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Copper Metallochaperones are Required for the Assembly of Bacteroid Cytochrome *c* Oxidase Which is Functioning for Nitrogen Fixation in Soybean Nodules

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Short Communication

***Bradyrhizobium japonicum*, a symbiotic nitrogen-fixing bacterium for *Glycine max*, has complex respiratory electron transport chains. Bll4880 contained a copper-binding motif for metallochaperone, H(M)X_{10}MX_{21}HXM. A mutant strain, Bj4880, induced nodules with lower acetylene reduction activity. A double mutant, Bj4880-1131, which had inserted mutations both in *blr1131*, a gene of the Sco1-like protein, and in *bll4880*, induced nodules of significant Fix$^-$ phenotype and low cytochrome *c* oxidase (Cco) activity in the bacteroid. Our data suggest that bll4880 protein is involved in copper ion delivery to Cco through blr1131 protein, and the expression of both proteins was induced under microaerobic conditions.**

Keywords: *Bradyrhizobium japonicum* • Cytochrome *c* oxidase • Metallochaperone.

Abbreviations: ARA, acetylene reduction activities; Cco, cytochrome *c* oxidase; qRT-PCR, quantitative real-time PCR.

Bradyrhizobium japonicum, a Gram-negative soil bacterium, can differentiate to a bacteroid that fixes nitrogen in the symbiotic tissue of soybean plants (*Glycine max*). As a free-living bacterium, *B. japonicum* presumably encounters an oxygen (O_2) concentration of around 250 μM in soil air space. In the nodules, *B. japonicum* bacteroids are believed to perform high respiration at an intracellular O_2 concentration of approximately 11 nM (Witty and Minchin 1990). To accommodate this wide range of oxygen tension, *B. japonicum* has a multiple branched electron transport system, with each branch terminating with an oxidase of varying affinity for O_2. The presence of four heme–copper cytochrome *c* oxidases (Ccos) has already been reported in *B. japonicum* with molecular and genetic evidence (Göttfert et al. 2005). The transition of respiration from aerobic to microaerobic mode in symbiosis is accompanied by massive changes in bacterial cytochrome composition, as was shown by comparative spectroscopic analysis of free-living aerobic cells vs. root nodule bacteroids (Appleby 1969).

Bacteroid proteins corresponding to microaerobic respiration in *B. japonicum* have been annotated using proteomics (Hoa et al. 2004, Sarma and Emerich 2006, Chang et al. 2007, Pessi et al. 2007). To determine the function of these proteins, a number of deficient mutants were constructed and inoculated into soybean plants. Bll4880 protein shared a potential metal-binding motif, $HX_{10}MX_{21}HXM$, and was identified as a class of hypothetical proteins in the database. Hydropathy and topological membrane analysis indicated that bll4880 is a putative periplasmic soluble protein or anchored to the inner membrane by a single N-terminal transmembrane helix where its water-soluble region faces the periplasm. To analyze the function of the gene, the bll4880::Ω insertion mutant was constructed (**Fig. 1**). Biparental mating was conducted on HM agar plates using *Escherichia coli* HB101 carrying pRK2013 (Figurski and Helinski 1979). Double crossover was verified by Southern hybridization (data not shown), and 2D-electrophoresis revealed that a bacteroid of the bll4880::Ω insertion mutant (strain Bj4880) did not express bll4880 protein although it was visible in the USDA110 (wild-type) bacteroid (**Supplementary Fig. S1**). When Bj4880 was inoculated onto soybean, it was observed that this mutant induced a deficiency in symbiotic N_2 fixation (**Fig. 1**). The soybean infected with Bj4880 exhibited growth inhibition and a yellowish foliar appearance.

In eukaryotes, copper is required within mitochondria for the function of a metalloenzyme, Cco. Studies in yeast have postulated that Cox17 is the copper chaperone implicated in copper trafficking to mitochondrial Cco (Cobine et al. 2006). Since it is suggested that bll4880 is a Cox17-like metallochaperone for Cco, another protein contributing to the function was investigated. Within the inner membrane space, Cox17 is reported to deliver copper to two mitochondrial inner membrane proteins, Sco1 and Cox11, which are thought to be copper donors to Cco Cu_A and Cu_B sites, respectively (Cobine et al. 2006). Although reports on metallochaperones for Cco have been carried out on eukaryotes, Banci et al. revealed that 50 protein sequences showed a conserved

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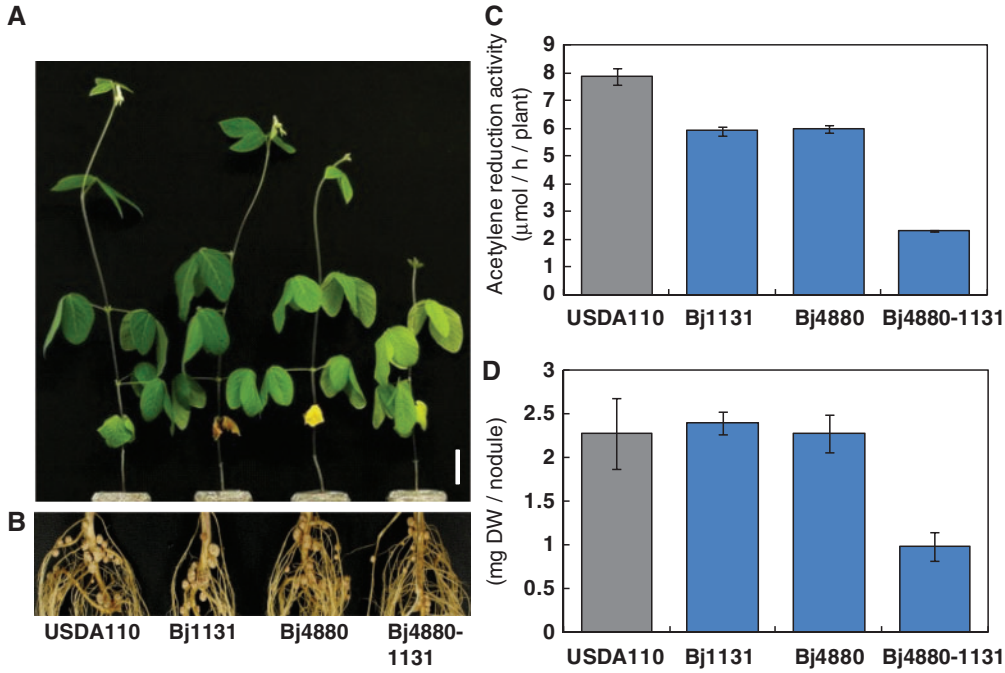

Fig. 1 The soybean plants (A) and nodules (B) inoculated with wild-type USDA110 (USDA110), Bj4880, Bj1131 and Bj4880-1131. Nodules were harvested 28 d after inoculation. Acetylene reduction activity (C) and nodule dry weight (D) of soybean were measured. Data are the mean ± SD for three individual experiments ($n = 3$).

potential metal-binding motif, $HX_{10}MX_{21}HXM$, in prokaryotes, suggesting that it can take on the role of Cox17 in the extracytoplasmic environment of bacteria (Banci et al. 2005). By surveying a BLAST search, blr1131 was identified as a Sco1 homolog protein, containing a CXXXC consensus motif. A DXXXD motif is also conserved (Arnesano et al. 2005). A Tc insertion mutant (Bj1131) of *blr1131* induced nodules with a Fix$^-$ phenotype (**Fig. 1**), and a double mutant of *bll4880* and *blr1131* (Bj4880-1131) showed remarkable reduction of acetylene reduction activity (ARA) and the dry weight of the nodules (**Fig. 1C, D**). We propose that bll4880 encodes a copper metallochaperone that is implicated in the assembly of the copper site of Cco and that blr1131 protein mediates the transfer of copper ion from bll4880 to Cco. A Cco encoded by *fixNOPQ* is induced at low oxygen concentrations and constitutes a *cbb3*-type Cco (Preisig et al. 1993), which might be a candidate Cco by which bll4880–blr1131 proteins transfer copper. *Bradyrhizobium japonicum* fixNOQP mutants are deficient in symbiotic nitrogen fixation, are affected in bacteroid development and exhibit decreased whole-cell oxidase activity when grown microaerobically (Preisig et al. 1993).

To investigate the conditions whereby the expression of *bll4880* is regulated, several genes corresponding to various living forms of *B. japonicum* were analyzed by quantitative real-time PCR (qRT-PCR) (**Fig. 2**). The expression of both *bll4880* and *blr1131* was induced dominantly under microaerobic conditions and in bacteroids (**Fig. 2**). These expression profiles were similar to those of *FixN* and *FixL*, which encode typical proteins with nitrogenase expression in bacteroids. In contrast, an *aa3*-type heme copper Cco (CoxA, CoxB; Bott et al. 1990) and an alternative heme–copper Cco (CoxN, CoxM; Bott et al. 1990) showed expression at a higher level with aerobically grown cells (**Fig. 2**). These data suggest that *bll4880* and *blr1131* play a role in the assembly of Cco in bacteroids or microaerobically grown cells.

In order to obtain biochemical evidence as to whether bll4880 is required for Cco activity, we compared Cco activities in free-living cells of wild-type *B. japonicum* and mutant cells (**Table 1**). In cells grown aerobically as free-living cells, no significant difference in Cco activity was observed in either the wild type or the mutants; however, in microaerobically grown cells, substantial decreases in Cco activity were detected with Bj4880 and Bj1131. The activities were remarkably decreased in bacteroids of Bj4880-1131. Enzymes that employ transition metals as cofactors are housed in a wide variety of intracellular locations or are exported to the extracellular environment. Specific metal cofactors were transported to diverse locations, and were subsequently sorted into the correct metalloenzyme via metallochaperones. Cco, a key mitochondrial enzyme in the respiratory chain, requires three copper ions to be inserted into two subunits in eukaryotes. Cox17 is a water-soluble copper metallochaperone in yeast (Beers et al. 1997). Such a water-soluble form of bll4880 protein could be produced if the N-terminal transmembrane segment is removed. The predicted cleavable site in the N-terminal region of bll4880

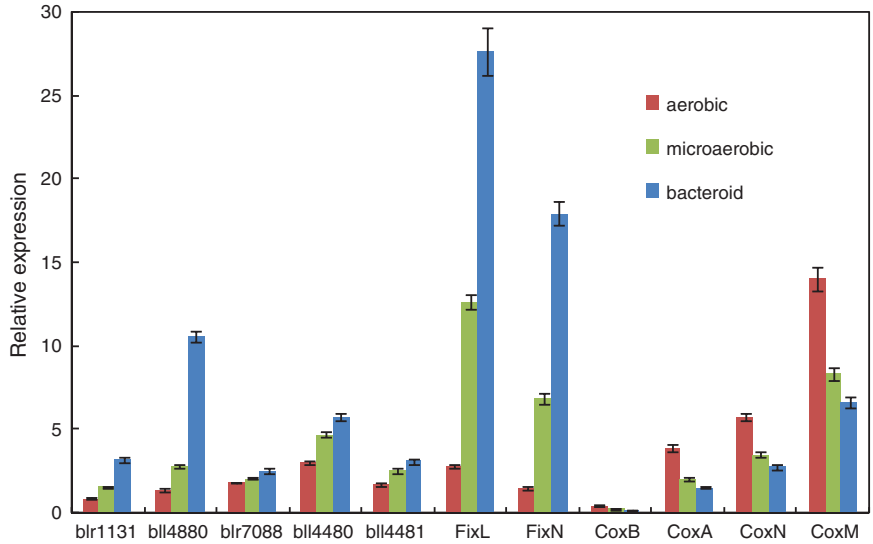

Fig. 2 Relative amounts of *B. japonicum* gene expression in free-living cells grown under aerobic (Aerobic) or microaerobic (Microaerobic) conditions. Bacteroid RNA was purified from the nodules at 28 d after inoculation. Transcript amounts of blr1131, bll4880, blr7088, bll4480, bll4481, FixL (bll2760), FixN (blr2763), CoxB (blr1170), CoxA (blr1171), CoxN (bll3784) and CoxM (bll3785) were normalized against *sigA*. Data are the mean ± SD for 10 individual experiments ($n = 10$).

Table 1 Cytochrome *c* oxidase activities in free-living cells and bacteroids of *B. japonicum*

Strain name	Cytochrome *c* oxidase activity (nmol min^{-1} mg protein^{-1})		
	Aerobic growth	Microaerobic growth	Bacteroid
USDA110	32.6 ± 5.74	76.6 ± 8.24	82.8 ± 11.43
Bj1131	33.3 ± 1.66	48.4 ± 13.98	72.5 ± 10.12
Bj4880	33.5 ± 1.17	41.4 ± 12.92	68.1 ± 18.01
Bj4880-1131	26.1 ± 1.47	22.3 ± 6.62	15.4 ± 0.96

Bacteroids were isolated from the nodules 28 d after inoculation. Values are means ± SD ($n = 3$).

can be found to be located between residues 24 and 25 using the software Signal IP (Bendtsen et al. 2004). Thus, bll4880 is proposed to be an accessory protein required for the correct assembly of Cco. The periplasm is a cell component of Gram-negative bacteria where Cco and other copper enzymes acquire their metal cofactors. Under normal conditions, the copper concentration in the periplasm is not a limiting factor (Finney and O'Halloran 2003); however, with a low copper supply, a more efficient copper uptake mechanism might be activated.

Bradyrhizobium japonicum has two genes, *bll4880* and *blr7088*, that conserve a metal-binding motif, $HX_{10}MX_{21}HXM$. Although blr7088 protein is also activated by $FixK_2$ which is required for microaerobic respiration, transcript formation was reported to be weak (Mesa et al. 2008). QRT-PCR showed that both *bll4880* and *blr7088* genes were induced in the bacteroid, but the induction of *blr7088* was significantly lower than that of *bll4880* (**Fig. 2**). Interestingly, other novel types of heme–copper Cco (bll4480, bll4481; Göttfert et al. 2005) are expressed under microaerobic conditions and in bacteroids (**Fig. 2**). To confirm the function of these metallochaperone proteins, a biochemical experiment to determine copper transfer to either Cco encoded by FixNOPQ or a novel type of heme–copper Cco would be necessary.

In this report, a new family of soluble metal receptor proteins, known as metallochaperones, is reported to be necessary to express the nitrogenase activity of nodules. It has been shown that soybean nodule bacteria contain a multigene family of chaperones, such as GroESL-like genes, which exhibited extremely high amino acid sequence similarity and were differentially expressed under a variety of environmental and physiological conditions (Fischer et al. 1993). bll4880 and blr7088 proteins might be involved in the complex regulation of nitrogenase activity in *B. japonicum*. To our knowledge, the function of a copper metallochaperone in prokaryotes has not been described previously. Additional studies are now being conducted to elucidate the mechanism of copper trafficking.

Materials and Methods

Soybean (*G. max* L. Merr cv. Akishirome) seeds were surface-sterilized and transferred on sterile vermiculite with liquid B&D medium (Broughton and Dilworth 1971) in sterile Leonard jar assemblies composed of two plant boxes, followed by inoculation with *B. japonicum*. Bacterial strains and plasmids are listed in **Supplementary Table S1**. Rhizobium strains

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were grown in HM medium (Cole and Elkan 1973) at 28°C. PSY medium was used for microaerobic growth in closed 500 ml flasks with 200 ml as culture and the remainder as the N_2 gas phase containing 0.5% O_2 that was replaced every 12 h.

Mutagenesis targeting the *bll4880* gene was performed according to Sameshima-Saito et al. (2006). The *bll4880* gene was excised as a 7.4 kb *Eco*RI fragment identified from the genome sequence of *B. japonicum* (Kaneko et al. 2002), and was inserted into the *Eco*RI site of pK18mob (Schäfer et al. 1994) to generate plasmid pK4880. For *bll4880* gene deletion, the omega cassette, which was excised from pHP45Ω (Prentki and Krisch 1984) digested with *Sma*I, was inserted into the *Eco*RV sites of pK4880 to generate pK4880::Ω. A portion of 3.2 kb of *Bam*HI and *Eco*RI fragments of *blr1131* was subcloned into pK18mob, generating the plasmid pK1131. The *Sma*I fragment containing the Tc cassette was excised from p34S-Tc (Dennis and Zylstra 1998) and then cloned into the *Nur*I site of pK1131, generating pK1131::Tc. Both pK4880::Ω and pK1131::Tc were introduced into *B. japonicum* USDA110 by triparental mating using pRK2013 as a helper plasmid (Figurski and Helinski 1979). The double mutant was generated by introducing a pK1131::Tc plasmid into the pK4880::Ω mutant (Bj4880). The double crossover event was further verified by Southern hybridization using a 270 bp fragment of bll4880 and a 972 bp fragment of the omega gene as probes. These fragments were amplified by PCR using the following primers: 4880-Fwm, 5′-CCGGACCATTCGGGAATGAAGATGT-3′; 4880-Rv, 5′-TGGCGCATCTTCATCACGCCATTGT-3′; 1131-Fw, 5′-TCGAATTCGACTGGGGCGGG-3′; 1131-Rv, 5′-GAAAGCCCCGGTTCCCTAGC-3′; Omega-Fw, 5′-CTTGACCTGATAGTTTGGCTGTGAG-3′; Omega-Rv, 5′-GGGTCGATGTTTGATGTTATGGAGC-3′; Tc-Fw, 5′-GCACTGTCCGACCGCTTTGG-3′; and Tc-Rv: 5′-CGGCGCCTACAATCCATGCC-3′.

RNA was isolated by RNAwiz (Applied Biosystems) and then purified with RNeasy spin columns (Qiagen). Isolated RNAs from *B. japonicum* were used as templates for qRT-PCR. Primer sequences were as shown in **Supplementary Table S2**. qRT-PCR was performed using a One Step SYBR® PrimeScript™ RT-PCR Kit II (TAKARA BIO INC.). PCRs were run with the ABI Prism 7000 sequence detection system (Applied Biosystems). The transcript of the primary sigma factor gene (*sigA*) was used as an internal reference for relative quantification.

Bacteroids were isolated from nodules as described previously (Dao et al. 2008). Proteins were extracted from excised nodules and the activity of Cco was assayed according to Neuburger et al. (1982). 2D-electrophoresis was performed according to the procedure of Hoa et al. (2004). For ARA assay, nodules at 28 d after inoculation were used. The ARA was measured by gas chromatography (Shimazu GC-8A) as previously described (Nomura et al. 2006).

Supplementary data

Supplementary data are available at PCP online.

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