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Thiosulfate-Dependent Chemolithoautotrophic Growth of *Bradyrhizobium japonicum*▽†

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Thiosulfate-oxidizing *sox* gene homologues were found at four loci (I, II, III, and IV) on the genome of *Bradyrhizobium japonicum* USDA110, a symbiotic nitrogen-fixing bacterium in soil. In fact, *B. japonicum* USDA110 can oxidize thiosulfate and grow under a chemolithotrophic condition. The deletion mutation of the $soxY_1$ gene at the *sox* locus I, homologous to the sulfur-oxidizing (Sox) system in *Alphaproteobacteria*, left *B. japonicum* unable to oxidize thiosulfate and grow under chemolithotrophic conditions, whereas the deletion mutation of the $soxY_2$ gene at *sox* locus II, homologous to the Sox system in green sulfur bacteria, produced phenotypes similar to those of wild-type USDA110. Thiosulfate-dependent O_2 respiration was observed only in USDA110 and the $soxY_2$ mutant and not in the $soxY_1$ mutant. In the cells, 1 mol of thiosulfate was stoichiometrically converted to approximately 2 mol of sulfate and consumed approximately 2 mol of O_2. *B. japonicum* USDA110 showed $^{14}CO_2$ fixation under chemolithotrophic growth conditions. The CO_2 fixation of resting cells was significantly dependent on thiosulfate addition. These results show that USDA110 is able to grow chemolithoautotrophically using thiosulfate as an electron donor, oxygen as an electron acceptor, and carbon dioxide as a carbon source, which likely depends on *sox* locus I including the $soxY_1$ gene on USDA110 genome. Thiosulfate oxidation capability is frequently found in members of the *Bradyrhizobiaceae*, which phylogenetic analysis showed to be associated with the presence of *sox* locus I homologues, including the $soxY_1$ gene of *B. japonicum* USDA110.

Bradyrhizobium japonicum exhibits two life styles: as a free-living soil bacterium and as a bacteroid that fixes atmospheric nitrogen in soybeans (2, 16, 17). The genomic sequence of *B. japonicum* USDA110 (20) is unusual and complex, containing numerous copies of genes for degradation, transport, C1 metabolism, transcriptional regulation, and respiratory chains (12), which might allow the bacterium to adapt to diverse and fluctuating environments (17). However, *B. japonicum* USDA110 expresses only a portion of the complicated gene sets for the degradation of aromatics and C1 compound metabolism in the presence of vanillate, a naturally occurring aromatic compound (17, 42).

Biological oxidation of reduced inorganic sulfur compounds is one of the major reactions in the global sulfur cycle (9, 26) and is the major reaction under extreme conditions such as those in deep-sea hydrothermal vents (31), solfataras (23), and volcanic environments (22). The oxidation reactions in these ecosystems are carried out by prokaryotes of the domains *Archaea* and *Bacteria* (9, 23). In the *Bacteria*, sulfur is oxidized by chemotrophic and phototrophic bacteria, which are phylogenetically and physiologically diverse (9).

A sulfur-oxidizing (Sox) enzyme system has been found and characterized in *Paracoccus versutus* and *Paracoccus pantotrophus* GB17 through the pioneering work of research groups associated with Lu et al. (28) and Friedrich et al. (8, 9, 46). Genes homologous to those encoding the Sox enzyme have been found from genomes of the members of the domain *Bacteria* but not in the domain *Archaea* (8, 9). However, the functional aspects of the Sox system based on genome information remain to be answered (9).

A survey of the *B. japonicum* USDA110 genome revealed multiple homologues of *sox* genes; however, it was not clear if these homologues were functional for the oxidation of inorganic sulfur compounds and chemolithotrophic growth in this bacterium (7). The *sox* gene cluster in *P. pantotrophus* GB17 comprises at least 15 genes, *soxTRSVWXYZABCDEFGH*, which are responsible for thiosulfate oxidation and chemolithotrophic growth (9). Thiosulfate has been regarded as crucial among the reduced inorganic compounds in Sox biochemistry (7) and the geochemical sulfur cycle (18).

B. japonicum is known to grow chemolithoautotrophically using the gaseous electron donors H_2 and CO (6, 13, 27). H_2 uptake-positive (Hup^+) strains of *B. japonicum* USDA122 (13) and USDA110 (6) carrying *hup* genes can grow chemolithoautotrophically in mineral salts medium with H_2, CO_2, and low concentrations of O_2. *B. japonicum* USDA110 is also reportedly able to grow chemolithotrophically with carbon monoxide as an electron donor (27). Thus, it is possible that *B. japonicum* USDA110 may utilize an inorganic sulfur compound, thiosulfate, as an electron donor and CO_2 as a carbon source for chemolithoautotrophic growth.

With this background, the present study addressed the following questions: (i) Does *B. japonicum* USDA110 grow chemolithoautotrophically with thiosulfate as an energy source? (ii) Which of the *sox* genes could be responsible for thiosulfate oxidation in *B. japonicum*? (iii) Do these features extend to other members of the *Bradyrhizobiaceae*?

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Reference or source
Strains		
B. japonicum		
USDA110	Soybean bradyrhizobia; sox^+	20
USDA110 $soxY_1$::Tc	USDA110 $soxY_1$ (*sox* locus I with a deletion or insertion of the Tc cassette); Tc^r	This study
USDA110 $soxY_2$::Ω	USDA110 $soxY_2$ (*sox* locus II with a deletion or insertion of the Ω cassette); Sm^r Sp^r	This study
NC6	Soybean bradyrhizobia; Sox^+	39
USDA122	Soybean bradyrhizobia; Sox^+	45
NK2	Soybean bradyrhizobia; Sox^+	39
NC4	Soybean bradyrhizobia; Sox^+	39
USDA124	Soybean bradyrhizobia; Sox^-	45
USDA6^T	Soybean bradyrhizobia; Sox^-	45
T7	Soybean bradyrhizobia; Sox^-	39
T9	Soybean bradyrhizobia; Sox^-	39
A. oligotrophica		
S58	Slow-growing oligotrophic bacteria; Sox^+	35
Bradyrhizobium sp.		
G14130	Slow-growing oligotrophic bacteria; Sox^-	35
BTAi1	Photosynthetic stem-nodulating bacteria; Sox^+	11
ORS278	Photosynthetic stem-nodulating bacteria; Sox^+	11
HW13	2,4-D-degrading bacteria; Sox^-	19
HWK12	2,4-D-degrading bacteria; Sox^-	19
R. palustris		
CGA009	Photosynthetic bacteria; Sox^+	24
B. elkanii		
USDA76^T	Soybean bradyrhizobia; Sox^-	45
P. pantotrophus		
GB17	Denitrifying, sulfur-oxidizing bacteria; Sox^+	46
E. coli		
DH5α	*recA*; cloning strain	Toyobo Inc.[b]
JM109	*recA*; cloning strain	Toyobo Inc.[b]
Plasmids		
pSAC20	pK18mob carrying 4.8-kb $soxVWX_1Y_1Z_1A_1B_1$ SalII fragment of brp14182; Km^r	This study
pSAC21	pK18mob carrying $soxY_1$ with a deletion or insertion of the Tc cassette; Tc^r Km^r	This study
pSAC22	pK18mob carrying 4.6-kb BamHI fragment $soxX_2Y_2Z_2A_2B_2$ of brp01133; Km^r	This study
pSAC23	pK18mob carrying $soxY_2$ with a deletion or insertion of the Ω cassette; Sm^r Sp^r Km^r	This study
brp14182	pUC18 carrying *sox* locus I	20
brp01133	pUC18 carrying *sox* locus II	20
p34S-Tc	Plasmid carrying 2.1-kb Tc cassette; Tc^r	3
pHP45Ω	Plasmid carrying 2.1-kb Ω cassette; Sp^r Sm^r Ap^r	32
pK18mob	Cloning vector; pMB1*ori* oriT; Km^r	41
PUC4-KIXX	Plasmid carrying 1.6-kb Km cassette; Km^r Ap^r $Bleo^r$	Amersham-Pharmacia[c]
pRK2013	ColE1 replicon carrying RK2 transfer genes; Km^r	5

[a] Sox^+ and Sox^- indicate the presence and absence of thiosulfate-oxidizing activity, respectively (see text). 2,4-D, 2,4-dichlorophenoxyacetic acid; Ap^r, ampicillin resistant; Tc^r, tetracycline resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant.
[b] Tokyo, Japan.
[c] Uppsala, Sweden.

MATERIALS AND METHODS

Search for *sox* genes. Using the amino acid sequences for the *sox* genes of *P. pantotrophus* GB17 and *Chlorobium tepidum* TLS (4), the BLASTP program was used to search the *Bradyrhizobium* section of Rhizobase (http://bacteria.kazusa.or.jp/rhizobase/Bradyrhizobium/index.html) for matching amino acid sequences of *sox* gene homologues belonging to *B. japonicum* USDA110. The amino acid sequences of the *sox* genes of *P. pantotrophus* GB17 and *C. tepidum* TLS were obtained from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) and Cyanobase (http://genome.kazusa.or.jp/cyanobase/), respectively. The signal peptide and transmembrane helices of the *sox* genes were predicted by the SignalP (version 3.0), TatP, and TMHMM programs available from the Center for Biological Sequence Analysis at the Technical University of Denmark (http://www.cbs.dtu.dk/services/). Molecular weights of *sox* genes were calculated by Genetyx, version 5.1, for Windows (Genetyx, Tokyo, Japan). We searched for conserved domains of *sox* genes with the InterProScan Sequence Search available from the European Bioinformatics Institute of the European Molecular Biology Laboratory (EMBL-EBI; http://www.ebi.ac.uk/Tools/InterProScan/). The amino acid sequences of $soxY_1$ genes were aligned using the ClustalX program (http://www.clustal.org/).

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. Cultures of *B. japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium* sp., and *Agromonas oligotrophica* were precultured aerobically at 30°C in HM salt medium (1) supplemented with 0.1% (wt/vol) arabinose and

0.025% Difco yeast extract (Becton, Dickinson and Co., Sparks, MD). *Rhodopseudomonas palustris* CGA009 (24) was precultured aerobically at 30°C in Difco nutrient broth (Becton, Dickinson and Company). For growth experiments, these strains were grown aerobically at 25°C in Taylor medium (43), which is a minimal medium for thiosulfate-oxidizing bacteria, supplemented with sodium thiosulfate ($Na_2S_2O_3$) or succinate (Na salt). Taylor medium contained the following components dissolved in 1 liter of distilled water (pH 6.8): 1.0 g of NH_4Cl, 2.0 g of KH_2PO_4, 0.8 g of $MgSO_4 \cdot 7H_2O$, and 1 ml of a trace metal solution consisting of 5.0 g of Na_2EDTA, 2.2 g of $ZnSO_4 \cdot 7H_2O$, 7.3 g of $CaCl_2 \cdot 2H_2O$, 2.5 g of $MnCl_2 \cdot 4H_2O$, 0.5 g of $CoCl_2 \cdot 6H_2O$, 0.5 g of $(NH_4)_6Mo7O_2 \cdot 7H_2O$, 5.0 g of $FeSO_4 \cdot 7H_2O$, and 0.2 g of $CuSO_4 \cdot 5H_2O$. Antibiotics were added to the medium for growing *B. japonicum* USDA110 as follows: tetracycline (Tc), spectinomycin (Sp), streptomycin (Sm), and kanamycin (Km) at 100 μg ml^{-1} and polymyxin B at 50 μg ml^{-1}. For *Escherichia coli* the concentrations were 15 μg ml^{-1} Tc, 50 μg ml^{-1} Sp, 50 μg ml^{-1} Sm, 50 μg ml^{-1} Km, and 100 μg ml^{-1} ampicillin.

Growth conditions. Cells were washed twice in Taylor medium before inoculations. For plate assays of thiosulfate oxidation, phenol red was added to the medium at 20 μg $liter^{-1}$ as a pH indicator. The color was monitored for a change from red to yellow because thiosulfate oxidation causes a drop in pH (46). The cells were grown at 25°C for 20 days on Taylor agar plates supplemented with various concentrations of thiosulfate. For chemolithotrophic growth experiments in liquid cultures, the cells were grown aerobically in 100 ml of Taylor medium in 500-ml Erlenmeyer flasks supplemented with 4 mM sodium thiosulfate for 28 days at 25°C. The number of CFU was monitored as a growth index by serial dilution of the culture with sterilized water and plating on HM salt medium. For mixotrophic growth conditions, the cells were grown aerobically in 100 ml of Taylor medium in 500-ml Erlenmeyer flasks supplemented with 4 mM sodium thiosulfate and 0.1% (wt/vol) sodium succinate for 6 days at 25°C. The turbidity (optical density at 660 nm [OD_{660}]) of cultures was measured with a UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan) as an indicator of cell growth.

Thiosulfate and sulfate determinations. Thiosulfate concentrations in the culture supernatants were measured by iodometric titration (21). Sulfate was determined gravimetrically (10). Fifty milliliters of the culture supernatant was acidified with 1 ml of 5 N HCl. Subsequently, sulfate was precipitated with 4 ml of 0.5 M $BaCl_2$. The precipitation reaction was allowed to proceed for 2 h, after which the $BaSO_4$ was collected by filtration and dried overnight at 90°C.

DNA manipulations. Isolation of plasmids, DNA ligation, and transformation of *E. coli* were performed as described by Sambrook et al. (37). Total bacterial DNA was isolated from cultured cells as described previously (30). Southern hybridization was carried out as described previously (39).

Construction of the *soxY*$_1$ and *soxY*$_2$ deletion mutants. A 4.8-kb SalI DNA fragment containing *soxVWX*$_1$*Y*$_1$*Z*$_1$*A*$_1$*B*$_1$ was isolated from brp14182, a clone from the pUC18 library for the sequences of *B. japonicum* USDA110, and inserted into the SalI site of pK18mob. The resulting plasmid, pSAC20, was double digested by AccIII and StuI. The Tc^r cassette was isolated from p34S-Tc at the SmaI site (3) and inserted into pSAC20 at the AccIII and StuI sites, yielding pSAC21. pSAC21 was introduced into *B. japonicum* USDA110 by triparental mating using pRK2013 as a helper plasmid (39).

A 4.6-kb BamHI DNA fragment containing *soxX*$_2$*Y*$_2$*Z*$_2$*A*$_2$*B*$_2$ was isolated from brp01133 and inserted into the BamHI site of pK18mob, resulting in the vector pSAC22. pSAC22 was double digested by BsiWI and BstPI. The Ω-cassette was isolated from pHP45Ω at the SmaI site (32) and inserted into pSAC22 at the BsiWI and BstPI sites, resulting in the vector pSAC23. pSAC23 was introduced into *B. japonicum* USDA110 by triparental mating. Double crossover events of *soxY*$_1$ and *soxY*$_2$ deletion mutations were verified by PCR.

O_2 respiration. The O_2 uptake rate of whole cells was determined polarographically with a Clark-type O_2 electrode (5300 Biological Oxygen Monitor; Yellow Springs Instrument Co. Inc., Tokyo, Japan). The cells were harvested by centrifugation and then washed twice and resuspended in 50 mM potassium phosphate buffer containing 2.5 mM $MgCl_2$ (pH 7.0) (13). The assay mixture contained the potassium phosphate-$MgCl_2$ buffer, about 10 mg of cells (wet weight), and 30 mM thiosulfate at 25°C to start the reaction (13, 46). The amount of dissolved oxygen in air-saturated water was calculated on the basis of 258 nmol of O_2 ml^{-1} in air-saturated water at 25°C. The rate of thiosulfate-dependent oxygen consumption was calculated using the rates of thiosulfate oxidation and endogenous respiration and expressed as nanomoles of O_2 consumed per minute per gram of wet cells (nmol O_2 min^{-1} g of wet $weight^{-1}$).

CO_2 fixation. $NaH^{14}CO_3$ (specific activity, 2.18 GBq $mmol^{-1}$) was obtained from GE Healthcare UK Limited (GE Healthcare, Tokyo, Japan). The CO_2 fixation assay was conducted using the method of Lepo et al. (25) and Maier (29). After the cells had been grown chemolithotrophically for 12 days at 25°C, $NaH^{14}CO_3$ (1.91 μmol) was added to 100 ml of culture in Taylor medium in 500-ml Erlenmeyer flasks. One milliliter of the culture was periodically transferred to scintillation vials and acidified with 0.3 ml of 60% trichloroacetic acid. CO_2 that had not been fixed was allowed to dissipate for 48 h into a fume hood. Fifteen milliliters of scintillation cocktail (ACS II; Amersham Biosciences, Tokyo, Japan) was added to each vial, and the radioactivity was determined by liquid scintillation counting (Aloka LSC-5100; Tokyo, Japan). As a negative control, cells were killed by placing the culture flask in a boiling water bath for 10 min just before the addition of $NaH^{14}CO_3$.

Resting cells were prepared to examine whether CO_2 fixation depends on thiosulfate. Cells were harvested from 1 liter of culture that had been grown chemolithotrophically for 12 days, washed twice, and suspended in 20 ml of Taylor medium without thiosulfate. The $^{14}CO_2$ uptake assay was started by the addition of $NaH^{14}CO_3$ (370 kBq) at a final concentration of 20 mM $NaHCO_3$ and 4 mM thiosulfate to cells at a density of $(4.6 \pm 0.7) \times 10^9$ CFU ml^{-1} at 25°C.

Gas chromatography. For the analysis of CO_2 concentrations in the flask headspace, 1-ml gas samples were injected into a GC-2014 gas chromatograph ([GC] Shimadzu), equipped with a thermal conductivity detection (TCD) detector (Shimadzu) and Parapak Q column (80/100 mesh; 0.3-mm diameter by 2-m length). The flow rate of the carrier gas (He) was 30 ml min^{-1}. Throughout the GC analysis, the temperatures of injection, column, and detector were 100, 40, and 100°C, respectively. Peak areas were calculated from chromatograms by an integrator (ChromatoPack C-R18; Shimadzu).

Phylogenetic analysis. Phylogenetic analysis was conducted using the neighbor-joining (NJ) method and ClustalW by the method of Saitou and Nei (36) and Thompson et al. (44).

RESULTS

Genomic survey for *sox* genes. We found four regions of the genomic sequence of *B. japonicum* USDA110 (20) that are homologous to well-known *sox* genes that mediate thiosulfate oxidation (9). In the present study, these regions are designated *sox* loci I (*soxTSRVWX*$_1$*Y*$_1$*Z*$_1$*A*$_1$*B*$_1$*C*$_1$*D*$_1$), II (*soxX*$_2$*Y*$_2$*Z*$_2$*A*$_2$*B*$_2$), III (*soxFY*$_3$*Z*$_3$*A*$_3$), and IV (*soxC*$_2$*D*$_2$) (Fig. 1A). The amino acid sequence analyses of the proteins deduced from these *sox* gene homologues suggest that most of them carry Tat- and Sec-dependent signal peptides so that they are excreted into the periplasm of the bacterium (see Table S1 in the supplemental material), where the Sox system functions (8).

As shown in Fig. 1B, the gene organization at *sox* loci I, II, and III in *B. japonicum* USDA110 was very similar to the organization of photosynthetic stem-nodulating bradyrhizobia, *Bradyrhizobium* sp. BTAi1 and *Bradyrhizobium* sp. ORS278 (11). The homology of the amino acid sequences was high (identities of 67% to 92%) between USDA110 and photosynthetic bradyrhizobia (see Table S2 in the supplemental material). *R. palustris* CGA009 (24) carried *sox* genes that were homologous to those at *sox* locus I in *B. japonicum* USDA110 (identities of 44% to 73%) (see Table S2). Beyond the above members (*Bradyrhizobium* and *Rhodopseudomonas* spp.), the deduced amino acid sequences for loci I and II were highly homologous to those of well-characterized *P. pantotrophus* GB17 (44% to 73% identities) (7) and the green sulfur bacterium *C. tepidum* (44% to 57% identities) (4), respectively (see Table S2).

Thiosulfate oxidation and cell growth under chemolithotrophic conditions. Plate assays showed thiosulfate-oxidation capability in *B. japonicum* USDA110 at thiosulfate concentrations below 4 mM (Table 2; see also Fig. S1 in the supplemental material). The presence of 20 mM sodium thiosulfate inhibited its oxidation, as indicated by color changes on the Taylor plate (Table 2; see also Fig. S1). Thus, we examined thiosulfate oxidation by *B. japonicum* USDA110 under chemolithotrophic conditions with 4 mM thiosulfate as the sole energy source (Fig. 2A). The numbers of CFU increased with time over 28

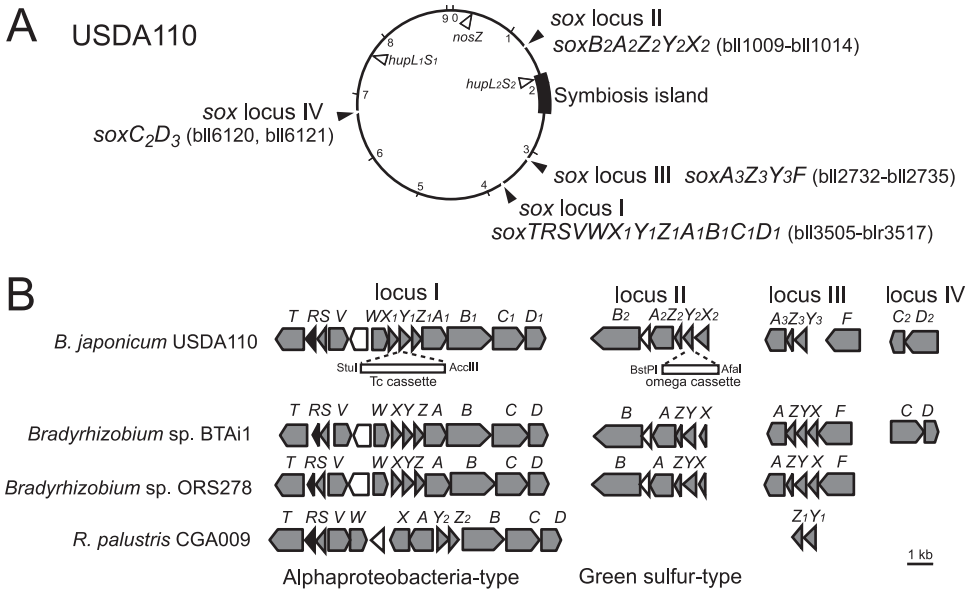

FIG. 1. (A) Genomic loci of *sox* gene homologues on *B. japonicum* USDA110. Four loci of the *sox* gene cluster (black arrowheads) were designated *sox* loci I, II, III, and IV. Parentheses indicate gene numbers on the USDA110 genome (20). White arrowheads inside the circle show the locations of *hupLS* genes (within or outside the symbiosis island) and the *nosZ* gene. (B) Physical maps of *sox* genes on the genome of *B. japonicum* USDA110, *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. ORS278, and *R. palustris* CGA009. Deletion of $soxY_1$ and $soxY_2$ was shown on *sox* loci I and II of *B. japonicum* USDA110, respectively.

TABLE 2. Thiosulfate oxidation and distribution of *sox* genes of *sox* locus I in members of *Bradyrhizobiaceae*

Strain	Thiosulfate oxidation at the indicated concn (mM)[a]					Hybridization with *sox* locus I[b]	
	0	0.4	2	4	20	*soxTRSV*	*soxWX*$_1$*Y*$_1$*Z*$_1$*A*$_1$*B*$_1$*C*$_1$*D*$_1$
P. pantotrophus							
GB17	−	++	++	++	++		
B. japonicum							
USDA110	−	++	++	++	−	+	+
USDA122	−	++	++	++	−	+	+
NC6	−	++	++	++	−	+	+
NC4	−	++	++	++	−	+	+
NK2	−	++	++	++	−	+	+
USDA124	−	−	−	−	−	−	−
T9	−	−	−	−	−	−	−
T7	−	−	−	−	−	−	−
USDA6^T	−	−	−	−	−	−	−
A. oligotrophica							
S58	−	++	++	++	−	+	+
Bradyrhizobium sp.							
G14130	−	−	−	−	−	−	−
HW12	−	−	−	−	−	−	−
HWK13	−	−	−	−	−	−	−
BTAi1	−	+	+	+	−	+	+
ORS278	−	++	++	++	−	+	+
B. elkanii							
USDA76^T	−	−	−	−	−	−	−
R. palustris							
CGA009	−	++	++	++	−	+	+

[a] Thiosulfate oxidation was evaluated in agar plates with phenol red according to the following criteria: ++, yellow (strong activity); +, orange (weak activity); −, red (no activity).

[b] Two sets of *sox* genes (*sox* locus I) in *B. japonicum* USDA110 were used as hybridization probes. +, positive; −, negative.

days; thiosulfate concentrations ($S_2O_3^{2-}$) in the culture decreased along with the cell growth (Fig. 2A). During chemolithotrophic cultivation, 3.5 ± 0.1 mM (average ± standard deviation [SD]) thiosulfate was consumed while 7.6 ± 0.1 mM sulfate was produced. Thus, the stoichiometry of thiosulfate oxidation is approximately 2 mol of sulfate generated by 1 mol of thiosulfate. No growth was observed when thiosulfate was not added to the medium (data not shown). These results indicate that *B. japonicum* USDA110 can grow chemolithotrophically using thiosulfate as a sole electron donor.

Thiosulfate oxidation and cell growth under mixotrophic conditions. *B. japonicum* USDA110 also oxidized thiosulfate (4 mM) in the presence of 0.1% (wt/vol) succinate as an energy source (Fig. 2B), indicating that the addition of succinate does not inhibit thiosulfate oxidation (Fig. 2A and B). Under these conditions, 1 mol of thiosulfate produced 1.9 mol of sulfate during growth (4.0 ± 0.0 mM [average ± SD] thiosulfate was consumed while 7.4 ± 0.1 mM sulfate was produced). *B. japonicum* USDA110 was unable to grow with 20 mM sodium thiosulfate under the mixotrophic condition (data not shown). This growth inhibition was not due to toxicity of the counter ion (Na^+) in the sodium thiosulfate added because 40 mM NaCl did not affect cell growth of USDA110 under the mixotrophic condition (data not shown).

Phenotypes of *sox* deletion mutants. SoxY, a subunit of the SoxYZ complex, plays a key role in thiosulfate oxidation in Sox systems (9). Sulfur-SoxY complexes are covalently bound to a cysteine residue ($Cys110^Y$ is on the carboxy terminus of SoxY in *P. pantotrophus* GB17) located within a characteristic GGCGG pentapeptide at the C terminus of the SoxY subunit of the SoxYZ complex. C-terminal glycine residues are conserved in SoxY orthologs and are presumably essential (33, 40). In the genome of *B. japonicum* USDA110, the GGCGG

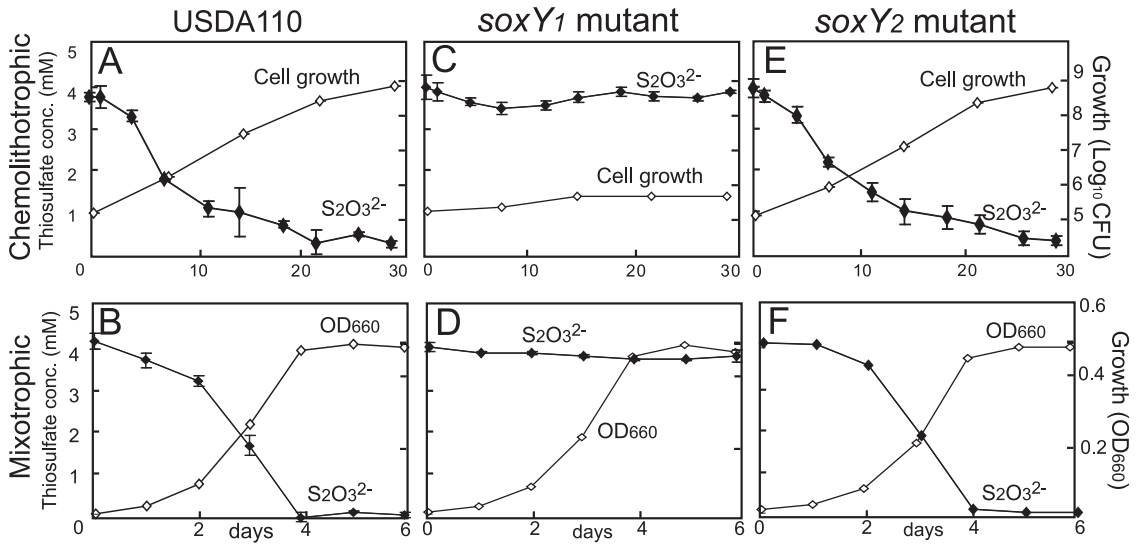

FIG. 2. Thiosulfate consumption and cell growth of *B. japonicum* USDA110 (A and B), *soxY*$_1$ mutant (C and D), and *soxY*$_2$ mutant (E and F) under chemolithotrophic and mixotrophic conditions. Cell growth under mixotrophic conditions was monitored by turbidity (OD_{660}). Cell growth under chemolithotrophic conditions was monitored by CFU counts because chemolithotrophic growth showed low cell turbidity (maximum OD_{660}, 0.04).

motif was not conserved in the C terminus of *soxY*$_3$ in locus III but was well conserved in the C terminus of *soxY*$_1$ and *soxY*$_2$ in loci I and II, respectively (see Fig. S2 in the supplemental material). Therefore, for mutation analysis of the *B. japonicum* USDA110 genome, we focused on *soxY*$_1$ and *soxY*$_2$ carrying the GGCGG motif. Deletion mutants for *soxY*$_1$ and *soxY*$_2$ were constructed as described in Materials and Methods (Fig. 1B).

The *soxY*$_1$ mutant culture showed no chemolithotrophic growth (expressed as CFU), and thiosulfate concentrations did not decrease over 28 days (Fig. 2C). Under mixotrophic conditions, the *soxY*$_1$ mutant was unable to oxidize thiosulfate even when growth was observed (Fig. 2D). The *soxY*$_2$ mutant was able to oxidize thiosulfate and grow under both chemolithotrophic and mixotrophic conditions (Fig. 2E and F), similar to wild-type USDA110 (Fig. 2A and B). These results indicate that *soxY*$_1$ is responsible for thiosulfate oxidation in *B. japonicum* USDA110.

Thiosulfate-dependent O_2 respiration. It is likely that *B. japonicum* USDA110 can grow chemolithotrophically using thiosulfate as an electron donor (Fig. 2A). If this is true, then addition of thiosulfate could enhance O_2 respiration under conditions deficient in electron donors. To test this, USDA110 cells from mixotrophic growth conditions with thiosulfate and succinate were subjected to a respiration assay with an O_2 electrode. To minimize endogenous respiration, cells were prepared from cultures in late log phase. The O_2 uptake rate appeared to increase just after the addition of thiosulfate to the cell suspensions (Fig. 3A, arrowhead). Five independent assays yielded 3.2 ± 0.1 (average ± SD) μmol of O_2 min^{-1} g of wet $cells^{-1}$ as the thiosulfate-dependent O_2 uptake rate for USDA110 cells. In contrast, the *soxY*$_1$ mutant showed no thiosulfate-dependent O_2 uptake (0.0 ± 0.1 μmol of O_2 min^{-1} g of wet $cells^{-1}$) (Fig. 3B). In cultures of the *soxY*$_2$ mutant, the O_2 uptake rate also increased just after the thiosulfate addition (2.8 ± 0.1 μmol of O_2 min^{-1} g of wet $cells^{-1}$) (Fig. 3C). These results show that *B. japonicum* USDA110 uses O_2 as an electron acceptor during thiosulfate oxidation and that the *soxY*$_1$ gene in *B. japonicum* USDA110 is required for this to occur. The molar ratio of thiosulfate added and O_2 consumed was 2.1 ± 0.2 in four independent determinations, suggesting that 2 mol of O_2 are stoichiometrically consumed during oxidation of 1 mol thiosulfate.

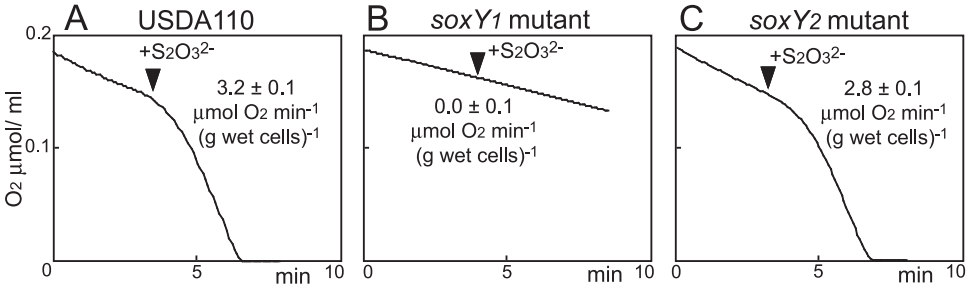

FIG. 3. Thiosulfate-dependent O_2 uptake of whole cells of *B. japonicum* USDA110, a *soxY*$_1$ mutant, and a *soxY*$_2$ mutant. Arrows indicate thiosulfate addition. O_2 concentrations are expressed as μmol ml^{-1} at 25°C. Each panel shows thiosulfate-dependent O_2 uptake calculated by subtracting the O_2 uptake rate of cells without thiosulfate from that of cells with thiosulfate. Five independent determinations yielded estimates (mean ± SD) of 3.2 ± 0.1 (USDA110), 0.0 ± 0.1 (*soxY*$_1$), and 2.8 ± 0.1 (*soxY*$_2$) μmol of O_2 min^{-1} g of wet $cells^{-1}$.

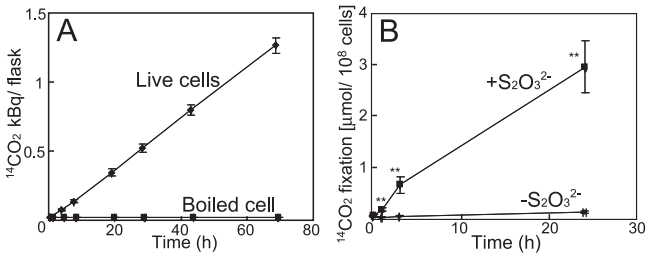

FIG. 4. CO_2 uptake rates in *B. japonicum* USDA110. (A) CO_2 fixation under chemolithotrophic growth conditions. $NaH^{14}CO_3$ (4.17 kBq) was added to 100 ml of chemolithotrophic culture 12 days after inoculation when the remaining thiosulfate was 1.9 mM (initial concentration, 4 mM) and the cell density was 8.7×10^6 CFU ml^{-1}. The CO_2 fixation activity was calculated as 7.7 μmol h^{-1} 10^8 CFU^{-1}, based on the $^{14}CO_2$ uptake rate (18.2 Bq h^{-1} $flask^{-1}$ from panel A), the CO_2 concentration in the flask (691 ppm), and the ^{14}C specific activity of $NaH^{14}CO_3$ (2.18 GBq $mmol^{-1}$). (B) Thiosulfate-dependent $^{14}CO_2$ uptake of chemolithotrophically cultivated whole cells (4.6×10^9 CFU ml^{-1}) in the presence and absence of 4 mM thiosulfate. The reaction was started by the addition of $NaH^{14}CO_3$ (370 kBq) at a final concentration of 20 mM $NaHCO_3$.

CO_2 fixation. We examined the fixation of $^{14}CO_2$ by USDA110 under chemolithotrophic growth conditions. $NaH^{14}CO_3$ was added into the culture flasks at a concentration of 1.91 μmol (equivalent to 0.087 ppm CO_2 in the flasks) 12 days after inoculation. The average ambient CO_2 concentration in the flasks was 691 ppm. $^{14}CO_2$ uptake by chemolithotrophically grown USDA110 cells increased linearly with time (Fig. 4A, live cells), whereas $^{14}CO_2$ uptake by heat-treated cells was not detected (Fig. 4A). The CO_2 fixation rate was calculated as 7.7 μmol h^{-1} 10^8 CFU^{-1} on the basis of the $^{14}CO_2$ uptake rate (18.2 Bq h^{-1} $flask^{-1}$) (Fig. 4A), the CO_2 concentration in the flask (691 ppm), and the ^{14}C specific activity of $NaH^{14}CO_3$ (2.18 GBq $mmol^{-1}$). The resting cells for this experiment (4.6×10^9 CFU ml^{-1}) were prepared from chemolithotrophic growth culture 12 days after inoculation. When thiosulfate was added to the cell suspensions (final concentration, 4 mM), the CO_2 fixation rate increased significantly compared with cells incubated without thiosulfate (Fig. 4B).

Distribution of *sox* genes in *Bradyrhizobiaceae*. When Southern hybridization was performed using two long probes of *sox* locus I (*soxTRSV* and *soxWX*$_1$*Y*$_1$*Z*$_1$*A*$_1$*B*$_1$*C*$_1$*D*$_1$) from *B. japonicum* USDA110, the positive signals were detected in only eight strains: *B. japonicum* USDA122, NC6, NC4, and NK2; *A. oligotropica* S58; photosynthetic bradyrhizobia BTAi1 and ORS278; and *R. palustris* CGA009 (Table 2); this suggests that the *sox* locus I genes are conserved in these strains. However, no signal was detected in four strains of *B. japonicum* (USDA124, T7, T9, and USDA6^T), *Bradyrhizobium* sp. (G14130, HWK12, and HW13), and *B. elkanii* USDA76^T.

Thiosulfate oxidation capability in *Bradyrhizobiaceae*. To determine whether the eight strains belonging to the *Bradyrhizobiaceae* that carry homologues of the *B. japonicum sox* locus I genes express thiosulfate-oxidizing activity, they were subjected to a plate assay for this activity. These strains consistently produced yellow halos on the medium containing less than 4 mM thiosulfate (Table 2). The remaining strains (*B. japonicum* USDA124, T9, T7, USDA6^T, *Bradyrhizobium* sp. G14130, HW12, HWK13, and *B. elkanii* USDA76^T) did not produce yellow halos on any medium containing thiosulfate. These results show that *Bradyrhizobiaceae* carrying the *sox* locus I gene homologues of USDA110 consistently oxidize thiosulfate at concentrations of less than 4 mM.

When a phylogenetic tree was constructed on the basis of 16S rRNA gene sequences (Fig. 5A), cluster BJ1 of *B. japonicum* (16) and a group of photosynthetic bradyrhizobia (PB) including *A. oligotrophica* S58 consistently carried *sox* locus I genes and expressed thiosulfate oxidation activity (Table 2).

Distribution of *soxY* in *Alphaproteobacteria*. To examine how the Sox system is distributed in the phylum *Alphaproteobacteria*, 50 amino acid sequences homologous to the *B. japonicum* *soxY*$_1$ gene with E-values lower than 5e−20 were collected from the NCBI database. Among these sequences, the functionally important cysteine residue and GGCGG motif (33, 40) were well conserved (see Fig. S3 in the supplemental material). A phylogenetic analysis of these SoxY sequences (Fig. 5B; see also Fig. S4) showed that major members of the order *Rhodobacterales*, including *P. pantotrophus* GB17, formed a single cluster in terms of SoxY homology (Fig. 5B, gray triangle). A group including *B. japonicum* USDA110 and photosynthetic bradyrhizobia BTAi1 and ORS278 and another group including five strains of *R. palustris* formed respective compact clusters with high bootstrap values (Fig. 5B, black triangles), which are distant from the well-characterized *Rhodobacterales* cluster.

DISCUSSION

Herein, we demonstrated that *B. japonicum* USDA110 can grow chemolithotrophically with low concentrations of thiosulfate as an electron donor (Fig. 2A) and with oxygen as an electron acceptor. In addition, ^{14}C experiments showed that USDA110 cells fixed ambient CO_2 under chemolithotrophic conditions, indicating that the cells used ambient CO_2 as a carbon source (Fig. 4A). This chemolithoautotrophic growth required the functional *soxY*$_1$ gene (Fig. 2C). The members of *Bradyrhizobiaceae* with homologues of the *sox* locus I genes of USDA110 also demonstrated thiosulfate oxidation (Table 2 and Fig. 5A).

Generally, there are two different end products of bacterial oxidation of thiosulfate: sulfate and tetrathionate (14). In *B. japonicum* USDA110, 1 mol of thiosulfate was converted to approximately 2 mol of sulfate and consumed approximately 2 of mol O_2. Thus, the stoichiometry of the reaction for thiosulfate ($S_2O_3^{2-}$) oxidation in *B. japonicum* USDA110 should be the following: $S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+$ ($\Delta G'^0 = -818.29$ kJ).

This reaction indicates that sulfate is a product of thiosulfate oxidation in *B. japonicum* USDA110, yielding energy for growth which is identical to that reported for *P. pantotrophus* GB17 (10).

B. japonicum is known to grow chemolithoautotrophically using the gaseous electron donors H_2 and CO (6, 13, 27). Thus, the present study demonstrates that *B. japonicum* USDA110 can utilize an inorganic sulfur compound, thiosulfate, as a nongaseous electron donor for chemolithoautotrophic growth.

Previously, thiosulfate-oxidizing bacteria such as aerobic sulfur-oxidizing bacteria have been isolated from sulfur-rich environments using enrichment culture. Therefore, high concentrations of thiosulfate (20 mM) have been added to the medium for cultivation and for biochemical and ecological analyses of these sulfur-oxidizing bacteria. Our results make it clear that these

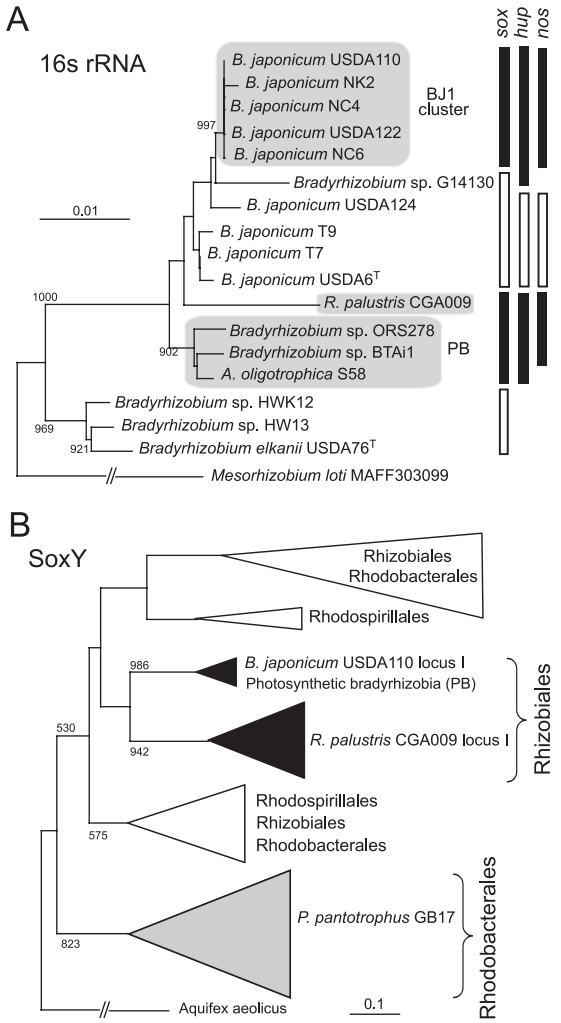

FIG. 5. Phylogenetic relationships of members of the *Bradyrhizobiaceae* based on 16S rRNA gene sequences (A) and of *Alphaproteobacteria* based on amino acid sequences of SoxY (B). The phylogenetic tree was constructed by the neighbor-joining method. Bar lengths represent the number of base substitutions per nucleotide. Numbers are bootstrap values from 1,000 replications and are shown if they are greater than 900 (A) or 500 (B). In panel A, *Mesorhizobium loti* MAFF303099 was used as the outgroup, and *sox*, *hup*, and *nos* indicate the presence (black line) or absence (white line) of genes for thiosulfate oxidation (*sox* locus I), uptake hydrogenase (*hupLS*), and N_2O reductase (*nosZ*), respectively. The *sox* genotype was determined in this study, and *hup* and *nos B. japonicum* genotypes were previously determined along with their positive correlations with hydrogenase and N_2O reductase activities (16, 35, 38). The *hup* and *nos* genotypes of *R. palustris* CGA009, *Bradyrhizobium* sp. ORS278, and *Bradyrhizobium* sp. BTAi1 were deduced from their published genome sequences. Phylogenetic positions and *sox* gene distributions show similar patterns with the BJ1 cluster in *B. japonicum* (16, 38), photosynthetic bradyrhizobia, and *R. palustris* carrying the *sox* locus I (Fig. 5A). Interestingly, the distribution of the *sox* locus I was closely associated with *hup* and *nos* genotypes. Panel B is a simple presentation of SoxY phylogenetic relationships based on the original analysis of SoxY phylogeny (see Fig. S4 in the supplemental material). Using the amino acid sequences of the SoxY protein at the *sox* locus I of *B. japonicum* USDA110, the NCBI database was searched for SoxY homologues by the BLASTP program. A cysteine residue (Cys110) was consistently conserved when the SoxY sequences with E-values lower than 5e−20 were aligned (see Fig. S3). The SoxY homologue of *Aquifex aeolicus* VF5 (NP_214241.1) was used as the outgroup.

copiotrophic procedures would fail to detect strains capable of bacterial thiosulfate oxidation only at low thiosulfate concentrations, such as *B. japonicum* USDA110. Therefore, it is possible that past studies have overlooked such phenotypes of sulfur-oxidizing bacteria in nature. In fact, this study has shown that several members of the *Bradyrhizobiaceae* such as *B. japonicum*, *A. oligotrophica*, *Bradyrhizobium* sp. (photosynthetic bradyrhizobia), and *R. palustris* can oxidize thiosulfate only at low concentrations (Table 2). A phylogenetic tree based on SoxY (Fig. 5B; see also Fig. S4 in the supplemental material) shows unique clusters that are distant from the well-characterized *Rhodobacterales* cluster. Taken together, it is likely that some members of the *Bradyrhizobiaceae* are a new type of thiosulfate-oxidizing bacteria that may adapt to oligotrophic sulfur environments.

A careful homology search for *sox* genes (Fig. 1; see also Table S1 and Fig. S2 in the supplemental material) initially suggested that *sox* loci I and II would be functional for thiosulfate oxidation. Subsequently, our deletion mutant analyses revealed that the $soxY_1$ gene is essential for thiosulfate oxidation (Fig. 1 and 2). Among the duplicate copies of oxygenase genes for aromatic compound degradation in *B. japonicum*, only one set of genes that included $pcaG_1H_1$ was functional for vanillate catabolism (17, 42). Therefore, it is likely that the *B. japonicum* genome carries one functional gene for specific biochemical traits among redundant copies.

In the present study, we frequently found thiosulfate oxidation capability in members of *Bradyrhizobiaceae*, which was associated with the presence of homologues for *sox* locus I genes of *B. japonicum*. So far, we do not know the phylogenetic or ecological implications of the coexistence of Sox^+ and Sox^- strains among *Bradyrhizobiaceae* and even within *B. japonicum* strains (Table 2 and Fig. 5A). However, it is interesting that the strains showing thiosulfate-oxidizing capability (Sox^+) were clustered with those with *hupSL* genes encoding uptake hydrogenase, and *nosZ* genes encoding N_2O reductase for denitrification (Fig. 5A). One possible explanation is that the genomic regions containing *sox*, *hup*, and *nos* genes behave similarly during the evolution of the *Bradyrhizobiaceae* owing to ecological advantages in low-nutrient environments. In particular, *B. japonicum* lineages may diverge into the BJ1 cluster and others for these inorganic metabolisms. However, this possibility cannot be explained by a single event of horizontal gene transfer because these genes (*sox*, *hup*, and *nos*) are scattered on different loci of the USDA110 genome (Fig. 1).

The possession of alternative respiration systems provides flexibility to bacteria for adapting to fluctuating environments (15, 34). It will be interesting to clarify the ecological role of *Bradyrhizobiaceae* that exhibit sulfur oxidation in soil environments such as paddy fields.

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REFERENCES

1. **Cole, M. A., and G. H. Elkan.** 1973. Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. Antimicrob. Agents Chemother. **4:**248–253.
2. **Dao, T. V., M. Nomura, R. Hamaguchi, K. Kato, M. Itakura, K. Minamisawa, S. Sinsuwongwat, H. T. Le, T. Kaneko, S. Tabata, and S. Tajima.** 2008. NAD-malic enzyme affects nitrogen-fixing activity of *Bradyrhizobium japonicum* USDA 110 bacteroids in soybean nodules. Microbes Environ. **23:**215–220.
3. **Dennis, J. J., and G. J. Zylstra.** 1998. Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. Appl. Environ. Microbiol. **64:**2710–2715.
4. **Eisen, A. J., K. E. Nelson, I. T. Paulsen, J. F. Heidelberg, M. Wu, R. J. Dodson, R. Deboy, M. L. Gwinn, W. C. Nelson, D. H. Haft, E. K. Hickey, J. D. Peterson, A. S. Durkin, J. L. Kolonay, F. Yany, I. Holt, L. A. Umayam, T. Mason, M. Brenner, T. P. Shea, D. Parksey, W. C. Nierman, T. V. Feldblyum, C. L. Hansen, M. B. Craven, D. Radune, J. Vamathevan, H. Khouri, O. White, T. M. Gruber, K. A. Ketchum, J. C. Venter, H. Tettelin, D. A. Bryant, and C. M. Fraser.** 2002. The complete genome sequence of *Chlorobium tepidum* TLS, a photosynthetic, anaerobic, green-sulfur bacterium. Proc. Natl. Acad. Sci. U. S. A. **99:**9509–9514.
5. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. U. S. A. **76:**1648–1652.
6. **Franck, W. L., W. S. Chang, J. Qiu, M. Sugawara, M. J. Sadowsky, S. A. Smith, and G. Stacey.** 2008. Whole-genome transcriptional profiling of *Bradyrhizobium japonicum* during chemoautotrophic growth. J. Bacteriol. **190:** 6697–6705.
7. **Friedrich, C. G., A. Quentmeier, F. Bardischewsky, D. Rother, G. Orawski, P. Hellwig, and J. Fisher.** 2008. Redox control of chemotrophic sulfur oxidation of *Paracoccus pantotrophus*, p. 139–150. *In* C. Dhal and C. G. Friedrich (ed.) Microbial sulfur metabolism, Springer, Berlin, Germany.
8. **Friedrich, C. G., D. Rother, F. Bardischewsky, A. Quentmeier, and J. Fischer.** 2001. Oxidation of reduced inorganic sulfur compounds by bacteria: emergence of a common mechanism? Appl. Environ. Microbiol. **67:**2873–2882.
9. **Friedrich, C. G., F. Bardischewsky, D. Rother, A. Quentmeier, and J. Fischer.** 2005. Prokaryotic sulfur oxidation. Curr. Opin. Microbiol. **8:**253–259.
10. **Friedrich, C. G., and G. Mitrenga.** 1981. Oxidation of thiosulfate by *Paracoccus denitrificans* and other hydrogen bacteria. FEMS Microbiol. Lett. **10:**209–212.
11. **Giraud, E., L. Moulin, D. Vallenet, V. Barbe, E. Cytryn, J. C. Avarre, M. Jaubert, D. Simon, F. Cartieaux, Y. Prin, G. Bena, L. Hannibal, J. Fardoux, M. Kojadinovic, L. Vuillet, A. Lajus, S. Cruveiller, Z. Rouy, S. Mangenot, B. Segurens, C. Dossat, W. L. Franck, W. S. Chang, E. Saunders, D. Bruce, P. Richardson, P. Normand, B. Dreyfus, D. Pignol, G. Stacey, D. Emerich, A. Verméglio, C. Médigue, and M. Sadowsky.** 2007. Legumes symbioses: absence of Nod genes in photosynthetic bradyrhizobia. Science **316:**1307–1312.
12. **Göttfert, M., H. Hennecke, and S. Tabata.** 2005. Facets of the *Bradyrhizobium japonicum* 110 genome, p. 99–111. *In* R. Palacios and W. E. Newton (ed.), Genomes and genomics of nitrogen-fixing organisms. Springer, Dordrecht, Netherlands.
13. **Hanus, J. F., J. M. Robert, and H. J. Evans.** 1979. Autotrophic growth of H2-uptake-positive strains of *Rhizobium japonicum* in an atmosphere supplied with hydrogen gas. Proc. Natl. Acad. Sci. U. S. A. **76:**1788–1792.
14. **Hensen, D., D. Sperling, H. G. Trüper, D. C. Brune, and C. Dahl.** 2006. Thiosulphate oxidation in the phototrophic sulphur bacterium *Allochromatium vinosum*. Mol. Microbiol. **62:**794–810.
15. **Hiraishi, A.** 2008. Biodiversity of dehalorespiring bacteria with special emphasis on polychlorinated biphenyl/dioxin dechlorinators. Microbes Environ. **23:**1–12.
16. **Itakura, M., K. Saeki, H. Omori, T. Yokoyama, T. Kaneko, S. Tabata, T. Ohwada, S. Tajima, T. Uchiumi, K. Honnma, K. Fujita, H. Iwata, Y. Saeki, Y. Hara, S. Ikeda, S. Eda, H. Mitsui, and K. Minamisawa.** 2009. Genomic comparison of *Bradyrhizobium japonicum* strains with different symbiotic nitrogen-fixing capabilities and other *Bradyrhizobiaceae* members. ISME J. **3:**326–339.
17. **Ito, N., M. Itakura, S. Eda, K. Saeki, H. Oomori, T. Yokoyama, T. Kaneko, S. Tabata, T. Ohwada, S. Tajima, T. Uchiumi, E. Masai, M. Tsuda, H. Mitsui, and K. Minamisawa.** 2006. Global gene expression in *Bradyrhizobium japonicum* cultured with vanillin, vanillate 4-hydroxybenzoate and protocatechuate. Microbes Environ. **21:**240–250.
18. **Jorgensen, B.** 1990. A thiosulfate shunt in the sulfur cycle of marine sediments. Science **249:**152–154.
19. **Kamagata, Y., R. R. Fulthorpe, K. Tamura, H. Takami, L. J. Fomey, and J. M. Tiedje.** 1997. Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. **63:**2266–2272.
20. **Kaneko, T., Y. Nakamura, S. Sato, K. Minamisawa, T. Uchiumi, S. Sasamoto, A. Watanabe, K. Idesawa, M. Iriguchi, K. Kawashima, M. Kohara, M. Matsumoto, S. Shimpo, H. Tsuruoka, T. Wada, M. Yamada, and S. Tabata.** 2002. Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res. **9:**189–197.
21. **Kelly, D. P., and A. P. Wood.** 1994. Synthesis and determination of thiosulfate and polythionates. Methods Enzymol. **243:**475–501.
22. **King, G. M.** 2007. Chemolithotrophic bacteria: Distributions, functions and significance in volcanic environments. Microbes Environ. **22:**309–319.
23. **Kletzin A., T. Urich, F. Muller, T. M. Bandeias, and C. M. Gomes.** 2004. Dissimilatory oxidation and reduction of elemental sulfur in thermophilic archaea. J. Bioenerg. Biomembr. **36:**77–912.
24. **Larimer, F. W., P. Chain, L. Hauser, J. Lamerdin, S. Malfatti, L. Do, M. L. Land, D. A. Pelletier, J. T. Beatty, A. S. Lang, F. R. Tabita, J. L. Gibson, T. E. Hanson, C. Bobst, J. L. Torres, C. Peres, F. H. Harrison, J. Gibson, and C. S. Harwood.** 2004. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. Nat. Biotechnol. **22:**55–61.
25. **Lepo, J. E., F. J. Hanus, and H. J. Evans.** 1980. Chemoautotrophic growth of hydrogen-uptake-positive strains of *Rhizobium japonicum*. J. Bacteriol. **141:**664–670.
26. **Li, X., H. Arai, I. Shimoda, H. Kuraishi, and Y. Katayama.** 2008. Enumeration of sulfur-oxidizing microorganisms on deteriorating stone of the Angkor monuments, Cambodia. Microbes Environ. **23:**293–298.
27. **Lorite, M. J., J. Tachil, J. Sanjuvan, O. Meyer, and E. J. Bedmar.** 2000. Carbon monoxide dehydrogenase activity in *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. **66:**1871–1876.
28. **Lu, W. P., E. P. Swoboda, and D. P. Kelly.** 1985. Properties of the thiosulfate-oxidizing multi-enzyme system from *Thiobacillus versutus*. Biochim. Biophys. Acta **828:**116–122.
29. **Maier, R. J.** 1981. *Rhizobium japonicum* mutant strains unable to grow chemolithoautotrophically with H2. J. Bacteriol. **145:**533–540.
30. **Minamisawa, K., T. Seki, S. Onodera, M. Kubota, and T. Asami.** 1992. Genetic relatedness of *Bradyrhizobium japonicum* field isolates as revealed by repeated sequences and various other characteristics. Appl. Environ. Microbiol. **58:**2832–2839.
31. **Nakagawara, S., and K. Takai.** 2008. Deep-sea vent chemoautotrophs: diversity, biochemistry and ecological significance. FEMS Microbiol. Ecol. **65:**1–14.
32. **Prentki, P., and H. M. Krisch.** 1984. *In vitro* insertional mutagenesis with a selective DNA fragment. Gene **29:**303–313.
33. **Quentmeier, A., and C. G. Friedrich.** 2001. The cysteine residue of the SoxY protein as the active site of protein-bound sulfur oxidation of *Paracoccus pantotrophus* GB17. FEBS Lett. **503:**168–172.
34. **Richardson, D. J.** 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology **146:**551–571.
35. **Saito, A., H. Mitsui, R. Hattori, K. Minamisawa, and T. Hattori.** 1998. Slow-growing and oligotrophic soil bacteria phylogenetically close to *Bradyrhizobium japonicum*. FEMS Microbiol. Ecol. **25:**277–286.
36. **Saitou, N., and M. Nei.** 1987. The neighbour-joining method: a new method for reconstructing phylogenic trees. Mol. Biol. Evol. **4:**406–425.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
38. **Sameshima-Saito, R., K. Chiba, J. Hirayama, M. Itakura, H. Mitsui, S. Eda, and K. Minamisawa.** 2006. Symbiotic *Bradyrhizobium japonicum* reduces N_2O surrounding the soybean root system via nitrous oxide reductase. Appl. Environ. Microbiol. **72:**2526–2532.
39. **Sameshima-Saito, R., K. Chiba, and K. Minamisawa.** 2006. Correlation of denitrifying capability with the existence of *nap*, *nir*, *nor* and *nos* genes in diverse strains of soybean bradyrhizobia. Microbes Environ. **21:**174–184.
40. **Sauvé, V., S. Bruno, B. C. Berks, and A. M. Hemmings.** 2007. The SoxYZ complex carries sulfur cycle intermediates on a peptide swinging arm. J. Biol. Chem. **282:**23194–23204.
41. **Schäfer, A., A. Tauch, W. J.äger, J. Kalinowski, G. Thierbach, and A. Pühler.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69–73.
42. **Sudtachat, N., N. Ito, M. Itakura, S. Masuda, S. Eda, H. Mitsui, Y. Kawaharada, and K. Minamisawa.** 2009. Aerobic vanillate degradation and C1 compound metabolism in *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. **75:**5012–5017.
43. **Taylor, B. F., and D. S. Hoare.** 1971. *Thiobacillus denitrificans* as an obligate chemolithotroph. Isolation and growth studies. Arch. Mikrobiol. **78:**193–204.
44. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
45. **van Berkum, P., and J. J. Fuhrmann.** 2000. Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence. Int. J. Syst. Evol. Microbiol. **50:**2165–2172.
46. **Wodara, C., F. Bardischewsky, and C. G. Friedrich.** 1997. Cloning and characterization of sulfite dehydrogenase, two *c*-type cytochromes, and a flavoprotein of *Paracoccus denitrificans* GB17: essential role of sulfite dehydrogenase in lithotrophic sulfur oxidation. J. Bacteriol. **179:**5014–5023.

Thiosulfate-dependent chemolithoautotrophic growth of *Bradyrhizobium japonicum*

Supplemental materials

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Running title: Chemolithoautotroph of *B. japonicum* by thiosulfate

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Table S1. Features of proteins encoded in *sox* loci of *B. japonicum* USDA110.

Protein		Calculated mol wt +/- signal peptide	Predicted cellular localization transport pathway	Conserved domains	Highest sequence similarity, accession number
sox locus I					
bll3505	*soxT*	36,788/ 34,996	Periplasm, soluble Sec-dependent	IPR007272: Protein of unknown function DUF395, YeeE/YedE	YeeE/YedE family membrane protein, *Bradyrhizobium* sp. BTAi1, YP_001239651
bll3506	*soxR*	13,437	Cytoplasm, soluble	IPR001845: Bacterial regulatory protein, ArsR	Ars family transcriptional regulator, *Bradyrhizobium* sp. ORS278, YP_001205206
bll3507	*soxS*	13,323/ 10,943	Periplasm, soluble Sec-dependent	IPR012336: Thioredoxin-like fold	putative thioredoxin, *Bradyrhizobium* sp. BTAi1, YP_001239653
blr3508	*soxV*	25,106	transmembrane protein	IPR003834: Cytochrome c assembly protein, transmembrane region	putative transmembrane cytochrome C biogenesis protein, putative SoxV protein, *Bradyrhizobium* sp. ORS278,
blr3510	*soxW*	23,161/ 18,919	Periplasm, soluble Tat-dependent	IPR012336: Thioredoxin-like fold	putative thioredoxin-like protein, *Bradyrhizobium* sp. BTAi1, YP_001239656
blr3511	$soxX_1$	17,689/ 13,718	Periplasm, soluble Sec-dependent	IPR009056: Cytochrome c, monohaem	SoxX, *Bradyrhizobium* sp. BTAi1, YP_001239657
blr3512	$soxY_1$	15,671/ 12,706	Periplasm, soluble Tat-dependent	IPR016568: Sulphur oxidation, SoxY	SoxY, *Bradyrhizobium* sp. BTAi1, YP_001239658
blr3513	$soxZ_1$	11,919	Periplasm, soluble Tat-dependent co-transport with	none	SoxZ, *Bradyrhizobium* sp. ORS278, YP_001205213
blr3514	$soxA_1$	31,006/ 28,573	Periplasm, soluble Sec-dependent	IPR009056: Cytochrome c, monohaem	SoxA, *Bradyrhizobium* sp. ORS278, YP_001205214
blr3515	$soxB_1$	61,445/ 58,333	Periplasm, soluble Tat-dependent	IPR004843: Metallophosphoesterase	SoxB, *Bradyrhizobium* sp. BTAi1, YP_001239661
blr3516	$soxC_1$	47,197/ 43,997	Periplasm, soluble Tat-dependent	IPR000572: Oxidoreductase,	SoxC, *Bradyrhizobium* sp. ORS278, YP_001205216
blr3517	$soxD_1$	25,722/ 22,830	Periplasm, soluble Sec-dependent	IPR009056: Cytochrome c, monohaem	SoxD, *Bradyrhizobium* sp. ORS278, YP_001205217
sox locus II					
bll1009	$soxB_2$	64,111/ 60,384	Periplasm, soluble Tat-dependent	IPR004843: Metallophosphoesterase	SoxB, *Bradyrhizobium* sp. BTAi1, YP_001236865
bll1011	$soxA_2$	32,251/ 29,358	Periplasm, soluble Sec-dependent	IPR009056: Cytochrome c, monohaem	SoxA, *Bradyrhizobium* sp. BTAi1, YP_001236867
bll1012	$soxZ_2$	11,143	Periplasm, soluble Sec-dependent co-transport with	none	SoxZ, *Bradyrhizobium* sp. ORS278, YP_001208658
bll1013	$soxY_2$	16,796/ 130,30	Periplasm, soluble Sec-dependent	IPR016568: Sulphur oxidation, SoxY	SoxY, *Bradyrhizobium* sp. BTAi1, YP_001236869
bll1014	$soxX_2$	21,057	Cytoplasm, soluble	IPR009056: Cytochrome c, monohaem	SoxX, *Bradyrhizobium* sp. ORS278, YP_001208656
sox locus III					
bll2732	$soxA_3$	30,612/ 26,186	Periplasm, soluble Sec-dependent	IPR009056: Cytochrome c, monohaem	SoxA, *Bradyrhizobium* sp. ORS278, YP_001204470
bll2733	$soxZ_3$	11,596	Periplasm, soluble Tat-dependent co-transport with	none	SoxZ, *Bradyrhizobium* sp. ORS278, YP_001204471
bll2734	$soxY_3$	16,684/ 13,449	Periplasm, soluble Tat-dependent	IPR006311: Twin-arginine translocation pathway signal	SoxY, *Bradyrhzobium* sp. BTAi1, YP_001238805
bll2735	*soxF*	45,058/ 41,678	Periplasm, soluble Sec-dependent	IPR015323 Flavocytochrome c sulphide dehydrogenase, flavin-binding IPR015904: Sulphide quinone-reductase	sulfide dehydrogenase, *Bradyrhizobium* sp. BTAi1, YP_001238807
sox locus IV					
bll6120	$soxC_3$	19,075/ 16,452	Periplasm, soluble Sec-dependent	IPR009056: Cytochrome c, monohaem	putative sulfite oxidase, *Methylobacterium nodulans* ORS2060, ZP_02122633
bll6121	$soxD_3$	46,367/ 42,634	Periplasm, soluble Tat-dependent	IPR000572: Oxidoreductase, molybdopterin binding	putative sulfite oxidase cytochrome subunit, *Methylobacterium nodulans* ORS2060, ZP_02122634

Molecular weights of *sox* genes were calculated by the Genetyx ver. 5.1 (for Microsoft Windows). The signal peptide and transmembrane helices of the *sox* genes were predicted by the SignalP 3.0, TatP and TMHMM programs available from the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/). The InterProScan Sequence Search at the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/Tools/InterProScan/) was used to search for conserved domains of *sox* genes. Those with the highest sequence similarity were searched for in the National Center for Biotechnology Information (NCBI) database.

Table S2. The similarity (identity percentage) of amino acid sequences of *sox* genes in *Bradyrhizobium japonicum* USDA110 with those of other bacteria.

Bradyrhizobium japonicum USDA110	*Bradyrhizobium* sp. BTAi1	*Bradyrhizobium* sp. ORS278	*Rhodopseudomonas palustris* CGA009	*Paracoccus pantotrophus* GB17	*Chlorobium tepidum* TLS	*Allochromatium vinosum* DSM180^T
locus I						
T	76	77		44		
R	91	91		64		
S	72	75	45	47		
V	70	69	46	53		
W	73	73	44	62		
X	81	82	45			
Y	80	81	43	58		
Z	86	90	60	56		
A	82	83	50			
B	89	88	63	64		
C	92	90	63	73		
D	73	72	48	53		
locus II						
B	74	72			57	56
A	84	86			50	49
Z	74	75			51	
Y	75	73			44	
X	81	77			46	47
locus III						
A	69	67	30			
Z	76	77	41	41		
Y	71	72	38	40		
F	70	68	44			
locus IV						
D	55		43			
C	61					

The BLASTP program in NCBI database was used for the comparison based on the amino acid sequences of *sox* genes in *B. japonicum* USDA110. Figures show as identity percentage of the amino acid sequences. Blank (no figure) indicates no hit in top 50 sequences by blast P search.

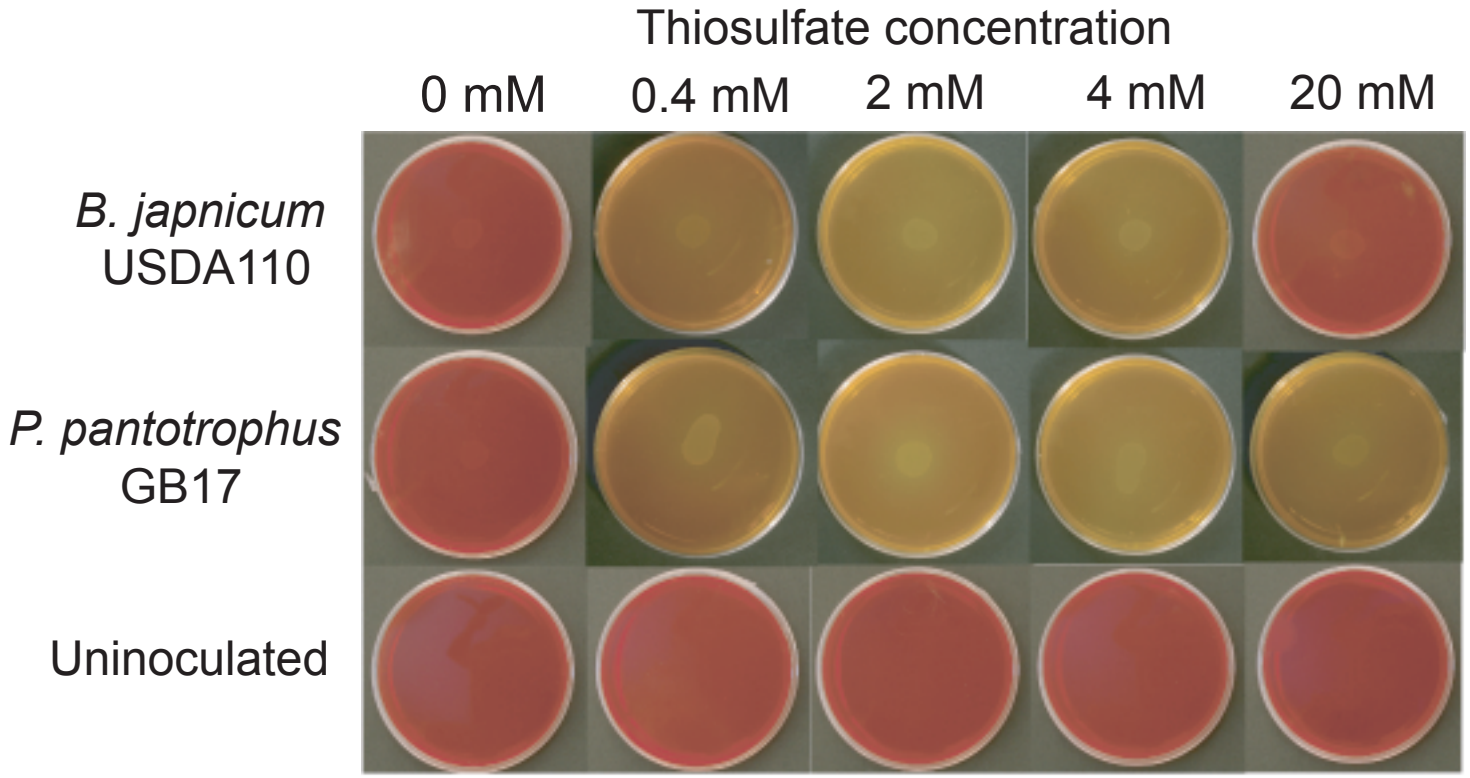

Fig. S1. Plate assays showing thiosulfate-oxidizing capability in *B. japonicum* USDA110 and *Paracoccus pantotrophus* GB17. Comparison of color changes of *B. japonicum* USDA110 with those of *P. pantotrophus* GB17 and an uninoculated control at thiosulfate concentrations from 0 to 20 mM. Phenol red was added to the growth media at 20 μg L^{-1} as a pH indicator. Thiosulfate oxidation causes a drop in pH, which can be recognized by the color change from red to yellow. The uninoculated control consistently showed a red color in plates supplemented with various concentrations of thiosulfate. *P. pantotrophus* GB17 formed yellow halos on the media at all thiosulfate concentrations as a positive control for the plate assay. In contrast, *B. japonicum* USDA110 cells formed apparently yellow halos on plates with 0.4, 2 and 4 mM thiosulfate, whereas the cells did not produce the halos on plates with 20 mM thiosulfate.

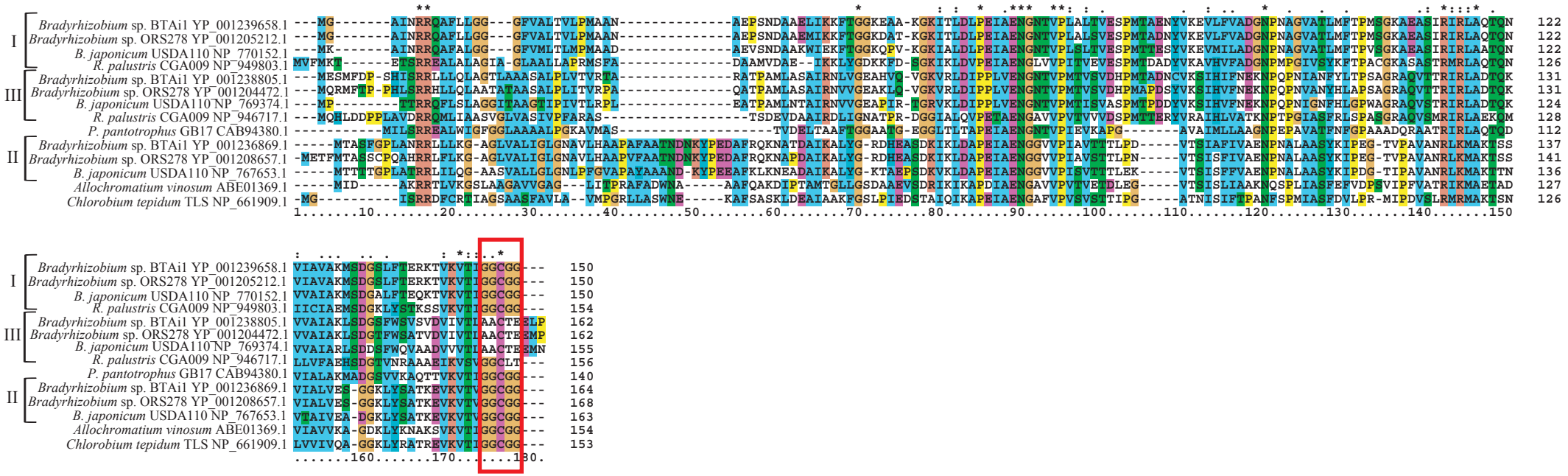

Fig. S2

Multiple sequence alignment of SoxY. The NCBI database was searched for the amino acid sequences of SoxY in *P. pantotrophus* GB17 and *A. vinosum*. The amino acid sequences of SoxY in *R. palustris* and *C. tepidum* were searched by the Cyanobase. The SoxY homologues of *Bradyrhizobium* sp. BTAi1 and *Bradyrhizbium* sp. ORS278 was searched by the BLASTP program in the *Bradyrhizobium* section of Rhizobase based on the amino acid sequences of SoxY protein (SoxY1, SoxY2 and SoxY3) in *B. japonicum* USDA110. Sulfur-SoxY are covalently bound to a cysteine residue located within a characteristic GGCGG pentapeptide at the C terminus (marked with red asterisks) of the SoxY subunit of the SoxYZ complex. C-terminal glycine residue are conserved in SoxY orthlogs and are presumably essentig.

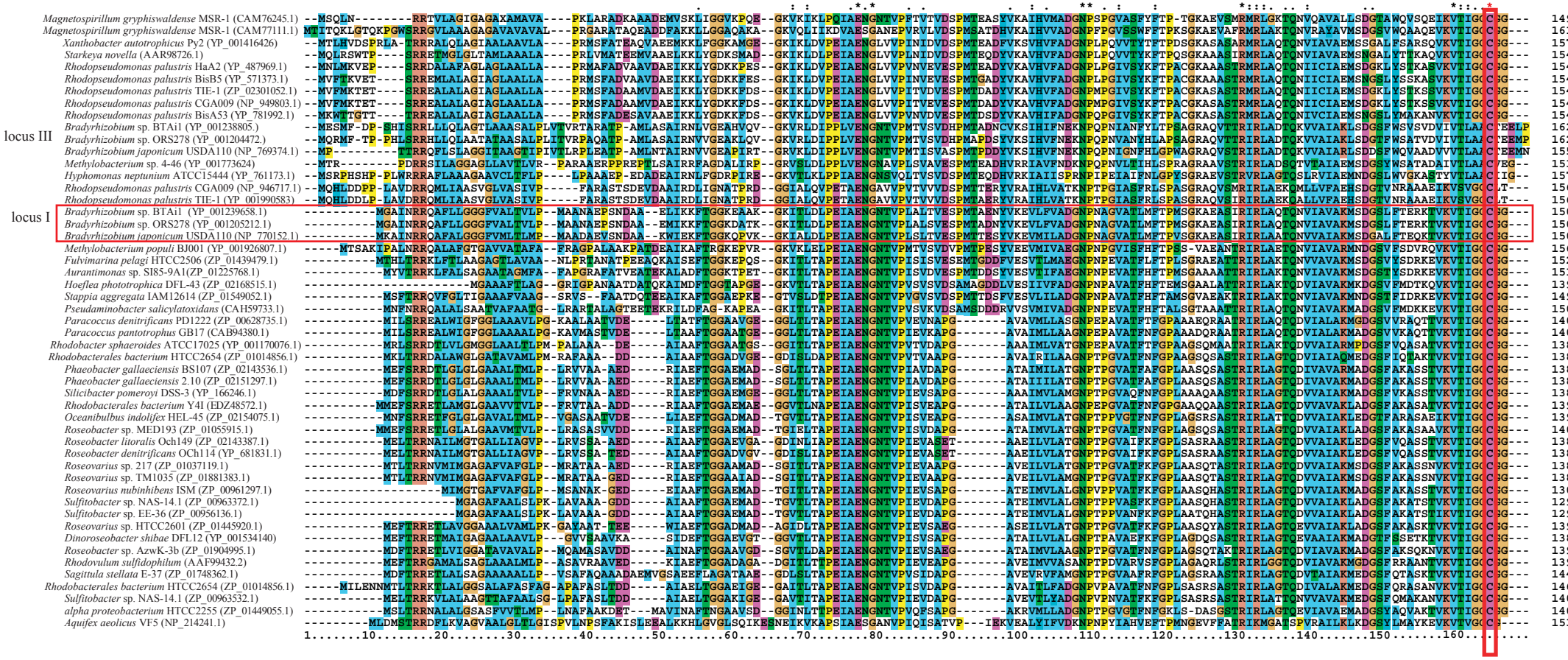

Fig. S3. Multiple sequence alignment of SoxY. Based on the amino acid sequences of SoxY protein in sox locus I of *B. japonicum* USDA110, SoxY homologs were searched by the BRASTP program in the NCBI database. Cysteine residue (Cys110 red asterisked) was consistently conserved in the Alphaproteobacteria when the SoxY sequences with E-values lower than 5e-20 were aligned.

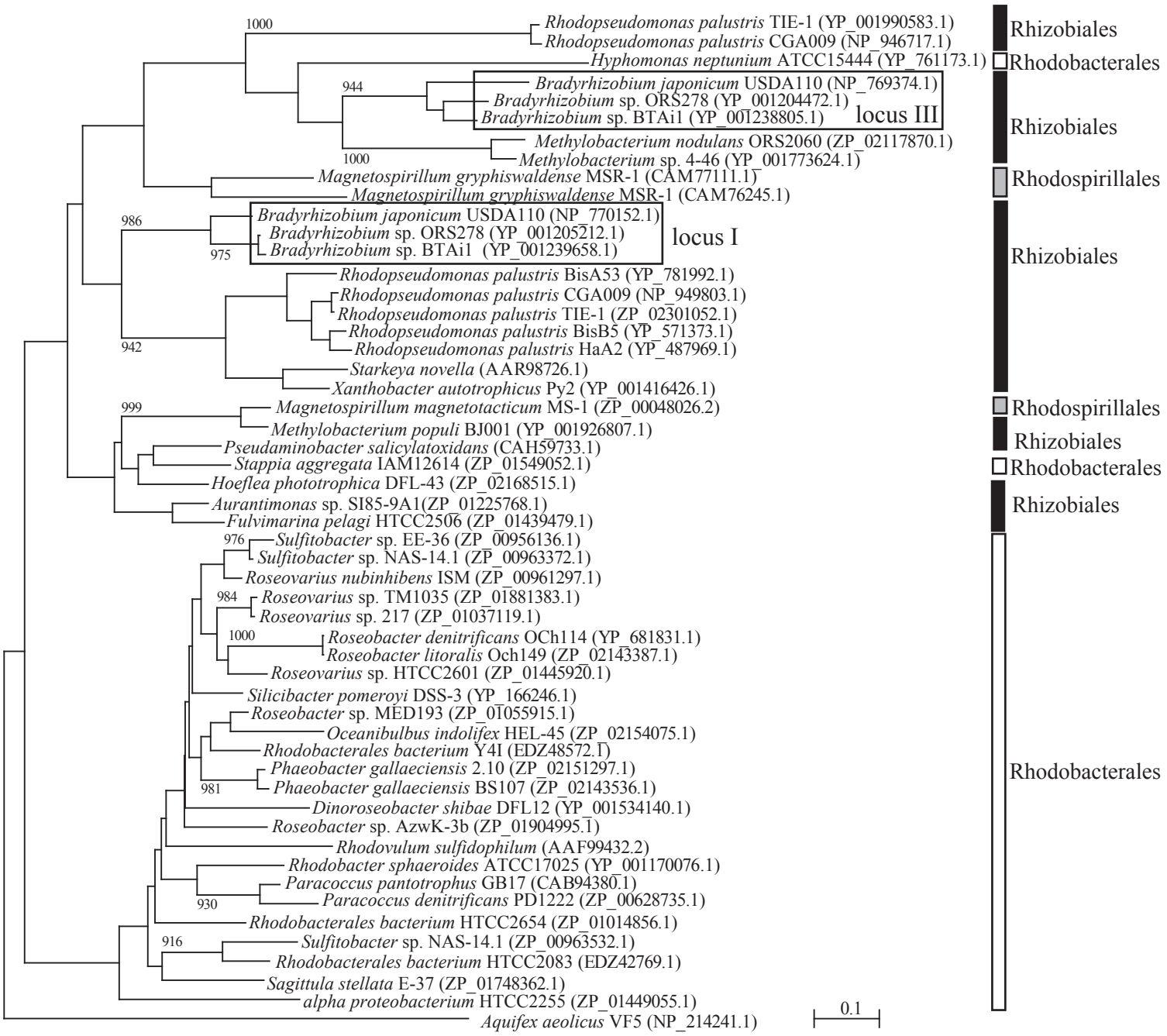

Fig. S4. Phylogenetic relationships of *B. japonicum* USDA110 and other members in Alphaproteobacteria based on SoxY amino acid sequences. Based on the amino acid sequences of SoxY in *sox* locus I of *B. japonicum* USDA110, we searched the SoxY amino acid sequences in the members of Alphaproteobacteria using the BRASTP program in the NCBI database. We constructed the phylogenetic tree of SoxY amino acid sequences that had the E-values lower than 5e-20 for amino acid similarity. Phylogenetic tree constructed by the neighbour-joining (NJ) method. *Aquifex aeolicus* VF5 was used as outgroup. Numbers are bootstrap values from 1000 replications. Bars show base substitutions per nucleotide. Right column shows the order of the bacteria.