

Copper Starvation-inducible Protein for Cytochrome Oxidase Biogenesis in *Bradyrhizobium japonicum**^[5]

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

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

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

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

Microarray analysis of *Bradyrhizobium japonicum* grown under copper limitation uncovered five genes named *pcuABCDE*, which are co-transcribed and co-regulated as an operon. The predicted gene products are periplasmic proteins (PcuA, PcuC, and PcuD), a TonB-dependent outer membrane receptor (PcuB), and a cytoplasmic membrane-integral protein (PcuE). Homologs of PcuC and PcuE had been discovered in other bacteria, namely PCu_AC and YcnJ, where they play a role in cytochrome oxidase biogenesis and copper transport, respectively. Deletion of the *pcuABCDE* operon led to a pleiotropic phenotype, including defects in the *aa*₃-type cytochrome oxidase, symbiotic nitrogen fixation, and anoxic nitrate respiration. Complementation analyses revealed that, under our assay conditions, the tested functions depended only on the *pcuC* gene and not on *pcuA*, *pcuB*, *pcuD*, or *pcuE*. The *B. japonicum* genome harbors a second *pcuC*-like gene (*blr7088*), which, however, did not functionally replace the mutated *pcuC*. The PcuC protein was overexpressed in *Escherichia coli*, purified to homogeneity, and shown to bind Cu(I) with high affinity in a 1:1 stoichiometry. The replacement of His⁷⁹, Met⁹⁰, His¹¹³, and Met¹¹⁵ by alanine perturbed copper binding. This corroborates the previously purported role of this protein as a periplasmic copper chaperone for the formation of the Cu_A center on the *aa*₃-type cytochrome oxidase. In addition, we provide evidence that PcuC and the copper chaperone ScoI are important for the symbiotically essential, Cu_A-free *cbb*₃-type cytochrome oxidase specifically in endosymbiotic bacteroids of soybean root nodules, which could explain the symbiosis-defective phenotype of the *pcuC* and *scoI* mutants.

This report deals with copper routing to membrane-bound heme-copper cytochrome oxidases in a Gram-negative bacterium. Based on the presence of different copper sites, two groups of heme-copper oxygen reductases must be distinguished (1, 2): (i) those that carry only the high-spin heme-Cu_B center on subunit I, which is buried in the membrane part and forms the active site for O₂ reduction to H₂O (3), and (ii) those that carry in addition a binuclear Cu-Cu center (Cu_A) on subunit II, which is exposed to the periplasm and serves as a recipient of the electrons delivered by periplasmic *c*-type cytochromes (4, 5). Hence, members of the second group are cytochrome oxidases, represented prominently by the mitochondrial and bacterial *aa*₃-type oxygen reductases, whereas most of the bacterial quinol oxidases belong to the first group. The bacterial *cbb*₃-type cytochrome oxidase is in a class of its own (6). Although it lacks the Cu_A center, electrons are delivered to the heme-Cu_B site on subunit I (FixN or CcoN) via the *c*-type cytochromes FixO/CcoO and FixP/CcoP, which constitute subunits II and III of the enzyme complex (7). In contrast to mitochondria, which have only one energy-conserving respiratory chain with one terminal oxidase, bacteria often employ a branched respiratory chain terminating with disparate oxidases, allowing them to efficiently adapt to a wide range of environmental oxygen concentrations (8, 9). The symbiotic nitrogen-fixing bacterium *Bradyrhizobium japonicum*, the organism investigated here, reflects this complexity most notably because it possesses up to eight different terminal oxidases. Two of these dominate (*i.e.* cytochrome *aa*₃ in oxically grown cells and cytochrome *cbb*₃ in symbiosis) (10).

The question of how copper is delivered to and assembled in the Cu_A and Cu_B centers has been addressed not only in mitochondria but also in Gram-negative bacteria, primarily in members of the α -proteobacteria (*e.g.* *Rhodobacter* species, *B. japonicum*, *Paracoccus denitrificans*) because they are phylogenetically the closest extant relatives of mitochondria. Certain conserved functions seem to emerge for each of the following four biogenesis factors. (i) CoxG, also termed CtaG, is a

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^[5] This article contains supplemental Tables S1–S4 and Fig. S1.

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homolog of the mitochondrial Cox11 protein (10–14). The protein is a membrane-anchored Cu(I) chaperone with the copper-binding domain facing the periplasm or the mitochondrial intermembrane space. It plays a role in the assembly of the Cu_B center of cytochrome aa_3 (12). For unknown reasons, however, CoxG does not appear to be required for the symbiotically essential cbb_3 -type oxidase in *B. japonicum*, although this enzyme also carries a Cu_B center (10). (ii) FixI, also called CcoI or CtpA, might be the unknown factor for Cu_B insertion into cytochrome cbb_3 . If it is a copper-translocating P_{1B} -type ATPase, as previously proposed (15, 16), this protein might work as an uptake system destined to insert copper into subunit I. Although copper import has yet to be proven unequivocally, the dependence of cytochrome cbb_3 assembly on FixI (or its paralogs) is well documented (16–18). (iii) ScoI, also called SenC or PrrC, is a homolog of the mitochondrial copper chaperone ScoI (for a review, see Ref. 19). Like CoxG, the ScoI protein is anchored to the bacterial cytoplasmic membrane or the mitochondrial inner membrane, where the copper-binding globular domain protrudes into the periplasm or the intermembrane space. Work with the *Bacillus subtilis* (20) and the mitochondrial ScoI proteins has suggested a role in the metallation of the Cu_A site on subunit II of the aa_3 -type cytochrome oxidase (19). Subsequent work with *B. japonicum* ScoI has adopted this view, supported by the fact that a *scoI* knock-out mutant was clearly defective in cytochrome aa_3 but not in cytochrome cbb_3 when the latter was tested in cells that had been grown micro-oxically or anoxically (10). The symbiosis defect caused by the *B. japonicum* *scoI* mutant remained enigmatic (10, 21). Other reports have shown, however, that SenC and PrrC play a role in the formation of active cytochrome cbb_3 (22–24). (iv) A more recently discovered copper chaperone is PCu_AC , which rivals ScoI in its function to metallate the Cu_A site. Abriata *et al.* (25) designed *in vitro* experiments that documented the direct transfer of Cu(I) to the Cu_A site of the *Thermus thermophilus* ba_3 oxidase. ScoI functioned in this assay as a disulfide reductase to maintain the correct oxidation state of the subunit II cysteine ligands. *In vivo* data with *Rhodobacter sphaeroides* confirmed that PCu_AC and ScoI co-participate in the assembly of a functional Cu_A center in cytochrome aa_3 (24). Intriguingly, the authors of this study noticed a role of PCu_AC also in the formation of the Cu_B center of cytochrome cbb_3 . Homologous genes for PCu_AC -like proteins had been recognized in the *B. japonicum* genome (10, 21), but detailed studies on their biochemical function had not been done.

The present work was initiated with the idea of finding new genes for copper acquisition in *B. japonicum*. Incidentally, transcriptome analyses of copper-starved cells uncovered an operon that also contained the gene for a PCu_AC -like protein. What ensued was an extensive genetic and biochemical investigation that proved its identity as a copper protein and provided evidence for its role in the biogenesis of both the aa_3 - and cbb_3 -type cytochrome oxidases.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions—*Escherichia coli* was grown in Luria-Bertani (LB) medium (26) containing the following concentrations of antibiotics, if necessary:

ampicillin, 200 $\mu\text{g}/\text{ml}$; kanamycin, 30 $\mu\text{g}/\text{ml}$; spectinomycin, 20 $\mu\text{g}/\text{ml}$; tetracycline, 10 $\mu\text{g}/\text{ml}$. *B. japonicum* was routinely cultivated in a peptone-salts-yeast extract medium supplemented with 0.1% L-arabinose (27, 28). Buffered Vincent's minimal medium (BVM),² here defined as vitamin-free modified Vincent's minimal medium (29, 30) supplemented with trace elements (31), 10 mM MOPS (final pH adjusted to 6.8 with 2 M NH_3), and 0.3% L-arabinose, was alternatively used. This medium contains 20 nM CuSO_4 . Glassware was treated overnight with 0.1 M HCl and rinsed thoroughly with double-distilled H_2O when used for experiments on copper limitation, and 10 μM BCS and 1 mM ascorbate were added. Yeast extract-mannitol medium (YEM) (32) supplemented with 10 mM KNO_3 was used for anoxic growth (nitrate respiration). Where appropriate, antibiotics were added to these final concentrations: kanamycin, 100 $\mu\text{g}/\text{ml}$; spectinomycin, 100 $\mu\text{g}/\text{ml}$; streptomycin, 50 $\mu\text{g}/\text{ml}$; tetracycline, 50 $\mu\text{g}/\text{ml}$ (solid media) or 25 $\mu\text{g}/\text{ml}$ (liquid media). *B. japonicum* strains used in this work are listed in Table 1; *E. coli* strains are listed in supplemental Table S1.

Mutant Constructions—Detailed information on plasmids and primers is given in supplemental Tables S1 and S2, respectively. For both the $\Delta\text{pcuABCDE}$ and $\Delta\text{blr7088}$ marker replacement mutants, the upstream and downstream flanking regions of the target genomic sequences were amplified and cloned into pBluescript SK(+) (Stratagene, La Jolla, CA). The *aphII* gene encoding kanamycin resistance or the Ω cassette encoding streptomycin resistance ($\Delta\text{pcuABCDE}$ and $\Delta\text{blr7088}$ mutants, respectively) was then inserted in both orientations between the upstream and downstream flanking regions. The DNA constructs were excised and inserted into the suicide plasmid pSUP202pol4 (35), yielding plasmids pRJ6611, pRJ6612, pRJ6620, and pRJ6621. Mobilization of these plasmids into *B. japonicum* 110*spc4* was carried out via *E. coli* S17-1 and followed by screening for double recombination events. The resulting strains 6611 ($\Delta\text{pcuABCDE}$, same orientation of the *aphII* gene) and 6612 ($\Delta\text{pcuABCDE}$, opposite orientation) carry a deletion between positions 5,408,844 and 5,414,217 of the genome (Fig. 1). Strains 6620 ($\Delta\text{blr7088}$, same orientation of the Ω cassette) and 6621 ($\Delta\text{blr7088}$, opposite orientation) are deleted between positions 7,804,884 and 7,805,644. For the generation of double mutants 6611-20 ($\Delta\text{pcuABCDE}$, same orientation; $\Delta\text{blr7088}$, same orientation) and 6611-21 ($\Delta\text{pcuABCDE}$, same orientation; $\Delta\text{blr7088}$, opposite orientation), the plasmid pRJ6611 was mobilized into strains 6620 and 6621, respectively.

Complementation of $\Delta\text{pcuABCDE}$ —The genome sequence comprising the *pcuABCDE* operon and its upstream region necessary for a single crossover event (corresponding to genome coordinates 5,408,585–5,414,850), with the addition of appropriate restriction sites, was cloned into vector pGEM-T Easy (Promega, Madison, WI), yielding plasmid pRJ6626. The restriction map of pRJ6626 allowed excision of operon fragments via simple digestions followed by self-ligation: FseI treatment deleted the entire operon, leaving its 5' region to give an

² The abbreviations used are: BVM, buffered Vincent's minimal medium; BCS, bathocuproine disulfonate; YEM, yeast extract mannitol medium; qRT-PCR, quantitative RT-PCR; TEV, tobacco etch virus.

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empty vector insertion for control (pRJ6631); SphI was used to excise *pcuE* (pRJ6627); MlsI/OliI were used to in-frame delete *pcuB* (pRJ6628). When in-frame deletion of genes was not possible with the aforementioned strategy, alternative approaches were applied. Substitution of the naturally occurring HindIII/FspAI fragment with a shorter PCR-generated fragment, including the natural HindIII site on its 3' end and an added FspAI site on its 5' end, was carried out to obtain an in-frame *pcuD* deletion (pRJ1651). Analogously, natural AbsI/EcoRV restriction sites were exploited for the *pcuC* in-frame deletion (pRJ6629). An overlapped extension PCR (36) was used to obtain a fragment carrying a *pcuA* in-frame deletion, which substituted the full-length gene via BamHI/SpeI restriction sites (pRJ1652). SpeI/PsiI fragments from pRJ6626, pRJ6631, pRJ6627, pRJ1651, pRJ6629, pRJ6628, and pRJ1652 were inserted into a SpeI/SmaI-linearized pSUP202pol4 suicide plasmid, yielding pRJ6633, pRJ6632, pRJ6630, pRJ1654, pRJ6634 (Fig. 1), pRJ1650, and pRJ1653, respectively. These plasmids were then mobilized into *B. japonicum* 6611 via *E. coli* S17-1, followed by screening for single recombination events (Fig. 1). Plasmid pRJ6632 (empty vector control) was mobilized into *B. japonicum* 110*spc4* as well. All of the resulting strains are listed in Table 1.

Determination of Copper Atoms in Copper-free BVM—The concentration of copper in CuSO₄-free BVM prepared with treated glassware was determined by graphite furnace atomic absorption spectroscopy. The instrument used was an AAAnalyst800 (PerkinElmer Life Sciences) equipped with a transversely heated graphite furnace and longitudinal Zeeman background correction system. A hollow cathode lamp was chosen as the radiation source. A temperature program was applied to the furnace for solvent drying (110 °C for 30 s followed by 130 °C for 30 s under 250 ml/min argon flow), matrix pyrolysis (1,200 °C for 20 s under 250 ml/min argon flow), and atomization (2,100 °C for 5 s). Copper atoms were detected during the last step by measuring absorption at 324.8 nm. A three-point standard addition procedure was applied for the quantification of copper in the analyzed samples. 10- μ l samples were mixed with a matrix modifier solution (~500 ng of MgNO₃ and PdNO₃/g of sample) and different amounts of a copper standard solution (0, 0.9, 1.8, and 2.9 ng of copper/g with samples containing up to 3.5 ng; 0, 9, 18, and 29 ng/g with samples containing more than 3.5 ng of copper) in a final volume of 30 μ l. Each measurement was repeated three times. All solutions were prepared in 0.5% HNO₃. The instrumental limit of detection was 0.05 ng of copper/g of sample.

RNA Isolation and cDNA Synthesis—Cell harvesting, RNA extraction, cDNA synthesis for microarray, and quantitative RT-PCR (qRT-PCR) were performed as described (37).

qRT-PCR—RNA from aerobically grown *B. japonicum* 110*spc4*, 6611-33, and 6611-34 was isolated in order to monitor the expression level of *pcuD* by qRT-PCR, similarly to what had been described previously (38). In order to analyze the influence of copper on the expression of genes *pcuA* and *pcuB*, RNA was extracted from *B. japonicum* 110*spc4* grown in standard BVM or in copper-free BVM supplemented with 10 μ M BCS and 1 mM ascorbic acid. Details of the primers used are listed in supplemental Table S2.

Primer Extension—Mapping of the transcription start site of the *pcuABCDE* operon was carried out as described previously (39) using the primer named “primer_extension_1” (supplemental Table S2). RNA was isolated from *B. japonicum* 110*spc4* grown aerobically in standard BVM or in copper-free BVM supplemented with 10 μ M BCS and 1 mM ascorbic acid. The sequencing ladder was obtained by sequencing plasmid pRJ6336 (supplemental Table S1) with the same primer that was used for the extension reaction.

Microarrays—Transcriptomics experiments were carried out as described previously (40) using a custom-designed Affymetrix GeneChip® (37). The chip was hybridized with cDNA obtained from *B. japonicum* 110*spc4* grown aerobically either in BVM containing 2 μ M CuSO₄, in copper-free BVM (5 nM copper), or in copper-free BVM supplemented with 10 μ M BCS and 1 mM ascorbic acid. Three biological replicates were prepared for each condition. Data were analyzed using GeneSpring GX 7.3.1 software (Agilent, Santa Clara, CA). The data were filtered for probe sets that were called “present” or “marginal” in at least two of three replicas, and Student's *t* test with a *p* value threshold of 0.01 was applied. Differential expression in a comparison of two conditions was defined when the -fold change value was larger than 2 or smaller than -2. Data sets generated in this work are deposited in the GEO database with the record number GSE40437.

Plant Growth—Sterilization of soybean seeds (*Glycine max* (L.) Merr. cv. Williams), cultivation of plants, and nitrogenase activity measurements were performed as described previously (10, 41–43). Plants were evaluated for nodulation and nitrogen fixation 21 days after the infection with the appropriate *B. japonicum* strains.

Bacteroid Isolation—Bacteroids were isolated from root nodules and separated from plant material as described previously (44, 45).

Nitrate and Nitrite Detection in the Growth Medium—Adapted versions of described methods were used (46). Cells were removed from the growth medium by centrifugation. For nitrate detection, a 0.1-ml sample of 10-fold diluted supernatant was mixed with 0.1 ml of 1% sulfamic acid and 0.8 ml of a 1:1 mixture of 98% H₂SO₄ and 85% H₃PO₄ (v/v). After a 10-min incubation at room temperature, 0.1 ml of 0.12% (w/v) 2,6-dimethyl phenol in 100% (v/v) acetic acid was added. Absorption at 334 nm was recorded after a 90-min incubation at room temperature. A standard curve was recorded from 0 to 10 mM KNO₃ in YEM. For nitrite determination, a 0.4-ml sample of 80-fold diluted supernatant was mixed with 0.4 ml of 1% (w/v) sulfanilamide in 7.4% (v/v) HCl and 0.4 ml of 0.02% (w/v) *N*-(1-naphthyl)ethylenediamine. Absorption at 540 nm was measured after a 30-min incubation at room temperature. A standard curve was taken from 0 to 10 mM NaNO₂ in YEM.

Determination of Cytochrome *c* Oxidase Activity in Membrane Fractions—Preparation of the membrane fraction and determination of cytochrome *c* oxidase activity were carried out as described previously (10, 47), starting either from culture-grown cells or from purified bacteroids. Concentration of solubilized membrane proteins was determined with the Bradford method (48), using a Bio-Rad assay with bovine serum albumin as the standard.

Immunological Techniques—Rabbit antibodies specific for CoxA and CoxB proteins were available from previous work (10, 49). Western blot analyses were carried out on membrane proteins (35 $\mu\text{g}/\text{lane}$) separated by SDS-PAGE (50) and blotted as described previously (49). Anti-rabbit IgG(H+L)-horseradish peroxidase conjugate (Bio-Rad) and a peroxidase chemiluminescence detection kit (Thermo Fisher Scientific, Waltham, MA) were used to detect bands containing primary antibodies bound to CoxA and CoxB.

Purification of PcuC and Its Derivatives—Details of the plasmids and primers are listed in supplemental Tables S1 and S2, respectively. For the periplasmic expression construct, the *B. japonicum* genomic sequence corresponding to the *pcuC* gene product (amino acids 25–173), excluding the predicted signal peptide, was amplified, adding a *Strep*-tag II sequence and a TEV protease recognition site (51) at the 5' end, and cloned into pEC415 (52). This resulted in a plasmid for expression of PcuC that was additionally fused to the *E. coli* OmpA signal peptide. For cytoplasmic expression, DNA for the soluble part of PcuC was cloned into pKL1 to fuse it with a His₁₀ tag followed by a TEV recognition site. Derivatives of PcuC in which His⁷⁹, Met⁹⁰, His¹¹³, Met¹¹⁵, and His¹¹³ plus Met¹¹⁵ residues are substituted by alanine were obtained by exchanging the wild-type sequence of the cytoplasmic expression construct with synthetic fragments (custom-synthesized by Eurofins MGW Operon, Ebersberg, Germany) bearing the point mutations. A shortened version of *pcuC* missing the coding region for the 15-amino acid, methionine-rich C terminus was created by PCR and by inserting a stop codon at the 3' end. This was cloned into pKL1. Dense *E. coli* BL21 (DE3) precultures containing the described expression vectors or the TEV protease expression vector pRK793 (53) were used to inoculate 1-liter main cultures. Cells were grown at 37 °C until they reached an optical density (A_{600}) of 0.6; the expression was then induced by adding a final concentration of 0.1% (w/v) arabinose or 0.1 mM isopropyl- β -D-1-thiogalactopyranoside for the periplasmic PcuC expression and for the other expression constructs, respectively. Cultures were transferred to 30 °C, and after 2 h, cells were collected by centrifugation and disrupted by means of three passages at 9,000 p.s.i. through a French press. The periplasmically expressed protein was purified with a Strep-Tactin-Sepharose column (IBA GmbH, Göttingen, Germany) according to the supplier's protocol. To obtain apo-PcuC, the purified protein was incubated with an \sim 1,000-fold molar excess of BCS, and the buffer was eventually exchanged with a PD-10 desalting column (GE Healthcare). The poly-His-tagged proteins were purified via nickel-nitrilotriacetic acid-agarose columns (Qiagen, Hilden, Germany). Cleavage of the tag was achieved by treatment with TEV protease, which was later removed with a nickel-nitrilotriacetic acid-agarose column. Purity of the protein and the copper binding status were verified with electrospray ionization MS measurements performed at the Functional Genomics Center Zurich.

Tests for Cu(I) Binding to PcuC—PcuC from cytoplasmic expression was used for the experiment, which proved to be copper-free according to electrospray ionization MS analysis. Alternatively, apo-PcuC was obtained from periplasmic expression as described above. The titration buffer was

obtained by filtering 100 mM HEPES, pH 7, 10 mM NaCl through a Chelex 100 chelating ion exchange resin (Bio-Rad) into 0.1 M HCl-treated glassware in order to ensure a maximally possible absence of free copper. The protein elution buffer was exchanged with titration buffer using a PD-10 desalting column. The preparation of $\text{Cu}(\text{BCS})_2^{3-}$ solution and the titration of apo-PcuC on the $\text{Cu}(\text{BCS})_2^{3-}$ complex was carried out as described (54), except that titration buffer was used. The experiment was performed in both anoxic and oxic conditions. In the first case, the solutions were prepared in an anaerobic glove box (Coy Laboratory Products, Grass Lake, MI) with a maximal oxygen concentration of 80 ppm, and the UV-visible spectra were recorded on an Agilent diode array photometer. In the latter case, CuCl was dissolved directly into a BCS solution, and UV-visible spectra were recorded on a Hitachi U-3300 spectrophotometer (Hitachi, Tokyo, Japan).

Bioinformatics Analyses—BLAST was used for homology searches. Multiple alignments were done with ClustalW and visualized with ESPript 2.2. The presence and cleavability of signal peptide were predicted with SignalP. The extinction coefficient of purified proteins was calculated by ProtParam. Homology modeling of the PcuC amino acid sequence on the *TtPCu_AC* three-dimensional structure (Protein Data Bank code 2K6W) was done using SWISS-MODEL.

RESULTS

Comparative Transcription Profile of Cells Grown at Different Copper Concentrations—Copper-limiting growth conditions were thought to cause an induction of genes possibly involved in copper uptake and sorting. This rationale in mind, we performed microarray analyses on *B. japonicum* cells grown in three variations of the BVM minimal medium. Variant 1 contained 2 μM CuSO_4 (copper excess). Variant 2 was prepared in HCl-treated glassware without any copper added (copper starvation). The residual copper concentration in this copper starvation medium was analyzed by graphite furnace atomic absorption spectroscopy and determined to be 5 nM. Variant 3 (extreme copper limitation) was prepared like variant 2 but with the addition of 10 μM BCS and 1 mM ascorbic acid where BCS chelates Cu(I) selectively, and ascorbic acid reduces any Cu(II) to Cu(I). Changes in the transcription profiles were recorded by the pairwise comparison of cells grown in variant 2 versus variant 1 and in variant 3 versus variant 2 (supplemental Tables S3 and S4, respectively). Only a small set of genes were differentially up- or down-regulated when copper-starved cells were compared with cells grown in copper excess (supplemental Table S3). Most notably, five genes located adjacent to each other on the *B. japonicum* genome displayed an increased expression: bll4882 to bll4878; supplemental Table S3). For reasons that will become evident from subsequent research (see below), the five genes were named *pcuA*, *pcuB*, *pcuC*, *pcuD*, and *pcuE* (mnemonic of proteins for Cu trafficking). The genes with decreased expression either are of unknown function or, not surprisingly, play a role in copper resistance. Extreme copper limitation (variant 3 versus variant 2) did not further enhance the expression of the five *pcu* genes. Instead, another cluster of adjacent genes was strongly up-regulated: bll0889 to bll0883, which code for unidentified transport functions (supplemental

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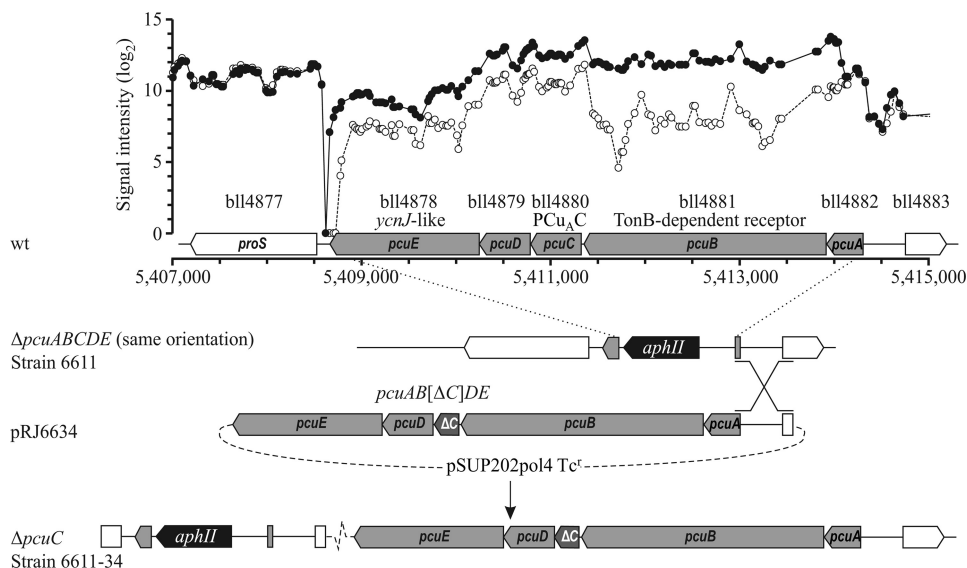


FIGURE 1. Map of the *pcuABCDE* gene cluster. Gene names and relevant homologies (if available) are given below the gene numbers. Genome coordinates are reported below the gene map. The top part of the scheme shows the transcription levels of *B. japonicum* wild-type cells grown in copper excess BVM ($2 \mu\text{M}$ CuSO_4 ; open circles) and in copper starvation BVM (5 nM copper; closed circles) obtained from microarray analyses. The bottom part shows the genotype of the $\Delta pcuABCDE::aphII$ strain (same orientation) and the strategy used to partially complement it with an in-frame *pcuC*-deleted version of the operon (*pcuAB*[ΔC]DE), resulting in a $\Delta pcuC$ genotype. Analogous strategies were used to construct $\Delta pcuA$, $\Delta pcuB$, $\Delta pcuD$, and $\Delta pcuE$ strains.

Table S4). Incidentally, the list also includes the copper chaperone ScoI. As expected, the pairwise comparison of variant 3 versus variant 1 (not shown) included again all of the differentially expressed genes listed in supplemental Table S3. Taken together, copper-limiting growth conditions have led to the derepression of genes potentially involved in copper acquisition. In this work, we have focused on the investigation of the *pcuABCDE* gene cluster.

The *pcuABCDE* Genes Are Transcribed as an Operon—The short distances between the ORFs of the *pcuABCDE* cluster already suggested an operon organization in which the five genes would be co-regulated and co-transcribed. The tiling-like design of the oligonucleotides on the gene chip used for microarrays (37) allowed us to confirm this inference because, consistently, transcript levels in copper-starved cells were higher throughout the entire *pcuABCDE* cluster as compared with copper-rich conditions (Fig. 1). Complementary information was obtained by applying qRT-PCR and primer extension techniques. For these experiments, RNA was extracted from wild-type cells grown in extreme copper limitation and from cells grown in standard BVM. When cDNA corresponding to *pcuA* and *pcuB* was produced thereof for qRT-PCR, the data reflected a respective up-regulation of 3.4 ± 0.8 - and 33.3 ± 6.2 -fold in copper limitation. The numbers compared well with the transcriptomics data. Likewise, a primer extension product of the mRNA 5' end was seen only in copper-limited cells (Fig. 2). The transcription start site was thus mapped at nucleotide position 5,414,300 in the *B. japonicum* genome, which lies at a distance of 21 bp upstream of the *pcuA* start codon. Sequence inspection of the associated promoter region did not allow predictions to be made on the possible mode of transcriptional regulation.

Predicted Proteins Encoded by the *pcuABCDE* Operon Suggest a Role in Copper Trafficking—The putative gene products can be grouped into two categories, soluble and membrane-bound

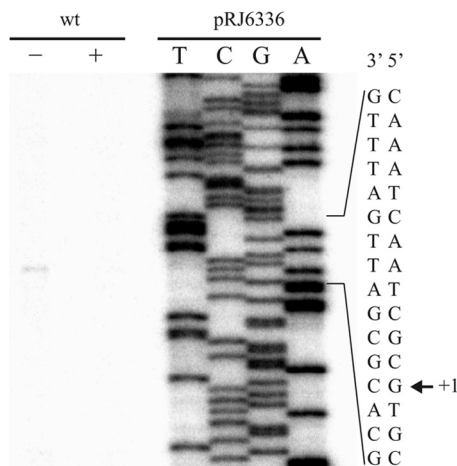


FIGURE 2. Transcription start site mapping of *pcuA*. Total RNA from *B. japonicum* wild-type strain grown in standard BVM ($0.02 \mu\text{M}$ CuSO_4 ; plus sign) or in extreme copper limitation BVM (containing $10 \mu\text{M}$ BCS and 1 mM ascorbic acid; minus sign). Extension products were obtained with the ^{32}P -labeled primer named "primer_extension_1" and separated on a 6% denaturing polyacrylamide gel. The sequencing ladder was generated with plasmid pRJ6336 and the same primer. Part of the promoter region is shown on the right, and the transcription start site (+1) is indicated (arrow).

proteins. PcuA, PcuC, and PcuD are predicted soluble, periplasmic proteins because all three have an N-terminal sequence for Sec-dependent secretion. PcuA is conserved only in the order Rhizobiales and does not contain a noteworthy amino acid motif other than a CXXC sequence. PcuC is clearly homologous to the known copper chaperone PCu_AC (e.g. sharing 36% identity with the *T. thermophilus* PCu_AC protein; Fig. 3). PcuD is well conserved in bacteria, occasionally fused N-terminally with PcuC, but neither is its function known, nor does it contain a conspicuous amino acid domain or motif. The PcuB and PcuE proteins are membrane-integral proteins. PcuB is a member of the TonB-dependent receptor protein family, which implies a location in the outer membrane by virtue of the β -barrel struc-

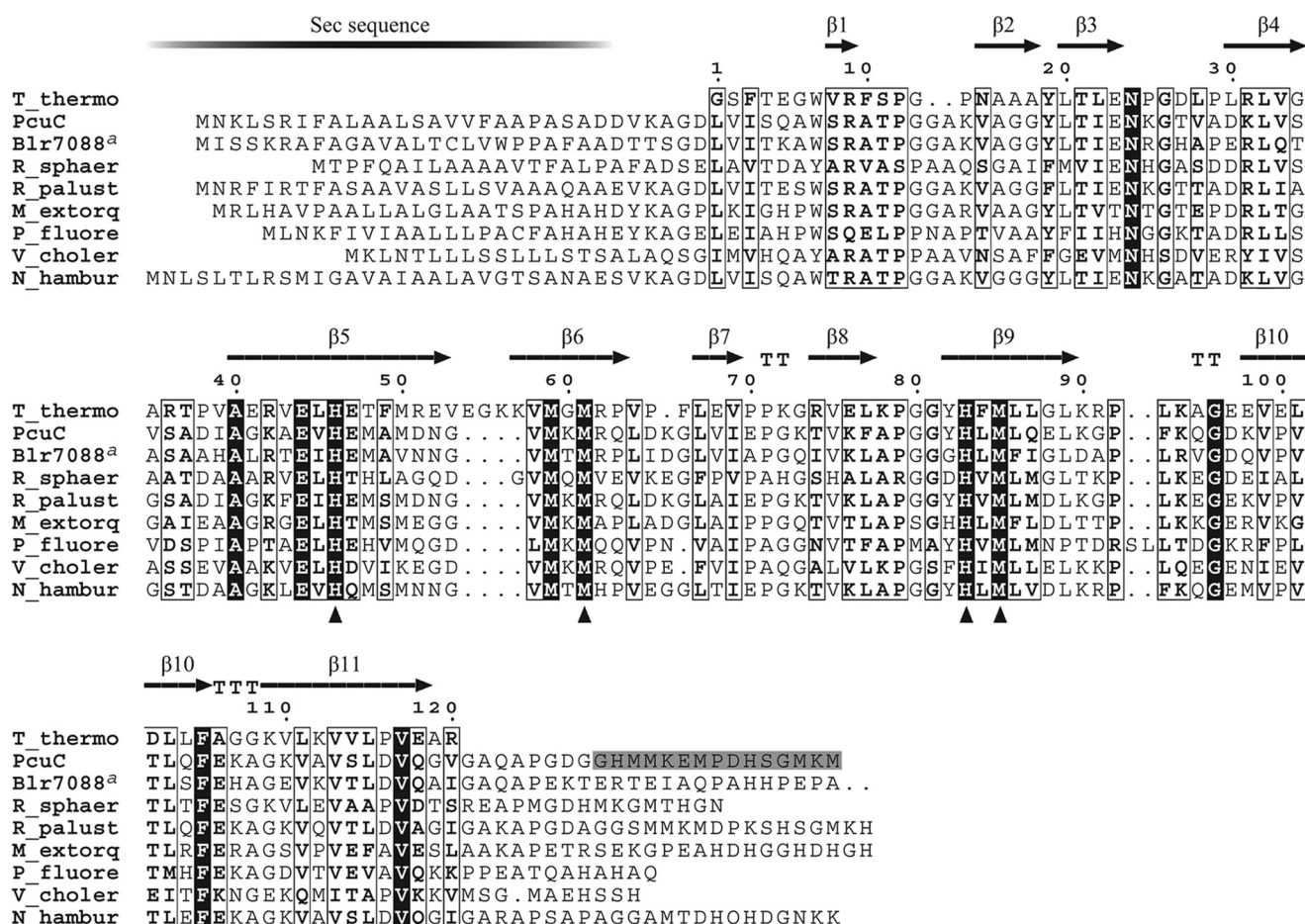


FIGURE 3. Multiple alignment of PCu_AC amino acid sequences. The secondary structure of *T. thermophilus* PCu_AC extracted from its crystal structure (Protein Data Bank code 2K6W) is shown above the alignment. The numbering refers to the *T. thermophilus* PCu_AC sequence. *B. japonicum* PcuC and Blr7088, *R. sphaeroides* 2.4.1 RSP_2017, *Rhodospseudomonas palustris* HaA2 RPB_2549, *Methylobacterium extorquens* PA1 Mext_1379, *Pseudomonas fluorescens* Pf0-1Pfl01_0598, *Vibrio cholerae* HC-02A1 VCHC02A1_2964, and *Nitrobacter hamburgensis* X14 Nham_2200 sequences are included in the alignment. Similarity and identity columns are shown as empty boxes and white on black type, respectively. Copper-binding residues of TtPCu_AC are marked with triangles; the corresponding residues of PcuC are His⁷⁹, Met⁹⁰, His¹¹³, and Met¹¹⁵. The gray-shaded amino acids at the C-terminal end of the PcuC sequence are those that were removed to test if they bind copper. *a*, the C-terminal 118-amino acid domain of Blr7088 was omitted in the alignment.

ture. The high majority of the characterized members of this family are siderophore-iron or tetrapyrrole-metal importers (55). It was tempting to speculate that PcuB might be a receptor for the uptake of a copper-chelate complex through the *B. japonicum* outer membrane. PcuE is most likely a cytoplasmic membrane protein made up of an N-terminal CopC-like domain and a C-terminal CopD-like domain. The conserved CopC and CopD proteins have first been described in *Pseudomonas syringae* as part of the copper resistance (export) system (56). By contrast, the *B. subtilis* YcnJ protein, which shares 27% identity with PcuE, is a CopCD-like fusion protein that was reported to have a copper acquisition function (57). In conclusion, the proteins encoded by the *B. japonicum* *pcuABCDE* operon appeared as strong candidates for playing a role in copper import and sorting.

The Δ pcuABCDE Strains Have a Pleiotropic Phenotype—Two deletion mutants were created by replacing the entire operon sequence with a kanamycin resistance cassette in both orientations relative to the deleted genes, yielding strains 6611 (same orientation; Fig. 1) and 6612 (opposite orientation; Table 1). Aerobic growth was measured in different copper concentrations. The *B. japonicum* wild type and the two Δ pcuABCDE

strains had an identical growth behavior in standard complete and minimal media or in copper starvation medium under oxic conditions (data not shown). However, growth of the mutants was impaired in extreme copper limitation (Fig. 4A; here shown for strain 6611). Also, under conditions of anoxic nitrate respiration (YEM plus NO₃⁻, 0.2 μM CuSO₄), the growth rate of the operon mutants was diminished when compared with the wild type (Fig. 5A). Concomitantly, a delayed consumption of nitrate and a transient accumulation of nitrite were observed in the mutants (Fig. 5B). This could be interpreted to mean that, in the mutants, the copper-dependent nitrite reductase (NirK) (58) is insufficiently supplied with the copper cofactor. In fact, attempts at limiting the copper concentration in such anoxic cultures led to a cessation of growth already with the wild type, making it impossible to further elaborate discriminative mutant phenotypes with respect to the denitrification pathway.

Another important phenotype observed with the Δ pcuABCDE mutants was the complete lack of activity of the aa₃-type cytochrome oxidase, which is the predominant respiratory oxygen reductase in oxically grown *B. japonicum* wild type. The membrane protein fraction was isolated from oxically grown strains, and the ability to oxidize reduced horse heart

TABLE 1

B. japonicum strains used in this work

Strain	Relevant genotype or phenotype	Source or Ref.
110 <i>spc4</i>	Sp ^r wild type	Ref. 27
COX132	Sp ^r Km ^r <i>coxA</i> ::Tn5	Ref. 33
3613	Sp ^r Km ^r <i>fixN</i> ::Tn5	Ref. 34
2574	Sp ^r Km ^r Δ <i>scol</i> :: <i>aphII</i> (same orientation)	Ref. 10
6611	Sp ^r Km ^r Δ <i>pcuABCDE</i> :: <i>aphII</i> (same orientation)	This work
6612	Sp ^r Km ^r Δ <i>pcuABCDE</i> :: <i>aphII</i> (opposite orientation)	This work
6620	Sp ^r Sm ^r Δ <i>blr7088</i> :: Ω (same orientation)	This work
6621	Sp ^r Sm ^r Δ <i>blr7088</i> :: Ω (opposite orientation)	This work
6611-20	Sp ^r Km ^r Sm ^r Δ <i>pcuABCDE</i> :: <i>aphII</i> (same orientation), Δ <i>blr7088</i> :: Ω (same orientation)	This work
6611-21	Sp ^r Km ^r Sm ^r Δ <i>pcuABCDE</i> :: <i>aphII</i> (same orientation), Δ <i>blr7088</i> :: Ω (opposite orientation)	This work
6632	Sp ^r Tc ^r pSUP202 <i>pol4</i> chromosomally integrated in 110 <i>spc4</i>	This work
6611-32	Sp ^r Km ^r Tc ^r pSUP202 <i>pol4</i> chromosomally integrated in 6611	This work
6611-33	Sp ^r Km ^r Tc ^r <i>pcuABCDE</i> on pSUP202 <i>pol4</i> chromosomally integrated in 6611	This work
6611-34	Sp ^r Km ^r Tc ^r <i>pcuA</i> [Δ C] <i>DE</i> on pSUP202 <i>pol4</i> chromosomally integrated in 6611	This work
6611-6630	Sp ^r Km ^r Tc ^r <i>pcuABC</i> [Δ E] on pSUP202 <i>pol4</i> chromosomally integrated in 6611	This work
6611-1654	Sp ^r Km ^r Tc ^r <i>pcuABC</i> [Δ D] <i>E</i> on pSUP202 <i>pol4</i> chromosomally integrated in 6611	This work
6611-1650	Sp ^r Km ^r Tc ^r <i>pcuA</i> [Δ B] <i>CDE</i> on pSUP202 <i>pol4</i> chromosomally integrated in 6611	This work
6611-1653	Sp ^r Km ^r Tc ^r <i>pcu</i> [Δ A] <i>BCDE</i> on pSUP202 <i>pol4</i> chromosomally integrated in 6611	This work

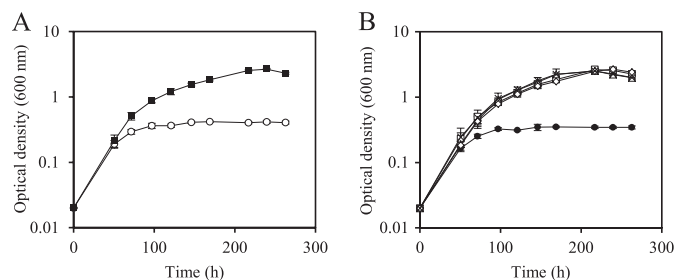


FIGURE 4. Decreased growth of the operon mutant Δ *pcuABCDE* under copper starvation is due to loss of *pcuC*. Strains were grown in BVM medium under extreme copper limitation (no copper added; 10 μ M BCS and 1 mM ascorbic acid added). Aerobic growth was measured from a starting optical density of 0.02 until stationary phase, and values represent the average optical density of cultures measured in triplicate. A, wild-type *B. japonicum* (filled squares) and Δ *pcuABCDE* strain 6611 (open circles). B, single-gene deletion strains Δ *pcuE* (open squares), Δ *pcuD* (open triangles), Δ *pcuC* (filled circles), Δ *pcuB* (open diamonds), and Δ *pcuA* (crosses). Error bars, S.D.

cytochrome *c* was measured. As shown in Fig. 6, oxidase activity in wild type is due to cytochrome *aa*₃ because membrane proteins extracted from a *coxA* mutant (Tn5 insertion in *aa*₃-subunit-I gene) have almost no activity. Cytochrome *c* oxidase activity of both Δ *pcuABCDE* strains (here shown for 6611) is as low as that of the *coxA* mutant, indicating an involvement of the operon in cytochrome *aa*₃ biogenesis. The wild-type

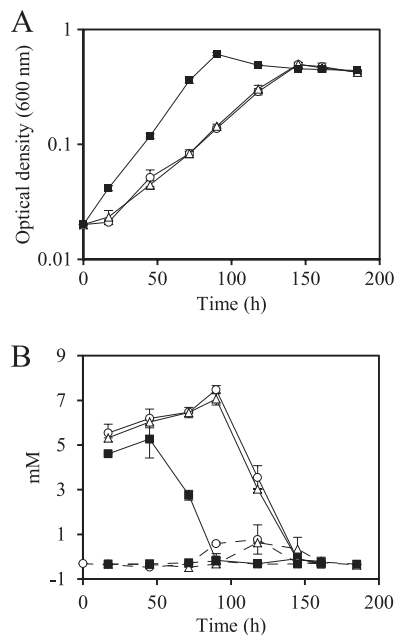


FIGURE 5. Anaerobic growth, NO_3^- consumption, and transient NO_2^- accumulation in the Δ *pcuABCDE* mutant. The following strains were tested: wild type (closed squares), 6611 (Δ *pcuABCDE* insert in the same orientation; open circles), and 6612 (Δ *pcuABCDE* insert in the opposite orientation; open triangles). A, growth curves in anaerobic YEM containing 10 mM KNO_3 . B, concentrations (in mM) of NO_3^- (continuous lines) and NO_2^- (dashed lines) in the medium during growth. All of the measurements were taken in triplicates. Error bars, S.D.

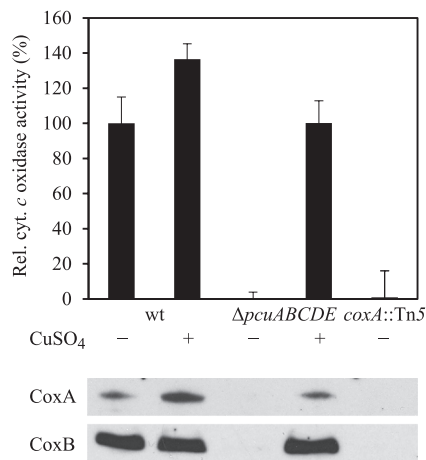


FIGURE 6. Effect of the *pcuABCDE* deletion on *aa*₃-type cytochrome *c* oxidase activity. The relative cytochrome *c* oxidase activity of aerobically grown wild type (*wt*) and strains 6611 (Δ *pcuABCDE*) and COX132 (*coxA*::Tn5) is shown in the top panel. Wild-type activity corresponds to 0.206 μ mol of horse heart cytochrome *c* oxidized/mg of membrane protein/min. 50 μ M CuSO_4 was added to the peptone-salts-yeast extract medium where indicated (+). Western blot analysis for the detection of subunits I and II of the cytochrome *aa*₃ (CoxA and CoxB, respectively) was carried out on the same membrane proteins (bottom panels). Error bars, S.D.

phenotype could be restored by growing the cells in medium containing 50 μ M CuSO_4 . Western blot analyses of all of these membrane extracts (Fig. 6, bottom) revealed an absence of both subunits I and II in the Δ *pcuABCDE* mutant, whereas they were detectable again in cells grown in copper excess. We tentatively concluded, therefore, that the *pcuABCDE* operon is involved in providing copper for the *aa*₃-type cytochrome *c* oxidase.

TABLE 2

Symbiotic properties of $\Delta pcuABCDE$ mutants inoculated on soybean

Mutants were tested in parallel with the wild type in three independent experiments. Shown here are data from one representative experiment.

Strain	Relevant genotype ^a	Number of nodules	Nodule dry weight	Nitrogenase activity ^b	Relative Fix activity ^c
110 <i>spc4</i>	Wild type	20.4 ± 4.0	2.4 ± 0.7 <i>mg</i>	3.0 ± 0.7 <i>%/min/g</i>	100 ± 22.3 <i>%</i>
6611	$\Delta pcuABCDE$ -a	25.8 ± 7.8	1.3 ± 0.3	0.8 ± 0.5	25.9 ± 16.9
6612	$\Delta pcuABCDE$ -b	23.3 ± 5.1	1.4 ± 0.4	0.7 ± 0.4	24.4 ± 12.5

^a Strain 6611 has insert in the same orientation (-a) and strain 6612 in the opposite orientation (-b).^b Nitrogenase activity is expressed as percentage of C₂H₄ formed/min/g.^c Relative nitrogen fixation activity is expressed as a percentage of wild type.

Finally, as we routinely tested any *B. japonicum* mutant generated in our laboratory for its symbiotic efficiency, a 75% decrease of symbiotic nitrogen fixation activity (measured with the acetylene reduction test) was recorded in soybean root nodules induced with the $\Delta pcuABCDE$ strains (Table 2). The possible reason for this strong defect will be addressed further below (see "PcuC and ScoI Are Required for the Symbiotically Essential *cbb*₃-Type Cytochrome Oxidase"). The addition of excess copper to the plant growth medium did not help to abrogate the defect.

pcuC Is the Only Essential Gene of the pcuABCDE Operon for All Tested Functions—Having established a pleiotropic phenotype associated with the operon deletion, it was necessary to find out how many and which of the five genes are responsible. For this purpose, a comprehensive complementation strategy was applied in which either all five genes or five minus one were recombined back into the deletion mutant 6611. The latter constructs consisted of operons in which a markerless in-frame deletion was generated in each of the five genes, leaving the other four intact (see the $\Delta pcuC$ example in Fig. 1). This resulted in a set of five strains with single gene deletions plus the controls (vector insertion, complete operon; Table 1). All strains were then tested for most of the phenotypes reported in the preceding section. Surprisingly, the $\Delta pcuC$ deletion proved to be the only responsible mutation for the decreased cytochrome *c* oxidase activity (Table 3), and this defect was restored to wild-type activity in cells grown with copper excess (Table 3). Deletions of *pcuA*, *pcuB*, *pcuD*, or *pcuE* did not cause a defect. Likewise, the $\Delta pcuC$ strain was the only one that exhibited an impaired aerobic growth in conditions of copper limitation (Fig. 4B), and it was the only one that had a decreased symbiotic nitrogen fixation activity (Table 3). The $\Delta pcuC$ phenotype was unlikely to be the result of polar effects of the deletion on the downstream operon genes, because the deletions of *pcuE* and *pcuD* themselves had no effect. Nevertheless, we ascertained by qRT-PCR that DNA downstream of the *pcuC* in-frame deletion was fully transcribed (data not shown).

B. japonicum Possesses a Second pcuC-like Gene (blr7088) without an Obvious Function—The predicted Blr7088 protein shares in its N-terminal half 40% identity with PcuC (cf. Fig. 3), whereas the C-terminal extension carries a weakly conserved domain. The blr7088 gene is located next to the respiratory nitrite reductase gene (*nirK*, blr7089), which might implicate a function of the Blr7088 protein in the biogenesis of the copper-containing nitrite reductase. However, $\Delta blr7088$ strains (6620 and 6621; Table 1) were constructed and shown to be unaffected in anoxic growth with nitrate as the terminal electron

TABLE 3

Complementation of $\Delta pcuABCDE$ (strain 6611) and effects of single-gene deletions on the phenotypes

Activities of the mutant strains are expressed as a percentage of wild-type activity.

Strain	Genes added for complementation	Relative cytochrome <i>c</i> oxidase activity ^a		Relative Fix activity ^a
		Without 50 μ M Cu(SO) ₄	With 50 μ M Cu(SO) ₄	
6611		16.8 ± 4.7 <i>%</i>	100.1 ± 12.8 <i>%</i>	34.7 ± 13.5 <i>%</i>
6611-32	Only vector insert	14.4 ± 5.1	90.3 ± 9.8	ND ^b
6611-33	<i>pcuABCDE</i> ⁺	106.6 ± 10.5	ND	ND
6611-6630	<i>pcuABCD</i> [ΔE] ⁺	113.4 ± 9.9	ND	112.8 ± 16.8
6611-1654	<i>pcuABC</i> [ΔD] <i>E</i> ⁺	120.5 ± 18.2	ND	127.8 ± 40.9
6611-34	<i>pcuAB</i> [ΔC] <i>DE</i> ⁺	24.8 ± 4.4	100.5 ± 10.1	36.1 ± 5.0
6611-1650	<i>pcuA</i> [ΔB] <i>CDE</i> ⁺	101.0 ± 17.0	ND	105.1 ± 8.5
6611-1653	<i>pcu</i> [ΔA] <i>BCDE</i> ⁺	111.8 ± 19.5	ND	99.7 ± 10.3

^a The average wild-type cytochrome *c* oxidase activity (100%) is ~0.31 μ mol of horse heart cytochrome *c* oxidized/mg of membrane protein/min. The average wild-type Fix activity (100%) is 2.82 ± 0.4% C₂H₄/min/g.^b ND, not determined.

acceptor. Also, these mutants had none of the phenotypes observed for the $\Delta pcuABCDE$ strains, except for a slight delay in aerobic, copper-limited growth. Furthermore, double deletion strains ($\Delta pcuABCDE$ plus $\Delta blr7088$; Table 1) had phenotypes that were not more severe than those observed for the $\Delta pcuABCDE$ strains, except for perhaps an additive impairment in aerobic, copper-limited growth (data not shown). Hence, the function of the Blr7088 protein remains elusive, and there was no indication that it might be able to functionally replace the PcuC protein in a *pcuC* mutant background. Therefore, research on blr7088 was discontinued.

PcuC Is a Cu(I)-binding PCu_AC-like Protein—Because *pcuC* turned out to be the only important gene of the *pcuABCDE* operon (under the conditions tested), we wished to obtain additional evidence for its role in copper metabolism. The PcuC protein was expressed in *E. coli* using both periplasmic and cytoplasmic overexpression systems (see "Experimental Procedures"), and the proteins were purified to apparent homogeneity from these compartments. Electrospray ionization MS analyses revealed that cytoplasmically produced PcuC was copper-free, whereas periplasmically expressed PcuC contained one copper ion bound to it. Copper could be removed from the latter by treatment with a >1,000-fold molar excess of BCS but not with EDTA (data not shown). Considering the high specificity of the BCS chelate for Cu(I) (59, 60), this pointed toward a preference of PcuC for Cu(I). Increasing amounts of copper-free PcuC were then mixed as competitors with a constant amount of the Cu(BCS)₂³⁻ complex, and the decrease of the characteristic 483-nm absorbance peak of the latter (54) was

Copper Protein for Cytochrome Oxidase Biogenesis

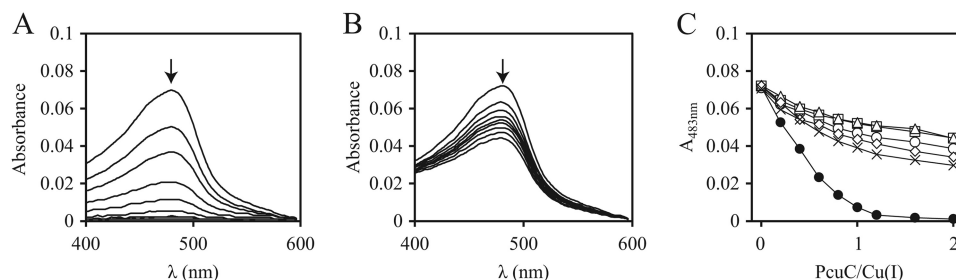


FIGURE 7. **PcuC binds one Cu(I) ion via His⁷⁹, Met⁹⁰, His¹¹³, and Met¹¹⁵.** A, UV-visible spectra of incremental addition of apo-PcuC (wild type) to a 7.5 μM $\text{Cu}(\text{BCS})_2^{3-}$ solution. Spectra were taken when 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0 protein equivalents/copper atom were added. The arrows indicate the direction of the absorption change after the protein addition. B, incremental addition of apo-PcuC(M90A) to a 7.5 μM $\text{Cu}(\text{BCS})_2^{3-}$ solution is shown as an example. An analogous behavior was observed for the other substituted versions of PcuC (data not shown). C, plot of A_{483} against the ratio of PcuC wild type (closed circles), PcuC(H79A) (open circles), PcuC(M90A) (squares), PcuC(H113A) (triangles), PcuC(M115A) (diamonds), and PcuC(H113A/M115A) (crosses) to total Cu(I). Saturation is observed only when PcuC (wild type) was used in the experiment.

recorded by UV-visible absorption spectroscopy until saturation occurred (Fig. 7A). The result showed that the affinity of PcuC for Cu(I) was higher than that of BCS. Applying the method elaborated by Zhou *et al.* (54), we arrived at an estimated K_D of $\leq 10^{-16}$ M. When the A_{483} values were plotted against the PcuC/Cu(I) ratio, we observed the disappearance of $\text{Cu}(\text{BCS})_2^{3-}$ after the addition of one equivalent of PcuC, indicating a PcuC/Cu(I) stoichiometry of 1:1 (Fig. 7C, curve with black circles). Similar data were obtained with a PcuC derivative in which the last 15, non-conserved amino acids had been deleted (data not shown). This C-terminal extension is rich in possible copper-binding amino acids (5 Met, 2 His, 1 Glu, 1 Asp; Fig. 3). The unaltered activity of the truncated form precluded a role of this peptide region in copper binding.

The copper-binding amino acid ligands described for the homologous *T. thermophilus* PCu_AC protein (25) are conserved in PcuC (Fig. 3), and their similar spatial orientation was confirmed by modeling the PcuC primary structure onto the three-dimensional structure of *T. thermophilus* PCu_AC (data not shown). Therefore, copper binding was also tested with purified mutant derivatives of PcuC in which each of the four presumed Cu(I)-binding residues had been substituted with alanine: H79A, M90A, H113A, and M115A. Further, a double replacement derivative was tested (PcuC(H113A/M115A)). The purity and identity of these proteins were documented by mass spectrometry (supplemental Fig. S1). All of the mutant proteins had an impaired capability of removing Cu(I) from the $\text{Cu}(\text{BCS})_2^{3-}$ complex, as shown with PcuC(M90A) as an example (Fig. 7B). The plot in Fig. 7C illustrates that saturation occurred only with wild-type PcuC, whereas a moderate decrease in A_{483} was observed for the substituted proteins; hence, they bound Cu(I) more weakly than wild-type PcuC. The experiments supported the notion that PcuC binds one Cu(I) ion with high affinity via His⁷⁹, Met⁹⁰, His¹¹³, and Met¹¹⁵.

PcuC and ScoI Are Required for the Symbiotically Essential *cbb*₃-type Cytochrome Oxidase—As described above, deletion of the *pcuC* gene caused a defect in symbiotic nitrogen fixation (Tables 2 and 3). Having shown that the PcuC protein is a periplasmic Cu(I) protein, we asked whether or not it is involved in the biogenesis of the symbiotically essential *cbb*₃-type oxidase. To assess its functionality, cells were first grown anoxically with nitrate as the terminal electron acceptor. Under these conditions, virtually all of the cytochrome *c* oxidase activ-

ity present in wild-type membranes stems from cytochrome *cbb*₃ and not from cytochrome *aa*₃ (Fig. 8A, compare *coxA* mutant with *fixN* mutant). The activity measured with membranes extracted from anoxically grown $\Delta\text{pcuABCDE}$ cells was as high as that from the wild type. It thus appeared as if PcuC was not important for the formation of cytochrome *cbb*₃. Prompted by recent findings of Thompson *et al.* (24), who reported that PCu_AC was a biogenesis factor for cytochrome *cbb*₃ in *R. sphaeroides*, we tested membrane proteins that had been extracted from endosymbiotic cells. For this purpose, root nodule bacteroids of the wild type and of the deletion mutants 6611 ($\Delta\text{pcuABCDE}$) and 6611-34 (ΔpcuC) were separated from plant material via sucrose gradient centrifugation prior to membrane isolation. Bacteroids of *coxA* and *fixN* mutants served as positive and negative controls, respectively. The *cbb*₃-type oxidase activity was strongly impaired in membranes of *pcuC* mutant bacteroids (Fig. 8B). In contrast to the result of Fig. 8A, this now suggested a role of PcuC in cytochrome *cbb*₃ biogenesis and provided a possible explanation for the symbiotic nitrogen fixation defect of *pcuC* mutants.

In previous work (10), we found that a *B. japonicum scoI* mutant had very similar phenotypes as described now for the *pcuC* mutant, such as the absence of an effect on *cbb*₃-type oxidase activity in cells grown anoxically. The unexpected condition-dependent difference (free-living, anoxic cells versus symbiotic bacteroids) led us to investigate the *cbb*₃ oxidase activity of the *scoI* mutant also in symbiosis. Indeed, oxidase activity was strongly impaired in membranes of *scoI* mutant bacteroids (Fig. 8B). Hence, the ScoI protein is important for the formation of cytochrome *cbb*₃ specifically in symbiosis, just like PcuC.

DISCUSSION

Our expectation to discover derepressed genes in *B. japonicum* cells grown in copper-limited conditions was fulfilled. Which and how many of these are in fact important for copper acquisition is a matter of ongoing and future research. Extreme copper limitation led to the expression of seven clustered genes (bll0889–bll0883), preceded by a predicted transcription activator (bll0890), which codes for two putative cytoplasmic membrane complexes of the ABC transporter family and a major facilitator superfamily transport protein (bll0889). Interestingly, Ekici *et al.* (61) have reported a major facilitator super-

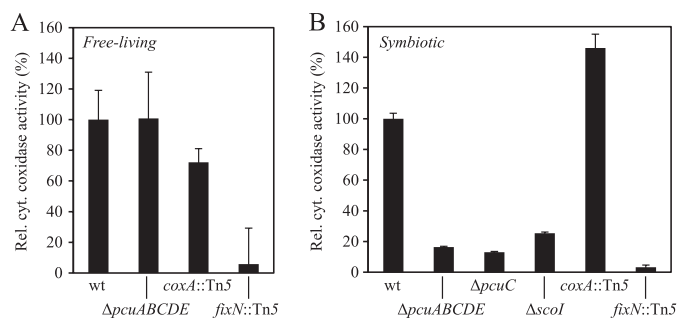


FIGURE 8. PcuC and ScoI are involved in cbb_3 oxidase biogenesis in bacteroids. A, relative cytochrome *c* oxidase activity of anaerobically grown wild type (wt) and strains 6611 ($\Delta pcuABCDE$), COX132 ($coxA::Tn5$), and 3613 ($fixN::Tn5$). Wild-type activity corresponds to 0.891 μ mol of horse heart cytochrome *c* oxidized/mg of membrane protein/min. B, relative cytochrome *c* oxidase activity of bacteroid membrane proteins from wild type (wt) and strains 6611 ($\Delta pcuABCDE$), 6611-34 ($\Delta pcuC$), 2575 ($\Delta scoI$), COX132 ($coxA::Tn5$), and 3613 ($fixN::Tn5$). Wild-type activity corresponds to 1.15 μ mol of horse heart cytochrome *c* oxidized/mg of membrane protein/min. Error bars, S.D.

family-type transporter (CcoA) that is specifically required for cytochrome cbb_3 biogenesis in *Rhodobacter capsulatus*. Although *B. japonicum* has several *ccoA* homologs, the bll0889 gene product might play a similar role because of its low-copper inducibility. Undoubtedly, this cluster of *B. japonicum* genes is worthy of further investigation.

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

Further investigations focused on *pcuC* and its product. The results have ascertained the cuproprotein nature of PcuC. The reduced form of copper (Cu^+) was found to be bound to purified PcuC in a 1:1 ratio, and the estimated affinity ($K_D \leq 10^{-16}$ M) compares well with data obtained for other copper chaperones (62). Deduced from the structure of a PcuC-homologous protein (PCu_AC) (25), the two histidines and two methionines for copper binding lie in an unusual protein motif (HX₁₀MX₂₂HXM) in which not only the four ligands but also the fairly large spacing is evolutionarily well conserved. We confirmed here by mutational analysis that all four liganding amino acids are needed for efficient copper binding to PcuC. Furthermore, we made sure by truncation that the 15 C-terminal amino acids, which are rich in Met and His, are dispensable. This truncated version might become useful in crystallization studies because efforts to crystallize the full-length PcuC protein have been unsuccessful. *In vivo*, the PcuC protein was shown here to be involved in the biogenesis of the aa_3 -type cytochrome oxidase. This was anticipated because Abriata *et al.* (25) had demonstrated the transfer of Cu(I) from *T. thermophilus* PCu_AC to the Cu_A site of the ba_3 -type oxygen reductase *in vitro*. Less expected was the role that PcuC appears to play in the formation of the cbb_3 -type oxygen reductase because this oxidase lacks the Cu_A center. Two independent studies (Ref. 24 and this work) have now arrived at this new function. With an expedient combination of genetic and biochemical approaches, Thompson *et al.* (24) have recently shown for *R. sphaeroides* that PCu_AC works both for the metallation of the Cu_A site of its aa_3 -type oxidase and for the metallation of the Cu_B site of its cbb_3 -type oxidase. Furthermore, these authors argued that PCu_AC might even be involved to some extent in the formation of the Cu_B center in the aa_3 -type oxidase despite the presence of Cox11 (see Introduction), which is still the major player in this process (14). Our own data with *B. japonicum* were less straightforward because the dependences of cytochrome cbb_3 formation on PcuC differed fundamentally in anoxic, culture-grown cells and in symbiotic bacteroids, where a *pcuC* mutant had no effect in the former but a strong defect in the latter. Formally, we cannot rule out the possibility that a PcuC-compensating protein is synthesized in free-living conditions but not in symbiosis. In any case, we showed that the PcuC-homologous Blr7088 protein was not involved. We rather suspect that the copper concentrations play a decisive role. In order to achieve optimal growth of *B. japonicum* under denitrifying conditions (even with the wild type), the copper concentration should not fall below 0.2 μ M, which might already be high enough to bypass the need for PcuC in the assembly of active cytochrome cbb_3 . In symbiosis, however, it is possible that such a functional compensation does not work if the copper concentration that is available for bacteroids in root nodules is very low. Unfortunately, it is practically difficult, if not impossible, to assess and distinguish copper contents in separate compartments, such as the cytosol of colonized plant cells in nodules and the symbiosome (*i.e.* bacteroids plus peribacteroid membrane-surrounded space). Our attempt to abrogate the symbiotic defect by increasing the copper content in the plant growth medium has not been successful. The bottom line of our investigations was that PcuC is important for cytochrome cbb_3 for-

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mation specifically in endosymbiotic bacteroids. This fact alone is sufficient to explain the low nitrogen fixation activity in *pcuC* mutant-infected soybean nodules because this bacteroid process depends on micro-oxic respiration via the high affinity *cbb₃*-type oxidase (63). Along the same lines, we now showed that the *B. japonicum scoI* mutant had a similar condition-dependent phenotype as the *pcuC* mutants (*i.e.* the *scoI* mutation negatively affected cytochrome *cbb₃* activity in bacteroids (Fig. 8B) but had no effect on this oxidase in cells grown under anoxic, denitrifying conditions (10)). Therefore, we must revise a previous interpretation according to which *ScoI* was not believed to be an important biogenesis factor for cytochrome *cbb₃* in *B. japonicum* (10). Other recent studies have provided good evidence for a role of *ScoI*-like proteins (SenC) in cytochrome *cbb₃* formation in *P. aeruginosa* (22), *R. sphaeroides* (24), and *R. capsulatus* (23).

In conclusion, a unifying concept seems to emerge, at least for members of the α -proteobacteria, according to which two proteins (*ScoI*-like and *PcuC*-like) are required collectively for the biogenesis of two types of cytochrome oxidases (*aa₃* and *cbb₃*). To what extent each of the two biogenesis factors is involved in the metallation of the Cu_A and Cu_B sites and how they cooperate with each other need to be explored. Lack of *ScoI* (10) or *PcuC* (*cf.* Fig. 6) leads to an apparent destabilization and/or degradation not only of subunit II but also of subunit I of cytochrome *aa₃*. Therefore, one cannot readily attribute an exclusive function to the chaperones in either Cu_A or Cu_B formation. Intuitively, one would like to propose that the soluble, periplasmic *PcuC* protein disseminates copper to the membrane-bound *ScoI* protein and perhaps to other membrane-bound recipients (*e.g.* *CoxG/Cox11*, *FixI/CcoI*, and *CcoA*), as has been suggested in part also by others (15, 21, 23, 24). However, more biochemical work, such as establishing a copper transfer assay with purified components *in vitro*, will be needed as proof.

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