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Disparate Pathways for the Biogenesis of Cytochrome Oxidases in *Bradyrhizobium japonicum*[*]

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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. Although 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 homologs 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.

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 (1–4).

There is diversity regarding the number and cofactor content of the other subunits, which relates to the substrates used as electron donor (5, 6). 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 (3, 4). The subclass of cbb_3-type oxidases is exceptional because its members have a subunit II (CcoO or FixO) that is a monoheme *c*-type cytochrome instead of the Cu_A-containing protein (3, 7). 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 (8, 9). Subunit III of all other heme-copper oxidases is cofactor-free, just like the non-conserved small subunit IV (1–4). 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 (10).

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. Whereas 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 (4, 10), which is equivalent to the intermembrane 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 (11, 12), the use of the comparatively simpler bacterial aa_3-type oxidases facilitates biogenesis studies. Members of the α-proteobacteria (*e.g. Paracoccus denitrificans*, *Bradyrhizobium japonicum*, and *Rhodobacter* sp.) are attractive model organisms for this purpose because they appear to be the closest extant relatives of a mitochondrial ancestor (2). 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 (13, 14); CtaG (or CoxG), a homolog of mitochondrial Cox11 (15), for copper trafficking to the Cu_B site in subunit I (16–18); Sco1 (also called SenC or PrrC), for copper delivery to the Cu_A site in subunit II (19–23); and TlpA, a protein dithiol:disulfide oxidoreductase with an unknown role in cytochrome aa_3 formation in *B. japonicum* (24, 25). 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 (26). 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. Because subunits II and III are *c*-type cytochromes, their synthesis requires the complete set of maturation proteins for the covalent attachment of heme

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TABLE 1
Terminal respiratory oxidases in *B. japonicum*

Gene number[a]	Gene name	Subunit (SU)	Cytochrome (cyt) type	Copper center	Phenotype[b]	-Fold change Bact/Air[c]	Source/Reference
						-fold	
Heme-copper cytochrome oxidases							
blr1170	*coxB*	SU II		Cu_A	Fix^+	–	Ref. 58
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	Ref. 61
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	Refs. 32 and 60
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[d]
bll4480		SU I	Diheme protein	Cu_B		−2.1	
bll4479[e]		SU III[e]	Diheme cyt *c*[e]			−2.8	
Heme-copper quinol oxidases							
blr2714	*coxW*	SU II			Fix^+	–	Refs. 82 and 83
blr2715	*coxX*	SU I	bb_3	Cu_B		–	
blr2716	*coxY*	SU III				–	
blr2717	*coxZ*	SU IV				–	
blr0149		SU II			NT	−2.8	Ref. 84
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^+	–	Ref. 85
bll0282	*cydB*	SU II				–	
blr3728		SU I	*bd*-like		NT	–	Ref. 84
blr3729		SU II				–	

[a] Gene number according to RhizoBase.
[b] Symbiotic Fix phenotype of mutants deleted for at least the subunit I and/or subunit II genes. Fix^+, 70–100% of wild-type activity; Fix^-, 0–5% of wild-type activity; NT, not tested.
[c] -Fold change of gene expression in soybean bacteroids (Bact; 21 days postinfection) compared with cells grown aerobically (Air). Data were taken from Pessi *et al.* (86). Positive values, increased expression; negative values, decreased expression; –, within threshold range of ±2.
[d] The genes had been reported previously (84), but the mutant phenotype was determined in this work.
[e] Sequence analysis predicts that this cytochrome c transfers electrons obtained from reduced PQQ. Therefore, the oxidase proper (bll4481/4480) is classified here as a cytochrome oxidase.

(27, 28). Additional assembly factors are needed whose biochemical functions have not been elucidated (29, 30). 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 (31) and six are heme-copper oxidases, the latter being further divided into two quinol oxidases and four cytochrome oxidases (Table 1). 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 (32, 33). 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 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 (34) that 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.

EXPERIMENTAL PROCEDURES

Media, Growth Conditions, and Strains—*Escherichia coli* was grown in Luria-Bertani (LB) medium (35) containing these concentrations of antibiotics for plasmid selection: ampicillin, 200 μg/ml; kanamycin, 30 μg/ml; tetracycline, 10 μg/ml. *B. japonicum* was cultivated in peptone salts-yeast extract (PSY)[2] medium (36, 37) supplemented with 0.1% L-arabinose. Aerobic

[2] The abbreviations used are: PSY, peptone salts-yeast extract; APS, ammonium persulfate; Fix, nitrogen fixation; NADI, 1-naphthol plus *N*,*N*-dimethyl-1,4-phenylendiamine.

TABLE 2
***B. japonicum* strains used in this work**

Strains	Relevant genotype or phenotype	Source/Reference
110*spc*4	Sp^r wild type	Ref. 36
COX132	Sp^r Km^r *coxA*::Tn*5*	Ref. 58
3613	Sp^r Km^r *fixN*::Tn*5*	Ref. 60
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	Ref. 64

[a] Equivalent to Δ*cox*-b, as shown in Fig. 1.
[b] Equivalent to Δ*cox*-a, as shown in Fig. 1.

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 (38, 39) 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: spectinomycin, 100 μg/ml; streptomycin, 50 μg/ml; kanamycin, 100 μg/ml; tetracycline, 50 μg/ml (solid media) or 25 μg/ml (liquid media). *B. japonicum* strains used in this work are listed in Table 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 (40). 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 (41, 42).

General DNA Biochemistry—Standard techniques were used for plasmid isolation, cloning, transformation, Southern blotting, hybridization, and sequencing (43). *E. coli* strain DH5α (Invitrogen) was the host for routine clonings, and strain BL21 (DE3) (44) was the host for heterologous protein expression. DNA probes for hybridization were labeled with the digoxigenin DNA labeling kit from Roche Applied Science.

Mutant Construction—Plasmids with the pRJ prefix are from our laboratory collection. Details on their genealogy and DNA content are available from the authors upon request.

For construction of the *coxG* deletion mutants, part of the *coxG* gene on plasmid pRJ3550 was excised, using restriction enzymes BbsI and BamHI, and replaced by the SmaI fragment from pUC4-KIXX (Amersham Pharmacia Biotech AB, 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 (45) using EcoRI sites, which yielded plasmids pRJ3583 and pRJ3586. Mobilization of these plasmids via *E. coli* S17-1 (46) 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 and Fig. 1 with genome coordinates).

The first step toward 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) and verified by sequencing. Both amplicons were then cloned tail-to-head into pBluescript II KS(+) (Stratagene, La Jolla, CA), resulting in pRJ2572. In two separate constructions, a 1,206-bp PstI kanamycin resistance cassette (*aphII*) of pBSL86 (47) was inserted in either orientation into the unique PstI site between the *scoI*-upstream and -downstream regions on pRJ2572. A 2,872-bp EcoRI-XbaI fragment with *scoI*-flanking DNA plus intervening *aphII* cassette was excised from each of the two constructs and cloned in pSUP202pol4 (48). 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). 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 SmaI-XhoI fragment containing *coxB*. A unique NcoI-NruI fragment from within *coxB* was excised and replaced by the HindIII-SmaI kanamycin resistance fragment of pUC4-KIXX (Amersham Biosciences), and then the *coxB*-flanking BamHI-SmaI and EcoRI fragments were added on both sides of the Δ*coxB* construct, resulting in pRJ3562K. This DNA construct was cloned in suicide vector pSUP202pol4 (48) 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). 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 (47) was inserted in between, using a PstI 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. The deletion end points are at genome coordinates 4,961,905 and 4,962,644.

Preparation of Membrane Fraction—*B. japonicum* cells were disrupted by three passages through a French pressure cell at 9,000 p.s.i. After the removal of cell debris by centrifugation at 28,000 × *g* for 30 min at 4 °C, membrane pellets were collected by ultracentrifugation at 129,000 × *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).

Determination of Protein Concentration—Concentration of solubilized membrane protein was determined with the Brad-

ford method (49), using a Bio-Rad assay with bovine serum albumin as the standard.

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. Similarly, dithionite-reduced minus APS-oxidized spectra were recorded. For the reduced spectrum, 2 μl of freshly prepared sodium 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 of APS of 0.1 M solution was added to a final concentration of 5 mM.

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 (50) with one modification; measurements were performed under continuous stirring in 50 mM HEPES buffer (pH 7.4) containing 1% (w/v) dodecylmaltoside.

Expression and Purification of ScoI and Its Mutant Derivative—The *scoI* codons for cysteines 74 and 78 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 (51), 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 liter. 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 the addition of arabinose to a final concentration of 0.1% (w/v). After induction, the cultures were transferred to 30 °C, and after 2–4 h, cells were collected by centrifugation and disrupted by three passages through a French press at 9,000 p.s.i. Purification was performed with Strep-Tactin Sepharose columns (IBA GmbH, Göttingen, Germany) according to the supplier's protocol.

UV-visible Spectroscopy of ScoI—The method was used to follow the binding of Cu(II) to reduced ScoI (52). Reduction of $ScoI_{sol}$ was achieved by incubation with 2 mM dithiothreitol for 4 h at 4 °C. Dithiothreitol removal and exchange against 50 mM sodium phosphate buffer (pH 7.0) was done by gel filtration over PD-10 columns (GE Healthcare). 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 molecular weight cut-off, miniunits) from Pierce was used. UV-visible spectra were recorded from 200 to 800 nm on an Agilent diode array photometer (Agilent Technologies, Santa Clara, CA).

Immunological Techniques—Rabbit antibodies specific for the FixO, FixP, and CoxA proteins were available from previous work (8, 24, 53). For the production of antibodies against CoxB, a peptide of the sequence NH_2-RVVEDKEFASWVETAKKK-COOH, corresponding to residues 243–260 of the predicted CoxB sequence, was synthesized. Both the peptide and the polyclonal antibodies from rabbits were custom-made by TANA Laboratories, LLC (Houston, TX). Polyclonal antibodies against the soluble part of ScoI were raised in rabbits. StreptagII-marked protein was used as antigen, and the immunization was performed by EUROGENTEC S.A. (Liege, Belgium). For Western blot analyses, membrane proteins (30 μg/lane) were separated by SDS-PAGE (54) and blotted as described previously (24). 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, and 1:5,000 for anti-FixP. Protein bands with bound immunoglobulins were detected with anti-rabbit IgG-POD (Roche Applied Science) or with anti-rabbit IgG (H+L)-horseradish peroxidase conjugate (Bio-Rad) and chemiluminescence detection kits from Roche Applied Science and Pierce.

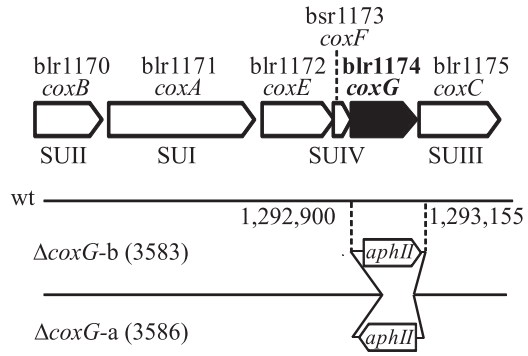

FIGURE 1. **Map of the *cox* gene cluster (blr1170–1178).** The *arrows* show the arrangement of the genes in the cluster. Gene names (if available) are given *below* the gene numbers. The *bottom part* of the scheme shows the orientation and precise chromosomal nucleotide position of the kanamycin resistance gene (*aphII*) inserted in the *coxG* deletion mutants. *wt*, wild type; *SUI–SUIV*, subunits I–IV, respectively.

RESULTS

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 Data base, accession number AJ242592) and in the course of the *B. japonicum* genome sequencing project (34). Three open reading frames (*coxEFG*) were found to be located between the subunit I (*coxA*) and subunit III (*coxC*) genes (Fig. 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 (55). There is evidence to suggest that Cox11-like proteins transfer copper to the Cu_B site in subunit I (56, 57). 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 and Fig. 1). Both mutants were

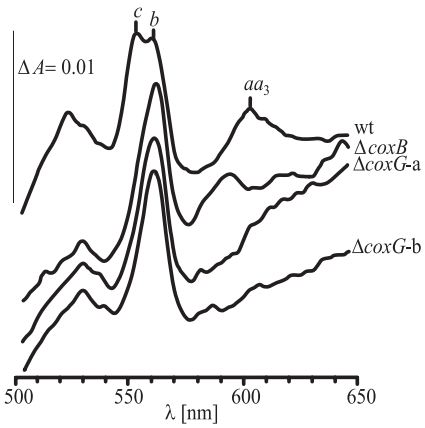

FIGURE 2. **Dithionite-reduced minus air-oxidized difference spectra.** The difference spectra were recorded with solubilized membrane protein fractions prepared from aerobically grown *B. japonicum* cells of the wild type (*wt*), the *coxB* deletion mutant 3563 (Δ*coxB*), and the two *coxG* deletion mutants (Δ*coxG*) with the *aphII* cassette inserted in orientation *a* (strain 3586) and *b* (strain 3583). The protein concentration was 2 mg/ml. 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.

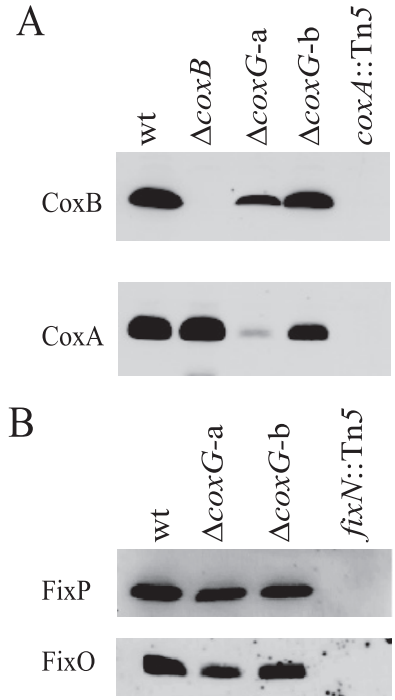

FIGURE 3. **Detection of aa_3- and cbb_3-type oxidase subunits by Western blot analysis.** *A*, antibodies against CoxB and CoxA were used. *B*, antibodies against FixP and FixO were used. Cells had been cultivated either aerobically (*A*) or microaerobically (*B*). Membrane proteins (30 μg/lane) were 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*).

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) and the parental wild type were included for comparison. With the wild-type membrane fraction, an activity of 0.469 μmol of cytochrome *c* oxidized/min/mg of 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 (58). 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) due to destabilization of the cytochrome bc_1-CycM-aa_3 supercomplex (58). Curiously, a small peak at 594 nm appeared with the *coxB* strain (Fig. 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. 3*A*). 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. 3*A*). Although 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. 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. However, 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). 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 of 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 1). However, the data in Table 3 show that both *coxG* strains were able to elicit a

TABLE 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.

Strain	Relevant genotype	Number of nodules	Nodule dry weight	Nitrogenase activity[a]	Relative Fix activity[b]
			mg	*%/min/g*	*%*
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

[a] Nitrogenase activity is expressed as percentage of C_2H_4/min/g.
[b] Relative nitrogen fixation activity is expressed as a percentage of wild type.

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.

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 (19–23). In many aerobic bacteria, its gene maps immediately adjacent to cytochrome oxidase structural and biogenesis genes (*cf.* String Data base; available on the EMBL web site). The *B. japonicum cox* gene cluster shown in Fig. 1, however, does not encode a Sco1-like protein. Instead, based on sequence similarity, the blr1131 gene (34) was identified as a likely candidate to encode a Sco1-like protein. The blr1131 open reading frame codes for a 196-amino acid protein that shows the typical hallmarks of prokaryotic 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) (20). For these reasons and further evidence given below and in keeping with the standard nomenclature for bacterial genes (59), blr1131 of *B. japonicum* was named *scoI*.

To approach ScoI function, two mutants were constructed (strains 2575 and 2576; Table 2) in which almost the entire *scoI* gene was deleted and replaced by a kanamycin resistance cassette in either orientation. Because 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) having an insertion in the subunit I gene (*coxA*::Tn*5*) was included in these assays for comparison. Fig. 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

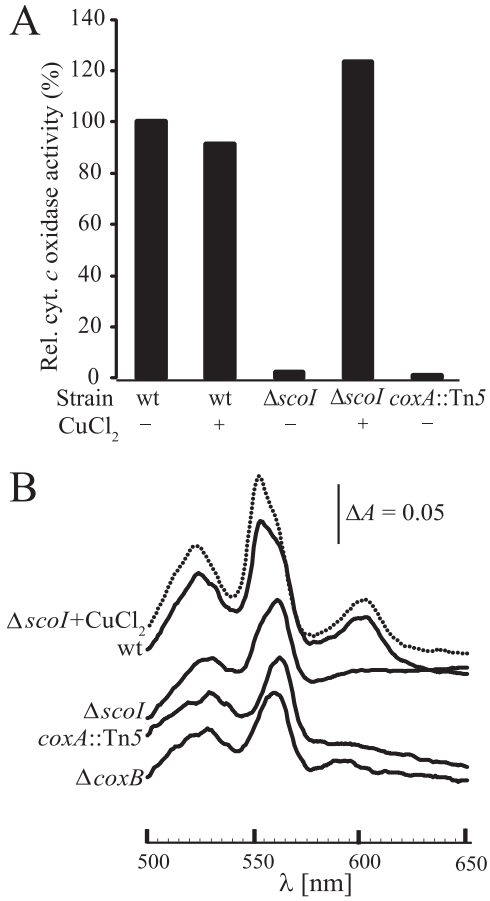

FIGURE 4. **The *scoI* mutation affects cytochrome aa_3.** *A*, relative cytochrome *c* oxidase activity of aerobically grown wild type (*wt*) and mutant strains 2575 (Δ*scoI*) and COX132 (*coxA*::Tn5). Wild-type activity (100%) corresponds to ~0.43 μmol horse heart cytochrome *c* oxidized/mg of membrane protein/min. Whether or not 50 μM $CuCl_2$ was added to the PSY medium is indicated with a *plus* or *minus sign*. *B*, dithionite-reduced minus APS-oxidized difference spectra of solubilized membrane protein fraction (61 mg/ml) from aerobically grown cells. As *labeled*, the continuous curves represent the wild type 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, the cytochrome *b* is not resolved here as a separate peak but as a shoulder (560 nm).

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. 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. 4*B*). Likewise, cytochrome *c* oxidase activity of the *scoI* mutant was restored to wild-type levels by the same $CuCl_2$ treatment (Fig. 4*A*). Assembly of cytochrome aa_3 in the membrane was then analyzed in Western blots using antibodies specific for CoxA (subunit I) (24) and CoxB (subunit II). Fig. 5 (*A* and *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. 5*B*).

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 fur-

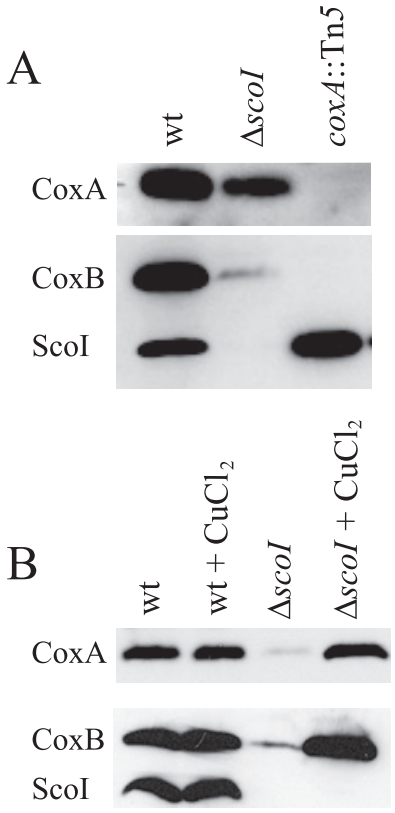

FIGURE 5. **Western blot analysis of cytochrome aa_3 subunits in the Δ*scoI* mutant.** *A*, comparison of *scoI* mutant with wild type and *coxA* mutant. *B*, restoration of subunit assembly by copper. As marked on the *left*, antibodies specific against CoxA, CoxB, and ScoI (control) were used. The *lower blots* in *A* and *B* were exposed to a mixture of anti-CoxB and anti-ScoI immunoglobulins. Membrane proteins (30 μg/lane) were separated on 14% polyacrylamide gels containing SDS. They were isolated from the following strains: wild type (*wt*), 2575 (Δ*scoI*), and COX132 (*coxA*::Tn5). For copper supplementation, PSY medium contained 50 μM $CuCl_2$.

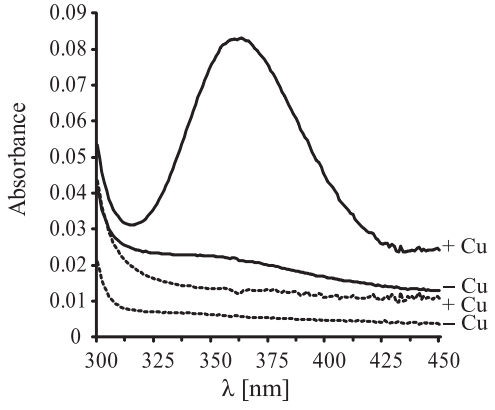

FIGURE 6. **Evidence for copper binding to ScoI.** UV-visible spectra were taken of 23 μM reduced $ScoI_{sol}$ (*solid curves*) and $ScoI[C74S/C78S]_{sol}$ (*dashed curves*), dissolved in 50 mM phosphate buffer, pH 7.0. −, no Cu(II) was added; +, Cu(II) was added to protein in molar equivalents of either 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.

ther 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-visible absorption spectroscopy. It was essential to reduce $ScoI_{sol}$ with dithiothreitol prior to the experiment. The addition of $CuCl_2$ to the protein resulted in an absorbance peak at ~360 nm (Fig. 6), reflecting the binding of Cu(II) to the protein (52). Upon incremental copper addition, the peak increased and reached a plateau at a ratio of ~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 Cys^{74} and Cys^{78} had been replaced by serines. By analogy with Sco1-like proteins from other organisms, our results indicate that Cys^{74} and Cys^{78} 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.

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 (*G. max*). Table 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 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 (58). On the other hand, although all of the *scoI* mutant phenotypes were not as severe as those of a cytochrome cbb_3-defective mutant (60), 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.

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 (32, 60). Fig. 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. The *in vitro* experiment of Fig. 7*A* was corroborated by a *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidation assay *in vivo* with cells that had been cultivated microaerobically. Again, only the *fixN* mutant exhibited an impaired *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine 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 anaerobi-

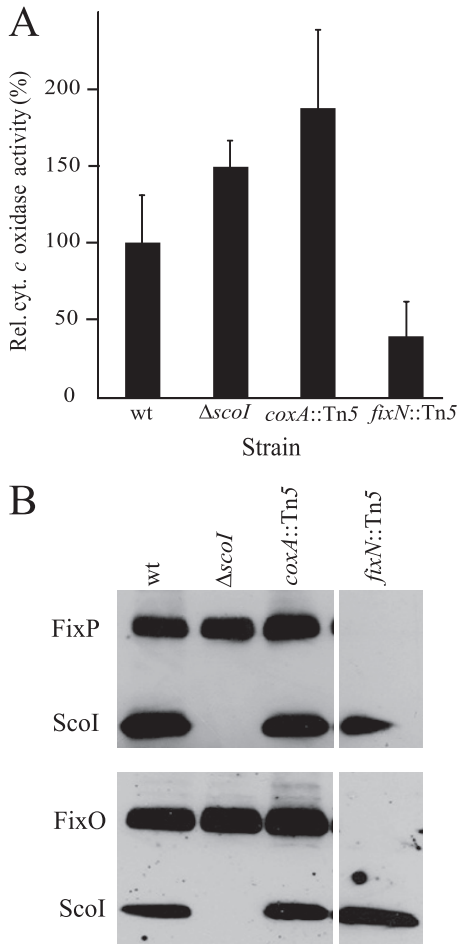

FIGURE 7. **The *scoI* mutation does not affect cytochrome cbb_3.** *A*, relative cytochrome *c* oxidase activity of the wild type (*wt*) and strains 2575 (Δ*scoI*), COX132 (*coxA*::Tn5), and 3613 (*fixN*::Tn5). Cells had been cultivated under conditions of anaerobic nitrate respiration. A total of 11 measurements with three independent biological replicas were done for each strain. Wild type activity (100%) corresponds to 1.2 μmol cytochrome *c* oxidized/mg of protein/min. *B*, Western blot analysis of cytochrome cbb_3 subunits. As marked on the *left*, antibodies specific against FixP, FixO, and ScoI (control) were used. The blots in the *upper* and *lower panels* were exposed to mixtures of anti-FixP plus anti-ScoI immunoglobulins and anti-FixO plus anti-ScoI immunoglobulins. Membrane proteins (30 μg/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. *Error bars*, S.D.

cally or microaerobically and tested by Western blot analysis with antibodies specific for the FixO and FixP proteins. As shown in Fig. 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 1). A *coxN* deletion mutant had previously been shown to be Fix^+ in symbiosis (61). Here we constructed and tested bll4481–4480 deletion mutants (strains 6532 and 6533; Table 2) and found that they also had a Fix^+ phenotype (Table 1). The *nosZ* gene codes for a periplasmic Cu_A-containing nitrous oxide reductase (62, 63). A *B. japonicum nosZ* mutant (strain GRZ3035 (64)) was reported by Mesa *et al.* (65) 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, although 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.

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 and Cox11) had been implicated in copper delivery to the Cu_B site on subunit I (15), one might have expected CoxG to serve 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 (55–57, 66), the mechanism of copper insertion into subunit I remains elusive (18, 67). 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 copper 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 (68–70). Especially if copper insertion occurs during membrane translocation, as proposed by Khalimonchuk *et al.* (56), the CoxG protein might be better suited to deliver copper 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 C*XXX*C 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 (19, 22, 71, 72) and some prokaryotic ScoI homologs (20, 23, 73) 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 copper chaperones has been challenged. Because they are thioredoxin-like, some were found to possess protein dithiol:disulfide oxidoreductase activity (26, 74, 75).

Also, a Cu^{2+}-reducing activity was reported (76). 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 copper chaperone (77). The latter appeared to depend on the activity of the former. We tested the *B. japonicum* $ScoI_{sol}$ in the standard insulin reduction assay (78) but did not detect any reducing activity. It is an attractive hypothesis, however, that the previously described thioredoxin-like TlpA protein (24, 25) 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 (24). 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 copper chaperone that is specific for the formation of the ba_3-type oxidase (26). We noticed that the *B. japonicum* genome harbors two genes for PCu_AC-like proteins (bll4880 and 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 *Rhodobacter capsulatus* (21) and *Pseudomonas aeruginosa* (79). These discoveries are intriguing in view of the fact that the cbb_3-type oxidase does not have a Cu_A center (3, 7). 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, however, 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 (29, 30). One of the genes (*fixI*/*ccoI*) codes for a potential metal-transporting P-type ATPase (80, 81), which is perhaps specific for copper. Rather than transporting copper through the cytoplasmic membrane (to which destination?), the role of FixI might be to sequestrate copper on the periplasmic side and deliver it directly to the membrane-embedded Cu_B site in subunit I (FixN/CcoN).

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