bll3782	*coxP*	SU IIIB				–	
bll3781	*coxQ*	SU IV				–	
blr2763	*fixN*	SU I	bb_3	Cu_B	Fix^-	30.6	(Preisig *et al.*, 1993, Preisig *et al.*, 1996b)
blr2764	*fixO*	SU II	monoheme cyt *c*			26.8	
blr2765	*fixQ*	SU IV				38.3	
blr2766	*fixP*	SU III	diheme cyt *c*			23.9	
bll4481		SU II		Cu_A	Fix^+	-2.3	This work[e]
bll4480		SU I	diheme protein	Cu_B		-2.1	
bll4479[d]		SUIII[d]	diheme cyt *c*[d]			-2.8	
Heme-copper quinol oxidases							
blr2714	*coxW*	SU II			Fix^+	–	(Surpin *et al.*, 1996; Surpin & Maier, 1999)
blr2715	*coxX*	SU I	bb_3	Cu_B		–	
blr2716	*coxY*	SU III				–	
blr2717	*coxZ*	SU IV				–	

TABLE 2.1 (continued)

blr0149		SU II			n.t.	-2.8	(Göttfert *et al.*, 2004)
blr0150		SU I	diheme protein	Cu_B		-4.1	
blr0151		SU III				-4.1	
blr0152		SU IV				-2.5	
***bd*-type quinol oxidases**							
bll0283	*cydA*	SU I	*b*, *bd*		Fix^+	−	(Arslan, 2001)
bll0282	*cydB*	SU II				−	
blr3728		SU I	*bd*-like			−	(Göttfert *et al.*, 2004)
blr3729		SU II				−	

[a]Gene number according to RhizoBase (http://genome.kazusa.or.jp/rhizobase).

[b]Symbiotic N_2 fixation (Fix) phenotype of mutants deleted for at least the SUI and/or SUII genes. Fix^+, 70-100% of wild-type activity; Fix^-, 0-5% of wild-type activity; n.t., not tested.

[c]Fold-change (FC) of gene expression in soybean bacteroids (Bact; 21 days post infection) compared with cells grown aerobically (Mattatall *et al., 2000*). Data were taken from Pessi *et al.* (Pessi *et al.*, 2007). Positive values, increased expression; negative values, decreased expression; –, within threshold range of ±2.

[d]Sequence analysis predicts that this cyt *c* tranfers electrons obtained from reduced PQQ. Therefore, the oxidase proper (bll4481/4480) is classified here as a cytochrome oxidase.

[e]The genes had been reported previously (Göttfert *et al.*, 2004) but the mutant phenotype was determined in this work.

The *coxBACF*-encoded cytochrome aa_3 is the predominant heme-copper oxidase for aerobic growth. Of particular interest is the *fixNOQP*-encoded cbb_3-type oxidase because it enables endosymbiotic *B. japonicum* cells (bacteroids) to conserve energy despite the very low free O_2 concentration in soybean root nodules (Preisig *et al.*, 1996b; Arslan *et al.*, 2000). Accordingly, *fixNOQP* mutants do not fix N_2 in symbiosis (Fix^- phenotype), whereas mutants of all other *B. japonicum* oxidase genes so far examined are Fix^+ (TABLE 2.1). This unique trait allows us to test by mutation analysis whether or not candidate biogenesis genes are essential for the maturation of active cbb_3-type oxidase.

The purpose of this work was to look for genes in the *B. japonicum* genome (Kaneko *et al.*, 2002) which code for CtaG- and Sco1-like proteins, construct knock-out mutations, and test them for defects in the formation of the aa_3-type cytochrome oxidase. If such mutants are also defective in symbiotic N_2 fixation, the subsequent test for cbb_3-type oxidase presence and activity was thought to reveal a possible role of either protein in the biogenesis of this oxidase. We show here that although the aa_3- and cbb_3-type oxidases have similar Cu_B-containing active sites, disparate pathways are used for their biogenesis.

2.3 Experimental procedures

2.3.1 Media, growth conditions, and strains

Escherichia coli was grown in Luria–Bertani (LB) medium (Miller, 1972) containing these concentrations of antibiotics for plasmid selection (μg ml^{-1}): ampicillin, 200; kanamycin, 30; tetracycline, 10. *B. japonicum* was cultivated in peptone salts-yeast extract (PSY) medium (Regensburger & Hennecke, 1983; Mesa *et al.*, 2008) supplemented with 0.1% L-arabinose. Aerobic cultures (21% O_2) were grown in Erlenmeyer flasks containing one fifth of their total volume of PSY medium and shaken vigorously (160 rpm) at 30°C. Microaerobic cultures (0.5% O_2 in the gas phase) and anaerobic cultures were grown as described previously (Hauser *et al.*, 2006; Hauser *et al.*, 2007) except that the volume was larger (up to 50 ml for microaerobic conditions, up to 400 ml for anaerobic conditions). Where appropriate, antibiotics were used at these concentrations (μg ml^{-1}): spectinomycin, 100; streptomycin, 50; kanamycin, 100; tetracycline, 50 (solid media) or 25 (liquid media). *B. japonicum* strains used in this work are listed in TABLE 2.2.

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

Strains	Relevant genotype or phenotype	Reference
110*spc*4	Sp^r wild type	(Regensburger & Hennecke, 1983)
COX132	Sp^r Km^r *coxA*::Tn*5*	(Bott *et al.*, 1990)
3613	Sp^r Km^r *fixN*::Tn*5*	(Preisig *et al.*, 1993)
2575	Sp^r Sm^r Δ*scoI*::*aphII* (same orientation)	This work
2576	Sp^r Sm^r Δ*scoI*::*aphII* (opposite orientation)	This work
3563	Sp^r Km^r Δ*coxB*::*aphII* (same orientation)	This work
3583	Sp^r Km^r Δ*coxG*::*aphII* (same orientation)[a]	This work
3586	Sp^r Km^r Δ*coxG*::*aphII* (opposite orientation)[b]	This work
6532	Sp^r Km^r bll4480–81::*aphII* (same orientation)	This work
6533	Sp^r Km^r bll4480–81::*aphII* (opposite orientation)	This work
GRZ3035	Sp^r Sm^r *nosZ*::Ω	(Velasco *et al.*, 2004)

[a]Equivalent to Δ*cox*-b as shown in FIG. 2.1.

[b]Equivalent to Δ*cox*-a as shown in FIG. 2.1.

2.3.2 Plant growth

Soybean seeds (*Glycine max* (L.) Merr. cv. Williams) were surface-sterilized as described previously except that treatment with 30% H_2O_2 for 15 min was used (Göttfert *et al.*, 1990b). The symbiotic phenotype of *B. japonicum* mutants was determined in infection tests using soybean as host, and whole-nodule nitrogenase activity was measured with the acetylene reduction assay (Göttfert *et al.*, 1990a; Hahn & Hennecke, 1984).

2.3.3 General DNA biochemistry

Standard techniques were used for plasmid isolation, cloning, transformation, Southern blotting, hybridization, and sequencing (Sambrook, 1989). *E. coli* strain DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, MD) was the host for routine clonings, and strain BL21 (DE3) (Studier & Moffatt, 1986) for heterologous protein expression. DNA probes for hybridization were labeled with the digoxigenin DNA labeling kit from Boehringer (Mannheim, Germany).

2.3.4 Mutant construction

Plasmids with the pRJ-prefix are from our laboratory collection. Details on their genealogy and DNA content are available from authors on request. For construction of the *coxG* deletion mutants, part of the *coxG* gene on plasmid pRJ3550 was excised, using restriction enzymes *Bbs*I and *Bam*HI, and replaced by the *Sma*I fragment from pUC4-KIXX (formerly Pharmacia, Uppsala, Sweden) carrying the *aphII* gene. The resulting plasmids contained the *aphII* gene either with the same (pRJ3581) or the opposite (pRJ3582) transcriptional direction as the *coxG* gene. The two DNA constructs were then cloned into the suicide vector pSUP202pol3 (Rossmann *et al.*, 1997) using *Eco*RI sites, which yielded plasmids pRJ3583 and pRJ3586. Mobilization of these plasmids via *E. coli* S17-1 (Simon, 1983) into *B. japonicum* 110*spc*4 was followed by screening for double recombination events. The resulting two chromosomal *coxG* deletion mutants carrying the *aphII* gene in different orientations were named 3583 and 3586 (TABLE 2.2, and FIG. 2.1 with genome coordinates).

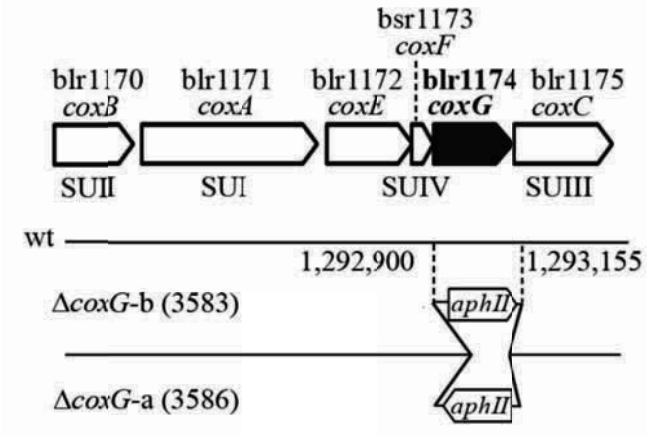


FIG.2.1. Map of the *cox* gene cluster (*blr1170-1178*). The arrows show the arrangement of the genes in the cluster. Gene names (if available) are given below the gene numbers. The bottom part of the scheme shows the orientation and precise chromosomal nucleotide position of the kanamycin resistance gene (*aphII*) inserted in the *coxG* deletion mutants.

The first step towards deleting *scoI* was the PCR-mediated amplification of *scoI*-flanking DNA using appropriate primers. The 881-bp upstream and 825-bp downstream regions were cloned in pGEM-T Easy (Promega, Madison, WI, USA) and verified by sequencing. Both amplicons were then cloned tail-to-head into pBluescript II KS(+) (Stratagene, La Jolla, CA, USA) resulting in pRJ2572. In two separate constructions, a 1,206-bp *Pst*I kanamycin resistance cassette (*aphII*) of pBSL86 (Alexeyev & Shokolenko, 1995) was inserted in either orientation into the unique *Pst*I site between the *scoI*-upstream and -downstream regions on pRJ2572. A 2,872-bp *Eco*RI-*Xba*I fragment with *scoI*-flanking DNA plus intervening *aphII* cassette was excised from each of the two constructs and cloned in pSUP202pol4 (Fischer *et al.*, 1993). The resulting plasmids pRJ2575 and pRJ2576 were mobilized individually by conjugation from *E. coli* S17-1 into *B. japonicum* 110*spc*4 for marker exchange, yielding mutants 2575 and 2676 with *aphII* in the same and opposite directions as *scoI*, respectively (TABLE 2.2). The correct chromosomal cassette integration was verified by PCR. The deletion end points are at genome coordinates 1,244,627 and 1,245,226.

Plasmid pRJ3563K was used to mutate the *coxB* gene. Construction of this plasmid started from pRJ3557, which has a 1.1-kb *Sma*I-*Xho*I fragment containing *coxB*. A unique *Nco*I-*Nru*I fragment from within *coxB* was excised and replaced by the *Hin*dIII-*Sma*I kanamycin resistance fragment of pUC4-KIXX (Pharmacia), and then the *coxB*-flanking *Bam*HI-*Sma*I and *Eco*RI fragments were added on both sides of the Δ*coxB* construct, resulting in pRJ3562K. This DNA construct was cloned in suicide vector pSUP202pol4 (Fischer *et al*., 1993) to give pRJ3563K, which was then mobilized via *E. coli* strain S17-1 into *B. japonicum* 110*spc*4. Kanamycin-resistant, tetracycline-sensitive exconjugants were selected and checked for double-crossover events by Southern blot analysis. An isolate containing the *coxB* deletion (with *aphII* in the same orientation as *coxB*) was named 3563 (TABLE 2.2). The deletion end points are at genome coordinates 1,288,938 and 1,289,556.

To delete the bll4480-81 genes, 665-bp upstream and 843-bp downstream regions were PCR-amplified with appropriate primer pairs, cloned in pGEM-T Easy (Promega), and verified by sequencing. Both amplicons were then cloned tail-to-head in pBluescript II KS(+) (Stratagene). The *aphII* gene from pBSL86 (Alexeyev & Shokolenko, 1995) was inserted in-between, using a *Pst*I restriction site, which resulted in plasmids pRJ6532 and pRJ6533. They were mobilized individually by conjugation from *E. coli* S17-1 into *B. japonicum* strain 110*spc*4 for marker exchange, yielding mutants 6532 and 6533 with the *aphII* orientations given in TABLE 2.2. The deletion end points are at genome coordinates 4,961,905 and 4,962,644.

2.3.5 Preparation of membrane fraction

B. japonicum cells were disrupted by three passages through a French pressure cell at 9,000 psi. After removal of cell debris by centrifugation at 28,000 x *g* for 30 min at 4°C, membrane pellets were

collected by ultracentrifugation at 129,000 x *g* for 90 min at 4°C. They were solubilized by slow stirring overnight at 4°C. If used for Western blots, the membrane pellets were solubilized in 50 mM Tris-HCl, pH 7.5. If used for cytochrome *c* oxidase measurements, the buffer additionally contained 1% (w/v) dodecylmaltoside (Glycon Biochemicals, Luckenwalde, Germany).

2.3.6 Determination of protein concentration

Concentration of solubilized membrane protein was determined with the Bradford method (Bradford, 1976), using a Bio-Rad assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard.

2.3.7 Cytochrome difference spectra

Dithionite-reduced minus air-oxidized spectra were recorded from 500 to 650 nm in a Hitachi U-3300 spectrophotometer using solubilized membrane proteins prepared from aerobically grown cells at a concentration of 2 mg ml^{-1}. Similarly, dithionite-reduced minus APS-oxidized spectra were recorded. For the reduced spectrum, 2 µl freshly prepared Na-dithionite solution (0.5 M in deionized H_2O) was mixed in to give a final concentration of 5 mM. For the oxidized spectrum, 10 µl APS of 0.1 M solution was added to a final concentration of 5 mM.

2.3.8 Determination of cytochrome *c* oxidase activity

Cytochrome *c* oxidase activity of solubilized membrane protein preparations from aerobically grown *B. japonicum* cells was determined as described (Gerhus *et al.*, 1990) with one modification: measurements were performed under continuous stirring in 50 mM HEPES buffer (pH 7.4) containing 1 % (w/v) dodecylmaltoside.

2.3.9 Expression and purification of ScoI and its mutant derivative

The *scoI* codons for cysteines C74 and C78 were mutated by QuikChange mutagenesis (Stratagene) into serine codons, resulting in pRJ8318. DNA coding for wild-type and mutated versions of the soluble part of ScoI (starting with a glycine at position 30) was cloned into the expression vector pEC425 (Schulz *et al.*, 1998) resulting in pRJ8331 (wild type) and pRJ8339 (C74S, C78S). *E. coli* BL21 (DE3) was transformed either with pRJ8331 or with pRJ8339. Dense precultures were used to inoculate the main cultures which had a volume of 200 ml or 1 L. The medium used was LB with ampicillin. Cultures were grown at 37°C until they reached an optical density (A_{600}) of 0.5. At this point, expression of the recombinant protein was induced by addition of arabinose to a final concentration of 0.1% (w/v). After induction, the cultures were transferred to 30°C, and after 2 to 4 h, cells were collected by centrifugation and disrupted by three passages through a French press at 9,000 psi. Purification was performed with Strep-Tactin Sepharose columns (IBA GmbH, Göttingen, Germany) according to the supplier's protocol.

2.3.10 UV/VIS spectroscopy of ScoI

The method was used to follow the binding of Cu(II) to reduced ScoI (Imriskova-Sosova *et al.*, 2005). Reduction of $ScoI_{sol}$ was achieved by incubation with 2 mM DTT for 4 h at 4°C. DTT removal and exchange against 50 mM sodium phosphate buffer (pH 7.0) was done by gel filtration over PD-10 columns (Amersham Biosciences, GE Healthcare Life Science, Waukesha, USA). Incremental Cu(II) was added in the following way: 5 µl of the solution added contained Cu(II) in amounts that corresponded to 10% of the stoichiometric amount of protein in 1 ml. With a protein concentration of 23 µM, for example, the requested copper solution was 460 µM. For removal of unbound copper by dialysis, a Slide-A-Lyzer® (10,000 MWCO, Mini Units) from Pierce Thermo Fisher Scientific (Rockford, USA) was used. UV-visible spectra were recorded from 200 to 800 nm on an Agilent Diode Array Photometer (Agilent Technologies, Santa Clara, USA).

2.3.11 Immunological techniques

Rabbit antibodies specific for the FixO, FixP, and CoxA proteins were available from previous work (Loferer *et al.*, 1993; Loferer & Hennecke, 1994; Zufferey *et al.*, 1996). For the production of antibodies against CoxB, a peptide of the sequence H_2N-RVVEDKEFASWVETAKKK-COOH corresponding to residues 243 to 260 of the predicted CoxB sequence was synthesized. Both, the peptide and the polyclonal antibodies from rabbits were custom-made by TANA Laboratories, L.C. (Houston, TX). Polyclonal antibodies against the soluble part of ScoI were raised in rabbits. Strep-tagII-marked protein was used as antigen, and the immunization was performed by EUROGENTEC S.A. (Liege, Belgium). To produce anti-FixN, the same procedure was applied as in Zufferey *et al*. (1996). Synthesis of the three peptides was performed by PickCell Laboratories (Lelystad, The Netherlands) and immunization by EUROGENTEC S. A. (Liege, Belgium). For Western blot analyses, membrane proteins (30 µg per lane) were separated by SDS-PAGE (Laemmli, 1970) and blotted as described previously (Loferer *et al.*, 1993). The dilutions of antibodies were 1:2,500 for anti-CoxA, 1:10,000 for anti-CoxB, 1:10,000 for anti-ScoI, 1:1,000 for anti-FixO, 1:1,000 for anti-FixN, and 1: 5,000 for anti-FixP. Protein bands with bound immunoglobulins were detected with anti-rabbit IgG-POD (Boehringer, Mannheim, Germany), or with anti-rabbit IgG (H+L)-HRP-conjugate (Bio-Rad Laboratories) and chemoluminescence detection kits from Boehringer (Mannheim, Germany) and Pierce (Rockford, IL).

2.4 Results

2.4.1 The *B. japonicum coxG* gene (*blr1174*) is important for the biogenesis of cytochrome aa_3, but not for that of cytochrome cbb_3

The genes for the aa_3-type cytochrome oxidase (*cox*) were sequenced in our laboratory (EMBL Nucleotide Sequence Database, accession number AJ242592) and in the course of the *B. japonicum* genome sequencing project (Kaneko *et al.*, 2002). Three open reading frames (*coxEFG*) were found to be located between the subunit-I (*coxA*) and subunit-III (*coxC*) genes (FIG.2.1). Based on amino acid sequence similarity, *coxE* codes for a putative protoheme IX farnesyltransferase. The *coxF* gene encodes a short transmembrane protein that probably corresponds to subunit IV. The *coxG* gene product shares 49% identity (60% similarity) with CtaG from *P. denitrificans* and 38% identity (52% similarity) with the *Saccharomyces cerevisiae* Cox11 protein. CoxG is predicted to have a hydrophobic N-terminal membrane anchor, and a hydrophilic periplasmic domain that carries the Cys-X-Cys motif implicated in Cu(I) ligation (Banci *et al.*, 2004). There is evidence to suggest that Cox11-like proteins transfer copper to the Cu_B site in subunit I (Khalimonchuk *et al.*, 2005; Banting & Glerum, 2006). We therefore tested the contribution of *B. japonicum coxG* to cytochrome oxidase activity.

Two *coxG*-internal deletion mutations were constructed, and the obtained mutant strains 3583 and 3586 carried the inserted *aphII* cassette in the same or the opposite orientation to *coxG*, respectively (TABLE 2.2, FIG. 2.1).

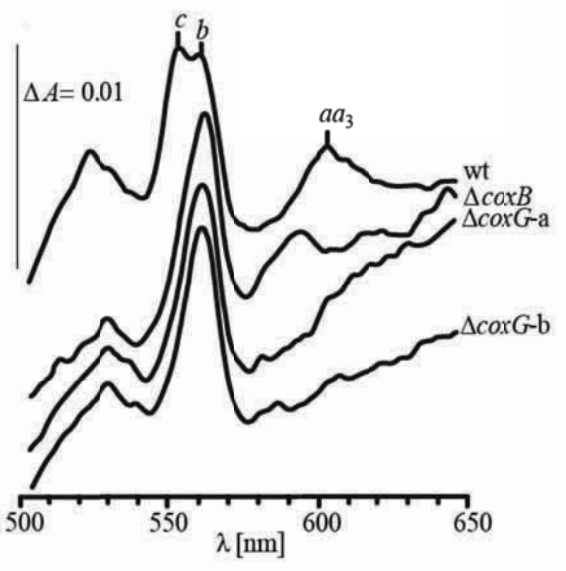
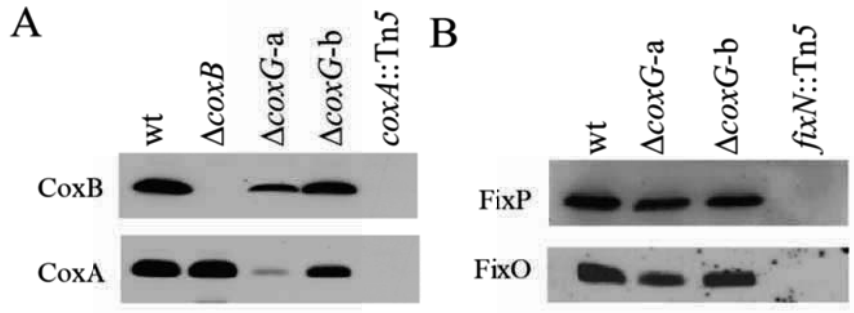


FIG. 2.2. Dithionite-reduced minus air-oxidized difference spectra**.** The difference spectra were recorded with solubilized membrane protein fractions prepared from aerobically grown *B. japonicum* cells of the wild type (wt), the *coxB* deletion mutant 3563 (Δ*coxB*), and the two *coxG* deletion mutants (Δ*coxG*) with the *aphII* cassette inserted in orientation *a* (strain 3586) and *b* (strain 3583). The protein concentration was 2 mg ml^{-1}. The vertical line on the left spans an absorption difference (Δ*A*) of 0.01. Peaks characteristic for cytochromes *c*, *b*, and aa_3 are marked.

Both mutants were NADI negative, meaning that whole-cell cytochrome oxidase activity was defective, and the activity could not be restored by supplementing the growth medium with at least 10 μM $CuCl_2$. The respiratory defect was then quantified in a cytochrome oxidase assay *in vitro*, using solubilized membrane proteins. A *coxB* mutant (subunit II gene deletion, TABLE 2.2) and the parental wild type were included for comparison. With the wild-type membrane fraction, an activity of 0.469 μmol cytochrome *c* oxidized per min and mg protein was measured, whereas both the *coxB* and the two *coxG* mutants yielded activities of only 0.01 μmol min^{-1} mg^{-1}, which corresponds to the background oxidation of cytochrome *c* by air during this assay. A similarly low value was previously reported for a *B. japonicum coxA* mutant (Bott *et al.*, 1990). Consistent with this finding was the absence in *coxG* mutants of the characteristic cytochrome aa_3 peak at 603 nm in dithionite-reduced minus air-oxidized difference spectra, and a strongly diminished cytochrome *c* peak at 552 nm (FIG. 2.2), due to destabilization of the cytochrome bc_1-CycM-aa_3 supercomplex (Bott *et al.*, 1990). Curiously, a small peak at 594 nm appeared with the *coxB* strain (FIG. 2.2) which might stem from an incompletely formed cytochrome aa_3 complex. The spectral defect seen with the *coxG* mutants could not be restored by the prior addition of 50 μM $CuCl_2$ into the growth medium.

The presence or absence of cytochrome aa_3 subunits I and II in *coxG* mutant membranes was tested by immunoblot analysis, including *coxA* and *coxB* mutants for control (FIG. 2.3.A).

FIG. 2.3. Detection of aa_3- and cbb_3-type oxidase subunits by Western blot analysis. A. Antibodies against CoxB and CoxA were used. B. Antibodies against FixP and FixO were used. Cells had been cultivated either aerobically (A) or micro-aerobically (B). Membrane proteins (30 μg per lane) were separated on 14% polyacrylamide gels containing SDS. Proteins from the following strains were analyzed (labeled here by the relevant genotypes): wild type (wt); 3536 (Δ*coxB*); 3586 (Δ*coxG* a); 3583 (Δ*coxG* b); COX132 (*coxA*::Tn*5*); 3613 (*fixN*::Tn*5*).



The controls show that subunits I (CoxA) and II (CoxB) are clearly detectable in the wild type but absent in the *coxA* mutant. In the *coxB* mutant, however, only subunit II is missing, as expected, whereas subunit I protein is detectable. We interpret this to mean that subunit II assembly in the membrane depends on the presence of subunit I, whereas subunit I may assemble in the absence of

subunit II. In the case of the *coxG* mutant, the outcome of this experiment is influenced by the orientation of the resistance cassette inserted in the *coxG* gene (FIG. 2.3.A). While the Δ*coxG*-b mutant (strain 3583) with the cassette in the same orientation as the *coxG* gene shows only a marginal diminution of the amounts of subunits I and II in its membrane, the Δ*coxG*-a mutant (strain 3586) with the cassette in the opposite orientation is strongly depleted for subunit I and, probably as a consequence, exhibits a substantial decrease of subunit II. The divergent behavior finds a likely explanation in the *cox* operon structure (FIG. 2.1). We infer that transcription of *coxC* (subunit-III gene) is disturbed in the Δ*coxG*-a mutant due to a polar effect of the inserted resistance cassette, and that the presence of subunit III is important for the stability of the entire oxidase complex. This polarity effect is largely masked in the Δ*coxG*-b mutant in which the out-reading transcription activity originating from the resistance cassette apparently leads to a sufficient expression of *coxC*. Hence, if *coxC* is expressed, the deletion in the *coxG* gene does not significantly interfere with assembly of the oxidase. Yet, as shown before, the same mutant 3583 had lost cytochrome *c* oxidase activity, which was also evidenced by the lack of the aa_3 peak in the difference spectrum (FIG. 2.2). The observed phenotype is in line with the proposed role of Cox11-like chaperones in the delivery of copper to the Cu_B site in subunit I.

Next, we addressed the question whether the biogenesis function of CoxG is also needed for other heme-copper cytochrome oxidases that possess the Cu_B-containing subunit I. If the cbb_3-type cytochrome oxidase, for example, would critically depend on CoxG, the *B. japonicum coxG* mutants might have a nitrogen fixation defect in symbiosis because this oxidase is essential for bacteroid respiration (*cf*. TABLE 2.1). However, the data in TABLE 2.3 show that both *coxG* strains were able to elicit a fully functional symbiosis (Fix^+ phenotype) indistinguishable from that of the *B. japonicum* wild type. Therefore, it is unlikely that CoxG is involved in the maturation of the cbb_3-type oxidase. To substantiate this notion, we confirmed the presence of cbb_3 oxidase subunits FixO and FixP in membranes of *coxG* mutant cells that had been grown under micro-oxic conditions (FIG. 3.B). Taken together, it appears as if the role CoxG plays for cytochrome aa_3 does not apply to cytochrome cbb_3.

TABLE 2.3. Symbiotic properties of Δ*scoI* and Δ*coxG* mutants inoculated on soybean**.** Mutants were tested in parallel with the wild type in two separate series of two (Δ*coxG*) and three (Δ*scoI*) independent experiments. Shown are the data of one representative experiment for each set of strains. N_2ase, nitrogenase; Fix, nitrogen fixation.

Strain	Relevant genotype	Number of nodules	Nodule dry weight (mg)	N_2ase activity (% C_2H_4 min^{-1} g^{-1})	Rel. Fix activity (% of wt)
110*spc*4	wild type	30.0 ± 3.3	0.93 ± 0.03	4.15 ± 2.16	100 ± 52
3586	Δ*coxG*-a	32.7 ± 16.4	0.82 ± 0.7	4.12 ± 2.11	99.3 ± 51.0
3583	Δ*coxG*-b	32.0 ± 13.3	0.88 ± 0.15	4.50 ± 0.93	108.4 ± 22.3
110*spc*4	wild type	35.7 ± 10.3	1.12 ± 0.29	2.31 ± 0.90	100 ± 29
2575	Δ*scoI*	40.7 ± 7.8	0.69 ± 0.15	0.82 ± 0.35	28.8 ± 11.9

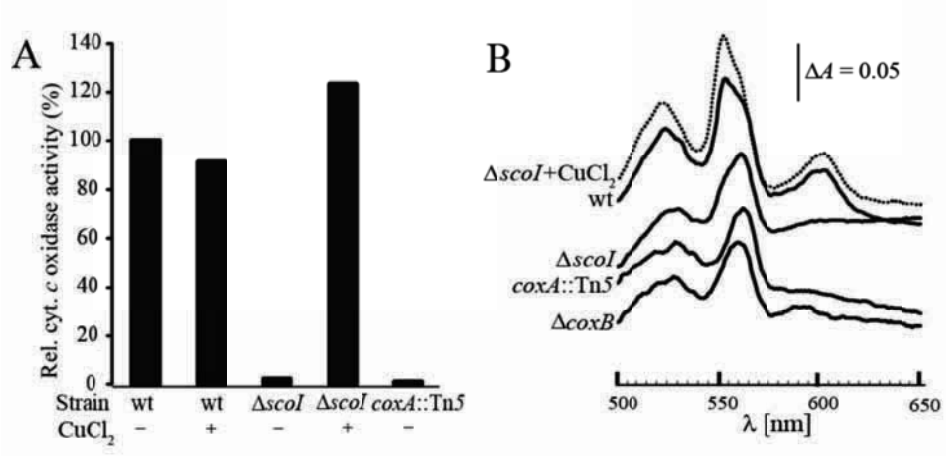
2.4.2 Gene *blr1131* encodes a Sco1-like protein

The second biogenesis protein that we considered to be relevant for this work is related to Sco1. A copper-chaperoning function specifically in the formation of the Cu_A site on subunit II has been attributed not only to mitochondrial but also to bacterial Sco1-like proteins (Nittis *et al.*, 2001; Balatri *et al.*, 2003; Swem *et al.*, 2005, Abajian & Rosenzweig, 2006; Cawthorn *et al.*, 2009). In many aerobic bacteria, its gene maps immediately adjacent to cytochrome oxidase structural and biogenesis genes (*cf*. String Database http://string-db.org/). The *B. japonicum cox* gene cluster shown in FIG. 2.1, however, does not encode a Sco1-like protein. Instead, based on sequence similarity, the *blr1131* gene (Kaneko *et al.*, 2002) was identified as a likely candidate to encode a Sco1-like protein. The *blr1131* ORF codes for a 196-amino-acid protein that shows the typical hallmarks of pro- and eukaryotic Sco proteins, *i.e.*, an N-terminal membrane anchor and a C-terminal, membrane-peripheral thioredoxin-like domain with a Cys-X_3-Cys motif and a conserved His for copper ligation (Balatri *et al.*, 2003). For these reasons and further evidence given below, and in keeping with the standard nomenclature for bacterial genes (Demerec *et al.*, 1968), *blr1131* of *B. japonicum* was named *scoI*.

To approach ScoI function, two mutants were constructed (strains 2575 and 2576, TABLE 2.2) in which almost the entire *scoI* gene was deleted and replaced by a kanamycin resistance cassette in either orientation. As no phenotypic difference was subsequently observed between strains 2575 and 2576, we report here only the results for strain 2575. The Δ*scoI* mutant exhibited wild type-like growth in complex medium on agar plates and in liquid cultures. A qualitative NADI test revealed a defect in cytochrome *c* oxidase activity. To further substantiate this defect, quantitative cytochrome *c* oxidase measurements were done. Mutant COX132 (TABLE 2.2) having an insertion in the subunit I

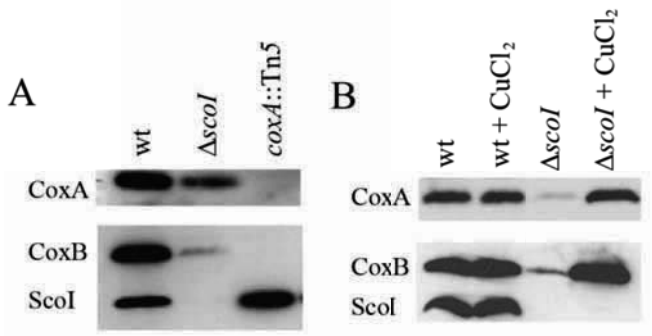
gene (*coxA*::Tn*5*) was included in these assays for comparison. FIG. 2.4.A shows that the *scoI* and *coxA* mutants have negligible cytochrome *c* oxidase activities below 2% of the wild type, which reflects spontaneous cytochrome *c* oxidation in air. Cytochrome aa_3 formation was analyzed by dithionite-reduced minus APS-oxidized difference spectroscopy of solubilized membranes. Again, *scoI* and *coxA* mutants showed the same phenotype, *i.e.*, absence of the diagnostic aa_3 peak at 603 nm, and a decrease of the cytochrome *c* peak at 552 nm (FIG. 2.4.B). In the *scoI* mutant, the 603-nm peak appeared again when cells had been grown in medium containing 50 µM $CuCl_2$ (FIG. 2.4.B). Likewise, cytochrome *c* oxidase activity of the *scoI* mutant was restored to wild-type levels by the same $CuCl_2$ treatment (FIG. 2.4.A).

FIG. 2.4. The *scoI* mutation affects cytochrome aa_3. A. Relative cytochrome *c* oxidase activity of aerobically grown wild type (wt), and mutant strains 2575 (Δ*scoI*) and COX132 (*coxA*::Tn*5*). Wild-type activity (100%) corresponds to approx. 0.43 µM horse heart cytochrome *c* oxidized per mg membrane protein per min. Whether or not 50 µM $CuCl_2$ was added to the PSY medium is indicated with + or –. B. Dithionite-reduced minus APS-oxidized difference spectra of solubilized membrane protein fraction (61 mg ml^{-1}) from aerobically grown cells. As labeled, the continuous curves represent the wild type (wt), and the Δ*scoI*, *coxA*::Tn*5*, and Δ*coxB* mutants (strains 2575, COX132, and 3536, respectively). The dotted curve represents the Δ*scoI* mutant grown in PSY supplemented with 50 µM $CuCl_2$. The vertical bar indicates a difference in absorption (Δ*A*) of 0.05. Note that, in contrast to FIG. 2.2, the cytochrome *b* is not resolved here as a separate peak but as a shoulder (560 nm).



Assembly of cytochrome aa_3 in the membrane was then analyzed in Western blots using antibodies specific for CoxA (subunit I) (Loferer *et al.*, 1993) and CoxB (subunit II). FIG. 2.5 (A, B) shows that both subunits are substantially depleted in *scoI* mutant membranes. When 50 µM $CuCl_2$ had been added to the growth medium, the amounts of both subunits in the *scoI* mutant were restored to wild-type levels (FIG. 5B).

FIG. 2.5. Western blot analysis of cytochrome aa_3 subunits in the Δ*scoI* mutant. A. Comparison of *scoI* mutant with wild type and *coxA* mutant. B. Restoration of subunit assembly by copper. As marked on the left, antibodies specific against CoxA, CoxB, and ScoI (control) were used. The lower blots in panels A and B were exposed to a mixture of anti-CoxB and anti-ScoI immunoglobulins. Membrane proteins (30 µg per lane) were separated on 14 % polyacrylamide gels containing SDS. They were isolated from the following strains: wild type (wt); 2575 (Δ*scoI*); COX132 (*coxA*::Tn*5*). For copper supplementation, PSY medium contained 50 µM $CuCl_2$.



The results suggest that the mutant defect was caused by a lack of copper-dependent maturation of the oxidase complex, and that ScoI acts as an aa_3-specific copper chaperone. To further support this idea, a soluble ScoI protein derivative ($ScoI_{sol}$) was purified (see Experimental procedures), and its ability to bind copper was tested by UV/VIS absorption spectroscopy. It was essential to reduce $ScoI_{sol}$ with DTT prior to the experiment. Addition of $CuCl_2$ to the protein resulted in an absorbance peak at approx. 360 nm (FIG. 2.6) reflecting the binding of Cu(II) to the protein (Imriskova-Sosova *et al*., 2005). Upon incremental copper addition, the peak increased and reached a plateau at a ratio of approx. 0.8 stoichiometric equivalents of Cu(II) per $ScoI_{sol}$ molecule. Copper binding did not occur to a $ScoI_{sol}$ mutant derivative in which the two predicted active-site cysteines C74 and C78 had been replaced by serines. By analogy with Sco1-like proteins from other organisms, our results indicate that C74 and C78 form part of the active site and that they need to be reduced to thiols or thiolate anions to allow complex formation with a copper cation.

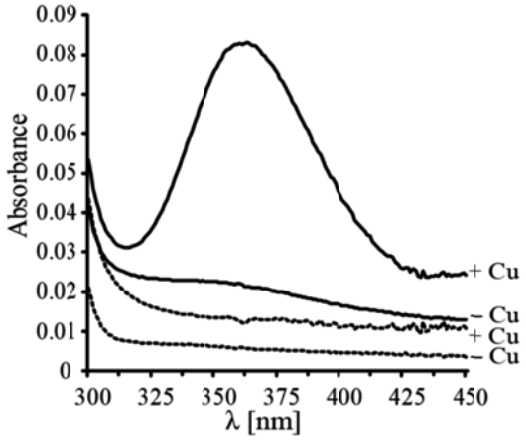


FIG. 2.6. Evidence for copper binding to ScoI. UV-VIS spectra were taken of 23 µM reduced $ScoI_{sol}$ (solid curves) and $ScoI[C74S/C78S]_{sol}$ (dashed curves), dissolved in 50 mM phosphate buffer, pH 7.0. (–) No Cu(II) was added; (+) Cu(II) was added to protein in molar equivalents of either 1 (in the case of $ScoI_{sol}$) or 1.4 (in the case of $ScoI[C74S/C78S]_{sol}$. The peak at 360 nm corresponds to the absorbance of protein-bound copper.

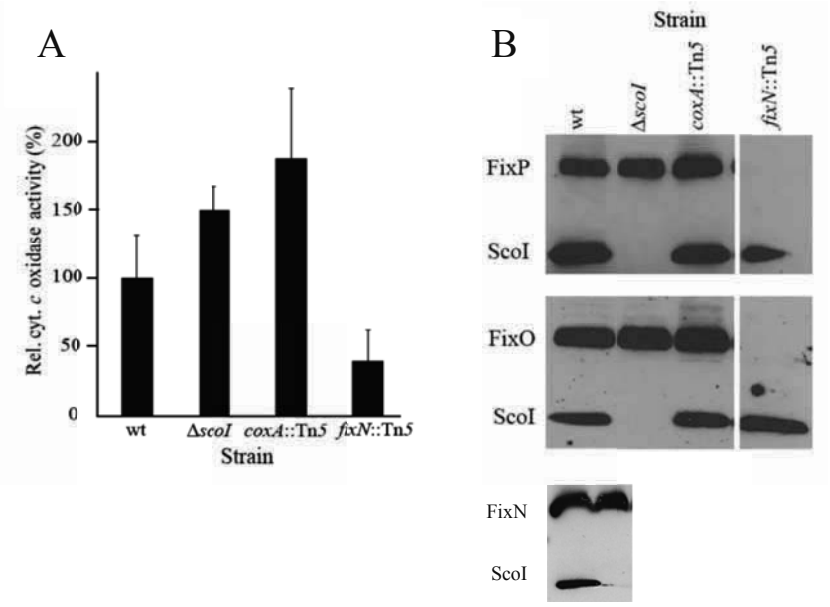
2.4.3 The *B. japonicum scoI* mutant is symbiotically defective

During anoxic growth with nitrate as the terminal electron acceptor, the *scoI* mutant (strain 2575) exhibited a slight delay in denitrification with a transient accumulation of nitrite, indicating that nitrite reduction or a subsequent N-oxide reduction step is impaired (data not shown). A more striking property of the *scoI* mutant was its inability to establish a fully functional symbiosis with soybean (*Glycine max*). TABLE 2.3 shows that it reaches only 29% of the nitrogen fixation (Fix) activity of the wild type, as determined by the acetylene reduction assay. Furthermore, plants inoculated with strain 2575 displayed signs of nitrogen starvation such as a decreased nodule dry weight (TABLE 2.3) and a pale-green leaf color. On the one hand, we can safely argue that the symbiotic defect in the *scoI* mutant was not caused by the described defect in cytochrome aa_3 biogenesis because a *coxA* knock-out mutant has a clear Fix^+ phenotype (Bott *et al.*, 1990). On the other hand, although all of the *scoI*-mutant phenotypes were not as severe as those of a cytochrome cbb_3-defective mutant (Preisig *et al.*, 1993), they would plausibly explain the symbiotic defect if ScoI served as a maturation factor for the Cu_B site of the symbiotically essential cytochrome cbb_3.

2.4.4 ScoI is not needed for the biogenesis of the cbb_3-type oxidase

Cytochrome oxidase activity was determined in membranes of the *scoI* mutant and compared with that of the wild type, the *coxA* mutant, and a cbb_3-defective *fixN* mutant. All strains were grown under conditions (micro-oxia, or anoxia with nitrate) in which the *fixNOQP* operon is strongly induced and its product is the predominant cytochrome oxidase expressed (Preisig *et al*.; 1993, Preisig *et al.*; 1996b). FIG. 2.7.A shows the results with membranes isolated from anoxically grown cultures, using reduced horse-heart cytochrome *c* as the electron donor. The *fixN* mutant had a more than 60% decreased cytochrome *c* oxidase activity as compared with the wild type, which confirms that the cbb_3-type oxidase is the most prominent oxidase under these growth conditions in the wild type. In contrast, neither the *scoI* nor the *coxA* mutant was impaired in this assay. Interestingly, they even showed higher activity than the wild type. Perhaps, the lack of competing cytochrome aa_3 in these mutants allows for a better substrate usage or a facilitated assembly of the cbb_3-type oxidase in the membrane.

FIG. 2.7. The *scoI* mutation does not affect cytochrome cbb_3. A. Relative cytochrome *c* oxidase activity of the wild type (wt), and strains 2575 (Δ*scoI*), COX132 (*coxA*::Tn*5*), and 3613 (*fixN*::Tn*5*). Cells had been cultivated under conditions of anaerobic nitrate respiration. A total of 11 measurements with three independent biological replicas were done for each strain. Wild-type activity (100%) corresponds to 1.2 μM cytochrome *c* oxidized per mg protein per min. B. Western blot analysis of cytochrome cbb_3 subunits. As marked on the left, antibodies specific against FixP, FixO, FixN and ScoI (control) were used. The blots in the three panels were exposed to mixtures of anti-FixP plus anti-ScoI immunoglobulins, anti-FixO plus anti-ScoI immunoglobulins, and anti-FixN plus anti-ScoI immunoglobulins. Membrane proteins (30 μg per lane) were separated on 14% polyacrylamide gels containing SDS. They were isolated from the strains indicated. The separate lane on the right was run on the same gel as the other lanes.



The *in vitro* experiment of FIG. 2.7.A was corroborated by a TMPD oxidation assay *in vivo* with cells that had been cultivated microaerobically. Again, only the *fixN* mutant exhibited an impaired TMPD oxidation activity as compared with the other three strains (data not shown). Finally, we examined membranes for the presence of some of the cbb_3-type oxidase subunits. For this purpose, membranes were isolated from wild-type and mutant cells that had been cultivated anaerobically or microaerobically, and tested by Western blot analysis with antibodies specific for the FixO, FixP, and FixN proteins. As shown FIG. 2.7.B, the *scoI* mutant contains these subunits in undisturbed amounts, just like the wild type and the *coxA* mutant, but in contrast to the control (*fixN* mutant) where both subunits are missing. Taken together, none of the phenotypic and biochemical tests performed with the *scoI* mutant revealed a function of the ScoI protein in the biogenesis of the cbb_3-type oxidase.

If not cbb_3, which protein might then be the symbiotically important target for ScoI? Using bioinformatics, we examined the *B. japonicum* chromosome for genes encoding potential periplasmic Cu_A-binding proteins other than CoxB, and found three: bll3785 (*coxM*), bll4481, and blr0315 (*nosZ*).

The first two are in operons for heme-copper cytochrome oxidases, *coxMNOPQ* and bll4481-4479 (TABLE 2.1). A *coxN* deletion mutant had previously been shown to be Fix^+ in symbiosis (Bott *et al.*, 1992). Here we constructed and tested bll4481-4480 deletion mutants (strains 6532 and 6533, TABLE 2.2) and found that they also had a Fix^+ phenotype (TABLE 2.1). The *nosZ* gene codes for a periplasmic Cu_A-containing nitrous oxide reductase (Haltia *et al.*, 2003, Zumft, 2005b). A *B. japonicum nosZ* mutant (strain GRZ3035; (Velasco *et al.*, 2004)) was reported by Mesa *et al*. (Mesa *et al.*, 2004) to be Fix^+ in symbiosis. We confirmed the phenotype of this mutant with the standard soybean infection test used in our laboratory (data not shown). In conclusion, while the biogenesis of all three proteins may well depend on ScoI, none of them is important for symbiosis. Future work on the identification of other periplasmic ScoI targets may help to explain the symbiotic *scoI* mutant phenotype.

2.5 Discussion

The relative exclusiveness of *B. japonicum* cytochromes aa_3 and cbb_3 for aerobic and microaerobic cells, respectively, was of tremendous help in our attempt to assess the contribution of CoxG and ScoI to the biogenesis of either oxidase.

It was somewhat surprising that *B. japonicum* CoxG was found to be essential for the biogenesis of cytochrome aa_3 but not for that of cytochrome cbb_3. Given that its pro- and eukaryotic homologs (CtaG, Cox11) had been implicated in copper delivery to the Cu_B site on subunit I (Cobine *et al.*, 2006), one might have expected that CoxG serves as an important chaperone for both oxidases. What are the reasons for the selectivity of CoxG? The question is difficult to answer because, despite the remarkable progress on the structure and function of Cox11-like proteins (Banci *et al.*, 2004; Khalimonchuk *et al.*; 2005, Banting & Glerum, 2006; Carr *et al.*, 2005), the mechanism of copper insertion into subunit I remains elusive (Greiner *et al.*, 2008; Hiser *et al.*, 2000). We could think of two possible, mutually not exclusive discriminating factors. The first is subunit III, which is conserved in almost all bacterial heme-copper oxidases except cytochrome cbb_3. A hypothetical CoxG–subunit-III interaction as a prerequisite for Cu insertion into subunit I would not be fulfilled by the completely dissimilar FixO subunit in cytochrome cbb_3. A second discriminator might be the reported difference in the heme-Cu_B active-site architecture between the two oxidase classes (Buse *et al.*, 1999; Hemp *et al.*, 2005; Rauhamaki *et al.*, 2009). Especially if Cu insertion occurs during membrane translocation, as proposed by Khalimonchuk *et al*. (Khalimonchuk *et al.*, 2005), the CoxG protein might be better suited to deliver Cu to subunit I of the aa_3- rather than cbb_3-type oxidase during folding and maturation.

The *B. japonicum scoI* gene was also shown here to be essential for cytochrome aa_3 formation and activity. The soluble ScoI domain devoid of its N-terminal membrane anchor was expressed and purified. It bound Cu(II) with a nearly 1:1 stoichiometry, and the reduced cysteines in a CXXXC motif were demonstrated to be essential for binding. All of these results are consistent with the purported role of the eukaryotic (*i.e.,* mitochondrial) ScoI-like proteins (Nittis *et al.*, 2001; Abajian & Rosenzweig, 2006; Horng *et al.*, 2005; Banci *et al.*, 2007b) and some prokaryotic ScoI homologs (Balatri *et al.*, 2003; Cawthorn *et al.*, 2009; Saenkham *et al.*, 2009) in the biogenesis of the membrane-peripheral, Cu_A-containing subunit II domain. Akin to Cox11/CtaG/CoxG, however, the direct transfer of copper from the ScoI chaperone to the target subunit remains to be demonstrated experimentally. Moreover, the model of ScoI-like proteins as Cu-chaperones has been challenged. Since they are thioredoxin-like, some were found to possess protein dithiol:disulfide oxidoreductase activity (Abriata *et al.*, 2008; Ye *et al.*, 2005; Williams *et al.*, 2005). Also, a Cu(II)-reducing activity was reported (Badrick *et al.*, 2007). An interesting case has emerged from work with human mitochondria, which have two ScoI homologs, one acting as a dithiol:disulfide oxidoreductase (Sco2), and the other (Sco1) as a Cu-chaperone (Leary *et al.*, 2009). The latter appeared to depend on the activity of the former. We tested the *B. japonicum* $ScoI_{sol}$ in the standard insulin reduction assay (Holmgren, 1979), but did not detect any reducing activity. It is an attractive hypothesis, however, that the previously described thioredoxin-like TlpA protein (Loferer *et al.*, 1993; Capitani *et al.*, 2001) interacts with ScoI in *B. japonicum*. Incidentally, all of the phenotypes described here for the *scoI* mutant are similar to those of a *tlpA* mutant (Loferer *et al.*, 1993). Finally, the biogenesis of the Cu_A center has experienced a new twist with the recent discovery of the *T. thermophilus* PCu_AC as a Cu-chaperone that is specific for the formation of the ba_3-type oxidase (Abriata *et al.*, 2008). We noticed that the *B. japonicum* genome harbors two genes for PCu_AC-like proteins (bll4880, blr7088). Future work will tell whether they are involved in copper trafficking.

The ScoI-like protein SenC was shown to be important for the cbb_3-type oxidase in *R. capsulatus* (Swem *et al.*, 2005) and *Pseudomonas aeruginosa* (Frangipani & Haas, 2009). These discoveries are intriguing in view of the fact that the cbb_3 type oxidase does not have a Cu_A center (Pereira *et al.*, 2001; Ducluzeau *et al.*, 2008). In our own studies, we have made a considerable effort to either prove or exclude an involvement of ScoI in cytochrome cbb_3 biogenesis and function, and the obvious necessity to do so was the partial Fix^- phenotype of the *scoI* mutant. The data provide clear evidence that ScoI is not a copper chaperone for the cbb_3-type oxidase in *B. japonicum*. The reason for the observed deviation between species is not known. The cbb_3-type oxidase is required for aerobic growth in *R. capsulatus* and *P. aeruginosa*, whereas *B. japonicum* needs it for respiration at very low oxygen tensions. It is difficult to understand, though, why such a physiological difference should be associated with a selective ScoI function.

Having ascertained that there are no additional *coxG* and *scoI* homologs in the genome, and having shown that the *B. japonicum* CoxG and ScoI proteins are involved only in the maturation of the aa_3-type cytochrome oxidase, we postulate a copper trafficking pathway for the maturation of cytochrome cbb_3 that is uncoupled from these two biogenesis factors. In this context, a previously made observation may now gain momentum. We and others had identified the *fixGHIS* operon (also called *ccoGHIS*) immediately downstream of the cytochrome cbb_3 structural genes, and had shown that it was essential for the assembly of the oxidase (Preisig *et al.*, 1996a; Koch *et al.*, 2000). One of the genes (*fixI*/*ccoI*) codes for a potential metal-transporting P-type ATPase (Solioz & Vulpe, 1996; Lu & Solioz, 2002) which is perhaps specific for copper. Rather than transporting Cu through the cytoplasmic membrane (to which destination?), the role of FixI might be to sequestrate Cu on the periplasmic side and deliver it directly to the membrane-embedded Cu_B site in subunit I (FixN/CcoN).

2.6. Acknowledgements

We gratefully acknowledge the expert technical assistance of Olivera Volarevic who sadly died in August 2009. The help of Patrizia Rossi in DNA sequencing, Sabine Haueter in mutant construction, and Rainer Follador in phenotypic analysis is greatly appreciated. Socorro Mesa is thanked for providing strain GRZ303.

CHAPTER 3

Search for ScoI targets

3.1 Abstract

This work addresses the question of what might be the symbiotic function of the *Bradyrhizobium japonicum* copper-binding protein ScoI. It was shown that ScoI exerts a pivotal function in the biogenesis of the aa_3-type cytochrome oxidase and that a Δ*scoI* mutant has a defect in establishing a fully functional symbiosis with its soybean host plant. The latter phenotype cannot be explained by the involvement of ScoI in the biogenesis of the aa_3-type cytochrome oxidase since deletion mutants of the subunits of this oxidase can undergo symbiosis like wild-type *B. japonicum*. In addition, it was excluded that ScoI is involved in the maturation of the symbiosis-relevant cbb_3-type cytochrome oxidase. We tested all remaining *B. japonicum* proteins predicted to comprise a copper A center (Cu_A) for their potential involvement in symbiosis and could also exclude them from being symbiotically relevant targets of ScoI. In a further approach, we combined data that was gathered in transcriptome and proteome analyses of *B. japonicum* bacteroids from soybean root nodules, motif predictions and topology predictions. This bioinformatics approach resulted in a list of 34 genes that are expressed in *B. japonicum* bacteroids; they encode proteins with a CXXXC motif that is predicted to be on the periplasmic side. An interesting family of five small proteins of unknown function that are only conserved among the closest relatives of *B. japonicum* emerged from this search.

3.2 Introduction

Sco-like proteins are thioredoxin-like proteins that are widespread in all kingdoms of life. In bacterial genomes, the genes encoding Sco-like proteins are very often associated with genes encoding copper enzymes. Frequently, a functional link of proteins is reflected at the gene level by genetic neighborhood. However, this is not the case in *B. japonicum* where *blr1131*, the gene encoding ScoI, has no gene for a recognizable copper protein in its vicinity (FIG. 3.1.A). While genetic neighborhood analysis in the *B. japonicum* genome is of no help to find putative ScoI targets, a String analysis (http://string-db.org/) may yield more valuable information (FIG. 3.1.B). The String analysis established several links between *scoI* and genes involved in aa_3 cytochrome oxidase biogenesis, namely *coxE*, *surf1* and *ctaA* which encode proteins involved in heme synthesis and insertion, and *coxG* which probably codes for a copper chaperone dedicated to deliver copper to the copper B center (Cu_B) (chapter 2). As expected, the genes encoding the three subunits of the oxidase *coxA*, *coxB* and *coxC* are also functionally linked to *scoI*. Among the proteins encoded by those genes, one is a direct or indirect target of ScoI, namely CoxB, the subunit II of the aa_3-type cytochrome oxidase. We believe that ScoI inserts copper into Cu_A of this oxidase. It cannot be fully excluded that there is another player between ScoI and CoxB. Nor can we exclude that there is a thiol-disulfide

oxidoreductase reaction taking place between ScoI and CoxB, although the redox potential of ScoI makes this reaction rather unlikely. Indeed, a redox potential of –155 mV positions ScoI among the dithiol oxidases (see chapter 5).

The biogenesis of the aa_3-type cytochrome oxidase cannot be the only function of ScoI because a Δ*scoI* mutant has a defect in symbiosis whereas a mutant that lacks aa_3 cytochrome oxidase can establish a fully functional symbiosis with the soybean host plant (Bott *et al.*, 1990). This chapter deals with this unknown function of ScoI, more precisely, with the search of other target proteins of ScoI.

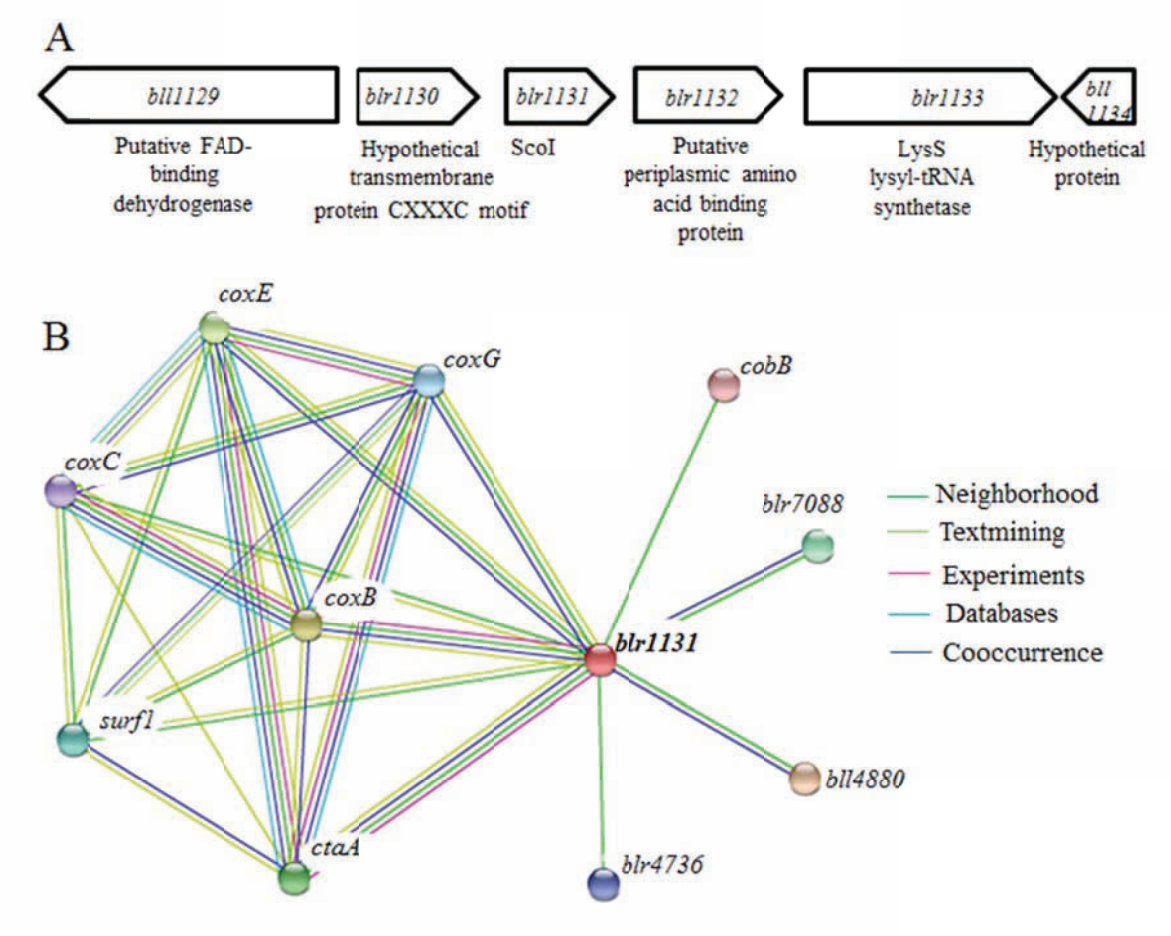
Activity of Sco-like proteins relies on the active site of the protein which consists of a CXXXCP motif. The cysteines of the active site can be either involved in copper binding or in thiol-disulfide oxidoreductase activities, or a combination of both, as was recently shown for the human protein Sco2 (Leary *et al.*, 2009). Therefore, targets of Sco-like proteins are likely to be copper-requiring proteins, or to possess redox-active cysteines, or both.

The *B. japonicum* genome encodes a total of 4 proteins that have a predicted Cu_A-binding site which is characterized by a H-X_{34}-CXEXCXXXHXXM signature sequence (Pereira *et al.*, 2001). Three of them are subunits II (SUII) of cytochrome oxidases (Göttfert *et al.*, 2004). CoxB is the SUII of the aa_3 cytochrome *c* oxidase, CoxM is the SUII of an alternative aa_3 cytochrome oxidase, and Bll4481 is a putative cytochrome oxidase SUII that had not yet been characterized. The fourth Cu_A-possessing protein is NosZ, the nitrous oxide reductase. The proper formation of Cu_A in CoxB relies on ScoI, as deduced from the spectral analysis and oxidase activity determination of a Δ*scoI* strain (Bühler *et al.*, 2010). However, *B. japonicum* is not defective in symbiosis when either of the two subunits II of the aa_3-type cytochrome oxidases is mutated (Bott *et al.*, 1990; Bott *et al.*, 1992). Neither is a Δ*nosZ* mutant impaired in symbiosis (Velasco *et al.*, 2004), and this phenotype was confirmed under our own plant cultivation conditions (Bühler *et al.*, 2010). This means that although these three Cu_A-containing proteins might be targets of ScoI, none of them is the symbiosis-relevant target that could explain the symbiotic defect of a Δ*scoI* mutant. Bll4481 is the only Cu_A-protein that had not been studied yet, and thus its potential involvement in symbiosis was investigated here.

Banci and coworkers are studying metal-binding-proteins, and, while searching for cadmium binding domains, they performed a bioinformatics approach where they combined a motif search (in that case a CXXC motif) with a requirement for a minimal identity with a query protein (Banci *et al.*, 2006b). In our approach, we also used in an *in silico* strategy; however, the motif search could not be

combined with a similarity search, and thus other criteria, such as protein topology and expression data were applied.

FIG. 3.1. A. Map of the *B. japonicum* gene region *bll1129-bll1134*. Gene numbers are indicated within the arrows and protein names or annotations according to Kazusa (http://genome.kazusa.or.jp) are indicated below.B. String analysis (http://string.embl.de/) of *scoI* (*blr1131*). The color code on the right side indicates the basis of the predicted relationships that were found in the database.



3.3 Material and methods

3.3.1 Media, growth conditions and strains

Escherichia coli was grown in Luria-Bertani (LB) medium (Miller, 1972) containing 200 μg/ml ampicillin for plasmid selection. Aerobic cultures of *B. japonicum* were grown in Erlenmeyer flasks containing one fifth of their total volume of PSY medium (Regensburger & Hennecke, 1983, Mesa *et al.*, 2008) supplemented with 0.1% of arabinose and shaken vigorously (160 rpm) at 30°C. When appropriate, antibiotics were used at the following concentrations: spectinomycin, 100 μg/ml; streptomycin, 50 μg/ml, kanamycin, 100 μg/ml.

3.3.2 Mutant construction

Construction of all mutant strains except 6536 and 6537 has been described previously and details can be extracted from the references indicated in TABLE 3.1. To construct strains 6536 and 6537, a 750-bp upstream and an 827-bp downstream region were PCR-amplified with appropriate primer pairs, cloned into pGEM-T Easy (Promega) and verified by sequencing. Both amplicons were then cloned tail-to-head in pBluescript II KS(+) (Stratagene). The *aphII* gene from pBSL86 (Alexeyev & Shokolenko, 1995) was inserted inbetween by using a *Xba*I restriction site. This construct was cloned into suicide vector pSUP202pol4, which resulted in plasmids pRJ6536 and pRJ6537. These plasmids were mobilized into *B. japonicum* 110*spc*4 via *E. coli* S17-1. The deletion end points are at genome coordinates are 4'962'621 and 4'963'931.

TABLE 3.1 Strains and plasmids used in this work.

Strains/ Plasmids	Relevant genotype or phenotype	Source
E. coli		
DH5α	*supE44* Δ*lacU169*(ϕ80 *lacZ*ΔM15) *hsdR17* *rec*A1 *gyrA96 thi*-1 *relA*1	Bethesda Research Laboratories, Inc., Gaitersburg, MD
S17-1	Sm^R Sp^R *hsdR* (RP4-2 *kan*::Tn7 *tet*::Mu, integrated in the chromosome)	(Simon *et al.*, 1983)

TABLE 3.1 (continued)

B. japonicum		Symbiosis phenotype	
110*spc*4	Sp^R wild type	Fix^+	(Regensburger & Hennecke, 1983)
2575	Sp^R Km^R Δ*scoI*::*aphII*	Fix^-	(Bühler *et al.*, 2010)
GRZ30035	Sp^R Sm^R nos*Z*: :Ω	Fix^+	(Velasco *et al.*, 2004)
6532	Sp^R Km^R Δ*bll4480-81*::*aphII* (same orientation)	Fix^+	(Bühler *et al*., 2010)
6533	Sp^R Km^R Δ*bll4480-81*::*aphII* (opposite orientation)	Fix^+	(Bühler *et al.*, 2010)
6536	Sp^R Km^R Δ*bll4482*::*aphII* (same orientation)	Fix^+	This work
6537	Sp^R Km^R Δ*bll4482*::*aphII* (opposite orientation)	Fix^+	This work
Plasmids			
pBSL86	Ap^R KmR		(Alexeyev & Shokolenko, 1995)
pBluescript II KS(+)	Ap^R cloning vector		Stratagene, La Jolla, CA
pGEM-T Easy	Ap^R cloning vector		Promega, Madison, WI
pSUP202pol4	Tc^R (pSUP202) oriT of RP4		(Fischer *et al.*, 1993)
pRJ6532	Km^R (pSUP2020pol4), 654-bp upstream *Eco*RI-*Pst*I fragment, 853-bp downstream *Pst*I-*Xba*I, *aphII* inserted in between (*Pst*I) same orientation		(Bühler *et al*., 2010)
pRJ6533	Km^R (pSUP2020pol4), 654-bp upstream *Eco*RI-*Pst*I fragment, 853-bp downstream *Pst*I-*Xba*I, *aphII* inserted in between (*Pst*I) opposite orientation		(Bühler *et al*., 2010)
pRJ6536	Km^R (pSUP2020pol4), 829-bp upstream *Pst*I-*Xba*I fragment, 755-bp downstream *Xba*I-*Not*I, *aphII* inserted in between (*Xba*I) same orientation		This work
pRJ6537	Km^R (pSUP2020pol4), 829-bp upstream *Pst*I-*Xba*I fragment, 755-bp downstream *Xba*I-*Not*I, *aphII* inserted in between (*Xba*I) opposite orientation		This work

3.3.3 Plant growth and nitrogenase activity measurement

The symbiotic phenotype of *B. japonicum* mutants was determined in infection tests using soybean as host, and whole nodule nitrogenase activity was measured with the acetylene reduction assay (Hahn & Hennecke, 1984; Göttfert *et al.*, 1990a). Soybean seeds (*Glycine max* (L.) Merr. Cv. Williams) were surface-sterilized as described previously (Bühler *et al*., 2010).

3.3.4 Predictions

Predictions of amino acid sequence motifs were done by Dr. Daniel Margadant from the Institute of Computational Sciences, ETH Zürich. The motif that we screened for was CXXXC. It is a minimized variant of the Cu_A site (H-X_{34}-**C**XEX**C**XXXHXXM (Pereira *et al.*, 2001)). Moreover it is a motif which can take part in copper binding, as ScoI itself is known to bind copper via this motif. The proteins for which CXXXC motifs were predicted to be either on the cytoplasmic side or within the membrane-inserted stretches were eliminated from the list. The same was done for the tentatively secreted proteins that harbored the motif on the N-terminal portion with respect to the cleavage site.

Topology predictions were done with TMHMM v2.0, SignalP v3.0 and TatP 1.0 as described previously (Delmotte *et al.*, 2010). The proteins were classified in three groups: periplasmic/secreted, transmembrane, and a third group that contained proteins which could not be assigned clearly to one of the two former groups. Proteins which are known to be cytoplasmic were eliminated in a manual editing step.

3.3.5 Transcriptome and proteome data of *B. japonicum*

Transcriptome and proteome data sets of *B. japonicum* bacteroids of soybean nodules were described previously (Delmotte *et al*., 2010).

3.4 Results

3.4.1 Bll4481 is not a symbiosis-relevant target of ScoI

The only Cu_A-possessing protein that has not been studied in any other context previously is Bll4481, a putative cytochrome oxidase subunit II. The gene region, including the genetic structure of the constructed mutants, is depicted in FIG. 3.2. *bll4480* encodes a putative cytochrome oxidase subunit I. As there is no subunit III encoded in the region, we suggest that one of the two cytochromes *c* (Bll4479 or Bll4483) that are encoded in the neighborhood replaces it, similarly as in cbb_3-type cytochrome oxidases. The second cytochrome *c* might be involved in electron shuttling between the putative PQQ-dependent dehydrogenase encoded by *bll4482* and the cytochrome oxidase. Like all other deletion mutants of genes encoding Cu_A-possessing proteins, the deletion mutants 6532 and 6533, lacking *bll4480* and *bll4481*, behaved like a wild-type strain in symbiosis with soybean. Moreover, the deletion mutants of *bll4482*, the associated putative dehydrogenase (6536 and 6537) were not impaired in symbiosis either. Strains 6532, 6533, 6536 and 6537 showed values similar to

the wild type with respect to nitrogenase activity, number of nodules and nodule weight (data not shown).

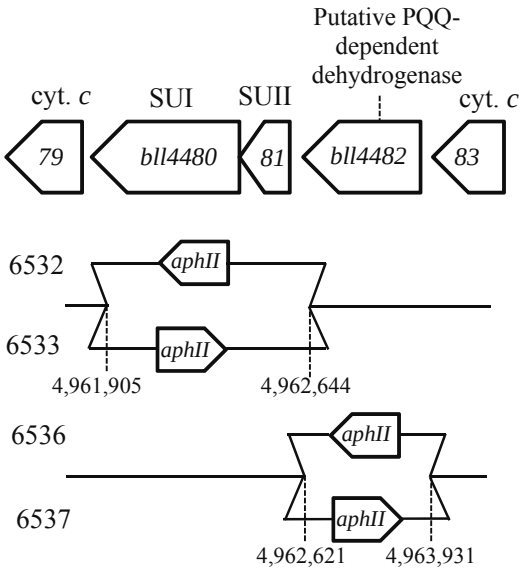


FIG. 3.2 Gene cluster *bll4479-bll4483*. ORF numbers are given within the arrows and annotations according to Kazusa (http://genome.kazusa.or.jp) are indicated above. The lower part of the figure shows which genes were deleted in the four mutant strains and in which orientation the kanamycin resistance cassette (*aphII*) was inserted in the respective deletion mutants.

3.4.2 Bioinformatics approach

Since the symbiosis-relevant target of ScoI does not seem to be a Cu_A-possessing protein, we decided to search for a protein that has a simplified version of the Cu_A binding site. The *B. japonicum* genome was browsed for all proteins encoding a CXXXC motif, which is involved in copper binding in ScoI. This resulted in a list containing 454 CXXXC motifs in a total of 379 proteins (SUPPLEMENTARY TABLE 1), meaning that some proteins encode more than one motif. For example, Blr6742, a glutamate synthase subunit, holds the record by encoding the motif five times. From this list, three types of proteins were eliminated in a first round, namely predicted cytoplasmic proteins, predicted transmembrane proteins with the motif not exposed to the periplasm, and predicted secreted proteins that have the CXXXC motif N-terminal to the predicted cleavage site. The next selection criterion was based on expression data. Proteins were retained if their expression was detected in soybean nodules either at the transcript or protein level. This left us with a list containing 16 predicted periplasmic/secreted proteins, 11 predicted transmembrane proteins, and 7 proteins for which the programs predicted a transmembrane domain and a cleavage site (TABLE 3.2). Among the retained proteins, the majority are hypothetical proteins. However, there are a few interesting candidates.

A family of five rather small proteins encoded by the genes *blr1130*, *blr4624*, *bll5314*, *blr5178*, *blr7166* caught our attention (FIG. 3.3 and shaded boxes in TABLE 3.2). Apart from the CXXXC motif, they harbor additional conserved residues resulting in an overall sequence identity ranging

between 40 and 60 %. Among those are two cysteines and one histidine which might be involved in metal complexing. Among the five proteins, Blr1130 is the only one that is clearly membrane-anchored, and its gene neighbors the ScoI-encoding *blr1131* gene on the chromosome.

TABLE 3.2. Genes encoding proteins with a periplasmic CXXXC motif and whose transcript and/or protein are detected in soybean nodules.

Gene number	Gene name	Annotation (Rhizobase)	Length protein	Detected in nodule	Start of CXXX motif (aa)	Motif
Predicted periplasmic/secreted proteins						
bll1366	-	hypothetical protein	201	tx	119	CDGGC
bll2191	-	hypothetical protein*	237	px	43	CSRVC
bll2411	-	hypothetical protein	257	px+tx	40	CAAPC
bll2736	-	putative aldehyde dehydrogenase protein*	729	tx	362	CEVWC
bll3611	-	hypothetical protein	624	tx	504	CPLIC
bll3914	-	Oxidoreductase	270	px	198	CNSIC
bll5184	-	putative cytochrome P460	195	tx	173	CGHAC
bll4909	*nuoI*	NADH ubiquinone oxidoreductase chain I*[#]	168	px+tx	74	CEAVC
bll5314	-	hypothetical protein	149	tx	56	CNSAC
blr0315	*nosZ*	nitrous oxide reductase* [Ψ]	650	px+tx	268	CFSTC
blr2671	-	hypothetical protein	120	tx	36	CVRMC
blr2995	-	hypothetical protein*	546	px+tx	207	CPYQC
blr5178	-	hypothetical protein	145	tx	57	CASAC
blr7038	*napA*	periplasmic nitrate reductase large subunit precursor*[#]	838	px+tx	54	CGTGC
blr7166	-	hypothetical protein	146	px	59	CLSAC
blr7816	-	probable mannitol-binding protein	364	tx	51	CEYFC
Predicted transmembrane proteins						
bll0892	-	unknown protein	324	tx	311	CIMQC
bll1715	*nodV*	two component regulator	890	tx	194	CIQTC
bll2213	-	unknown protein	199	tx	151	CAMMC
bll3785	*coxM*	cytochrome *c* oxidase subunit II [Ψ]	278	tx	225	CAELC
bll6457	-	unknown protein	255	px+tx	93	CPIIC
bll7976	-	hypothetical protein	211	tx	77, 99	CVRSC, CKAFC
blr1130	-	hypothetical protein	220	tx	103	CASAC
blr1131	*scoI*	cytochrome *c* oxidase assembly factor	197	px+tx	74	CPDVC
blr1170	*coxB*	cytochrome *c* oxidase subunit II [Ψ]	280	tx	229	CSELC

TABLE 3.2 (continued)

blr2506	-	putative chemotaxis protein	749	tx	38	CPPLC
blr7502	-	unknown protein	191	tx	173	CSFLC
Proteins which have predicted transmembrane domains and predicted secretion signal sequence						
bll2410	-	unknown protein	386	tx	42	CESEC
bll4882	-	unknown protein	129	tx	71	CALCC
bll5500	-	hypothetical protein	160	px+tx	40	CNTGC
blr1165	-	hypothetical protein	191	px+tx	58,142	CALLC, CKAAC
blr3941	-	hypothetical protein	473	px+tx	190	CGFAC
blr4624	-	hypothetical protein	177	tx	58	CVSAC
blr8135	-	hypothetical protein	269	tx	89,111	CVRSC, CQAFC

* Proteins that have a predicted TAT secretion motif.

[#] Proteins in which the CXXXC is part of a 4Fe-4S cluster.

[Ψ] Proteins in which the CXXXC is part of a Cu_A-binding site (Bll4481 is missing because it appears not to be expressed in symbiosis).

The genes encoding the proteins which belong to the family of five conserved proteins are shaded.

FIG. 3.3. Alignment of a family of five similar CXXXC-motif-possessing periplasmic proteins performed with ClustalW2. The cysteines of the CXXXC motif are marked in bold and are underlined. Other conserved cysteines and a histidine are highlighted in bold. Residues conserved in all five proteins are shaded.

Protein Alignment

```
Blr4624         ---------------------------------------------MDMRACLIAVVVST 14
Bll5314         -----------------------------------------------MRITLLSLLLLC 12
Blr1130         MQRGRNTGFEFVLSQPACLDKTSVTTDASRASPPDTQIRIRLGRGTMKFLTGLLAAVVLI 60
Blr5178         ---------------------------------------------MRCWVGLVIAALLT 14
Blr7166         ---------------------------------------------MQRAV-LVKALMLA 13

Blr4624         ALSVTSAS--ATVIISADIGGKMRDYTTRFQQVRDSGESVVIAGTCVSACTMVLGLVPSD 72
Bll5314         LAAATPAR--AELHITRDHGGYVEEYKAKYKRVREKGERVIIDGICNSACTLVLGIVPMN 70
Blr1130         ACMGASH---AVVRIADDRGGRIGTYVDKYQDLRQSGETVIIDGLCASACTIVLGAIPHD 117
Blr5178         TGSRASD---AALRITEDRGGPIEKYISRYERLRASGQPVIIDGFCASACTIVLAAIPSS 71
Blr7166         TALLASAPVAAEIRIIQSPGGRVGPFLDLFEKVRESGERVVIDGPCLSACTLVLSIVPGE 73

Blr4624         RICVTPDAVLGFHAAWMFDSS--GKRVVSASGTQDLMQTYPAAVRAWIARHGGLTPKMMY 130
Bll5314         KICVTPRASLGFHQAYYDKAFTFGIKVTSSEGTSDLMSYYPDTVKDWIRRNGGLTTDMKK 130
Blr1130         RICVTSSATLGFHAAWDFGTN--GRAVTNSEATQMLYAMYPSQVRRWISQRGGLTPHMLF 175
Blr5178         RICVTSQATLAFHAAWDFGHR--GRPVTNSGATRMLYSMYPVRVRQWIADRGGLTPRTIF 129
Blr7166         RICVTKRAVLGFHAARSVDRR--GRFYAEPEASDAVLQAYPGPVRDWISHRGGLTSRLLL 131

Blr4624         LR-GRDLAAIVAPCNSSRMASVSRAKRFGELHQLDDTNTPRASFDGH 176
Bll5314         IKNGVELWKIIDPCPEEW---------------------------- 148
Blr1130         LR-GKQLQAMYKPCYMDAQASAIKPTRR-SLPQSDQLESARGQLLH- 219
Blr5178         LH-GKPLKAMYRSCSS------------------------------ 144
Blr7166         LK-GRDLAAIYPRCR------------------------------- 145
```

3.5 Discussion

3.5.1 Alternative ScoI targets

In addition to SUII of cytochrome oxidase, a few additional targets of Sco-like proteins have been described in other organisms. In *Rhodobacter capsulatus*, the Sco-homolog SenC is required for cytochrome *c* oxidase activity. *R. capsulatus* only has one cytochrome *c* oxidase; it is a cbb_3-type cytochrome oxidase that lacks a Cu_A center. Since the mutant phenotype can be complemented by addition of copper it is believed that, in this case, the Sco-like protein SenC is a copper donor for a Cu_B center (Swem *et al.*, 2005). As symbiotic nitrogen fixation of *B. japonicum* relies on a cbb_3-type cytochrome oxidase also lacking a Cu_A center (Preisig *et al.*, 1996b), copper delivery to the Cu_B center of cbb_3 cytochrome oxidase could explain the symbiosis defect of a Δ*scoI* mutant. However, a Δ*scoI* mutant does not have an impaired cytochrome oxidation activity under conditions in which cbb_3-type cytochrome oxidase is the dominant oxidase (Bühler *et al.*, 2010).

PrrC, the Sco-homolog of *Rhodobacter sphaeroides* was described as a redox sensory protein. PrrC transduces the signal to PrrA, the sensor kinase of the PrrAB two-component regulatory system that controls expression of photosynthesis genes (Eraso & Kaplan, 2000). Cu(I)-binding was also observed for PrrC; however, spectroscopic data suggested that Cu(I) is not specifically bound by the CXXXC motif. Because 0.5 molar equivalent of bound copper was found per PrrC monomer, it was hypothesized that copper might induce dimerization of PrrC (Badrick *et al.*, 2007). The RegSR two-component regulatory system of *B. japonicum* is homologous to the aforementioned PrrAB system and to the *R. capsulatus* RegAB system (Buggy & Bauer, 1995). The copper dependency of RegS was demonstrated *in vitro* (H.M. Fischer, unpublished). However, a Δ*regS* mutant neither showed a defect in symbiosis nor did it have an impaired *fixR-nifA* P2-promoter activity. Therefore, the tools to test the dependency of RegSR on ScoI activity are missing.

Finally, in human, the two Sco paralogs Sco1 and Sco2 interact with each other in a thiol-disulfide oxidoreduction reaction (Leary *et al.*, 2009). As there is only one copy of ScoI in *B. japonicum*, this option cannot be considered. However, homodimers of purified $ScoI_{sol}$C74S or $ScoI_{sol}$C78S could be detected on non-reducing SDS-PAGE gels (own unpublished data), meaning that the thiol groups are exposed and can interact, a prerequisite for potential thiol-disulfide oxidoreduction activity. This could be a potential mechanism to release copper from the binding site and deliver it to the acceptor apoprotein.

In summary, cbb_3-type cytochrome oxidase FixNOQP and a possible ScoI paralog can be dismissed from the list of putative ScoI targets, whereas it is, at this stage, not feasible to test ScoI-dependency of RegS.

3.5.2 *bll4481*

Here we showed that a deletion mutant in *bll4481* can undergo a fully functional symbiosis with soybean. Therefore Bll4481 can be excluded from being the symbiosis-relevant target of ScoI. Nevertheless, this does not exclude that ScoI delivers copper to Bll4481.

bll4482 shares significant homology with methanol dehydrogenases (50% identity with *Methylobacterium extorquens* MxaF). Although the residues that participate in PQQ- and Ca(II)-binding in *M. extorquens* MxaF (Schmidt *et al.*, 2010) are only partly conserved in Bll4482, nevertheless, it can be postulated that *bll4482* encodes a putative PQQ-dependent dehydrogenase. Accordingly, we speculate that Bll4482 and Bll4481are functionally linked. Bll4482 perhaps relies on Bll4481 to be reoxidized. The substrate of Bll4482 remains unknown, but the range of substrates that are generally oxidized by PQQ-dependent dehydrogenases includes primary alcohols and a few secondary alcohols (Anthony, 2001). Since Lidstrom and coworkers found C1-catabolizing strains belonging to the *Agromonas*/*Bradyrhizobium* group in sediments of Lake Washington (Lidstrom *et al.*, unpublished data), and since C1 metabolism was also observed *in vitro* for *B. japonicum* (Sudtachat *et al.*, 2009), we hypothesize that Bll4482 plays a role in C1 metabolism. Since a *bll4482* mutant is now available, this hypothesis could be tested by cultivating the wild-type and the mutant strain on a minimal medium only containing, for example, methanol as a C-source, provided the wild type is able at all to grow on this type of C-source.

3.5.3 The limits of the bioinformatics approach

The bioinformatics approach that was chosen here in order to narrow down the number of potential interaction partners for ScoI is limited in many aspects. First, because we know that copper coordination does not necessarily rely on cysteines. It is known that coordination of Cu(I) ions prefers sulfur donor ligands such as cysteine or methionine residues, whereas Cu(II) prefers nitrogen donors such as histidine or oxygen donors such as glutamate or aspartate (Bertini *et al.*, 2007). Not to mention that the spacing of three amino acids between the cysteines that we have chosen here is just one of several possibilities. CXXC can also efficiently undergo thiol-disulfide oxidoreduction and can also be involved in copper binding, *e.g.* in P-type ATPases (Huffman & O'Halloran, 2001). CueR, a MerR-type sensor with a zeptomolar affinity for copper, has a CX_7CX_8CC motif in which the first two cysteines, with a spacing of seven amino acids, contribute mostly to copper binding (Chen *et al.*, 2003). Cox17, a eukaryotic copper chaperone that shuttles copper from the cytoplasm into mitochondria, is known to bind copper via a CCXC motif (Srinivasan *et al.*, 1998). Finally, the CXXXC motif is also known to bind other cations and is part of the 4Fe-4S cluster binding motif which is characterized by the CXXXCXXC sequence (Duin *et al.*, 1997; Ruzicka *et al.*, 2000).

3.5.4 Potential ScoI targets of particular interest

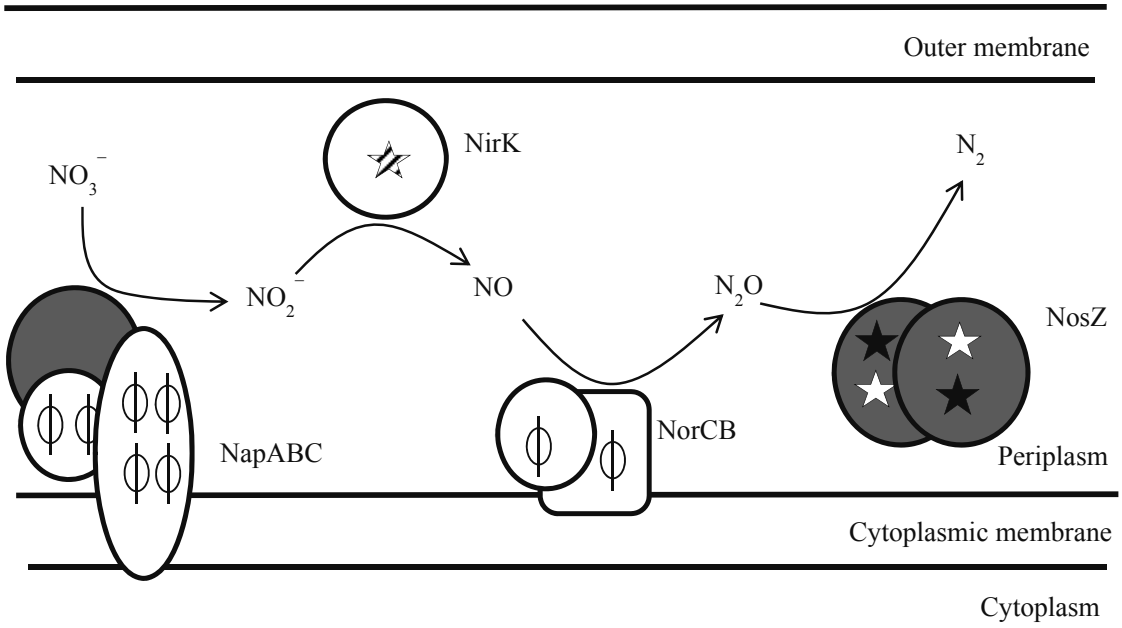
3.5.4.1 Blr1130 family

Blr1130 is one of the five members of a protein family that was found in our *in silico* target search and is encoded next to *scoI* (*blr1131)*. String analysis shows that *blr1130* and *scoI* neighborhood is not conserved. It is only observed in *B. japonicum*, *Nitrobacter winogradskii* and a few *Rhodopseudomonas palustris* strains. Moreover, Blast searches with the five proteins did not yield any hits besides unknown or hypothetical proteins in *R. palustris* and various *Nitrobacter* species, which are incidentally among the closest relatives of *B. japonicum* (Teske *et al.*, 1994). It also turns out that the five members of the family are strikingly well conserved in their overall amino acid sequence, and that the five-fold occurrence of a gene of this gene family is unique for *B. japonicum*. *R. palustris* CGA009 or *R. palustris* BisB18, for example, encode two of them whereas *Nitrobacter hamburgensis* X14 or *N. winogradskii* Nb-255 have only one. Thus, we face here proteins of unknown function(s) and whose occurrence is restricted to only a few related bacterial species.

3.5.4.2 Blr0315 and Blr7038

Two proteins involved in anaerobic nitrate respiration (FIG. 3.4) namely NosZ (nitrous oxide reductase, Blr0315) and NapA (large subunit of nitrate reductase, Blr7038) fulfilled the selection criteria of our *in silico* search. They possess a CXXXC motif as well as a TAT-recognition motif and are detected in nodules at both the transcript and protein level (TABLE 3.2). While NosZ has already been investigated, NapA is of interest in its own right, because it has been observed that a Δ*scoI* mutant is slightly impaired in denitrification and accumulates nitrite in the growth medium (Landolt, 2005). However, the accumulation of nitrite rather points towards a defect in an enzyme further downstream within the denitrification pathway. Another argument speaking against NapA being a target of ScoI is the fact that the CXXXC is part of a CXXXCXXC motif which contains 3 of the 4 cysteines that build up the 4Fe-4S cluster. Therefore an involvement of ScoI in NapA maturation, be it as a copper donor or as thiol-disulfide oxidoreductase, is rather unlikely.

FIG. 3.4. Anaerobic nitrate respiration. Enyzmes of the anaerobic nitrate respiration are depicted. Nitrate reductase (NapABC), nitrite reductase (NirK), nitric oxide reductase (NorCB) and nitrous oxide reductase (NosZ). Black asterisks represent Cu_A centers, white asterisks represent Cu_Z centers, striped asterisks represent type II copper centers (for definition of the different types of copper centers see chapter 6) whereas ϕ represents heme moieties. The proteins that are highlighted in grey passed all the selection criteria of our search for putative ScoI targets.



3.5.4.3 Blr5184

Cytochrome P460 has received its name from the typical absorption that it exhibits at 460 nm (Zahn *et al.*, 1994). Cytochrome P460 is studied in *Nitrosomonas europaea* where it is involved in oxidation of hydroxylamine (Bergmann & Hooper, 1994). The orthologous protein of *B. japonicum* (Blr5184) has not yet been studied; however, in a study by Elmore and colleagues, the sequence of the *B. japonicum* putative cytochrome P460 was included in a multiple alignment. The $CX_{2-3}C$ motif appears to be fully conserved in the 58 proteins that were aligned in the study, although the spacing between the cysteines appears to be X_2 rather than X_3 in most of the cases (Elmore *et al.*, 2007). Interestingly, *B. japonicum* encodes another putative cytochrome P460 (Blr1198) in which the spacing is X_2. The proposed function for cytochrome P460 is oxidation or reduction of some N-oxides for detoxification

or energy generation (Elmore *et al.*, 2007). In case the catalyzed reaction is an oxidation, and if CXXXC is involved in catalysis, a re-oxidizing recycling system would be required. In this case, a thiol-oxidizing function for ScoI might be necessary. However, this is very speculative especially as *B. japonicum* encodes a putative DsbA protein (Chapter 5). DsbA proteins are known to efficiently catalyze the formation of disulfide bonds in the periplasm (Ito & Inaba, 2008).

3.5.5 Is it possible that ScoI function in symbiosis does not involve any target?

In *Neisseria meningitis* and *Neisseria ghonorroeae*, two Gram-negative pathogenic bacteria that lack Cu_A-possessing cytochrome oxidases, Δ*sco* mutants suffer from increased sensitivity towards oxidative stress (Seib *et al.*, 2003). If such a function is also assumed for the *B. japonicum* homolog, one might speculate that ScoI could play a copper buffering role. In the absence of ScoI, free copper levels might rise and thereby induce oxidative stress via Fenton-like chemistry (copper-mediated Fenton reaction: $Cu(I) + H_2O_2 \rightarrow Cu(II) + OH^- + {}^{\bullet}OH$). Since it is known that establishment of symbiosis involves exposure of the bacteria to increased levels of H_2O_2 and other ROS (Chang *et al.*, 2009), it is possible that absence of free copper in the cell is even more critical when *B. japonicum* is in symbiosis compared to free-living conditions. However, at least two issues argue against this "copper-buffer"-hypothesis. First, oxidative stress sensitivity of a Δ*scoI* mutant has been tested in free-living conditions and found to be unaltered from the wild type (Landolt, 2005). Second, in a transcriptome analysis, we could show that *scoI* transcription is not increased under copper excess conditions; on the contrary, *scoI* transcription was increased when the cells were starved for copper (Chapter 6).

3.5.6 Is the symbiotic defect of Δ*scoI* the result of an additive effect?

If indeed ScoI is involved in the biogenesis of several Cu_A centers, it is possible that at the level of the single mutants in those Cu_A-binding site-possessing proteins, no defect in symbiosis is visible while the simultaneous presence of unliganded Cu_A-sites might be deleterious for symbiosis. This could only be verified in a symbiosis test with a strain in which all the genes encoding Cu_A-possessing proteins are deleted.

3.5.7 Future experiments

In order to determine whether Bll4481 and Bll4482 are involved in C1 metabolism it would be insightful to test the respective deletion mutants on minimal medium containing C1-compounds like methanol as carbon source. In case the hypothesis can be confirmed, the Δ*scoI* mutant should also be tested under those conditions. If it is similarly impaired in growth, ScoI might be involved in the biogenesis of the predicted Cu_A center of Bll4481.

Constructing mutants of some of the candidates obtained in the bioinformatics approach would certainly be worthwhile. The most intriguing candidates, namely the family of five small proteins encoded by *blr4624*, *bll5314*, *blr1130*, *blr5178*, *blr7166* are probably difficult to tackle in *B. japonicum*. As they are highly conserved, it is likely that they can functionally complement each other. Therefore, it is likely that no defect can be observed in single mutants. It would be a good idea to switch the organism and to construct a mutant in one of the *Nitrobacter* or *Rhodopseudomonas* strains which encode only one and two, respectively, of those proteins. The constructed mutant should be phenotypically characterized with respect to growth and resistance towards different stresses, for example. Since such proteins have not been described before, their function is interesting *per se*. In the present context, the goal of such a characterization would be to find a functional link between these five proteins, the *B. japonicum* symbiotic lifestyle, and possibly a copper-requiring process in order to find the missing element between ScoI and a symbiotic function.

An alternative approach to find potential targets of ScoI would be to assess the proteome of a Δ*scoI* strain. Indeed, for other cuproproteins it has been shown that the presence of the copper cofactor markedly increases the stability of the proteins (Hussain & Wittung-Stafshede, 2007). We made the same observation with CoxB, the subunit II of the aa_3-type cytochrome *c* oxidase. This enzyme relies on ScoI function for proper biogenesis and in the absence of ScoI, the subunits are largely absent. As this can be reverted by addition of copper to the medium, the absence of CoxB in a Δ*scoI* mutant is most probably due to the destabilization provoked by the absence of the copper cofactor. If other targets depending on ScoI behave similarly in the absence of ScoI they could be identified in a proteome analysis comparing wild-type and Δ*scoI* strains.

3.6 Acknowledgments

We gratefully acknowledge the expert help of Dr. Daniel Margadant who generated the list containing proteins with the CXXXC motif and Dr. Christian Ahrens who performed the topology predictions.

3.7 Supplementary material

SUPPLEMENTARY TABLE 3.1. The 379 *B. japonicum* proteins that carry at least one CXXXC motif are listed. Motif start indicates the amino acid position at which the motif starts.

ORF number	Gene name*	Annotation*	Motif start	Motif
bll0008		unknown protein	69	CGHLC
bll0157		ferredoxin	17, 21, 46	CVEVC, CPVDC, CEPEC
bll0198		amidase	141	CEHFC
bll0263		putative malonyl CoA-acyl carrier protein transacylase	256	CLQAC
bll0280	*fdhF*	formate dehydrogenase alpha subunit	179	CSNMC
bll0292		putative sulfur regulated plasmid-encoded protein precursor	64	CGAAC
bll0334		hypothetical protein	41	CIPGC
bll0406		unknown protein	163	CIWTC
bll0416	*leuC*	3-isopropylmalate dehydratase large subunit	167	CLIVC
bll0476		hypothetical protein	148	CDHRC
bll0523		hypothetical protein	157	CVRNC
bll0525		hypothetical protein	145	CTLNC
bll0596		hypothetical protein	797	CEEGC
bll0661		hypothetical protein	137	CPAAC
bll0672	*hemN*	oxygen-independent coproporphyrinogen III oxidase	6	CLSKC
bll0700		heavy-metal transporting P-type ATPase	92	CSAGC
bll0714		iron-sulfur cluster binding protein	213, 263	CLDIC, CLAAC
bll0795		hypothetical protein	166	CDKFC
bll0892		unknown protein	311	CIMQC
bll0966		two-component hybrid sensor and regulator	522, 532	CSLCC, CHDLC
bll1004		hypothetical protein	447	CGIIC
bll1083		hypothetical protein	210	CHTLC
bll1145		hypothetical protein	105	CKDTC
bll1231		unknown protein	26	CSGAC
bll1358		putative hydrolase	214	CYRRC
bll1366		unknown protein	119	CDGGC
bll1368		hypothetical protein	231	CALCC

bll1491		hypothetical protein	144	CEKLC
bll1517	*suhB*	extragenic suppressor protein	153	CVVAC
bll1523	*gapA*	glyceraldehyde 3-Phosphate dehydrogenase	153	CTTNC
bll1568		unknown protein	164	CHTRC
bll1694		unknown protein	201	CELSC
bll1715	*nodV*	two component regulator	194	CIQTC
bll1824		putative transposase	148, 152	CRYRC, CGSRC
bll1855		putative transposase	148	CPGYC
bll1872		hypothetical protein	574	CGNGC
bll1980		hypothetical protein	155	CSVGC
bll2007	*hemN1*	coproporphyrinogen III dehydrogenase	35	CRAIC
bll2021	*nodD2*	transcriptional regulatory protein LysR family	172	CVGCC
bll2040		unknown protein	133	CSFGC
bll2126		unknown protein	193	CQWPC
bll2128		unknown protein	11	CGAKC
bll2154		unknown protein	126	CSRIC
bll2213		unknown protein	151	CAMMC
bll2376		probable glycosyl transferase	156	CKASC
bll2377		probable glycosyl transferase	136	CAQRC
bll2410		unknown protein	42	CESEC
bll2411		unknown protein	40	CAAPC
bll2482		putative 6-pyruvoyl tetrahydrobiopterin synthase	110	CNESC
bll2515		similar to pyruvate phosphate dikinase	461	CVVGC
bll2553	*tag*	3-methyladenine-DNA glycosylase	192	CHASC
bll2645		hypothetical protein	266	CFFDC
bll2682	*macA*	maleylacetate reductase	85	CDADC
bll2704		unknown protein	118	CRLAC
bll2736		putative aldehyde dehydrogenase protein	362	CEVWC
bll2743		hypothetical protein	14	CSTCC
bll2793		unknown protein	122	CPAAC
bll2795		hypothetical protein	475	CTVTC
bll2817		gluconolactonase precursor	288	CANLC
bll2871		hypothetical protein	100	CVDWC
bll2956		gluconolactonase precursor	338	CANVC
bll3006		hypothetical protein	38, 304	CNLAC, CMAHC
bll3089		unknown protein	405	CCMLC
bll3136	*fdhF*	formate dehydrogenase alpha subunit	184, 227, 258, 379	CVRAC, CVQAC, CGVGC, CARVC
bll3138	*nuoE*	NADH dehydrogenase I chain E	128	CLGLC
bll3255	*cobV*	cobalamin (5'-phosphate) synthase	151	CAAHC
bll3259	*cbiP*	cobyric acid synthase	250	CKIAC
bll3282		unknown protein	377	CLALC
bll3579		unknown protein	43	CVQRC
bll3611		hypothetical protein	504	CPLIC
bll3638		acetyl-CoA carboxylase, biotin carboxylase	260	CVEAC
bll3651		probable O-antigen export system ATP-binding	223	CRKWC

	rfbA	protein		
bll3660		putative shikimate 5-dehydrogenase	78	CYEFC
bll3717	*lipA*	lipoic Acid Synthetase	93	CTRAC
bll3752	*moaA*	molybdenum cofactor biosynthesis protein	35	CDLRC
bll3753		hypothetical protein	70	CKTPC
bll3785	*coxM*	cytochrome c oxidase	225	CAELC
bll3788		hypothetical protein	223, 250	CVRTC, CKALC
bll3914		oxidoreductase	198	CNSIC
bll4205		unknown protein	292	CIAGC
bll4207		unknown protein	249	CLAHC
bll4220		hypothetical protein	74	CNGKC
bll4269		unknown protein	602	CRTTC
bll4274	*nrdE*	ribonucleoside-diphosphate reductase 2 alpha chain	848	CANPC
bll4395		unknown protein	20	CWNRC
bll4423		hypothetical protein	256	CIKLC
bll4481		hypothetical protein	149	CQEFC
bll4510		putative proline dipeptidase	219	CLLAC
bll4535		hypothetical protein	23	CRVYC
bll4536	*ilvD*	dihydroxy-acid dehydratase	118	CISNC
bll4570		probable sulfite reductase [NADPH] flavoprotein alpha-component	99	CGYNC
bll4571		putative ferredoxin--nitrite reductase	524	CHHSC
bll4648		hypothetical protein	96	CRFRC
bll4771		unknown protein	124	CDQDC
bll4882		unknown protein	71	CALCC
bll4909	*nuoI*	NADH ubiquinone oxidoreductase chain I	74, 114	CEAVC, CQEAC
bll4914		NADH ubiquinone oxidoreductase chain E	141	CLGAC
bll4927		hypothetical protein	65	CADCC
bll4943	*clpX*	ATP-dependent Clp protease ATP-binding	40	CVELC
bll5030	*ribE*	riboflavin synthase alpha chain	45	CNGVC
bll5046		adenylate cyclase 1	147	CLYSC
bll5119		transcriptional regulator	800	CLKHC
bll5184		putative cytochrome P460	173	CGHAC
bll5189		hypothetical protein	168	CDPTC
bll5204		oxidoreductase	38	CHLLC
bll5255		unknown protein	87	CRARC
bll5314		hypothetical protein	56	CNSAC
bll5354		probable transmembrane protein	106	CRIYC
bll5457		hypothetical protein	83	CNVYC
bll5476		formate dehydrogenase iron-sulfur subunit	19, 63, 67, 93, 151	CVTAC, CAAVC, CPVNC, CFYAC, CAEMC
bll5478		similar to formate dehydrogenase	79	CSVGC
bll5500		hypothetical protein	40	CNTGC
bll5524		hypothetical protein	42, 128	CEPNC, CESMC
bll5527		probable oxidoreductase	270	CGGIC

bll5559		hypothetical protein	91	CNGPC
bll5657		hypothetical protein	64	CKGDC
bll5666		hypothetical protein	31	CIPGC
bll5695		unknown protein	543	CPLVC
bll5696		unknown protein	533	CPLVC
bll5719	*purL*	phosphoribosylformylglycinamidine synthetase II	691	CEVPC
bll5740		hypothetical protein	343	CGPAC
bll5885	*fdx*	ferrodoxin	40	CGGAC
bll5912	*glyA*	serine hydroxymethyltransferase	438	CVEMC
bll5942		flavin-binding family monooxygenase	402, 406	CDLTC, CEYVC
bll5958		hypothetical protein	31	CGFPC
bll6038		hypothetical transport protein	232	CYGWC
bll6044		dimethyl sulfoxide reductase	14	CPHDC
bll6044		dimethyl sulfoxide reductase	18	CPSAC
bll6159		putative hydroxydechloroatrazine ethylaminohydrolase	308	CCPVC
bll6194		hypothetical protein	135	CLADC
bll6301		unknown protein	103	CATSC
bll6303		putative lipopolysaccharide biosynthesis protein	389	CHALC
bll6314		unknown protein	47	CFPVC
bll6337		unknown protein	115	CKRSC
bll6376		hypothetical protein	209	CRQRC
bll6407		ABC transporter substrate-binding protein protein	15	CEVYC
bll6439		hypothetical protein	39	CNNRC
bll6457		unknown protein	93	CPIIC
bll6491		unknown protein	26	CRTSC
bll6491		unknown protein	192	CKLAC
bll6558		oxidoreductase	393	CSLVC
bll6621	*emrB*	multidrug resistance protein B	250	CAWIC
bll6702		glutathione S-transferase	123	CRSIC
bll6709		probable ATP-binding protein	2	CRGLC
bll6792		putative transmembrane protein	275	CHGTC
bll6957		hypothetical protein	590	CANSC
bll7012		putative Cytochrome P450	5, 44, 46	CASRC, CLCVC, CVCRC
bll7086	*hemN*	anaerbic coproporphyrinogen III oxidase	57	CREMC
bll7103		ABC transporter permease protein	172	CNPVC
bll7196		ABC transporter ATP-binding protein	324	CDSEC
bll7271		carbon monoxide dehydrogenase large chain	29	CGTAC
bll7286	*poxB*	pyruvate oxidase	71, 125, 211	CAGSC, CSHYC, CGRGC
bll7307		two-component response regulator	141	CSESC
bll7365		unknown protein	24	CANWC
bll7413		similar to sulfate transporter	4	CIHAC
bll7540	*glcF*	glycolate oxidase iron-sulfur subunit	31, 82	CTATC, CMTTC
bll7572		unknown protein	4	CALGC

bll7626		hypothetical protein	191	CRFVC
bll7687		unknown protein	126	CFGHC
bll7793		similar to PEP2 protein	115	CDVVC
bll7837		hypothetical protein	112	CFAQC
bll7911		unknown protein	180	CGTDC
bll7928		putative fatty-acid--CoA ligase	130	CRTRC
bll7941		aminopeptidase	359	CYSKC
bll7953		hypothetical protein	5	CGVRC
bll7976		hypothetical protein	77,99	CVRSC
bll8086		unknown protein	163	CALAC
bll8141	*pckA*	phosphoenolpyruvate carboxykinase	273	CYAKC
bll8156		hypothetical protein	77	CLKTC
bll8171		transcriptional regulatory protein AraC family	46	CRPVC
bll8232		hypothetical protein	238	CASYC
bll8308		unknown protein	216	CLTQC
bll8309		unknown protein	26	CHQNC
blr0012		unknown protein	117	CWRLC
blr0107		probable coenzyme A ligase	60	CAEAC
blr0237		transcriptional regulatory protein LysR family	166	CEVPC
blr0298		unknown protein	103	CRGLC
blr0314	*nosR*	nitrous oxide reductase expression regulator	546, 645, 673, 680	CGWLC, CRYMC, CGSPC, CANEC
blr0315	*nosZ*	nitrous oxide reductase	268, 628	CFSTC, CSWFC
blr0341		hypothetical protein	26	CSYEC
blr0366		unknown protein	8	CCASC
blr0373		hypothetical aminotransferase	326	CKDKC
blr0515	*sdhB*	succinate dehydrogenase iron-sulfur protein subunit	177, 234	CSTSC, CAKAC
blr0528		probable oxidoreductase	6	CTQYC, ,
blr0538		hypothetical protein	86, 111, 390	CPYDC, CNLTC, CELLC
blr0577		unknown protein	275	CDLSC
blr0608		similar to ammonium transporter	115	CYWAC
blr0613	*amtB*	ammonium transporter	390	CLFFC
blr0853	*bchE*	Mg-protoporphyrin IX monomethyl ester oxidative cyclase 66kD subunit	202	CPHLC
blr0909		hypothetical protein	251	CGKVC
blr0938		putative dioxygenase	85	CGIRC
blr0944		hypothetical protein	478	CPLVC
blr1088		putative GNAT family acetyltransferase	66	CAYLC
blr1107		unknown protein	308	CHRAC
blr1130		hypothetical protein	103	CASAC
blr1131	*scoI*	cytochrome c oxidase assembly factor transmembrane protein	74	CPDVC
blr1165		hypothetical protein	58	CALLC
blr1165		hypothetical protein	142	CKAAC
blr1170	*coxB*	cytochrome c oxidase subunit II	229	CSELC
blr1300		hypothetical protein	25	CPAVC

blr1393	*plsC*	1-acylglycerol-3-phosphate O-acyltransferase	175	CGVPC
blr1444		hypothetical protein	123	CMPIC
blr1469		hypothetical protein	78, 101	CVRGC, CKSFC
blr1479		ferredoxin-nitrite reductase	457	CINAC
blr1513		hypothetical protein	4	CDATC
blr1556		unknown protein	61, 84	CSTRC, CETDC
blr1614		hypothetical protein	118	CTTSC
blr1626		ketopantoate hydroxymethyltransferase	222	CFMTC
blr1627		unknown protein	109	CSTWC
blr1645		putative transposase	155, 159	CRYRC, CGSCR
blr1659		unknown protein	192	CRGKC
blr1671		hypothetical protein	339	CIHGC
blr1672		hypothetical protein	199	CGQGC
blr1700		putative transposase	143, 180	CPCGC, CAKGC
blr1717		putative transposase	148	CRYRC
blr1730	*hypA*	hydrogenase nickel incorporation protein	88	CCPCC
blr1759	*nifB*	FeMo cofactor biosynthesis protein	63	CNIQC
blr1765	*fer2*	ferredoxin	64	CLGFC
blr1789		hypothetical protein	112	CMTDC
blr1822	*rhcU*	RhcU protein	175	CGMGC
blr1833		unknown protein	59, 109	CNLKC, CRTRC
blr1859		unknown protein	17	CGQNC
blr1879		hypothetical protein	115	CDAFC
blr1914		putative transposase	103, 107	CRYRC, CGSRC
blr1933		hypothetical protein	614	CHAEC
blr1975		unknown protein	59	CNLKC
blr2022		unknown protein	78	CSSAC
blr2027	*nodC*	chitin synthase	207	CCGPC
blr2030	*nodI*	transporter of Nod factors	205, 265	CDRLC, CYARC
blr2054		putative transposase	125	CTREC
blr2068		5-methyltetrahydropteroyltriglutamate-homocysteine S-methyltransferase	646	CFRIC
blr2077		rtxA homolog	162	CFVHC
blr2089		unknown protein	38	CRCGC
blr2095	*bioB*	biotin synthetase	68	CPEDC
blr2135		hypothetical protein	146	CSSPC
blr2156		hypothetical protein	9	CRLQC
blr2191		hypothetical protein	43	CSRVC
blr2238		putative adenylate cyclase	1010	CWFRC
blr2306		hypothetical protein	53	CGPCC
blr2308		putative molybdopterin biosynthesis protein	313	CLAVC
blr2314		MFS permease	40	CIHLC
blr2316		probable NADH-ubiquinone oxidoreductase chain F	145	CVGLC
blr2317		formate dehydrogenase alpha subunit	153, 196, 232, 366	CVRAC, CVQAC, CGVGC, CTRLC
blr2322	*citB*	citrate utilization protein B	23, 57	CEGLC, CYVDC

blr2368		unknown protein	333	CGLGC
blr2412		hypothetical protein	223	CAAAC
blr2455		isocitrate lyase	395	CVLDC
blr2506		putative chemotaxis protein	38	CPPLC
blr2519		hypothetical protein	106	CEHGC
blr2538	*ppdK*	pyruvate,orthophosphate dikinase	569	CVSGC
blr2671		unknown protein	36	CVRMC
blr2705		hypothetical protein	24	CAWGC
blr2719		hypothetical protein	255	CTPAC
blr2739		hypothetical methyltransferase	183	CPFTC
blr2767	*fixG*	iron-sulfur cluster-binding protein	116, 225, 281, 305	CGYLC, CTYMC, CVAVC, CIDAC
blr2809	*nasA*	nitrate reductase large subunit	18	CGVGC
blr2818		hypothetical protein	56	CEAIC
blr2828		unknown protein	31	CGVLC
blr2989		acyl-CoA dehydrogenase	150	CEIKC
blr2995		hypothetical protein	207	CPYQC
blr3026	*dnaE*	DNA polymerase III alpha chain	547	CIRKC
blr3103		hypothetical protein	36	CGRCC
blr3159		hypothetical protein	472	CATFC
blr3183		ABC transporter substrate-binding protein	132	CVHTC
blr3209		sugar ABC transporter ATP-binding protein	515	CDRIC
blr3226		ribitol kinase	182	CTVTC
blr3252		unknown protein	3643	CASAC
blr3262		hypothetical protein	48, 52	CLSAC, CSQGC
blr3264		unknown protein	333	CAKGC
blr3304		acyl-CoA dehydrogenase	71	CVEHC
blr3305		unknown protein	109	CRVTC
blr3321		putative aspartate aminotransferase related protein	94	CRARC
blr3358		putative ferredoxin	54	CDRAC
blr3429		hypothetical protein	211	CVMPC
blr3431		hypothetical protein	144	CVIDC
blr3445		putative enoyl-CoA hydratase	91	CIREC
blr3446		probable 6-carboxyhexanoate-COA ligase	220	CLEAC
blr3493		unknown protein	19, 25	CSPEC, CRPDC
blr3517	*soxD*	probable sulfite oxidase cytochrome subunit	202	CMRDC
blr3644		hypothetical protein	42	CALCC
blr3677		putative monooxygenase component	429	CLVPC
blr3681		hypothetical protein	165	CLELC
blr3682		hypothetical protein	255	CGSGC
blr3726	*tme*	NADP-dependent malic enzyme	595	CDTHC
blr3822		hypothetical protein	28	CPVMC
blr3839		putative dicarboxylic acid hydrolase	28	CDAHC
blr3857		putative monooxygenase	392	CKRPC
blr3858		putative shikimate 5-dehydrogenase	77	CYQAC
blr3879		major facilitator superfamily	32, 230,	CAWGC, CFRGC,

			425	CGVLC
blr3920		ABC transporter permease protein	233	CFTLC
blr3922	*kdgK*	2-dehydro-3-deoxygluconokinase	13	CIGEC
blr3941		hypothetical protein	190	CGFAC
blr4156		probable acetylornitine deacetylase	285	CDVDC
blr4161		hypothetical protein	4	CGRRC
blr4245		unknown protein	30	CFATC
blr4255		transcriptional regulatory protein AraC family	135	CADLC
blr4259		hypothetical protein	589	CANSC
blr4306		putative N-acetylmuramoyl-L-alanine amidase precursor	13	CVLLC
blr4337		1hypothetical protein	147	CPNPC
blr4388		hypothetical protein	107	CAVYC
blr4422	*hmgL*	hydroxymethylglutaryl-CoA lyase	142	CVLGC
blr4477	*lipA*	lipoic acid synthetase	86	CTRAC
blr4513		putative medium-chain-fatty-acid--CoA ligase	241	CNGWC
blr4575		hypothetical protein	146	CNNRC
blr4624		hypothetical protein	58	CVSAC
blr4773	*nwsA*	two-component hybrid sensor and regulator	127	CVALC
blr4809	*trpD*	anthranilate phosphoribosyltransferase	142	CVREC
blr4872		unknown protein	52, 155	CGPGC, CNSAC
blr5118		unknown protein	222	CNGGC
blr5152		hypothetical protein	69	CTTPC
blr5178		hypothetical protein	57	CASAC
blr5211		dehydrogenase	355	CALEC
blr5484		putative ferredoxin	271, 300, 511, 542	CLDLC, CAAAC, CVSAC, CQSTC
blr5515		hypothetical protein	36	CRPGC
blr5563		unknown protein	12	CTLCC
blr5707		putative threonine aldolase	256	CAAAC
blr5778	*fixG*	nitrogen fixation protein	108, 215, 271, 295	CGYMC, CIYMC, CINVC, CIDAC
blr5832		transcriptional regulatory protein MarR family	8	CDGDC
blr6041		putative glycolate oxidase	634	CRHEC
blr6215	*adhC*	alcohol dehydrogenase class III	165	CYIGC
blr6218		putative oxidoreductase protein	131	CRCGC
blr6253		hypothetical protein	214	CTLIC
blr6255		hypothetical protein	544	CRERC
blr6285		putative long-chain fatty acid transport protein	294	CSMLC
blr6437		gluconolactonase precursor	422	CGNIC
blr6659	*thiC*	thiamine biosynthesis protein	472	CAMLC
blr6719		hypothetical protein	442	CTVLC
blr6739	*pqqE*	probable pyrroloquinoline quinone synthesis protein E	45	CPLQC
blr6742		glutamate synthase small subunit	18,61, 65, 506, 510, 566	CQWAC, CDRPC, CEPAC, CVDIC, CPMDC, CAERC
blr6748		unknown protein	129	CGVDC

blr6818		hypothetical protein	207	CTNMC
blr6840		hypothetical protein	288	CGGAC
blr6894		unknown protein	85	CNACC
blr7038	*napA*	periplasmic nitrate reductase large subunit precursor	54	CGTGC
blr7166		hypothetical protein	59	CLSAC
blr7208		transcriptional regulatory protein LysR family	224	CNTAC
blr7339		unknown protein	192	CGGGC
blr7377	*carB*	carbamoylphosphate synthase large subunit	278, 638	CIIVC, CCHAC
blr7470	*uvrC*	excinuclease ABC chain C	241	CAGPC
blr7491		putative 2-keto-gluconate dehydrogenase	199	CFQGC
blr7502		unknown protein	173	CSFLC
blr7716		probable adenylate cyclase	286	CPSWC
blr7731		inositol monophosphatase family protein	190	CYSYC
blr7743	*gltB*	glutamate synthase large subunit	223	CSMSC
blr7744	*gltD*	glutamate synthase small subunit	106, 110	CPAPC, CEASC
blr7807		probable acyl-CoA ligase	227	CADMC
blr7816		probable mannitol-binding protein	51	CEYFC
blr7852		hypothetical protein	28, 32	CCQSC, CGACC
blr7878		unknown protein	119	CVRKC
blr7888		putative 3-oxoacyl-[acyl-carrier-protein] reductase	233	CAFLC
blr7926		unknown protein	307	CTGTC
blr7973		hypothetical protein	346	CFGGC
blr8017		hypothetical protein	198	CCYAC
blr8135		hypothetical protein	89	CVRSC
blr8135		hypothetical protein	111	CQAFC
blr8151		unknown protein	561	CPLVC
blr8155		hypothetical protein	88	CLGQC
blr8172		hypothetical protein	173	CASLC
blr8217		putative transposase	191	CCWTC
blr8292		hypothetical protein	47	CNRRC
blr8298		hypothetical protein	615, 644	CHAEC, CAAQC
bsl0041		unknown protein	35	CWRSC
bsl0296		unknown protein	8	CVVAC
bsl1161		unknown protein	46	CLAVC
bsl3737		hypothetical protein	7	CVAVC
bsl4812		probable fimbriae associated protein	3	CEHAC
bsl6675		unknown protein	12	CDDVC
bsl6681		putative bacterioferritin	37, 59, 63	CSAEC, CAKSC, CCSGC
bsl7391		unknown protein	72	CAPGC
bsr1260		hypothetical protein	30	CSSRC
bsr1553		unknown protein	87	CQCPC
bsr1739		ferredoxin	16, 51	CEFEC, CAVVC
bsr1750	*fer3*	ferredoxin	30, 80, 84	CFKVC, CDRVC, CPASC
bsr1760	*frxA*	ferredoxin-like protein	16, 51	CEPLC, CAAAC

bsr1775	*fixX*	probable ferredoxin	46	CPARC
bsr2672		hypothetical protein	5	CRNWC
bsr2802		unknown protein	46	CLGGC
bsr3197		putative ferredoxin	28, 59	CVDVC, CEADC
bsr3674		unknown protein	69	CSPFC
bsr3718		unknown protein	67	CLMIC
bsr4099		unknown protein	83	CDWPC
bsr4225		unknown protein	4	CDAAC

*Gene names and annotations according to Rhizobase

CHAPTER 4

Role of the conserved histidine 162 of ScoI

4.1 Abstract

The aim of this project was to assess whether the conserved histidine 162 of *Bradyrhizobium japonicum* ScoI is required for Cu(II) binding. Previously, it has been shown that the two conserved cysteines C74 and C78 are essential for Cu(II) binding, and structural data of ScoI orthologs has shown that the cysteines from the CXXXC motif, called Sco-motif, and the histidine located about 86 amino acids C-terminal to the Sco-motif are indeed involved in the ligation of copper. Spectroscopic analysis of a mutant ScoI derivative revealed that when histidine 162 is replaced by an alanine, Cu(II) can still be bound. However, at this stage, we do not know whether the *in vivo* function of the protein is affected by the mutation.

4.2 Introduction

Between 84 and 88 amino acids downstream of the CXXXC motif, a histidine residue is conserved in Sco proteins. This histidine residue has been shown to be essential for Sco activity in *Bacillus subtilis* (Siluvai *et al.*, 2009), in *Agrobacterium tumefaciens* (Saenkham *et al.*, 2009) and in yeast (Rentzsch *et al.*, 1999; Lode *et al.*, 2000). In human Sco1, NMR studies have shown that residues His260, Cys169 and Cys173 (corresponding to His162, Cys74 and Cys78 in *B. japonicum* ScoI) participate in the binding of Cu(I). Binding of Cu(II) was also observed; however, the authors do not indicate whether the same residues are implicated (Banci *et al.*, 2006a). In yeast, at least two studies have dealt with the conserved His239 of Sco1p. First, it was observed that a mutant allele of Sco1p in which the active-site His239 was changed to a threonine and transformed into a Δ*sco1* null mutant was unable to complement for the mutant phenotype. The transformed yeast cells remained respiration deficient, and the amount of CoxB, the subunit II of the oxidase that could be detected in the cells was lowered similarly as in a Δ*sco1p* null mutant. However, purified Sco1pH239T was still able to interact with CoxB as shown by co-immunoprecipitation (Lode *et al.*, 2000). A second study characterized the coordination of Cu(I). It was found that three residues are involved, namely Cys148, Cys152 and His239. Single exchange of each of these residues resulted in a respiration-deficient phenotype, and a H239A mutant was as impaired in Cu(I) binding as was a C148A/C152A double mutant (Nittis *et al.*, 2001).

The organism in which the contribution of the conserved histidine of Sco has been studied in most detail is *B. subtilis*; however, the available data conflict. While Imriskova-Sosova and coworkers found that His135 is critical for Cu(II) binding (Imriskova-Sosova *et al.*, 2005), a more recent study showed that although His135 is essential for *B. subtilis* Sco's contribution in aa_3-type cytochrome oxidase biogenesis, *B. subtilis* ScoH135A can still bind Cu(II) with a stoichiometry of 1:1 (Siluvai *et al.*, 2009). Nevertheless, Cu(II) bound to *B. subtilis* ScoH135A is prone to autoreduction. In detail,

when bound to a H135A variant of purified *B. subtilis* Sco, two thirds of the bound Cu(II) was spontaneously reduced to Cu(I). The electrons for copper reduction originated from the thiols of the Sco-motif, meaning that, in the process, one third of the *B. subtilis* Sco active site cysteines oxidized to form disulfide bridges. Thus, His135 is essential to stabilize the valence of Cu(II) (Siluvai *et al.*, 2009).

In *Thermus thermophilus*, Sco was shown to reduce the copper-liganding cysteines of the Cu_A site of the subunit II of the cytochrome oxidase ba_3. However, *T. thermophilus* Sco is also capable of binding Cu(II) and Cu(I), and the usual residues, namely Cys49, Cys53, and His137 are involved in the coordination of the metal. Nevertheless, *T. thermophilus* Sco cannot transfer the bound copper atom to the Cu_A site *in vitro*. Thus, the biological function of *T. thermophilus* Sco copper binding is currently not understood (Abriata *et al.*, 2008).

4.3 Material and Methods

4.3.1 Media, growth conditions and strains

Escherichia coli was grown in Luria-Bertani (LB) medium (Miller, 1972) containing 10 µg/ml tetracycline or 200 µg/ml ampicillin for plasmid selection. *E. coli* strain DH5α (Invitrogen) was used for cloning work and *E. coli* strain S17-1 (Simon *et al.*, 1983) was used for mobilization of plasmids into *B. japonicum*. *E. coli* strain BL21 (DE) (Studier & Moffatt, 1986) was used for overexpression of ScoI variants.

Aerobic cultures of *B. japonicum* were grown in Erlenmeyer flasks containing one fifth of their total volume of PSY medium (Regensburger & Hennecke, 1983; Mesa *et al.*, 2008) supplemented with 0.1% of arabinose and shaken vigorously (160 rpm) at 30°C. When appropriate, antibiotics were used at the following concentrations: spectinomycin, 100 µg/ml; kanamycin, 100 µg/ml, and tetracycline, 50 µg/ml (solid media) and 25 µg/ml (liquid media).

4.3.2 DNA biochemistry and strain construction

Plasmids pRJ8331 and pRJ8339 were constructed with PEC415 backbone as described previously (Landolt, 2005). To construct pRJ6548, a synthetic gene piece of 371 bp that started with a natural *Bgl*II site contained within the *scoI* gene and a natural *Eco*RI site located 25 bp after the stop codon of the *scoI* gene, in which the His162 codon was exchanged to an alanine codon, was synthetized and cloned into a PCR2.1-TOPO vector (Eurofins, Ebersberg, D) leading to plasmid pRJ6546. The 365 bp *Bgl*II-*Eco*RI fragment was then subcloned into a *Bgl*II-*Eco*RI 5628 bp fragment derived from pRJ8331. This led to plasmid pRJ6548 from which Strep-tagged $ScoI_{sol}$H162A could be periplasmically expressed.

A stable plasmid that can be maintained under non-selective conditions in *Rhodopseudomonas palustris* pMG103 (Inui *et al.*, 2000) was used here. To perform a complementation in *B. japonicum* two methods are generally used: either the gene is brought into the genome via double homologous recombination or the gene can be expressed *in trans* from pRKPolSca. However, double homologous recombination is time-consuming and pRRKPolSca derivatives can only be maintained in *B. japonicum* under antibiotic pressure, which renders the strains unsuitable for symbiotic tests because bacteroids in root nodules cannot be subjected to antibiotic pressure. Therefore, a tetracycline resistant variant of this plasmid, called pMG103tet, was used to construct an expression system for ScoIWT and ScoIH162A. This resulted in plasmids pRJ6545 and pRJ6547. The 944 bp long inserts of these plasmids correspond to chromosomal region ranging from nucleotide position 1,244,319 to 1,245,263. This means that the 330 bp long natural upstream region of the *blr1131* gene has been included in the construct in order to ensure that the genuine *scoI* promoter is most likely included. pRJ6545 contains the wild-type *scoI* sequence and in pRJ6547 the CAC codon corresponding to chromosomal position 1,245,132-1,245,134 is changed to a GCG, thereby His162 was changed to an alanine. pRJ6547 was constructed by ligating a 655 bp *Bgl*II-*Eco*RI fragment from pRJ6546 into a 7269 bp *Bgl*II-*Eco*RI fragment from pRJ6545. Plasmids pRJ6545 and pRJ6547 were mobilized into *B. japonicum* 110*spc*4 and 2575 with *E. coli* S17-1.

TABLE 4.1. Strains, plasmids and primers used in this work

Strains	**Relevant genotype**	**Reference**
E. coli		
DH5α	*supE44* Δ*lacU169*(ϕ80 *lacZ*ΔM15) *hsdR17 rec*A1 *gyrA96 thi*-1 *rel*A1	Bethesda Research Laboratories, Inc., Gaitersburg, MD
S17-1	Sm^R Sp^R *hsdR* (RP4-2 *kan*::Tn7 *tet*::Mu, integrated in the chromosome)	(Simon *et al.*, 1983)
BL21 (DE3)	F^- *ompT gal dcm lon hsdS B*(r_B^- m_B^-) λ(DE3)	(Studier & Moffatt, 1986)
B. japonicum		
110*spc*4	Sp^R wild type	(Regensburger & Hennecke, 1983)
2575	Sp^R Km^R Δ*scoI*::*aphII*	(Bühler *et al.*, 2010)

TABLE 4.1 (continued)

Plasmids		
pMG103tet	TC^R (pMG103tet) (Inui *et al.*, 2000)TC resistance cassette *Pst*I from pBR322 (Bolivar *et al.*, 1977)	Masloboeva, unpublished
pRJ6545	TC^R (pMG103tet) 957 bp *Xba*I-*Ecor*I from pRJ8315 containing ScoIWT and 330 bp upstream region	This work
pRJ6546	Ap^R , Km^R original name: pCR2.1-ScoI H162A synthetized by Eurofins MWG Operon (pCR2.1-TOPO) 371 bp *Bgl*II- *EcoR*I fragment starting at genomic position 1'244'899 (natural *Bgl*II site at codon 83-84), codon 162 CAC was exchanged to GCG	This work
pRJ6547	Tc^R (pRJ6546) 365 bp *Bgl*II-*EcoR*I fragment from pRJ6545	This work
pRJ8331	Ap^R, expression plasmid for $ScoI_{sol}$ WT pEC415 with 555 bp *Stu*I/*EcoR*I fragment from pRJ8326	(Landolt, 2005)
pRJ8339	Ap^R, expression plasmid for $ScoI_{sol}$ (C74S, C78S) pEC415 555 bp *Stu*I/*EcoR*I fragment from pRJ8330	(Landolt, 2005)
pRJ6548	Ap^R, expression plasmid for $ScoI_{sol}$ (H162A) pRJ8331 with 365 bp *Bgl*II/*EcoR*I fragment from pRJ6546	This work
pRJ8315	Ap^R, (pUC18) *BamH*I-*EcoR*I 951 bp fragment containing *scoI*	(Landolt, 2005)

Primers	**Sequence**	**Binding site**
44 (pMG-sco-for)	gat tgt agg cgc cgc cct ata cc	79 nucleotides proximally of 3' end of *tet* *
45 (pMG-sco-rev)	ccc atg gcc cag aac atg atc agc	89 nucleotides distally of 5' end of *scoI* *

* see SUPPLEMENTARY FIG. 4.1

4.3.3 Protein purification

Plasmid pRJ8331 was used to overexpress $ScoI_{sol}$, plasmid pRJ8339 to overexpress $ScoI_{sol}$ C74S/C78S, and plasmid pRJ6548 to overexpress $ScoI_{sol}$ H162A. Overexpression was performed with 1L cultures of *E. coli* BL21 (DE3) containing the respective plasmids. Overexpression and purification were done as previously described (chapter 2)

4.3.4 UV-VIS spectroscopy of $ScoI_{sol}$

Copper binding was studied with spectroscopic analysis. Upon copper binding, several electronic transitions that can be observed at 360, 480 and 610 nm in human Sco1 (Horng *et al.*, 2005) and in *B.*

subtilis BSco (Imriskova-Sosova *et al.*, 2005). With *B. japonicum* ScoI, we could observe a peak at 360 and a second, yet more modest, peak at 510 nm upon copper binding. Purified ScoI (in 100 mM Tris-HCl (pH 8), 150 nM NaCl, and 2.5 mM desthiobiotin [elution buffer after Strep-Tactin purification]) was reduced by incubation with 2 mM dithiothreitol for 4h at 4°C. Dithiothreitol was removed and exchanged against 50 mM sodium phosphate buffer (pH 7.0) by gel filtration over PD-10 columns (GE Healthcare). The buffer has been first depleted of copper and other cations by running it over a Chelex 100 resin column (BioRad). UV-VIS spectra from 200 to 800 nm were recorded on an Agilent diode array photometer (Agilent Technologies, Santa Clara, CA). Cu(II) was added in small volumes ranging from 5-40 µl of 1 mM $CuCl_2$ dissolved in 50 mM phosphate buffer (pH 7.0). The background spectra of buffer containing corresponding amounts of copper were subtracted from the protein spectra.

4.3.5 Western blot

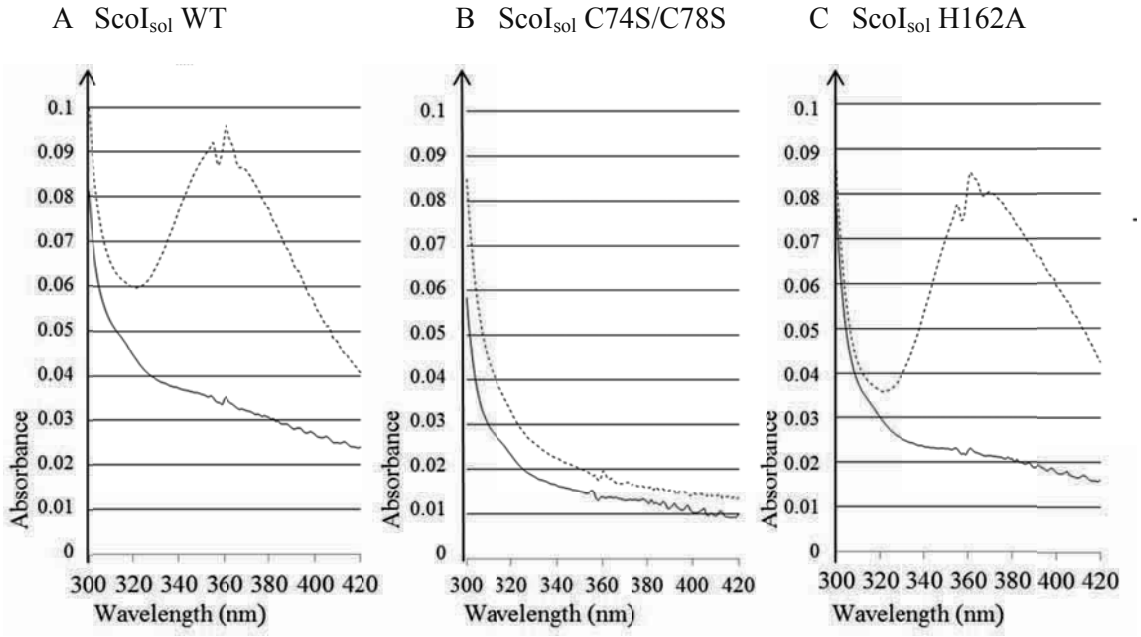
Western blots with *B. japonicum* cell extracts were performed as previously described (chapter 2) except that a concentration of α-ScoI serum of 1:5'000 was used.

4.4 Results

4.4.1 UV-VIS spectroscopy of $ScoI_{sol}$, $ScoI_{sol}$ C74S/C78S, and $ScoI_{sol}$ H162A

It has been shown previously that $ScoI_{sol}$ is capable to bind Cu(II), but that $ScoI_{sol}$C74S/C78S is not (Bühler *et al*., 2010). These proteins were thus used as positive (FIG. 4.1.A) and negative controls (FIG. 4.1.B). $ScoI_{sol}$H162A appears to behave like wild-type $ScoI_{sol}$. Its spectrum shows a peak at around 360 nm that is commonly attributed to a thiolate-to-Cu(II) charge transfer transition (FIG. 4.1). Thus, $ScoI_{sol}$H162A is able to bind copper. Two independent biological replicas of this experiment were performed, and the correct identity of the protein was verified with mass spectroscopy (Functional Genomics Center, Zürich).

FIG. 4.1. UV-VIS spectra of three variants of $ScoI_{sol}$ protein in the absence (plain lines) and the presence of ~0.2 molar equivalents Cu(II) of copper (dashed lines). The peak at ~360 nm is diagnostic for copper binding and can be observed for $ScoI_{sol}$ (panel A) and $ScoI_{sol}$H162A (panel C). Panel B depicts the spectrum of $ScoI_{sol}$C74S/C78S in which the characteristic peak at 360 nm is missing. The concentrations of proteins used here were 55 µM ($ScoI_{sol}$), 69 µM ($ScoI_{sol}$H162A) and 59 µM ($ScoI_{sol}$C74S/C78S).



4.4.2 In vivo complementation with a pMG103tet derivative

The *in vivo* complementations of Δ*scoI* with plasmids pRJ6545 and pRJ6547 were not successful. Nodules of plants infected with 2575 x pRJ6545 showed the same nitrogen fixation defect as nodules of plants infected with 2575. A qualitative TMPD test performed with 2575 x pRJ6545 and 2575 x pRJ6547 showed the same symptoms of respiratory deficiency as for 2575 (data not shown). Since pRJ6545 encodes a wild-type variant of *scoI*, we expected that in the 2575 x pRJ6545 complementation, the mutant phenotype of 2575 would have been reverted. Western blots of cell extracts of those strains showed that ScoI is not expressed from the pMG103tet derivatives (supplementary FIG. 4.2).

4.5 Discussion

When the conserved histidine 162 of ScoI is mutated, the Cu(II) binding ability of the protein is not impaired. This is in accordance with other observations in *B. subtilis* Sco where His135 is also not essential for Cu(II) binding. However, a role in stabilizing Cu(II), as it was described for His135 in *B. subtilis* Sco (Siluvai *et al*., 2009) seems unlikely for His162 in *B. japonicum*. In *B. subtilis*, autoreduction of Cu(II) in ScoH135A leads to a loss of intensity in the three spectral peaks that characterize Cu(II) binding to the cysteines of the CXXXC motif. After 5 minutes incubation, Siluvai and coautors observed an almost complete disappearance of the three peaks. This is due to two reasons: first, cysteine-liganded Cu(I) does not have the same spectral properties as cysteine-liganded Cu(II), and, secondly, one third of the Sco protein undergoes disulfide bridge formation in the active-site cysteines, thereby losing its bound cofactor (Siluvai *et al*., 2009). In the Cu(II)-binding experiment performed in our study, we left the samples in the cuvette for at least 15 minutes. During this time, the peak did not disappear neither in the case of $ScoI_{sol}$WT nor in the case of $ScoI_{sol}$H162A. If His162 in *B. japonicum* fulfilled a Cu(II)-stabilizing role, we would expect that, in the absence of this histidine, the peak would gradually disappear over time.

The complementation experiment in which Δ*scoI* was provided with an expression plasmid encoding a H162A variant of ScoI did technically not work. At the moment, we do not understand why this expression was not successful. The bacteroids that were reisolated from 2575 x pRJ6545 and 2575 x pRJ6547 infected plants were tested positively by for presence of the *tet* gene and of the *scoI* gene. Moreover, in an additional PCR reaction depicted in SUPPLEMENTARY FIG. 4.1, it was also verified that the pMG103tet derivatives did not insert into the chromosome. This confirms that pMG103tet derivatives can be maintained *in trans* without antibiotic pressure. In a further step, reisolated strains and the original strain used for infection were tested for the presence of ScoI proteins by immunodetection. It turned out that the ScoI variants are not expressed from the pMG103tet derivatives (SUPPLEMENTARY FIG. 4.2). As WT x pRJ6545 expresses ScoI, we can exclude an effect of pMG103tet on the expression of ScoI.

To gather further insights into the properties of ScoI, the understanding of the function the highly conserved H162 residue seems compelling, and thus the construction of a Δ*scoI* strain complemented with a H162A variant of ScoI is mandatory. One might probably choose a traditional, time-consuming double homologous recombination strategy to achieve this. One of the most burning questions is whether or not both the function in aa_3-type cytochrome oxidase biogenesis and the symbiotic function of ScoI are affected by the exchange of H162 to A.

In yeast, the conserved histidine is located on a flexible loop which can move into the vicinity of the conserved cysteines of the CXXXC motif or away from it in the apo-form of Sco1p. It has been

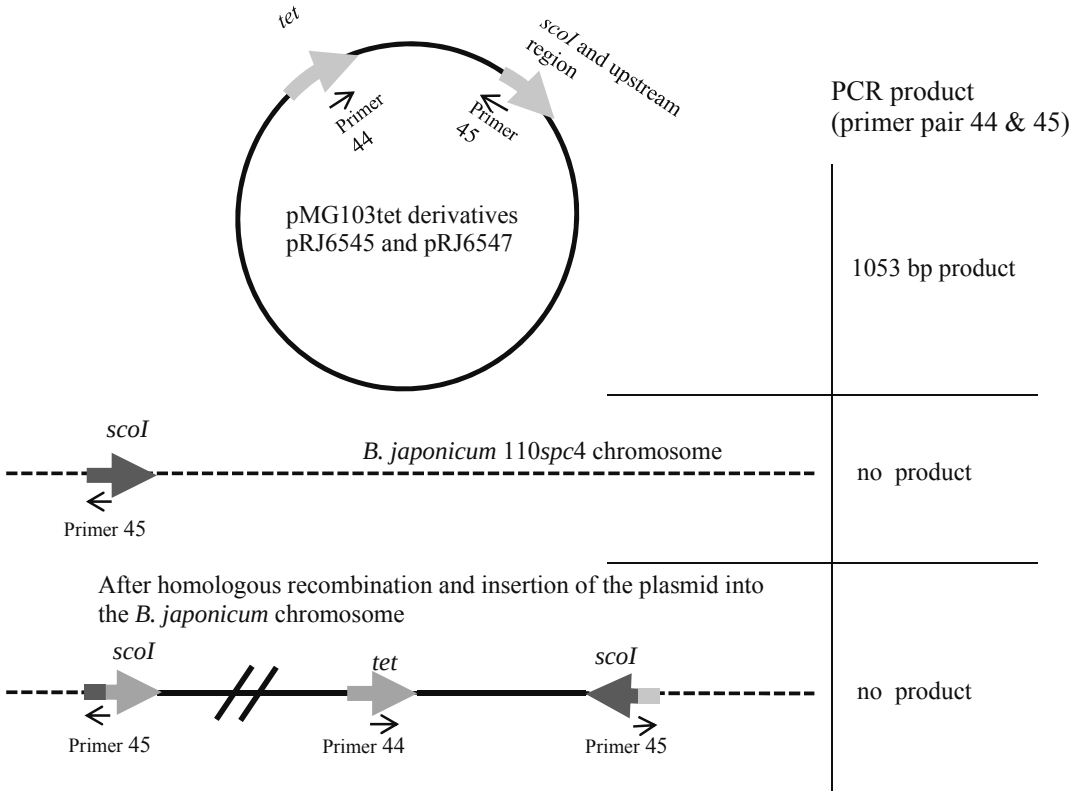
suggested that the conserved histidine plays a role in the releasing process of the bound copper (Abajian & Rosenzweig, 2006). This hypothesis would also be very attractive for *B. japonicum* and in complete accordance with the fact that $ScoI_{Sol}$H162A is perfectly capable to bind copper.

Cu(I) binding has been shown for the ScoI homologs of yeast (Nittis *et al.*, 2001), human (Banci *et al*., 2006a), and *B. subtilis* (Andruzzi *et al.*, 2005) but has never been assayed in *B. japonicum* due to technical difficulties. Indeed, such a study can only be done under anoxic atmosphere as Cu(I) is strongly prone to oxidation to Cu(II) upon contact with atmospheric oxygen. This experiment would be interesting for $ScoI_{sol}$H162A but also for the wild-type variant. One longstanding mystery in the area of Cu_A biogenesis of cytochrome oxidases is how the two copper atoms are delivered, and how their overall mixed valence of 1.5 is achieved. If ScoI is a copper-donating chaperone for Cu_A of the aa_3-type cytochrome oxidase SUII, does it deliver the two atoms sequentially? And if yes, what is the valence of the copper atoms at the moment of delivery? In *T. thermophilus*, which is, so far, the only case where direct transfer of copper to Cu_A could be documented *in vitro*, it was tested whether the Cu(II)-binding Sco protein and the Cu(I)-binding PCu_AC chaperone would cooperatively deliver copper atoms to Cu_A. This would result in a mixed valence of 1.5. However, this hypothesis was rejected. The data suggest that PCu_AC is the only one of the two which actually delivers copper to Cu_A centers (Abriata *et al*., 2008).

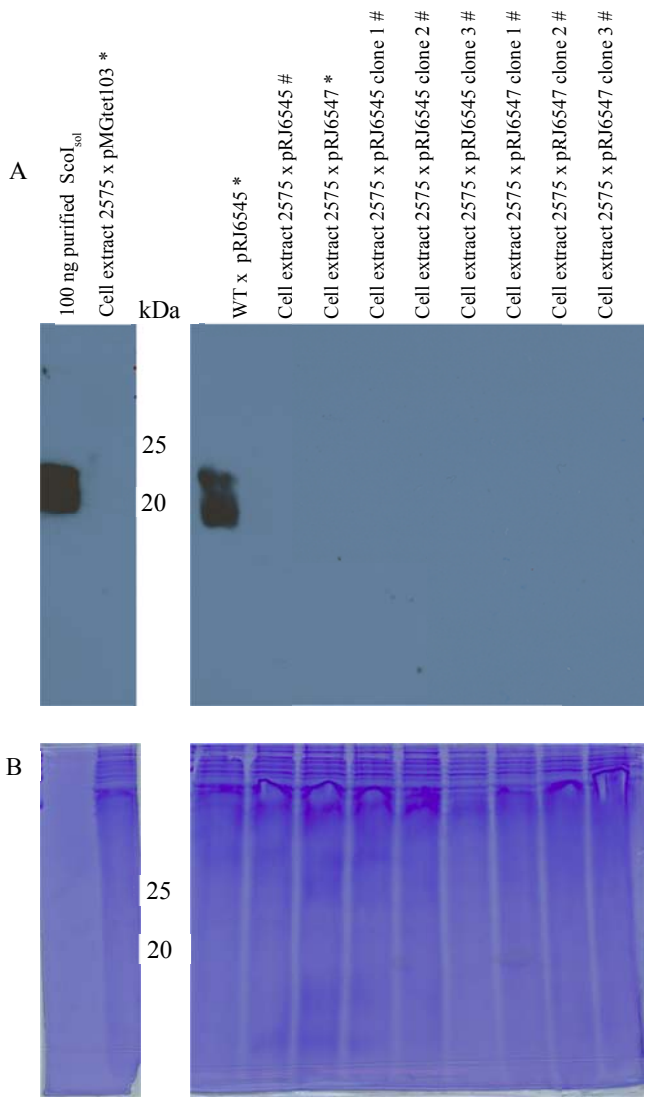
To summarize, the role of the conserved histidine, corresponding to H162 of *B. japonicum* ScoI, does not seem to be uniform in all the studied organisms. Although it is generally involved in copper complexing, its contribution is not compulsory in several cases. Its role might rely in the stabilization of a particular valence of the metal or in its delivery.

4.6 Supplementary material

SUPPLEMENTARY FIG. 4.1. Application of the primer pair 44&45 that were used to determine whether pRJ6545 and pRJ6547 were still present *in trans* or whether they underwent homologous recombination and integrated into the genome.



SUPPLEMENTARY FIG. 4.2. A: Western blot analysis of 100 ng purified $ScoI_{sol}$ protein and of 150 µg cell extract from different strains. Samples marked with * were cultivated under antibiotic pressure. Samples marked with # originate from bacteroid reisolations and were cultivated without antibiotic pressure (the presence of the plasmids was verified with PCR). Separation was done on a 14 % polyacrylamide gel containing SDS. B: Coomassie stained gels after the transfer for the Western blots; it shows that equal amounts of cell extracts were loaded and that, in the lower molecular-mass range, the transfer was efficient.



CHAPTER 5

Redox properties of ScoI

In collaboration with Prof. Dr. Rudolf Glockshuber and Elisabeth Mohorko

5.1 Abstract

Sco-like proteins are generally described to function as copper chaperones; however, their structural similarity with thioredoxins suggested that they might also assume disulfide-reducing functions, and for some of them, this has been experimentally demonstrated. That the active-site cysteines of Sco-proteins can undergo intermolecular disulfide bridging has been shown before and here we assess the redox potential of this cysteine pair. The redox potential of *Bradyrhizobium japonicum* ScoI was determined to be approximately –155 mV which challenges the disulfide-reductase hypothesis.

5.2 Introduction

Sco-like proteins are not exclusively involved in copper A center (Cu_A) biogenesis via copper insertion. Banci and coworkers propose a framework for the action of Sco-like proteins where metal transport and thiol-disulfide oxidoreductase activity are either coupled directly or where only one of those two is carried out (Banci *et al.*, 2007a). 128 out of 311 prokaryotic genomes encode a total of 254 Sco-like proteins, and in almost one third of the cases they do not have a predicted membrane anchor. In 18 of the 128 organisms however, genes encoding cytochrome oxidase subunit II or nitrous oxide reductase, the two proteins known to harbor Cu_A sites, are missing, further suggesting that Sco function is not restricted to Cu_A biogenesis (Banci *et al*., 2007a). In three cases, thiol-disulfide oxidoreductase activity of a Sco protein has been demonstrated biochemically. PrrC, the Sco homolog *of Rhodobacter sphaeroides*, is capable to reduce disulfides as well as to reduce Cu(II) to Cu(I) (Badrick *et al.*, 2007). In this organism, PrrC may be involved in sensing oxygen levels via the high-affinity cbb_3-type cytochrome oxidase and to transduce this signal to the PrrAB two-component regulatory system which in turn induces transcription of the photosynthesis genes. Another Sco-like protein for which disulfide-reducing activity has been shown is the *Thermus thermophilus* ScoI homolog. Despite its ability to bind copper atoms, *T. thermophilus* Sco1 is not capable to deliver them but instead reduces the cysteines of the Cu_A site of the cytochrome oxidase subunit II (Abriata *et al.*, 2008). Finally, the two human Sco-like proteins, undergo a thiol-disulfide-oxidoreductase reaction, the oxidizing partner being Sco1 and the reducing one being Sco2. The Sco1-mediated disulfide bridge in Sco2 induces the release of the Sco2-bound copper atom that was complexed by the free thiols of the latter (Leary *et al.*, 2009). Given the spectrum of different functions and activities that have been reported for Sco-like proteins, it was of interest to find out whether the aa_3 cytochrome oxidase assembly defect of a *scoI* mutant in *B. japonicum* could be assigned to a loss of

metallochaperone function, a disulfide reductase function, or a combination of both. The fact that excess copper in the growth medium could complement the defect of a *scoI* mutant, and the negative result of an insulin reduction test with purified ScoI supported the copper chaperone hypothesis (Bühler *et al.*, 2010). Nevertheless, we were keen to learn more about the redox properties of its active-site cysteines.

Another reason raised our interest to determine the redox potential of ScoI. *B. japonicum* encodes a periplasmic thioredoxin-like protein, named TlpA. Having a characteristic CXXC motif and other similarities, it resembles cytoplasmic thioredoxins. TlpA has a standard redox potential of –213 mV (Loferer *et al.*, 1995) and is able to reduce insulin (Loferer & Hennecke, 1994), demonstrating tha t, most probably, TlpA assumes a reducing function in *B. japonicum*. Although many efforts were undertaken, the substrate(s) of TlpA remained unknown. A Δ*tlpA* mutant has strikingly similar defects as a *scoI* mutant. Both fail to form aa_3 cytochrome oxidase and fail to establish a fully functional symbiosis with soybean (Loferer *et al.*, 1993; Bühler *et al*., 2010). These converging phenotypes led to the hypothesis that TlpA and ScoI are involved in the same pathway(s). More specifically, it was proposed that TlpA and ScoI interact directly in a disulfide exchange reaction via their active-site cysteines. To gain insight into the possible direction of such a reaction, *i.e.* which one is likely to be the reducing and which one the oxidizing partner, we wanted to assess the redox potential of ScoI. Since TlpA has a rather negative redox potential and because the cysteines of ScoI must be in a reduced form in order to bind copper, it appears plausible to predict that ScoI is reduced by TlpA.

5.3 Material and Methods

5.3.1 Purification

B. japonicum ScoI was purified as previously described (Bühler *et al.*, 2010) except that a size exclusion chromatography step was added to increase protein purity. The affinity-purified Strep-tagII-ScoI fusion protein in Buffer E (100 mM Tris/HCl, pH 8, 150 mM NaCl, 2.5 mM desthiobiotin) was dialysed against 500-1000 volumes of 100 mM NaH_2PO_4/NaOH pH, 7.0, 1 mM EDTA with GeBAflex midi dialysis tubes (Gene Bio-Application Ltd. (GeBA), Yavne, Israel). After dialysis, DTT was added to a final concentration of 10 mM, and the solution was incubated overnight at 4 °C. Subsequently, it was loaded on a Superdex 75 16/60 column (GE Healthcare, Chalfont St. Giles, UK) equilibrated with degassed 100 mM NaH_2PO_4/NaOH, pH 7.0, 1 mM EDTA. Chromatography was run on an Aekta purifier (GE Healthcare, Chalfont St. Giles, UK) at a flow rate of 1 ml/min. ScoI eluted as a monomer at the expected retention volume. Peak fractions were analyzed with SDS-PAGE and ScoI-containing fractions were pooled.

5.3.2 CD spectra measurements

Far-UV CD spectra from 200 to 250 nm were recorded on a JASCO J-710 CD spectropolarimeter in a 20 mM NaH_2PO_4/NaOH, pH 7.0 buffer containing 1 mM EDTA. The analyzed ScoI samples were either oxidized with 1 mM diamide (Sigma-Aldrich) or reduced with 1 mM DTT.

5.3.3 Separation of $ScoI_{red}$ and $ScoI_{ox}$ using reversed-phase HPLC

$ScoI_{ox}$ and $ScoI_{red}$ were separated with reversed-phase high performance liquid chromatography under the following conditions:

a) with a prepacked Nucleosil 120-5 C18 RP-HPLC column (Macherey-Nagel, Düren, D) a gradient from 40 to 46 % acetonitrile in 15 minutes allowed separation of the peaks (FIG. 5. 1).
b) with a Chromolith C18 RP-HPLC column (Merck, Darmstadt, D) a gradient from 38 to 40% acetonitrile in 16 minutes allowed separation of the two redox species, the oxidized being again the first one to elute.

5.3.4 Reversed-phase HPLC conditions to separate $ScoI_{red/ox}$ and $DsbA_{red/ox}$

Neither the Nucleosil 120-5 C18 column nor the Chromolith C18 column that were used to separate $ScoI_{red}$ and $ScoI_{ox}$ allowed separation of the two redox species of $DsbA_{red}$ and $DsbA_{ox}$. On the contrary, a Chromolith C8 HPLC column (Merck, Darmstadt, D) allowed separation of the redox species of both ScoI and of DsbA (FIG. 5.2). Elution conditions were isocratic at 38 % acetonitrile for 12 minutes for $ScoI_{red}$ and $ScoI_{ox}$ and isocratic at 40 % acetonitrile for 12 minutes for $DsbA_{red}$ and $DsbA_{ox}$.

5.3.5 Determination of the redox potential of the active-site cysteines 74 and 78 of ScoI

The redox potential of the active-site cysteines of ScoI was determined through equilibration with an excess of DTT_{ox} and DTT_{red} at different ratios. Reduced ScoI (2 µM final concentration) was incubated in 100 mM NaH_2PO_4, pH 7.0, 1 mM EDTA (containing 100, 10, 1, 0.5, 0.1, 0.01 or 0.001 mM DTT_{red} (final concentration) and between 19 and 114 mM DTT_{ox} (final concentration), in order to cover a redox potential range between –290 and –160 mV. Samples were incubated overnight under Argon atmosphere to minimize O_2-mediated oxidation, quenched with 10 % formic acid (v/v) the next day and subsequently separated with reversed-phase HPLC (injection volume 100 µl). HPLC elution profiles were quantified with the PeakFit software by integration of the peak area of the oxidized and

reduced ScoI. This allowed us to determine the fraction of reduced and oxidized ScoI of defined sample conditions with known reference redox potentials.

The equilibrium data were fitted according to the equation

$$R=\frac{[DTTred]/[DTTox]}{Keq+[DTTred][DTTox]} \qquad [1]$$

Where R is the fraction of reduced ScoI and K_{eq} is the equilibrium constant.

The equilibrium constant K_{eq} was then inserted into the Nernst equation [2] from which the redox potential of ScoI could be deduced.

$$E_0'= E_0-\frac{RT}{nF}*\ln K \qquad [2]$$

Where E_0' is the redox potential of ScoI and E_0 the redox potential of the reference redox couple, namely $DTT_{red/ox}$ which corresponds to –307 mV.

The same approach was applied with another reference redox couple, namely GSH/GSSG, except that a concentration of 4 µM ScoI was used and that the reference redox potentials ranged from –240 to –60 mV. Final concentration of GSSG was constantly 1.5 mM, and the final concentration of GSH varied between 38 µM and 47.4 mM.

Finally, in a third approach, we measured the redox potential of ScoI by determining the equilibrium constant between ScoI and purified *E. coli* DsbA which has a known redox potential of –122 mV (Wunderlich & Glockshuber, 1993). $DsbA_{ox}$ and $ScoI_{red}$ were incubated overnight at different ratios (from 2:1 to 1:8) under Argon atmosphere. After quenching with 10 % formic acid, the two redox species of ScoI were separated on a Chromolith C18 HPLC column as described before.

$$Keq=\frac{[ScoIox]*[DsbAred]}{[ScoIred]*[DsbAox]} \qquad [3]$$

Mathematical rephrasing leads to:

$$(b-1)\,x^2 - K_{eq}x\,(a+b) + K_{eq}\,(ab) = 0 \qquad [4]$$

Where a is initial $[ScoI_{red}]$, b is initial $[DsbA_{ox}]$, and x is the concentrations of both products $[ScoI_{ox}]$ and $[DsbA_{red}]$

Further rephrasing leads to:

$$x = \frac{k(a+b)-\sqrt{(k(a+b))^2-4(k-1)*k(ab)}}{2(k-1)} \qquad [5]$$

Despite performing the incubation under Argon atmosphere, spontaneous oxidation takes place at a low level. This required the introduction of a correction term into the calculation. As an approximation, it was assumed that the amount of air oxidation can be treated as if more $DsbA_{ox}$ had been added. This term was determined to be $1*10^{-6}$ M and was added to b, resulting in

$$x = \frac{k(a+b+0.000001)-\sqrt{(k(a+b+0.000001))^2-4(k-1)*k(ab+0.000001)}}{2(k-1)} \qquad [6]$$

Function [6] was used to fit the obtained data.

5.3.6 Determination of free thiols

Free thiol groups of purified protein were determined with Ellman's reagent (DTNB). Measurements were carried out as previously described (Wunderlich & Glockshuber, 1993) except that the used protein concentrations was lower (21 µM).

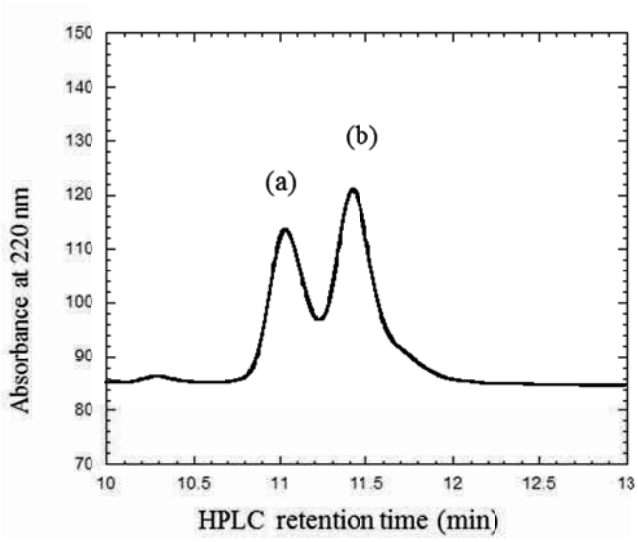
5.3.7 Fluorescence spectroscopy

A HITACHI F 4500 fluorescence spectrometer was used to record the spectra between 300 and 460 nm. *E. coli* $DsbA_{ox}$ and $ScoI_{red}$ were mixed in equimolar amounts in 100 mM NaH_2PO_4, pH 7.0, 1 mM EDTA, and fluorescence increase at 325 nm was followed over time.

5.4 Results

5.4.1 Separation of $ScoI_{ox}$ and $ScoI_{red}$ Separation of the two redox species of ScoI is shown in FIG. 5.1.

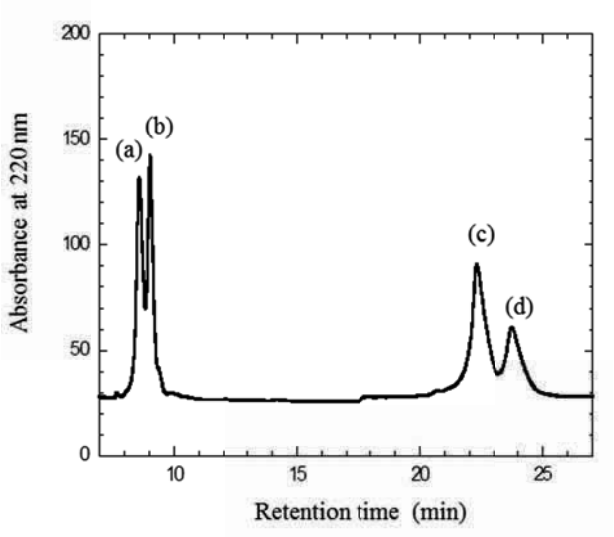
FIG. 5.1. HPLC elution profile of a mixture of $ScoI_{ox}$ (a) and $ScoI_{red}$ (b) from a Nucleosil 120-5 C18 RP- HPLC column. The oxidized ScoI elutes approximately 30 seconds before the reduced.



5.4.2 Separation of $ScoI_{ox}$, $ScoI_{red}$, $DsbA_{ox}$, and $DsbA_{red}$

Separation of the redox species of ScoI and DsbA is shown in FIG. 5.2.

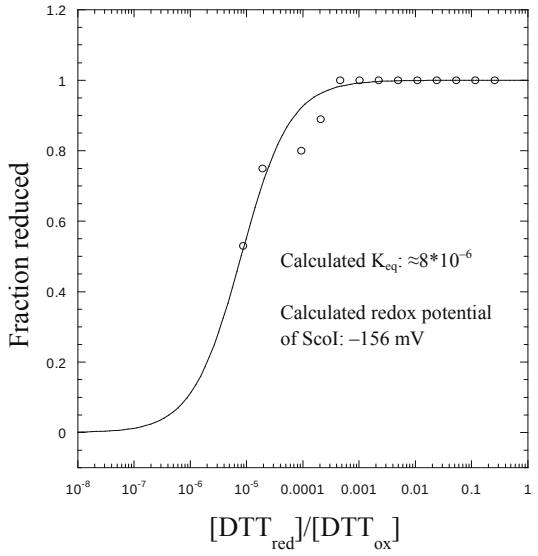
FIG. 5.2. HPLC elution profile of $ScoI_{ox}$ (a), $ScoI_{red}$ (b), $DsbA_{ox}$ (c) and $DsbA_{red}$ (d). The oxidized species of both proteins elute shortly before the reduced. Separation was performed on a Chromolith C8 HPLC column.



5.4.3 Redox potential determination of ScoI with DTT

The far-UV CD spectra of reduced and oxidized ScoI show that both redox species are folded, and they display the features that would be expected from a protein composed of α–helices and β–sheets (data not shown). Moreover, the actual redox state of $ScoI_{red}$ and $ScoI_{ox}$ was confirmed with an Ellman's test (data not shown). The redox potential of ScoI was found to be –156 mV when determined with the DTT redox couple. However, due to the low redox potential of DTT (–307 mV), it was not possible to adjust redox equilibria below –160 mV. Meaning that measuring points in the less reducing range are lacking. Nevertheless, it was possible to obtain a good estimation of the redox potential of ScoI with a $K_{eq} \approx 8*10^{-6}$ and $E_0 \approx -156$ mV (FIG. 5.3).

FIG. 5.3. Determination of the redox potential of ScoI from its redox equilibrium with DTT_{red}/DTT_{ox}. The fraction of reduced ScoI is plotted against the reference redox couple, total concentration of ScoI was 2μM. Equilibration was performed overnight at pH 7.0 and 25°C.



$$K_{eq} = \frac{[ScoIox]*[DTTred]}{[ScoIred]*[DTTox]}$$

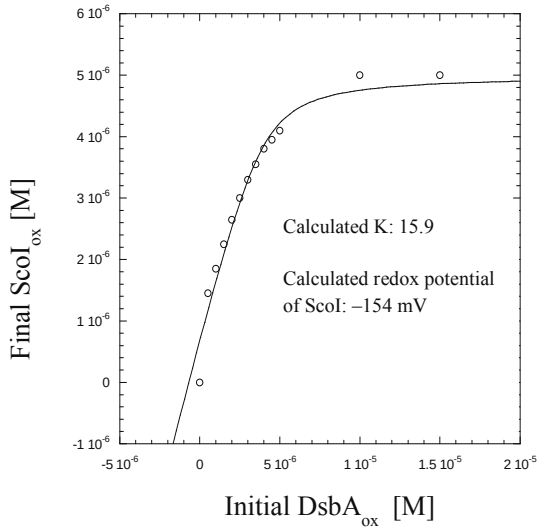
5.4.4 Redox potential determination of ScoI with GSH/GSSG

The redox potential of ScoI could not be determined with glutathione. It was not possible to oxidize more than approximately 45% of ScoI with glutathione. The reason for that is not clear, possibly the reaction is disturbed by the formation of mixed disulfides.

5.4.5 Redox potential determination of ScoI with $DsbA_{ox}$

In a preexperiment we ensured that ScoI and DsbA are actually capable of undergoing disulfide exchange. To do so, we made use of the tryptophan fluorescence properties of DsbA which increases about threefold upon reduction of the disulfide (Wunderlich & Glockshuber, 1993; Zapun *et al.*, 1993), and indeed, after mixing $ScoI_{red}$ and $DsbA_{ox}$, an increase of fluorescence could be observed (data not shown). By inserting the various concentrations of educts and the measured concentrations of the products (FIG. 5.4) into equation [6], we could determine K_{eq} of the redox pairs $ScoI_{red}/ScoI_{ox}$ and $DsbA_{ox}/DsbA_{red}$ to be 15.9 and the redox potential to be –154 mV. This result is very similar to what was found in the redox potential determination with DTT_{red}/DTT_{ox}.

FIG. 5.4. Determination of the redox potential of ScoI from a redox titration of $ScoI_{red}$ with $DsbA_{ox}$. The final concentration of $ScoI_{ox}$ is plotted against the initial concentration of $DsbA_{ox}$. Total concentration of ScoI was 5µM. Equilibration was performed overnight at pH 7.0 and 25°C.



$$K_{eq} = = \frac{[ScoIox]*[DsbAred]}{[ScoIred]*[DsbAox]}$$

5.5 Discussion

To our knowledge, this is the second study that has determined the redox potential of a Sco-like protein. The first Sco-protein for which this was done was the human Sco1 and in that case the redox potential was determined to be –277 mV (Banci *et al.*, 2007c). This is in contrast with the value that was determined in our study which is more oxidizing and thus in accordance with the observed properties of the respective proteins. We determined the redox potential of ScoI by two different approaches. When the redox potential determination was done with DTT_{red}/DTT_{ox}, the redox potential of ScoI was measured to be –156 mV and when it was done with $DsbA_{ox}$ it turned out to be –154 mV. However, both measurements have a flaw. When DTT is the reference redox pair, the difficulty resides in the technical impossibility to establish a reference redox potential more oxidizing than –160 mV (which corresponds to a $[DTT_{ox}]/[DTT_{red}]$ of $1.145 * 10^5$). And, when DsbA is used, a correction factor for the atmosphere-mediated, exogenous oxidation needs to be introduced for the quantification. To overcome these flaws, another approach was designed. As it was possible to establish a HPLC protocol to separate all four redox species of ScoI and DsbA in one single run of HPLC (FIG. 5.2), it will be possible in the future to determine the redox potential of ScoI avoiding the need of a correction factor for exogenous oxidation, because this will affect both partners similarly.

In their study, Abriata and colleagues showed that in *T. thermophilus* SUII of cytochrome oxidase, the cysteine residues Cys149 and Cys153, two of the highly conserved residues forming the Cu_A binding site, can undergo disulfide bridging. If this occurs, the copper cofactor cannot bind and no functional cytochrome can be made (Abriata *et al.*, 2008). Since the Cu_A-binding consensus sequence is universally conserved, it can be expected that the corresponding residues Cys229 and Cys233 of *B. japonicum* cytochrome oxidase SUII (CoxB) can also undergo disulfide bonding. It has been proposed that TlpA, a periplasmic thioredoxin-like protein that shows insulin reduction activity, is the enzyme that assumes this reducing function in *B. japonicum* (Loferer *et al.*, 1995), but so far no experimental data substantiated this hypothesis. Another putative candidate to assume this function is ScoI. First, because a Δ*scoI* mutant has a aa_3 cytochrome oxidase assembly defect. Secondly, because ScoI has a thioredoxin-like fold, which suggests disulfide-reducing function. And, thirdly, because this function has been shown for the *T. thermophilus* Sco homolog (Abriata *et al.*, 2008). Unfortunately, at the level of the oxidase, we cannot experimentally discriminate between a cytochrome oxidase which is not properly formed because of an undesired disulfide bridge in the Cu_A-binding site and a cytochrome oxidase that is defective because the copper cofactor has not been delivered. The goal was therefore to gather more information on the redox properties of ScoI in order to substantiate either the copper-chaperone hypothesis or the Cu_A cysteine disulfide reductase hypothesis. The ScoI redox potential of about –155 mV makes the latter possibility more unlikely because reducing thioredoxins typically

have redox potential below –200 mV. Thus, our results are more in favor of the copper-chaperone hypothesis. This is in accordance with the reversibility of the aa_3 cytochrome oxidase assembly defect of a Δ*scoI* mutant by addition of Cu(II) into the medium.

Another important question regarding ScoI concerns its function in symbiosis with soybean. It has been shown that a Δ*scoI* deletion mutant shows a defect in symbiosis whereas an aa_3 cytochrome oxidase deletion mutant does not. Therefore, ScoI must assume another function under symbiotic conditions that is unrelated to its contribution in the biogenesis of aa_3 cytochrome oxidase. Whether this unknown target relies on a metallochaperone function of ScoI or a thiol-disulfide oxido-reductase activity is unclear. However, a redox potential of –155 mV argues against ScoI being a disulfide reductase and, thus, it seems more likely that this symbiotically-relevant protein requires a copper-chaperone activity, or perhaps a dithiol-oxidase activity of ScoI. Nevertheless, the latter appears unlikely as *B. japonicum* encodes a putative DsbA (Bll2497) protein (FIG. 5.5) which is, at least in the case of the *E. coli* homolog, highly reactive and capable of introducing disulfide bridges at a rate near to the diffusion limit (Zapun *et al.*, 1993).

As outlined in the introduction, the similar phenotype of Δ*scoI* and of Δ*tlpA* mutants is striking. TlpA and ScoI also share other properties: both of them are periplasmic and their active sites consist of cysteines. In the case of TlpA, these cysteines are separated by two amino acids and they exhibit thiol-disulfide oxidoreductase activity. It has been proposed that the respiratory defect of a Δ*tlpA* mutant can be explained by disulfide-reducing activity of TlpA on the cysteines of the Cu_A center of SUII of the aa_3 cytochrome oxidase (Loferer *et al.*, 1993; Loferer & Hennecke, 1994). This hypothesis can now be expanded. TlpA might act not only on the cytochrome oxidase subunit II itself, but as well (or alternatively) on ScoI. The cysteine pairs of both the subunit II of the oxidase and ScoI must be in a reduced state in order to bind copper. There is convincing evidence that cysteine oxidation by DsbA happens cotranslationally. It is suggested that DsbA randomly introduces cysteine bridges into the nascent target proteins upon their entry into the periplasm (Kadokura & Beckwith, 2009). The *B. japonicum* gene bll2497 encodes a putative DsbA protein. Bll2497 shares 39% similarity with the *E. coli* DsbA, and the active-site residues CPVC are conserved (FIG. 5.5). If *B. japonicum* Bll2497 functions similarly as *E. coli* DsbA, then it is likely that upon entering the periplasm, ScoI and cytochrome oxidase subunit II active-site cysteines get oxidized. Since they are incapable of assuming their cellular functions in that redox state, they rely on a system which would reduce these cysteines. The thioredoxin-like protein TlpA is an ideal candidate to assume such a function, although there are also other related proteins which might play this role as well. CcmG (Blr0471) is another putative thiol-disulfide reductase with a CXXC motif, and the same holds true for the protein encoded by blr0124 (FIG. 5.6).

FIG. 5.5. ClustalW alignment of *E. coli* DsbA and *B. japonicum* Bll2407. The active-site residues are highlighted in bold.

```
E.c. DsbA         --------------------MKKIWLALAGLVLAFSASAAQYED---GKQYTTLEKPVAG 37
B.j. Bll2497      MGTSGKTTPIREPTLIITRRAFTTMLSLTGLAAVAGFSPLRFISDAMAQAAGDVAKPVSL 60

E.c. DsbA         ----------APQVLEFFSFFCPHCYQFEEVLHISDNVKKKLPEGVKMTKYHVNFMGGDL 87
B.j. Bll2497      PDMALGPKDAAVTITEFASMTCPHCAAFNEQVFP--KIKAEYIDTGKIRYVFREFP-LDI 117

E.c. DsbA         GKDLTQAWAVAMALGVEDK---VTVPLFEGVQKTQTIRSASDIRDVFINAGIKGEEYDAA 144
B.j. Bll2497      KAAAGSMLSRCIANGDAPKYFAVTDMLFRQQNDWVMKNTTETLTRIGKQAGLTQQQVEAC 177

E.c. DsbA         WNSFVVKSLVAQQEKAAADVQLRG-VPAMFVNGKYQLNPQGMDTSNMDVFVQQYADTVKY 203
B.j. Bll2497      LKDQALLDKIAADQKYASDVLKVDSTPTFFINGEKIKG---------EASFEEFAKKINP 228

E.c. DsbA         LSEKK 208
B.j. Bll2497      LLKS- 232
```

FIG. 5.6. ClustalW alignment of three paralogous thioredoxins of *B. japonicum* namely TlpA, CcmG and Blr0124. The active-site cysteines are highlighted in bold.

```
TlpA  bll1380         MLDTKPSATRRIPLVIATVAVGGLAGFAALYGLGLSRAPTGDPACRAAVATAQKIAPLAH 60
      blr0124         ------------------------------------------------------------
CcmG  blr0471         MSEQSTSANPQRRTFLMVLPLIAFIGLALLFWFRLG---SGDPSR-----------IPS 45

TlpA  bll1380         GEVAALTMASAPLKLPDLAFEDADGKPKKLSDFRGKTLLVNLWATWCVPCRKEMPALDEL 120
      blr0124         ---------------PQLRLQDLHGKDVVLTARPGRITLVNFWATWCAACRLDLPVLASL 45
CcmG  blr0471         ALIGRPAPQTALPPLEGLQADNVQVPGLDPAAFKGKVSLVNVWASWCVPCHDEAPLLTEL 105

TlpA  bll1380         QGKLSGPNFEVVAINIDTRDPEKPKTFLKEANLTRLGYFNDQKAKVFQDLKAIGRALGMP 180
      blr0124         VGSRPD-RLDVVAICTDTKDLRKIRAFLGGLAVQNLACYVDAYDTTAEASASAFTLVGMP 104
CcmG  blr0471         G---KDKRFQLVGINYKD-AADNARRFLGRYGNP----FGRVGVDANGRASIEWGVYGVP 157

TlpA  bll1380         TSVLVDPQGCEIATIAGPAEWASEDALKLIRAATGKAAAAL 221
      blr0124         VTYLVGASNHVEGYITGAPDWLSPAGARLLQFYREQA---- 141
CcmG  blr0471         ETFVVGREGTIVYKLVGPITPDNLRSVLLPQMEKALK---- 194
```

The hypothesis of TlpA playing a role in reducing the disulfide bridges that are unspecifically formed by DsbA is quite attractive because this function would allow for a variety of targets, as it was postulated from the pleiotropic phenotype of a Δ*tlpA* mutant. It would also explain why the symbiotic defect of a Δ*tlpA* mutant is more pronounced than the phenotype of a Δ*scoI* mutant: TlpA might also have symbiotically relevant targets other than ScoI.

In the present study, the redox potential of ScoI was determined to be about –155 mV which makes ScoI far less reducing than TlpA for which the redox potential was determined to be –230 mV

(Loferer *et al.*, 1995). Therefore, a thiol-disulfide oxidoreduction reaction in which TlpA is the reducing partner and ScoI the oxidizing partner is, from an electrochemical point of view, well possible. However, this is no proof. Therefore, a direct interaction between the two partners should be investigated. It has been attempted to trap mixed disulfides of the two proteins by mixing purified variants of them having one of the two active-site cysteines mutated to alanine. However, so far, the results were not reproducible and thus difficult to interpret. Another approach to prove that an oxidoreduction is taking place between ScoI and TlpA active-site cysteines is to assess this reaction *in vitro*. To do so, purified $TlpA_{red}$ and purified $ScoI_{sol}$ will be mixed and the reaction, if it takes place, can be followed fluorimetrically, because the intrinsic fluorescence of TlpA at 355 nm decreases 10-fold upon formation of the disulfide bridge in the active site (Loferer *et al.*, 1995).

CHAPTER 6

Effect of copper on genome-wide gene expression in *Bradyrhizobium japonicum*

6.1 Abstract

B. japonicum is the intracellular nitrogen-fixing symbiont of soybean; however, it is also capable of undergoing a free-living lifestyle in the soil. Agricultural soils feature variable concentrations of copper (Jo & Koh, 2004). Moreover, copper is simultaneously essential and toxic for *B. japonicum*. Thus, tight copper homeostasis is necessary. In our transcriptional profiling approach we identified genes that are involved in adaptation to either excess copper or to copper deprivation. A putative multi-copper oxidase (CopA) and other putative copper tolerance proteins were induced under copper excess. On the other hand, bacteria that were grown in copper-depleted medium induced the expression of two putative copper import operons. For one of them, namely *bll4878-82*, preliminary results of the phenotypical analysis of the deletion mutant seem to confirm the proposed function (F. Serventi, unpublished). The other one, *bll0883-89,* has not been studied yet. Moreover, when copper is poorly available, *B. japonicum* induces synthesis of a heme-copper quinol oxidase (encoded by *blr0149-blr0152*) and of an associated SURF-like protein, possibly involved in heme insertion into the oxidase. This is surprising because this oxidase depends on copper for its function. Rather one might have expected the derepression of respiratory oxidases that lack a copper site (e.g. *bd*-type quinol oxidases). Expression of a recently described gene encoding a copper trafficking protein, ScoI, was also induced under copper starvation, suggesting that not only the machinery for copper import is positively upregulated under these conditions, but also at least parts of the copper distribution machinery in the periplasm.

6.2 Introduction

The Earth's crust is composed of approximately 0.00007 % of copper (Kim *et al.*, 2008) and it is regarded as a "modern biometal" as it was only very poorly bioavailable before the atmosphere became oxic. Cu(II) is soluble in water whereas Cu(I) is insoluble. Its fairly positive redox potential implies that in a reducing atmosphere before the onset of photosynthesis, reduced (insoluble) forms of copper dominated (Ochiai, 1983). Copper is a trace element essential to most organisms (Linder, 2001) although there are exceptions as there are in many archaeal and a few bacterial phyla that lack genes for all known cuproenzmes in their genomes (Ridge *et al.*, 2008). There is a correlation between oxic life style and copper utilization. While 73% of the available genomes from anaerobes lack genes encoding copper-dependent proteins, 94% of the available genomes from aerobes encode such genes (Ridge *et al.*, 2008). With regard to its use as a biometal, copper is the second most utilized transition metal; only iron is more frequently used (Solioz & Stoyanov, 2003). The Cu(I) (cuprous)/ Cu(II) (cupric) pair has a redox potential of +200-750 mV when liganded by proteins, making it an ideal

metal for electron transfer events and thus a convenient cofactor for enzymes catalyzing redox reactions (Crichton & Pierre, 2001). A total of 10 cuproproteins have been characterized in prokaryotes: cytochrome *c* oxidase, NADH dehydrogenase-2, Cu,Zn-superoxide dismutase, nitrocyanin, plastocyanin, Cu-containing nitrite and nitrous oxide reductase, Cu amine oxidase, particulate methane monooxygenase, Cu-dependent laccase and tyrosinase (Ridge *et al.*, 2008). Two classes of enzymes utilize copper, namely transferases and oxidoreductases, the latter being the more prominent cuproenzymes since about 90% of the oxidoreductases feature one or several copper binding site (Waldron *et al.*, 2009). The most widespread copper-dependent oxidoreductases are the heme-copper oxidases and the Cu,Zn-superoxide dismutases. Heme-copper oxidases are the terminal enzymes of the respiratory chain (complex IV) and they harbor at least one, sometimes two, copper centers. Copper center B (Cu_B) is common to all heme-copper oxidases, its copper atom is closely associated with a high-spin heme and is the site of O_2 reduction. Copper center A (Cu_A) is present in subclass A of heme-copper oxidases; it is binuclear and of mixed valence (García-Horsman *et al.*, 1994; Castresana *et al.*, 1994; Iwata *et al.*, 1995; Pereira *et al.*, 2001). Cu,Zn-superoxide dismutase converts superoxide radical to oxygen and hydrogen peroxide and plays an essential role in the resistance against oxidative stress (Bertini *et al.*, 1998). Heme-copper oxidases and Cu,Zn-superoxide dismutases catalyze reactions that are essential for energy conservation and oxidative stress resistance, respectively. These are two crucial cellular functions; therefore, it is comprehensible that copper is indispensable for most organisms. An *in silico* search of the *B. japonicum* genome totalizes 19 genes encoding homologs of known cuproenzymes (TABLE 6.1). Nine of them are subunits (I or II) for heme-copper oxidases, two of them are involved in anaerobic respiration namely nitrous oxide reductase and nitrite reductase. Another two are putative multi-copper oxidases, there is one NADH dehydrogenase 2, and finally there are 5 members of the plastocyanin family (Ridge *et al.*, 2008). RegS, and Blr0154, two homologous sensor kinases belonging to two-component regulatory systems were added to TABLE 6.1. Copper dependency was shown in *R. sphaeroides* for RegB, (Swem *et al.*, 2003) and for RegS in *B. japonicum* (Fischer, unpublished data). Copper binding has been demonstrated for RegB *in vitro* (Swem *et al.*, 2003) and seems very likely for RegS and Blr0154 as well. FIG. 6.1 depicts an alignment of RegB, RegS and Blr0154. The putative copper chaperones that are discussed in chapters 2 and 7 as well as the putative copper-transporting P-type ATPases that are mentioned in chapter 6 and 7 were also added to TABLE 6.1.

TABLE 6.1. List of putative copper-binding proteins of *B. japonicum.*

ORF number	Gene name	Description	Copper site type
blr1171	*coxA*	SUI of heme-copper cytochrome oxidase (aa_3-type)	Cu_B
bll3784	*coxN*	SUI of heme-copper cytochrome oxidase (aa_3-type)	Cu_B
blr0150	*cyoB*	SUI of heme copper quinol oxidase	Cu_B
blr2715	*coxX*	SUI of heme copper quinol oxidase	Cu_B
bll4480		SUI of heme copper oxidase	Cu_B
blr2763	*fixN*	SUI of heme-copper cytochrome oxidase (cbb_3-type)	Cu_B
blr1170	*coxB*	SUII of heme-copper cytochrome oxidase (aa_3-type)	Cu_A
bll3785	*coxM*	SUII of heme-copper cytochrome oxidase (aa_3-type)	Cu_A
bll4481		SUII of heme copper oxidase	Cu_A
bll2292	*petE*	Probable plastocyanin	Type I *
bll3015		Putative amicyanin	Type I *
blr2559	*amcY*	Putative amicyanin	Type I *
bll1555		Putative plastocyanin	Type I *
bll2209	*copC*	Copper tolerance protein	Type I *
blr0315	*nosZ*	Nitrous oxide reductase	Cu_A and Cu_Z
blr3727		NADH dehydrogenase, putative copper reductase	CXXC **
blr7089	*nirK*	Nitrite reductase	Type I * and Type II***
bll2210	*copA*	Multicopper oxidase	Type I and trinuclear #
bll2293		Putative multicopper oxidase	Type I and trinuclear #
blr0905	*regS*	Two-component sensor histidine kinase	##
blr0154		Two-component sensor histidine kinase	##
blr1131	*scoI*	Cytochrome *c* oxidase assembly factor	CXXXC
blr1174	*coxG*	Cytochrome *c* oxidase assembly factor	CXC
bll4480		PCu_AC-like protein	$H(M)X_{10}MX_{21}HXM$ motif Ψ
bll7088		PCu_AC-like protein	$H(M)X_{10}MX_{21}HXM$ motif Ψ
blr0319	*nosL*	Putative nitrous oxide assembly factor	unknown
blr2769	*fixI*	Putative copper-importing ATPase	CXC ΨΨ
bll0700		Putative copper-importing ATPase	CXC ΨΨ

* Type 1 copper center: binding is mediated by 2 histidines, 1 cysteine + another residue

** Proposed Cu-binding site, in analogy with *E. coli* copper reductase NADH2 (Rapisarda *et al.*, 2002)

*** Type 2 copper center: binding is mediated by 4-5 residues, among those, 1-4 can be histidines, the remaining residues are O or S ligands

Type 1 + trinuclear site mediated by a total of 10 histidines, 1 methionine and 1 cysteine , (Lawton *et al.*, 2009; Fernandes *et al.*, 2010)

For the homolog RegB, a pyramidal or tetragonal geometry with O- and N-ligands is proposed. (Swem *et al.*, 2003)

Ψ As previously described (Abriata *et al.*, 2008)

ΨΨ In analogy with the *E. hirae* copper-ATPase CopA (Magnani & Solioz, 2005)

FIG. 6.1. ClustalW alignment of the two component sensor kinases RegB from *R. capsulatus* and the two *B. japonicum* homologs RegS and Blr0154, the latter is encoded in the vicinity of the copper-starvation induced oxidase. The site of disulfide-bridge mediated dimerization, called redox-box, and the site of autophosphorylation, called H-box, are highlighted and underlined.

```
RegB            --------------------------------MADMMRAVDRPEFDMSASLSHQEWVR-L 27
RegS            --------------------------------MTEIAASDFRP---------AQRHIR-L 18
Blr0154         MLPPAGRDQMLERSSNRPDDGRTPPQAFGELAVTLQSQADARSELIGAAPTDDETNRKNM 60

RegB            RTLILLRWAAVVGQLAALIAAYGYYDIALNLPMCIGTIGFAVAANIAAIYLYPESRRLSQ 87
RegS            DTILRLRWLAVLGQLAAIFIVAQGLEFNVEIVPCVSIIALSASLNLALQTVSNPLQRLEP 78
Blr0154         ALLIQLRWTAVVGQIVTIGGVHFWLGIPLPLERMGAVIGALVLLNVSSLVWVRHRAAISS 120

RegB            AEVTATLLFDTAQLALLLSLTGGLNNPFALLILVPVTIAATALKLRPTLLLGGATIAMIT 147
RegS            MQAAGLLALNIVELAGLLFFTGGLQNPFSFLFLAPVLISATALPARFTFGLGVLAVACAS 138
Blr0154         NELLVALMLDVAALTAQLYLSGGATNPFTSLFLLQVTLGAVLLDARSTWSLVALTCASFV 180

RegB            FVAVFNEPLQTRDGAHIGVPPMIEFGSWVAIVIGVIFLGAYAHRIAQEIHSMSDALFATQ 207
RegS            VLFFFHLPLPWDSDDPLVLPPIYLVGVWLSIVLAIGVTSLYSFQVTEEARKLADALAATE 198
Blr0154         WLTLAYRPLDLPPNPISEAYSLTVAGMLLGFVLNAVLLVVFVTRINRNLRERDAHLAALR 240

RegB            MALSREQKLTDLGGVVAAAAHELGTPLATIKLVSTELA--EELVDDPELHDDAVLIREQA 265
RegS            LVLTREQHLTQLDGLAAAAAHELGTPLATIFLISREL---EKTVKDPIFAADLKTLREQT 255
Blr0154         QHAAEQDHIVRMGLLASGAAHELGTPLASLSVILSDWRRMPDLAADQELAEDLAEMETSL 300
                                  H-box
RegB            ERCRDILRSMGRAG--KDDVHLRTAPLLAVLREAAEPHLDRGKMIYFDVVPGEGGSERQP 323
RegS            QRCRDILSKITQLS--STGAPFDRMKLSELIEEVVAPHRDFGVDIKVRIAVA---AFAEP 310
Blr0154         QRCKSIVTGILVSAGEARGEGSSPTTVTAFVTALVDEWRNARSARTLYFVNTFG---EDV 357
          Redox-box
RegB            TIYRYPELVHALRNLIQNAVDFAQTTVWVDAEWTDRSIIVRVTDDGRGYSPNVLNRIGDP 383
RegS            VGSRNPAILYGVGNIVENAVDFAHTTVEVNAWWNKDTIELLISDDGPGIPPDILNRIGEP 370
Blr0154         AIASDVALKQVIFNVLDNAYEVSRDWVELLAEREGDNLVLSISDRGPGFAPEMLAQLGKP 417

RegB            FISTR---SAERKEYEGMGLGLFIAKTLLERTGAKLRFANGSEPYQKNAPVPRRSGAVVE 440
RegS            YLSRRRTPDDAGGERRGLGLGVFIARTLLERTGAKVSFTN---------RIFPEHGAVVQ 421
Blr0154         YHSSK--------GRAGGGLGLFLVVNVVRKLGGSVTAEN-----------HRKRGATVR 458

RegB            LRWHIGRLIAPETGPLGENVPITA 464
RegS            ITWPRQRFEAIET--LEETIG--- 440
Blr0154         LTLPLATLAIGGS--FDA------ 474
```

Although being essential, copper is also very toxic. The widespread use of copper sulfate pentahydrate as an unspecific bactericidal and fungicidal agent in agriculture illustrates this well. Therefore, the concentration of free copper in living cells is generally very low. In eukaryotes, the concentration was determined to be in the range of 10^{-13} M; for example, in *Sacharomyces cerevisiae*, free copper is restricted to less than one atom per cell (Rae *et al.*, 1999). However, copper toxicity is not fully understood. The most commonly accepted hypothesis is that copper toxicity occurs by production of hydroxyl radicals via Fenton-type reactions (Halliwell & Gutteridge, 1985). However, this type of reaction is unlikely to happen under aerobic conditions because the concentration of hydrogen peroxide is very low in aerobiosis, unlike in hypoxia (Cadenas, 1983). Another widespread opinion is that copper might displace other cations and thereby disturb enzymatic functions (Peña *et al.*, 1999; Macomber & Imlay, 2009). To cope with toxicity, copper resistance mechanisms have evolved early, thus they are highly conserved among the phyla, and similar mechanisms can be found in a range of organisms as different as bacteria and humans (Solioz & Stoyanov, 2003).

To date, *Enterococcus hirae* is the best studied prokaryote with respect to its copper homeostasis. This Gram-positive bacterium is able to grow under conditions ranging from copper limitation to copper stress induced by 8 mM Cu. Copper tolerance relies on the *cop* operon which encodes four genes. CopA is a copper-importing P-type ATPase whereas CopB is a copper-exporting P-type ATPase. CopZ is a copper chaperone sequestering free copper inside the cells and routing it to CopY. The latter is a transcriptional repressor that gets released from the promotor of the *cop* operon upon copper binding (Magnani & Solioz, 2005). In addition to the aforementioned P-type ATPases, the Cus RND efflux systems (Munson *et al.*, 2000; Outten *et al.*, 2001; Franke *et al.*, 2003, Yamamoto & Ishihama, 2005) are the other players achieving an efficient copper export strategy. But exporting copper is not the only way to counteract copper toxicity; another strategy to accomplish this is to sequester copper atoms. This strategy can be pursued either intracellularly via metallothioneins (Sato & Bremner, 1993; Gold *et al.*, 2008) and multi-copper oxidases (Grass & Rensing, 2001; Outten *et al.*, 2001) or extracellularly via exopolysaccharide secretion (Ordax *et al.*, 2010).

Copper export mechanisms have been studied to a certain extent. However, copper import mechanisms only start to become unraveled. Several Gram-positives like *E. hirae*, *Staphylococcus aureus and Listeria monocytogenes*, appear to express copper-importing P-type ATPases (Francis & Thomas, 1997a, Francis & Thomas, 1997b, Lowe *et al.*, 1998, Solioz & Stoyanov, 2003). In the case of non-photosynthetic Gram-negatives, the need for active copper import systems has been doubted for a while. First, because to date there is no identified cytoplasmic cuproprotein in those bacteria; secondly, it was thought that copper can reach the periplasm, where it is known to be needed, by passive diffusion across the outer membrane through porins (Lutkenhaus, 1977). Currently, the view is being wholly revised. Rosenzweig and collegues reported on the existence of methanobactin, a copper-specific equivalent of siderophores that is synthetized and secreted by methanotrophs to sequester copper in their environment (Balasubramanian & Rosenzweig, 2008, Hakemian *et al.*, 2005). Furthermore, we and others had suspected for several years that there are P-type ATPases involved in the biogenesis of cbb_3-type cytochrome oxidase by assisting copper to attain the Cu_B site, which is buried inside the cytoplasmic membrane of *B. japonicum* and *Rhodobacter capsulatus* (Preisig *et al.*, 1996a; Koch *et al.*, 2000; Swem *et al.*, 2005) but this has never been demonstrated in those organisms so far. However, evidence for such a function has been presented recently for CtaP, the *Rubrivivax gelatinosus* homolog of those P-type ATPases (Hassani *et al.*, 2010). In the photosynthetic cyanobacterium *Synechocystis*, the existence of copper-importing P-type ATPase has also been described (Phung *et al.*, 1994). This case is special since it is the only known example where copper crosses the cytoplasmic membrane in a Gram-negative because it needs to reach the thylakoid membrane.

Transcriptional profiling under copper stress has been performed on several bacteria, like *Bacillus subtilis* (Moore & Helmann, 2005) and *Cupriavidus metallidurans* (Monchy *et al.*, 2006). Much less literature data is available for copper starvation. A transcriptome study in *Pseudomonas aeruginosa* showed that the transcription of the copper-independent and cyanide-resistant oxidase was strongly induced when copper was depleted in the growth medium. Moreover, the expression of many genes encoding the iron uptake machinery was repressed; however the autors found no evidence for copper uptake mechanism (Frangipani *et al.*, 2008). Unlike in *B. subtilis*, where copper depletion led to the upregulation of genes enoding YcnJ, a membrane embedded putative copper transporter, and its putative regulator YcnK (Chillappagari *et al.*, 2009). A copper starvation study performed in *S. cerevisiae* and *Schizosaccharomyces pombe* showed that genes encoding metal ion transporters are upregulated whereas genes encoding oxidoreductases and dehydrogenases are downregulated (Rustici *et al.*, 2007).

In the present work we wanted to assess the range of tolerance of *B. japonicum* towards copper and secondly to get an impression of the regulated genes in response to different copper concentrations, the emphasis being on import and export mechanisms and on possible metabolic adaptations.

6.3 Material and Methods

6.3.1 Media and growth conditions

Strains were grown on PSY agar plates supplemented with 0.1% of arabinose and 100 µg/ml of spectinomycin and grown in a first preculture in PSY liquid medium as established in our laboratory (Regensburger & Hennecke, 1983; Mesa *et al.*, 2008). When the late exponential/early stationary phase was reached, the cells were washed twice in Vincent's minimal medium, and subsequently another preculture was inoculated at a starting OD of 0.02 in a buffered Vincent's minimal medium containing copper (referred to as BVM). The medium was prepared according to Vincent (Vincent, 1970) except for the $CuSO_4$ that was omitted, if not stated otherwise, and 10 mM MOPS that were added for buffering at pH 6.8. The exact composition of BVM, including $CuSO_4$, is given in TABLE 6.2.

TABLE 6.2. Composition of BVM (buffered Vincent minimal medium)

Component	**Amount per liter**	**Final concentration**
* KH_2PO_4	2 g	11.5 mM
* K_2HPO_4	2 g	14.7 mM
* NH_4Cl	0.84 g	15.7 mM
Trace element stock solution (1000x)	1 ml	

**** Trace element stock solution (1000x)**			
Component	**Amount per liter**		
H_3BO_3	145 mg		
$ZnSO_4$ * 7 H_2O	108 mg		
$CuSO_4$ * 5 H_2O	5 mg		
Na_2MoO_4 * 2 H_2O	125 mg		
$MnCl_2$ * 4 H_2O	4 mg		
$FeSO_4$ * 7 H_2O	125 mg		
$CoSO_4$ * 7 H_2O	70 mg		
Nitrilotriacetate (NTA)	7 g		
Component	**Stock solutions**	**Final concentration**	**Volume (stock solution) added per liter of medium**
$MgSO_4$ * 7H_2O	*** 1 M	1 mM	1 ml
$CaCl_2$ x 2 H_2O	*** 1 M	0.46 mM	460 µl
$FeCl_3$ x 6 H_2O	*** 1 M	0.037 mM	370 µl
MOPS	*** 1 M	10 mM	10 µl
L-arabinose	*** 30 %	0.3 %	1 ml
NH_3	*** 2 M		**** ~0.833 ml

* these components are mixed and autoclaved separately
** trace element stock solution can be autoclaved
*** these stock solutions are sterile filtered
**** volume is variable, NH_3 is added for pH adjustment to pH 6.8

Main cultures were inoculated at a starting OD of 0.02 with exponentially growing cells that were washed twice in BVM. Main culture volume was 50 ml, and cells were grown in 500 ml Erlenmeyer flasks, at 30°C under vigorous shaking (160 rpm). All glassware used for the main cultures were soaked in 0.1 M HCl (Fluka) for 24 h and rinsed twice with double distilled water to render them copper-free (Frangipani *et al.*, 2008). Copper concentration in BVM without any copper salt added was determined by GF-AAS (Graphite Furnace Atomic Absorption Spectroscopy). The Perkin Elmer AAnalyst 800 with Transversely Heated Graphite Furnace (THGA) with longitudinal Zeeman-effect background corrector was used for the measurement. Copper limiting conditions were obtained by adding the Cu(I) chelator bathocuproine disulphonic acid (BCS; Sigma-Aldrich; final concentration 10 µM) and ascorbic acid (Fluka, final concentration 1 mM) to the liquid cultures. 10 µM BCS was the concentration chosen to perform the transcriptional profiling because at this concentration growth rate was not affected. Since we were interested in the mechanisms that enable *B. japonicum* to adapt to scarce copper we wanted to avoid general stress responses interfering with our specific copper response. This is why we decided to work under relatively mild copper-depletion stress; for simplicity this condition will be called "copper starvation" in the rest of this chapter.

6.3.2 RNA isolation, cDNA synthesis and microarray analysis

Cultures of *B. japonicum* were grown to mid-exponential phase (optical density at 600 nm of 0.4 to 0.5), which was reached after approximately 70 to 80 hours. Cell harvest, RNA extraction, reverse transcription to cDNA, fragmentation, and labeling were done as previously described (Hauser *et al.*, 2006, Hauser *et al.*, 2007). Details of the custom-designed Affymetrix *B. japonicum* gene chip BJAPETHa520090 (Affymetrix, Santa Clara, CA) have also been described previously (Hauser *et al.*, 2007). For each strain, three independent biological replicates were performed. Primary data evaluation was performed with the Affymetrix MAS5.0 algorithm (Agilent Technologies, Palo Alto CA, U.S.A.). Further, the data was processed, normalized and analyzed as previously described (Pessi *et al.*, 2007). Only those probe sets that were detected in at least two thirds of the measurements were further considered for analysis. Genes that had passed the statistical test were considered as differentially expressed if the relative change in expression was ≥2 or ≤–2.

6.4 Results

6.4.1 Copper toxicity in *B. japonicum*

A PSY gradient plate assay showed that *B. japonicum* is not able to grow when the concentration reaches approximately 4 mM Cu (data not shown). In order to determine the maximal concentration of Cu at which *B. japonicum* can sustain normal growth, *B. japonicum* was grown in liquid cultures with different copper concentrations. These measurements are depicted in Fig. 6.2.A. When 250 µM copper was added to the growth medium, the lag phase was slightly longer but the same growth rate was observed. When 1.25 mM of copper was added to the medium, the lag phase was approximately 100 hours longer, probably reflecting time needed for physiologic adaptations the cells have to undergo in order to acquire tolerance. Another interesting observation is that *B. japonicum* is more susceptible to copper stress under anaerobic conditions compared to aerobic conditions (Serventi, unpublished observation), which is in accordance with the more potent generation of hydroxyl radicals via free copper under anoxic conditions (Cadenas, 1983).

6.4.2 Minimal copper concentration required to sustain *B. japonicum* growth

In collaboration with Robert Brogioli and Prof. Detlef Günther (Institute of Inorganic Chemistry, ETH Zürich), a Graphite Furnace Atomic Absorption Spectroscopy experiment was done to determine the copper concentration in BVM medium without any copper salts added. 4.7 nM Cu +/– 3.1 nM was detected and probably this reflects contaminating copper that is introduced with other medium components and double distilled water. *B. japonicum* has a generation time of 19.6 +/–0.5 h when grown with this basal copper concentration of ~4.7 nM Cu. If 2 µM copper was added to BVM, which is considered a standard copper concentration (Regensburger & Hennecke, 1983; Mesa *et al.*, 2008),

B. japonicum had a generation time of 20.4+/– 0.4 h. This means that with ~5 nM Cu, *B. japonicum* can fully cover its needs. This observation made the use of BCS, a copper chelator, obligatory to induce copper starvation, as observed in previous studies with *P. aeruginosa* (Rustici *et al.*, 2007; Frangipani *et al.*, 2008). *B. japonicum* growth was assayed under different BCS concentrations (FIG. 6.2.B). Addition of 10 µM of BCS did not affect growth, whereas 50 µM affected growth as the doubling time increased by approximately 5 hours compared to growth in BVM with ~4.7 nM Cu. The addition of ascorbate did not affect growth (FIG. 6.2.B).

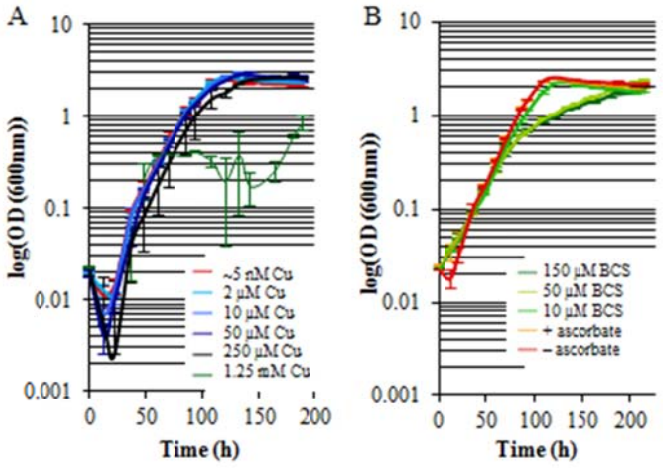


FIG. 6.2. Growth curves of *B. japonicum* in BVM (buffered Vincent minimal medium). *A*, with ~5 nM, 2 µM, 10 µM, 50 µM, 250 µM or 1.25 mM $CuSO_4$ added into the growth media. *B*, the three growth curves represented with green lines contained different BCS concentrations and 1 mM ascorbate to reduce Cu(II) to Cu(I). The culture represented with a red line do not contain BCS, those cultures were done in presence and absence of ascorbate, to ensure that it did not affect *B. japonicum* growth on its own.

6.4.3 Transcription profiling of *B. japonicum* under different conditions

We generated complete transcriptome data sets for 3 copper conditions, namely 10 µM BCS (thereafter called copper starvation), ~4.7 nM Cu (thereafter called basal copper level) and 2 µM Cu (thereafter called copper excess). In our analysis we performed two comparisons. First we compared copper starvation with basal copper levels (TABLE 6.3) A total of 46 genes were increased and 42 decreased in expression under copper-starved conditions compared with basal copper. Thus about 1% of all *B. japonicum* genes are differentially expressed in response to copper starvation. Among the upregulated genes we find *scoI* (4.6x), *surf1* (*blr0153*) (3.4x) and the subunits of a heme-copper quinol oxidase (*blr0149- blr0152*) (between 2.7 and 3.3x). ScoI is, like CoxG, essential for biogenesis of the aa_3-type cytochrome *c* oxidase (Bühler *et al.*, 2010) and Surf1 is one of the two copies of SURF-like proteins that are encoded by *B. japonicum*. Surf1 is predicted to be inserted in the cytoplasmic membrane and its gene neighbors the genes of a heme-copper quinol oxidase (*blr0149-52*). The *shb1* gene (*bll1177*), encoding the other SURF-like protein, is associated with the aa_3 cytochrome oxidase encoded by the *coxBAEFGC* operon. The most highly upregulated genes in copper starvation belong to the putative operon *bll0883-bll0889* and mainly encode components of an ABC-transporter.

TABLE 6.3. Genes differentially expressed by a factor ≥2 or ≥–2 under copper starvation (10 µM BCS) compared with basal copper level (5 nM Cu). The genes in bold belong to putative operons, the most strongly regulated gene of each operon is shifted to the right.

Gene number*	Gene name*	Description*	Fold change
Genes with increased expression in copper starvation			
bll0889		putative transport protein belonging to MFS superfamily	68.44
bll0888		hypothetical protein	12.72
bll0884		ABC transporter permease protein	5.60
bll0883		ABC transporter permease protein	5.54
bll0885		ABC transporter ATP-binding protein	4.02
bll0887		ABC transporter substrate-binding protein	4.02
bll0886		ABC transporter ATP-binding protein	3.83
bll1200		5-aminolevulinic acid synthase	5.15
bll1199		two-component hybrid sensor and regulator	2.35
blr1201		3-dehydroquinate dehydratase	2.13
trnT-UGU		tRNA-Thr(TGT)	7.84
blr1131	*scoI*	cytochrome *c* oxidase assembly factor	4.58
blr0241		1-aminocyclopropane-1-carboxylate deaminase	4.22
trnR-CCU		tRNA-Arg(CCT)	4.11
blr4637		probable HspC2 heat shock protein	3.79
trnL-UAA		tRNA-Leu(TAA)	3.64
trnA-GGC		tRNA-Ala(GGC)	3.63
bll1418		methionine synthase	3.57
bll1419		5,10-methylenetetrahydrofolate reductase	3.46
blr0153	*surf1*	probable surfeit locus protein 1	3.44
blr0150		heme-copper oxidase subunit I	3.26
blr0149		heme-copper oxidase subunit II	3.16
bll0148		MFS permease	3.06
blr0151		heme-copper oxidase subunit III	2.66
blr0152		heme-copper oxidase subunit IV	2.36
bll2757		transcriptional regulatory protein Crp family	3.39
blr2221		adenosylmethionine-8-amino-7-oxononanoate aminotransferase	3.15
blr2222		D-amino acid dehydrogenase, small subunit	2.13
bsr5148		unknown protein	2.96
blr7274		serine protease	2.94
bll4983		hypothetical protein	2.91
blr0581		bifunctional purine biosynthesis protein	2.76
bll5912		serine hydroxymethyltransferase	2.71
blr4417		unknown protein	2.69
bll4303		putative amidase	2.61
blr2476		hypothetical protein	2.43
blr4994		hypothetical protein	2.27
bll4302		unknown protein	2.23
bll8048		unknown protein	2.22
blr2641		hypothetical protein	2.22
bsl2596		unknown protein	2.22
blr3045		unknown protein	2.21
blr2763		cytochrome-c oxidase	2.16
bll0507		hypothetical protein	2.15
blr1093		phosphate ABC transporter permease protein	2.13
bll5476		formate dehydrogenase iron-sulfur subunit	2.05

TABLE 6.3 (continued)

Genes with decreased expression in copper starvation		
blr2891	putative phenylacetic acid degradation protein	-2.01
blr5226	heat shock protein	-2.01
bll4429	hypothetical protein	-2.04
blr1402	hypothetical protein	-2.07
blr2316	probable NADH-ubiquinone oxidoreductase chain	-2.11
blr4948	nitrogen regulatory protein PII	-2.13
blr4687	aspartate-semialdehyde dehydrogenase	-2.19
blr1123	ABC transporter sugar-binding protein	-2.25
bll4057	probable substrate-binding protein	-2.27
bll3146	hypothetical protein	-2.28
bsl5891	hypothetical protein	-2.28
blr2317	formate dehydrogenase alpha subunit	-2.31
bll3876	aldehyde dehydrogenase	-2.42
bll5219	small heat shock protein	-2.44
bsr6573	unknown protein	-2.96
bll6513	hypothetical protein	-2.96
blr5860	transcriptional regulatory protein GntR family	-2.21
bll5859	unknown protein	-3.05
bsr0350	unknown protein	-3.77
bll0334	hypothetical protein	-2.01
blr0335	putative carbon monoxide dehydrogenase small chain	-2.09
blr0336	carbon monoxide dehydrogenase large chain	-2.16
blr0337	putative carbon monoxide dehydrogenase medium chain	-2.33
bll0330	two-component response regulator	-2.86
bll0331	two-component response regulator	-2.58
bll0333	probable alcohol dehydrogenase precursor	-3.60
bll0332	unknown protein	-4.50
bll3149	putative oxalate:formate antiporter	-2.67
bll3148	hypothetical protein	-3.18
bll3150	putative oxalate:formate antiporter	-5.49
blr6334	dihydrolipoamide dehydrogenase	-4.85
blr6332	2-oxoisovalerate dehydrogenase beta subunit	-4.24
blr6333	lipoamide acyltransferase component	-4.72
blr6331	2-oxoisovalerate dehydrogenase alpha subunit	-5.59
blr3167	putative hydroxypyruvate isomerase protein	-4.95
blr3166	putative glyoxylate carboligase protein	-5.32
blr3168	oxidoredutase	-6.33
blr3160	transcriptional regulatory protein IclR family	-4.07
bll3156	probable formyl-CoA transferase	-4.83
bll3157	oxalyl-CoA decarboxylase	-5.21
bll3158	transcriptional regulatory protein GntR family	-3.50
blr3159	hypothetical protein	-10.0

*according to Rhizobase

Secondly, we compared basal copper level with copper excess (TABLE 6.4). Twelve genes were upregulated and 5 were downregulated under copper excess. Among the upregulated genes we find the genes of the *copBAC* operon, these genes encode a putative multi-copper oxidase (CopA), and two other putative copper tolerance proteins (CopB and CopC). It is remarkable that all 5 genes that are repressed under copper excess, or perhaps induced under basal copper level, belong to the *bll4878*-

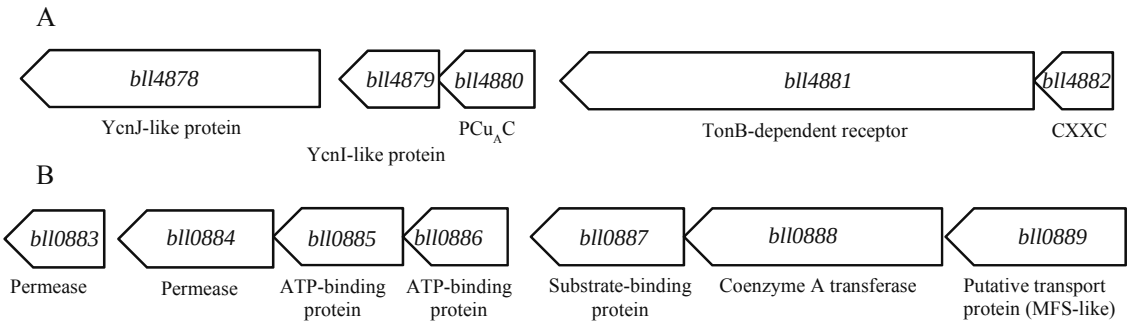
bll4882 operon (FIG. 6.3.A). The operon was confirmed with a tiling array analysis (data not shown). Bll4480 is homologuous to PCu_AC which has been described as a copper chaperone that specifically delivers Cu(II) to the Cu_A of *Thermus thermophilus* ba_3 oxidase (Abriata *et al.*, 2008). Bll4881 was identified as a Ton-B dependent receptor, Bll4882 has a CXXC motif which is often involved in copper binding, and Bll4878 is homologous to YcnJ, a *B. subtilis* copper importer (Chillappagari *et al*., 2009). Both Bll4878 and YcnJ, have a CopC domain in their N-terminus and a CopD-like domain in the C-terminus. The neighbor of *bll4878*, namely *bll4879* is homologous to the neighbor of *ycnJ*. The latter is called *ycnI* and is, like *ycnJ*, a low-copper responsive gene of *B. subtilis*; but the function of the encoded proteins is unknown (Chillappagari *et al.*, 2009). Given the homologies and motifs that were found in the *bll4878-bll4882* gene products it is very tempting to hypothesize that we are dealing here with a copper-importing system.

TABLE 6.4. Genes differentially expressed by a factor ≥2 or ≥–2 under copper excess (2 μM Cu) compared with basal copper level (5 nM Cu). The genes in bold belong to putative operons, the most strongly regulated gene of each operon is shifted to the right.

ORF number*	Gene name*	Description*	Fold change
Genes with increased expression in copper excess conditions			
bll2211	*copB*	copper tolerance protein	13.38
bll2210	*copA*	multicopper oxidase	9.13
bsl2212		hypothetical protein	8.23
bll2209	*copC*	copper tolerance protein	5.94
bll2208		hypothetical protein	5.82
bll4820		unknown protein	9.21
bsr4821		unknown protein	3.84
bll4816		unknown protein	4.69
bll4814		unknown protein	2.85
bll4815		unknown protein	2.26
bll5511		unknown protein	4.18
blr4931		hypothetical protein	2.68
Genes with decreased expression in copper excess conditions			
bll4878		possible copper import protein	-4.61
bll4879		hypothetical protein	-5.29
bll4880		PCu_AC-like	-5.38
bll4882		unknown protein	-5.62
bll4881		putative TonB dependent receptor	-20.88

*according to Rhizobase

FIG. 6.3. Genetic maps of two clusters encoding putative copper import system. A. depicts the cluster *bll4878-bll4882* and B depicts the cluster *bll0883-bll0889*. The arrows show the arrangements of the genes in the cluster, ORF numbers are given in the arrows, and homologies or interesting motifs are indicated below.



6.5 Discussion

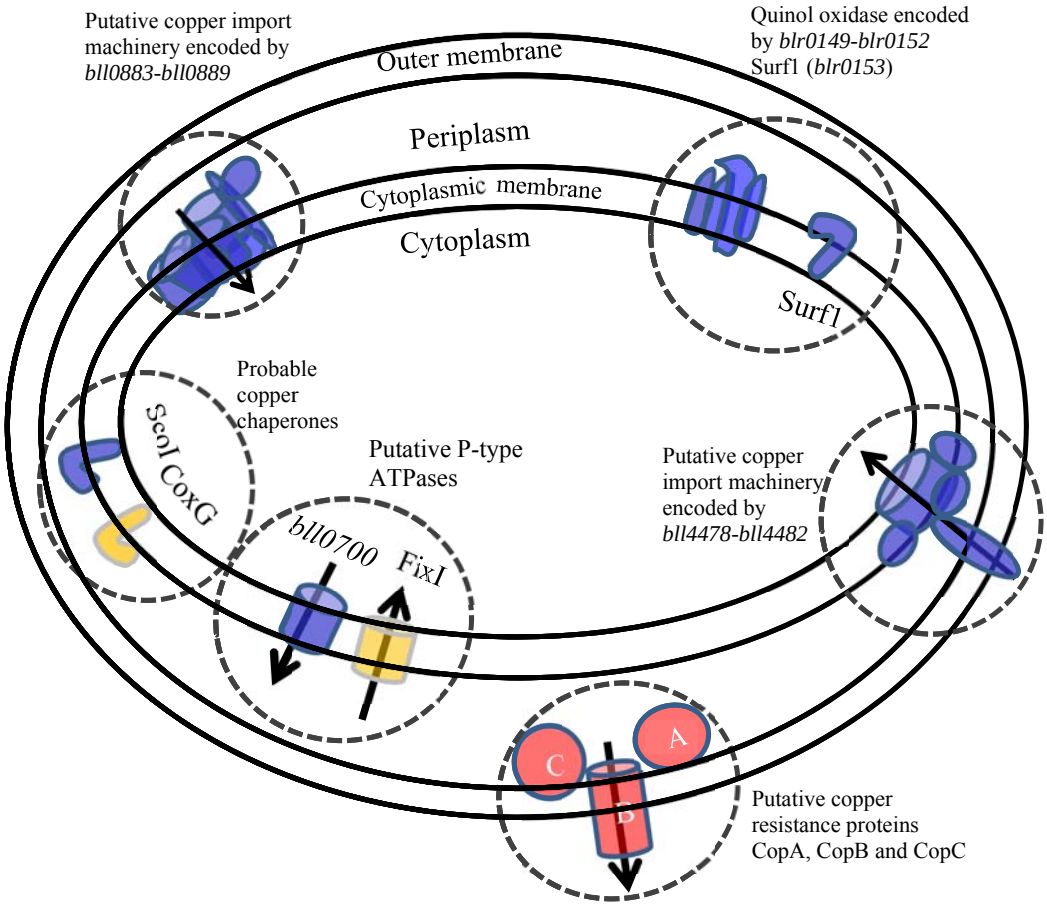
The degree of tolerance of *B. japonicum* towards copper is comparable to what was found for soil isolates of *Rhizobium leguminosarum*. The study by Laguerre and colleagues (Laguerre *et al.*, 2006) is, to our knowledge, the only study that had tackled the topic of rhizobia and copper tolerance in laboratory conditions. 226 out of 246 soil isolates of *R. leguminosarum* had a tolerance towards copper that ranged between 1 and 4 mM Cu. Only 20 of the soil isolates tolerated more than 4 mM Cu. In growth assays we observed a slightly longer lag phase at 1 .25 mM and no growth above approximately 4 mM Cu.

In the present work, we discovered a series of genes which are differentially expressed depending on the copper concentration contained in the growth medium. FIG. 6.3 gives a schematic overview of those and a few others proteins probably involved in copper metabolism. The *bll4878-bll4882* operon which encompasses a PCu_AC-like protein, a putative TonB-dependent receptor, two proteins homologous to *B. subtilis* YcnJ and YcnI respectively, and a protein with a CXXC motif was identified. Given their homologies it was postulated that this operon is dedicated to copper uptake. Phenotypical analysis of the deletion mutant of the entire operon confirmed the proposed function (Fabio Serventi, unpublished observation). However, it remains elusive how the copper atoms get recruited to the TonB-dependent receptor. An attractive hypothesis would be that *B. japonicum* produces chalkophores similar to the methanobactin synthetized by the methane-oxidizer *Methylosinus trichosporium* (Hakemian *et al.*, 2005), but further work would be necessary to confirm this. The transcription of the *bll4878-4882* operon is repressed in copper excess (2 μM Cu) as compared with basal copper level (~4.7 nM Cu). This operon is not differentially regulated when the

basal copper level (~4.7 nM Cu) is compared with copper starvation (10 µM BCS); therefore, this import system can be considered as a "house-keeping" copper importer that is expressed whenever copper is not present in excessive amounts. When copper gets scarce, the expression of the putative *bll0883-0889* operon is increased. The products of this gene cluster harbor homologies with components of ABC-transporters. For example, Bll0886 shares 24% identity and 44% similarity with ZnuC from *E. coli* which is the model protein for cation importing ABC-transporters (Davidson *et al.*, 2008). ABC-transporters pump their freights into the cytoplasm. However it seems unlikely that *B. japonicum* overcomes copper starvation via an ABC-transporter since there is no apparent need for copper in the cytoplasm. Nevertheless, it would be attractive to construct a mutant in the *bll0883-0889* region (FIG. 6.3.B) and maybe also a double mutant in the two putative copper homeostasis systems, i.e. *bll0883-0889* and *bll4878-bll4882*. If the hypothesis of *bll4878-bll4882* being a "house-keeping" copper importer and *bll0883-0889* of being an "emergency" copper importer which is needed in case of copper depletion is correct, then the two mutants would exhibit different phenotypes. A Δ*bll4878-bll4882* mutant would display a defect at milder copper starvation conditions than a Δ*bll0883-0889* mutant except if the two can take over each other's function. The double mutant would most probably display a more severe phenotype than the single mutants. If there are no passive import routes for copper it is conceivable that such a double mutant might even lethal.

Earlier data showed that *fixI* transcription is upregulated when *B. japonicum* is in symbiosis with soybean compared to free-living conditions (fold change: 11) (Pessi *et al.*, 2007) and that the *fixGHIS* operon is required for cbb_3 cytochrome *c* oxidase formation (Preisig *et al.*, 1996a). Given its homology to other P-type ATPases, we hypothesized that FixI is a copper uptaking P-type ATPase. *fixI* appears not to be upregulated in copper starved aerobic cultures. However, this is not in contradiction with the postulated copper-importing function because FixI is essential in symbiosis and is probably not required under free-living, aerobic conditions. On the other hand, the closest homolog of *fixI* in the *B. japonicum* genome, namely *bll0700* turns out to be slightly upregulated in case of copper excess (fold change 2.2). This suggests that *bll0700* encodes a copper ATPase working in the inside-out orientation, pumping out excess copper. Since *bll0700* was previously shown to be upregulated in free-living condition compared to symbiotic conditions (fold change: 11) (Pessi *et al.*, 2007) we can now expand our hypothesis: *B. japonicum* encodes two P-type ATPases, one importing copper in the symbiotic lifestyle, the other one exporting copper under free-living conditions. The presence of both copper importing and exporting P-type ATPases has been reported before for *E. hirae* (Solioz & Stoyanov, 2003). In the case of *E. hirae*, the proteins are encoded in the same operon, whereas in *B. japonicum* they are genetically not closely linked.

FIG. 6.3. Proteins encoded by genes that were induced in copper depletion are colored in blue, proteins encoded by genes that were induced in copper excess are represented in red. Proteins colored in yellow are encoded by genes that did not appear to be regulated in conditions chosen for the present study but that are also putative copper chaperones or transporters. The arrows indicate the presumed direction of copper transport. It should be noted that *fixI* is poorly expressed in aerobic conditions.



Copper and iron metabolism have been shown to be linked in *P. aeruginosa* (Frangipani *et al.*, 2008), yeast (van Bakel *et al.*, 2005) and in mammals (Winzerling & Law, 1997). The reason for this connection is not fully understood. One linkage between the two metals are the multi-copper oxidases that catalyze the oxidation of Fe(II) to Fe(III) and that rely on copper cofactors to carry out their function (Huston *et al.*, 2002). But whether this is sufficient to explain why iron uptake machineries are downregulated under copper depletion is unclear. Whatever the reason(s) for this link is, we were wondering whether we find it in *B. japonicum* as well. Unlike in *P. aeruginosa*, copper depletion did not lead to differential expression of genes involved in iron metabolism in *B. japonicum*. However, when we overlapped the regulons that were generated in a study that compared low and high iron (Rudolph, 2006) and the present study, we made an interesting finding. The gene region *blr0148-blr0153* encoding a heme-copper oxidase, an MFS permease and Surf1 is upregulated in low iron and

in low copper. Interestingly, these genes are positively regulated by *blr0154*, a sensor kinase that is homologous to RegS (Dominic Eicher, unpublished observation). Whether Blr0154 is copper dependent, like RegS, is unknown. Moreover, Blr0154 appears to positively regulate the *copABC* region which codes for putative copper stress proteins. To put these informations together in a hypothesis seems premature at the moment. But, in some way, the cell response towards the two biologically relevant transition metals iron and copper and the heme-copper oxidase encoded by *blr0148-blr0152* as well as the associated regulator Blr0154 are entangled.

This study sheds a new light on the composition of the full media that are usually used for cultivation of *B. japonicum*. 0.5 mg $CuSO_4$* 5 H_2O is commonly added per liter of PSY medium, which corresponds to a final concentration of 2 µM Cu. In this study we demonstrate, that at this concentration, the expression of a putative copper export machinery gets already upregulated, therefore this concentration can be regarded as a copper excess, maybe even a slight copper stress. It might be worth considering to lower the copper concentration in our standard PSY medium.

6.6 Acknowledgments

Simona Huwiler is acknowledged for her significant contribution to the results presented in this chapter.

CHAPTER 7

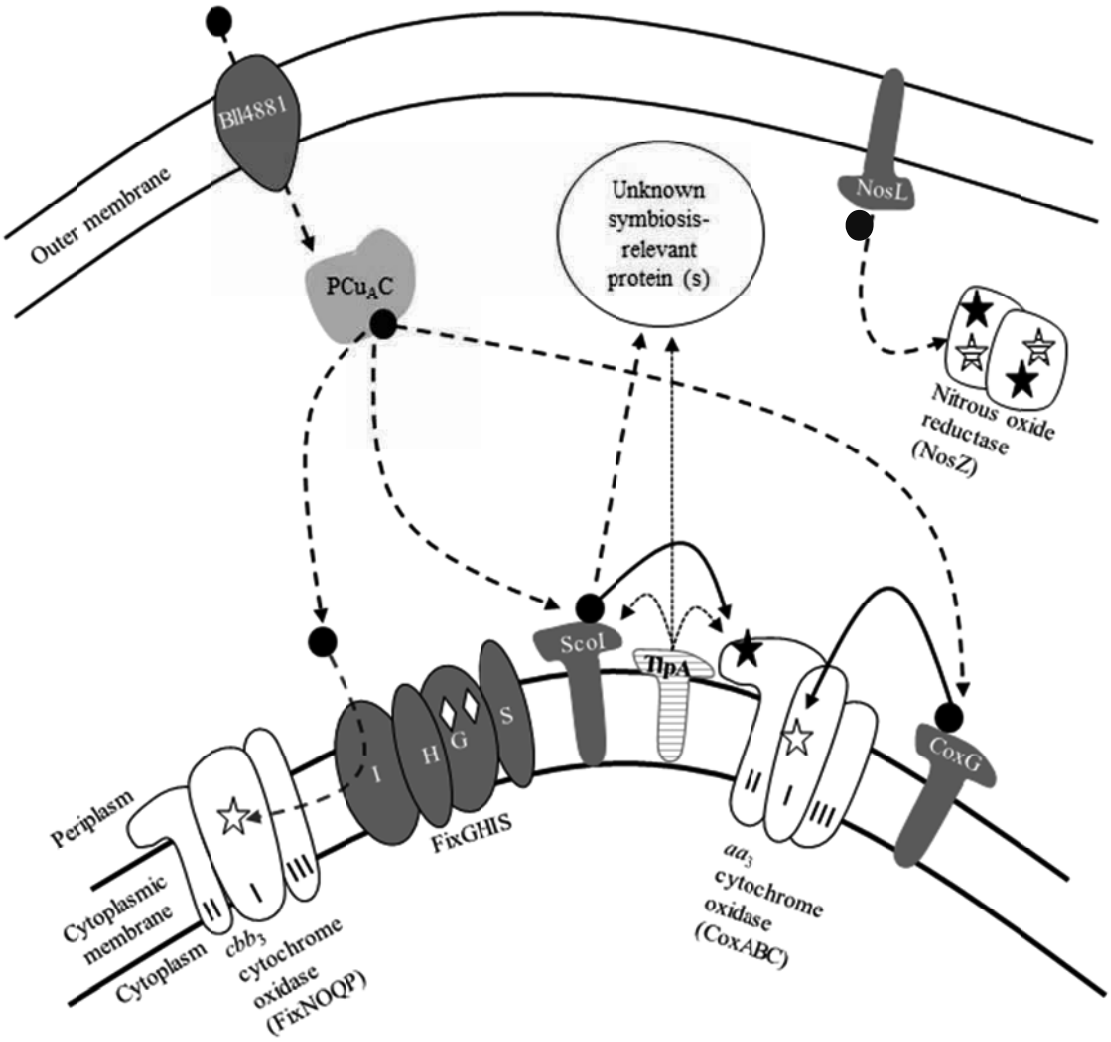
Future perspectives

The transcription of the genes encoding Bll4881, PCu_AC (Bll4880), and ScoI was found to be induced when copper was scarce, or alternatively repressed when copper was available. This suggested that they are involved in copper acquisition or distribution pathways (chapter 6). PCu_AC is a predicted soluble periplasmic protein that, by analogy with *Thermus thermophilus* PCu_AC, (Abriata *et al.*, 2008) is thought to bind copper via a $H(M)X_{10}MX_{21}HXM$ signature (Serventi *et al.*, manuscript in preparation). We hypothesize that PCu_AC is a copper-chaperone that is located upstream of ScoI in the copper distribution pathway. ScoI was shown to bind copper via the cysteines of the conserved CXXXC motif, and to be involved in aa_3 cytochrome oxidase biogenesis, probably via copper insertion into Cu_A, but not in biogenesis of cbb_3 cytochrome oxidase (chapter 2). The redox potential of ScoI is approximately –155 mV; it is therefore unlikely to function as a disulfide reductase but is a possible substrate for TlpA (chapter 5). Moreover, ScoI fulfills an unknown function in symbiosis, and a putative target protein has not yet been identified (chapter 3). CoxG is thought to deliver copper to Cu_B of the aa_3 cytochrome oxidase, but, similarly to ScoI, is not required for formation of the cbb_3 cytochrome oxidase (chapter 2). The proteins encoded by the *fixGHIS* operon are required for formation of the cbb_3 cytochrome oxidase (Preisig *et al.*, 1996b). Given its homology to P-type ATPases, FixI might be a copper importer. FixG encodes two sites for 4Fe-4S clusters; it might therefore be a copper reductase. TlpA is a periplasmically exposed thioredoxin that is involved in aa_3 cytochrome oxidase formation and in symbiosis. Its redox potential was determined to be –213 mV (Loferer *et al.*, 1995), and we hypothesize that it reduces critical cysteine disulfide pairs of ScoI and of the subunit II of cytochrome oxidase in order to allow copper binding (chapter 5). NosL has not been studied so far in *B. japonicum*, but given its sequence identity of 29 % (similarity 48 %) with *Pseudomonas stutzeri* NosL, we suggest that it might assume the same function as in the latter, which is a NosZ-specific copper chaperone function (Zumft, 2005a). These findings and hypotheses are summarized in FIG. 7.1.

7.1 Cu transfer in vitro

An attempt was made recently to follow copper transfer *in vivo* from a Cu-binding chaperone of the Cox11/CoxG/CtaG family (chapters 1 and 2) to cytochrome oxidase subunit I. *Paracoccus denitrificans* CtaG was introduced into a heterologous host, *Escherichia coli*, in which the Cu_B-possessing subunit I of *P. denitrificans* was coexpressed. To detect copper loading of subunit I, the authors performed total reflection X-ray fluorescence and a Cu(I)-specific 2,2' bicinchoninic acid colorimetric assay. However, copper loading could not be observed, possibly because other factors are needed for efficient transfer from the putative cuprochaperone to the cognate target protein (Greiner *et al.*, 2008).

FIG. 7.1. Working model for the biogenesis of copper A (Cu_A) and copper B (Cu_B) centers in *Bradyrhizobium japonicum.* This model integrates data that was presented in different chapters of this work. Some features that were presented in FIG. 6.3 were omitted here for clarity. Target proteins are represented in white, proteins putatively involved in copper chaperoning or import are colored in grey, and TlpA, a thioredoxin that might catalyze disulfide reduction is striped. Full black circles represent copper atoms. White asterisks represent Cu_B, black asterisks represent Cu_A, and striped asterisks represent Cu_Z. Diamonds represent 4Fe-4S clusters. Black thick arrows represent copper trafficking routes that are supported by evidence that was presented in chapter 2, although they are not yet proven. Thick dashed arrows represent hypothetical copper trafficking routes, and thin dashed arrows represent disulfide reducing activity.



The authors of this study propose an experimental setup in which copper transfer might be observed *in vitro*. In their approach, even cotranslational events can potentially be observed as protein expression is done in cell-free extracts.

The case of Cu_B is difficult to tackle because the metal binding site is deeply buried inside the membrane. However, biogenesis of Cu_A which is exposed on the soluble domain of the subunit II, is easier to study. Abriata and coworkers purified the soluble part of *Thermus thermophilus* subunit II of the cytochrome oxidase and soluble variants of the two protein factors involved in Cu_A biogenesis, namely PCu_AC and Sco. After mixing the purified subunit II with PCu_AC and Sco, copper transfer could be observed by NMR spectroscopy (Abriata *et al.*, 2008).

In the case of *B. japonicum*, several transfer steps of copper are suggested from indirect evidence, and we would be keen to observe them directly. These are: Cu-delivery from CoxG to Cu_B of subunit I, Cu-delivery from ScoI to Cu_A of subunit II, and copper transfer from PCu_AC to CoxG and to ScoI (FIG. 7.1). As illustrated by the *P. denitrificans* study that was described above, metal transfer to Cu_B is technically very challenging. However, we think it should be possible to observe the presumed transfer from PCu_AC to ScoI. The easiest case is if the transferred copper is of the Cu(II) valence. Indeed, Cu(II) complexed by the active-site cysteines of ScoI can be detected by spectroscopic means (chapters 2 and 4). The experimental set up for such an experiment would be to purify soluble variants of ScoI and PCu_AC and to deplete ScoI from copper. This can be done by reduction of the protein with DTT and subsequent gel filtration over a PD-10 column (GE Healthcare). PCu_AC should be loaded with copper, and it would be essential that this sample is devoid of non-protein-bound copper. This can be achieved by dialysis. Then, the two samples could be mixed, and a peak at 360 nm that is diagnostic for cysteine-mediated Cu(II) binding to ScoI should appear. In case of Cu(I) transfer, the procedure would be the same, except for the last detection step. As Cu(I) cannot be detected spectroscopically, the sample would be checked by mass spectrometry analysis. In case of Cu(I) (or Cu(II)) binding a difference in atomic mass of 63 Da would be observed. This procedure could be applied for copper loading of the soluble domains of subunit II, CoxG and ScoI. As mentioned in chapter 4, it should be noted that handling of Cu(I) is complicated by the fact that it tends to oxidize to Cu(II) in the presence of atmospheric O_2 and, therefore, these manipulations should be performed in anoxia.

7.2 How does the symbiotic *cbb*$_3$ cytochrome oxidase obtain its copper cofactor?

The energetic needs of *B. japonicum* in symbiosis with legume host plants are very high and can only be covered with oxic respiration. On the other hand, nitrogenase, the central enzyme for nitrogen fixation, is highly O_2 sensitive. This seemingly opposed relationship of *B. japonicum* towards O_2 is referred to as the oxygen paradoxon. To cope with this paradox, *B. japonicum* evolved several strategies; one of them is the use of a high-affinity cytochrome oxidase in symbiosis (Preisig *et al.*, 1996b). In chapter 2, we discussed the fact that this oxidase and the mitochondria-like *aa*$_3$ cytochrome oxidase use disparate pathways for their respective biogenesis. The *aa*$_3$ cytochrome oxidase depends on the activity of ScoI and CoxG whereas the *cbb*$_3$ cytochrome oxidase does not. Which are the factors involved in biogenesis of the latter? It is known that the factors encoded by the *fixGHIS* operon play a pivotal role the in biogenesis of *cbb*$_3$ cytochrome oxidase (Preisig *et al*., 1996a). Recently, it was suggested that the biogenesis of the *cbb*$_3$ cytochrome oxidase relies on PCu_AC (Bll4880) and ScoI (Arunothayanan *et al.*, 2010). This hypothesis relies on the finding that deletion mutants of *bll4880*, of *scoI*, and a double mutant are impaired in nitrogen fixation. We also found that Δ*bll4880* and Δ*scoI* are impaired in nitrogen fixation. The nodules of plants infected with mutants have acetylene reduction activities of 25 % (Serventi *et al.*, manuscript in preparation) and 20 % (Bühler *et al.*, 2010) compared to the wild type, respectively. However, in the case of Δ*scoI*, we have strong evidence from immunodetection of the oxidase subunits and from oxidase activity measurements that *cbb*$_3$ cytochrome oxidase biogenesis is not impaired in a Δ*scoI* mutant. Therefore, in the context of these contradictory results, we challenge the findings of Arunothayanan and coworkers. Our favored hypothesis to explain the biogenesis pathway of the *cbb*$_3$ cytochrome oxidase involves the proteins encoded by the *fixGHIS* operon (FIG. 7.1). In collaboration with Prof. Marc Solioz (Department of Clinical Pharmacology, University of Berne), we invested some efforts in unraveling the function of FixI, a putative copper-transporting P-type ATPase. We constructed pRJ6526, a pProExHTa-derived expression plasmid for purification of a 6His-tagged version of $FixI_{sol}$. The idea was that Solioz and coworkers would test copper-dependent ATPase activity of purified $FixI_{sol}$ *in vitro*. However, the expression of this construct appeared to be difficult in *E. coli* and in *Enterococcus hirae*, because the growth of the strains was decreased, suggesting that the expressed protein might have deleterious effects. $FixI_{sol}$ could not be purified and we now consider to try another *in vivo* approach, for example by complementing an *E. hirae* Δ*copA* mutant with *fixI*, CopA being a copper-importing ATPase (Magnani & Solioz, 2005).

Another way to prove the involvement of FixI in biogenesis of the *cbb*$_3$ cytochrome oxidase would be to compare expression of *fixI* in microaerobic cultures of *B. japonicum* grown under normal copper concentrations and its expression in copper-starved microaerobic cultures. Microoxia is required in order to induce transcription of *fixGHIS* and *fixNOQP* which encodes the high-affinity *cbb*$_3$ oxidase. If indeed FixI is a copper-importing P-type ATPase that specifically delivers copper to Cu_B of *cbb*$_3$

cytochrome oxidase, then transcription of *fixI* should be increased under copper starvation. As we could show that *fixI* transcription is not increased under copper starvation in oxic conditions (chapter 6), this adaptation would be specific for the microaerobic life style and would support our hypothesis.

7.3 TlpA and ScoI

As described in chapter 5, Δ*tlpA* and Δ*scoI* mutants have similar phenotypes and, as illustrated in FIG. 7.1, we suggest that they are involved in the same pathway(s). We hypothesize that TlpA reduces the active-site cysteines of ScoI and other targets, like the cysteines that are involved in the copper complexing of Cu_A of the aa_3 cytochrome oxidase. There are several approaches that can be considered to observe the disulfide exchange between TlpA and ScoI. As fluorescence of TlpA is heavily influenced by the redox state of its active-site cysteines (Loferer *et al.*, 1995), the reaction could be observed by fluorescence spectroscopy. On the other hand, the reaction can also be followed from the side of the presumed oxidizing partner, namely ScoI. Here we could make use of the copper binding property of reduced ScoI which can be detected spectroscopically. Since oxidized ScoI does not bind copper, the appearance of a peak at 360 nm upon mixing of $TlpA_{red}$ and $ScoI_{ox}$ in the presence of Cu(II) could be considered as a direct evidence of a disulfide exchange between the two partners. A step further might be taken by experimentally demonstrating that both ScoI and TlpA are involved in biogenesis of subunit II of aa_3 cytochrome oxidase in an *in vitro* approach. If it is possible to purify the soluble domain of subunit II and to oxidize its copper-liganding cysteine residues, it would be very attractive to show that copper loading of Cu_A relies on the concerted contributions of TlpA and ScoI. To detect copper loading of the soluble subunit II domain, one might consider to analyse the sample by mass spectrometry, as already suggested in paragraph 7.1. In addition, the increase in fluorescence of TlpA could be monitored, as well as the disappearance of the 360 nm peak of ScoI.

It should be noted here that the symbiotic defect of a Δ*tlpA* mutant is more severe than of a Δ*scoI* mutant. In detail, nodules induced by a Δ*tlpA* mutant are greenish and their acetylene reduction capacity is fully abrogated (Loferer *et al.*, 1993) whereas a Δ*scoI* mutant retains ~20 % of the acetylene reduction activity of the wild type and elicits nodules which contain leghemoglobin, although less than a wild type. Therefore, TlpA likely possesses additional symbiosis-relevant targets and even if we can prove the interaction of TlpA and ScoI, TlpA function may not be fully elucidated.

Finally, it should also be noted that in the course of this study the construction of a Δ*scoI* Δ*tlpA* double mutant was repeatedly tried but has not been successful. Given the phenotypes of the respective single mutants under free-living conditions, it seems rather unlikely that the double mutant is not viable, however this cannot be fully excluded.

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CURRICULUM VITAE

Doris Bühler

Born on August 16th, 1981 in Biel/Bienne

Citizen of Switzerland

2006-2010 Assistant Researcher at the Institute of Microbiology, ETH Zürich; PhD thesis

2001-2006 Studies at the University of Berne, Master of Science in Biology with major in Cell Biology

1997-2001 High school, Gymnase de la Rue des Alpes, Biel/Bienne

1987-1997 Primary and secondary schools in Tavannes

PUBLICATIONS

Bühler D., Rossmann R., Landolt S., Balsiger S., Fischer H.M., Hennecke H., (2010). Disparate pathways for the biogenesis of cytochrome oxidases in *Bradyrhizobium japonicum*. *J. Biol. Chem.* 285:15704-15713.

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