Expression and Functional Roles of *Bradyrhizobium japonicum* Genes Involved in the Utilization of Inorganic and Organic Sulfur Compounds in Free-Living and Symbiotic Conditions

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Submitted 13 August 2010. Accepted 14 December 2010.

Strains of *Bradyrhizobium* spp. form nitrogen-fixing symbioses with many legumes, including soybean. Although inorganic sulfur is preferred by bacteria in laboratory conditions, sulfur in agricultural soil is mainly present as sulfonates and sulfur esters. Here, we show that *Bradyrhizobium japonicum* and *B. elkanii* strains were able to utilize sulfate, cysteine, sulfonates, and sulfur-ester compounds as sole sulfur sources for growth. Expression and functional analysis revealed that two sets of gene clusters (bll6449 to bll6455 or bll7007 to bll7011) are important for utilization of sulfonates sulfur source. The bll6451 or bll7010 genes are also expressed in the symbiotic nodules. However, *B. japonicum* mutants defective in either of the sulfonate utilization operons were not affected for symbiosis with soybean, indicating the functional redundancy or availability of other sulfur sources in planta. In accordance, *B. japonicum* bacteroids possessed significant sulfatase activity. These results indicate that strains of *Bradyrhizobium* spp. likely use organosulfur compounds for growth and survival in soils, as well as for legume nodulation and nitrogen fixation.

Bacteria assimilate sulfur, an essential element, from various inorganic and organic compounds in the environment. Although bacteria utilize inorganic sulfur (sulfate) for growth in laboratory media, organic sulfur in the form of ester-sulfur and sulfonates constitute approximately 95% of the total sulfur in aerobic soils (Autry and Fitzgerald 1990). The detailed molecular mechanisms for sulfonate sulfur utilization (*ssu*) has been determined in the gram-negative bacteria *Escherichia coli* (van der Ploeg et al. 2001), *Pseudomonas aeruginosa* (Kertesz 1999), *P. putida* (Kahnert et al. 2000; Mirleau et al. 2005), and *Burkholderia cenocepacia* (Iwanika-Nowicka et al. 2007), as well as in the gram-positive bacteria *Bacillus subtilis* (van der Ploeg et al. 1998) and *Corynebacterium glutamicum* (Koch et

al. 2005). The *ssu* genes, that encode ABC transport proteins (*ssuABC*) and an (FMNH₂)-dependent sulfonate monooxygenase (*ssuD*), are usually found organized in an operon. In *E. coli*, *P. aeruginosa*, and *P. putida*, the *ssu* gene cluster also contains *ssuE*, encoding an NADPH-dependent FMN-reducing enzyme that provides FMNH₂ to SsuD (Kahnert et al. 2000; van der Ploeg et al. 2001). In addition, *E. coli* also contains the *tauABCD* genes that are required for the utilization of taurine (aminoethane sulfonate) (van der Ploeg et al. 1997), and *P. aeruginosa* contains an *msu* gene cluster that is required for the utilization of methanesulfonate (Kertesz et al. 1999). In contrast to alkane sulfonates, utilization of aromatic sulfonates by *P. putida* requires both the *ssu* genes as well as the electron transport components AsfA and AsfB (Vermeij and Kertesz 1999). The sulfonates are transported and degraded to sulfite, which then enters central metabolism via the cysteine biosynthetic pathway (Kertesz 1999).

Genes required for assimilation of sulfur from inorganic and organic sources in *E. coli* and *P. putida* are regulated by CysB, an LysR-type protein, which is highly conserved among the γ and β proteobacteria (Iwanika-Nowicka et al. 2007; Kertesz 1999; Kredich 1996; van der Ploeg et al. 2001). In addition, many bacteria require another LysR-type protein, Cbl (for CysB-like), for the utilization of sulfonates (Iwanika-Nowicka and Hryniewicz 1995). The expression of *cbl* requires CysB, resulting in the expression of sulfonate utilization only in the absence of cysteine and sulfate (Kertesz 1999; van der Ploeg et al. 2001). In contrast, the α -proteobacteria to which rhizobia belong does not contain homologues of CysB or Cbl (Iwanika-Nowicka et al. 2007).

A recent study reported the utilization of taurine as a sulfur source by *Sinorhizobium meliloti* (Wilson and Kappler 2009). In addition, studies using transcriptomics and proteomics have shown increased expression of putative sulfonate monooxygenases and sulfatases in the soybean nodules induced by *Bradyrhizobium japonicum* USDA 110 (Delmonte et al. 2010; Mesa et al. 2008; Pessi et al. 2007), and in the nodules of *Sesbania rostrata* formed by *Azorhizobium cualinodans* ORS278 (Tsukada et al. 2009). Furthermore, a mutant of *A. cualinodans* ORS578 defective in AZC_3159, a homolog of *E. coli ssuE*, formed nodules that were defective in nitrogen fixation (Suzuki et al. 2007). These results suggest that assimilation

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*The e-Xtra logo stands for “electronic extra” and indicates that seven supplementary tables are published online.

lation of these organosulfur compounds might play an important role not only during symbiosis and nitrogen fixation but also in bacterial survival and competitiveness in soils. Despite these initial studies, however, the details of sulfonate metabolism and its regulation in rhizobia are poorly understood.

In this study, we report on the ability of *B. japonicum* and *B. elkanii* strains to utilize various inorganic and organic sulfur compounds as sole sulfur sources for growth and identify the role of putative sulfonate monooxygenases in sulfonate utilization. We also show that *B. japonicum* expresses sulfonate monooxygenases and sulfatases in soybean nodules and discuss the potential role of organosulfur utilization in nodulation and nitrogen fixation.

RESULTS AND DISCUSSION

Putative sulfonate and sulfate-ester utilization genes in the *B. japonicum* USDA 110 genome.

The *B. japonicum* USDA 110 genome (Kaneko et al. 2002) contains two open reading frames (ORF) annotated as alkane-sulfonate monooxygenases (bll6451 and bll7010) that are flanked by genes annotated as subunits of ATP binding cassette (ABC) transporters. The genomic organization of the bll7007-to-bll7011 gene cluster in *B. japonicum* is similar to the sulfonate utilization gene operons in *E. coli* (van der Ploeg et al. 2001) and *P. putida* (Kahnert et al. 2000; Vermeij and Kertesz 1999) (Fig. 1). In contrast, the bll6449-to-bll6455 cluster contained two ORF of undefined function (bll6449 and bll6450) and a gene (bll6452) annotated as an acyl-CoA dehydrogenase. BLASTP analysis revealed that it is a DszC monooxygenase family member with 27% identity and 45% simi-

larity to DszC of *P. putida* KT2440. The DszC is involved in the desulfurization of the heterocyclic S compound dibenzothiophene. In addition, the genome contains six ORF (bll2665, bll2667, bll3900, bll4738, bll4741, and bll5074) described as putative sulfatases. Further analysis of genome sequences of all legume-nodulating rhizobia using Rhizobase revealed genes annotated as sulfonate transporters, sulfonate monooxygenases, and sulfatases (Supplementary Table S1). The widespread occurrence of organic sulfur utilization genes in rhizobia suggests that organic sulfur compounds might be an important source of S for rhizobia in soil, as well as during symbioses with legume hosts.

Utilization of organic sulfur by *Bradyrhizobium* spp. and transcriptional analysis of *B. japonicum* USDA 110 grown on organic and inorganic sulfur sources.

B. japonicum sp. strains USDA 110 and USDA 4 and *B. elkanii* sp. strain USDA 94 were able to utilize inorganic sulfate and the various sulfonates (butanesulfonate, octane sulfonate, methylmethane sulfonate, 4-[2-hydroxyethyl]-1-piperazine-ethanesulfonic acid [HEPES], 2-[N-morpholino] ethanesulfonic acid [MES], N-Tris[hydroxymethyl] methyl-2-aminoethanesulfonic acid [TES], CHAPS, 3-morpholinopropanesulfonic acid [MOPS], Ethyl methanesulfonate [EMS], and tarunine), sulfur-ester compound (p-nitrochetachol sulfate), and heterocyclic sulfur compounds (dibenzothiophene) as sole sources of sulfur for growth (Supplementary Table S2).

In order to examine the global control of gene expression by inorganic and organic sulfur sources, whole-genome transcriptional profiles of *B. japonicum* cells grown with cysteine, sulfate, HEPES, and taurine as sole sulfur sources were com-

B. japonicum USDA110

E. coli

P. putida

Fig. 1. Genetic organization of sulfonate utilization genes in *Bradyrhizobium japonicum* USDA 110 and their corresponding genes in *Escherichia coli* (van der Ploeg et al. 2001) and *Pseudomonas putida* (Kahnert et al. 2000). Clusters contain gene coding for alkane sulfonate monooxygenase (black bars), FMN reductases (hatched bars), and subunits of ABC transporters (white bars). The bll6449-to-bll6455 cluster lacks the FMN reductase (*ssuE*) gene and contains a gene with similarity to dibenzothiophene-degradative enzyme DszC (bll6452). Note that the putative FMN reductase in *B. japonicum* is the last gene in the operon whereas it is first gene in *E. coli* and *P. putida*.

pared. Three sets of comparisons were made: i) cells grown on cysteine were compared with cells grown on sulfate, HEPES, or taurine; ii) cells grown on sulfate were compared with cells grown on HEPES or taurine; and iii) cells grown on HEPES were compared with those grown on taurine. The complete data generated in the study reported here set is available in the Gene Expression Omnibus database (accession number GSE19039).

The microarray data were analyzed for determining the genes that showed more than twofold induction during growth with sulfate or sulfonates compared with cysteine as S source (Supplementary Table S3), and of genes that showed induction only during growth with sulfonates compared with growth with sulfate (Supplementary Table S4). In addition, we used K-means clustering to analyze the microarray data as described earlier (Gunasekera et al. 2008; Gyaneshwar et al. 2005). In total, 12, 16, or 20 clusters were generated. Separation of the data into 16 clusters gave the best average expression patterns that were distinct. Because the genes involved in utilization of cysteine, sulfate, and sulfonate utilization were expected to be induced during growth with their respective S sources, we focused our attention on cluster 5 and cluster 8 containing 165 and 82 genes, respectively (Fig. 2). Genes in cluster 5 showed enhanced expression during growth with cysteine compared with sulfate and sulfonates, and were also highly expressed when cells were grown with taurine compared with sulfate. In contrast, genes in cluster 8 showed enhanced expression during growth with sulfate or sulfonates compared with cysteine.

As expected from the expression pattern, many genes in cluster 8 were annotated as being involved in sulfate and sulfonate metabolism (Table 1; Supplementary Table S5). These included the putative alkane sulfonate monooxygenases and the closely linked ABC transporters (bll6449 to bll6455 and bll7007 to bll7011). This cluster also contained genes putatively involved in uptake and assimilation of sulfate (blr1478

to blr1485), *nodQ*, and *cysD* (Table 1). The expression pattern of these genes suggests that, in *B. japonicum* USDA 110, cysteine likely represses the expression of genes involved in sulfate and sulfonate metabolism.

An examination of genes in cluster 5 (Supplementary Table S6) revealed an unexpected link between sulfur metabolism and the regulation of nitrogen fixation. Of the 165 genes in cluster 5, 142 (85%) were recently shown to be regulated by the FixLJ-FixK₁-FixK₂ cascade (Mesa et al. 2008). FixK₂ regulates these operons and is part of a regulatory cascade involving FixLJ, a two-component regulatory system that responds to a moderate decrease in oxygen levels (Gilles-Gonsalvez et al. 1991; Gong et al. 1998). However, it was recently shown that FixK₂ is present in the *B. japonicum* cells grown under aerobic conditions but it is oxidized at one of its cysteine residues (C183). Under microaerobic conditions, C183 gets reduced, thus activating FixK₂ (Mesa et al. 2009). In contrast to the genes regulated by FixK₂ that showed increased expression on cysteine, expression of FixK₂ itself was increased during growth with sulfate and sulfonates. Although our results suggests that FixK₂ can activate its target genes even under aerobic conditions, we favor the view that growth with cysteine could lead to a high intracellular cysteine pool which can help in maintaining FixK₂ in active configuration by reducing the C183 residue. This view is further supported by increased expression of FixK₂ in sulfate- and sulfonate-grown cells. Growth on sulfate or sulfonates can result in oxidative stress (Gyaneshwar et al. 2005) leading to the oxidation of C183 in FixK₂, rendering it inactive.

Quantitative reverse-transcription polymerase chain reaction analysis of sulfonate monooxygenase expression in free-living and symbiotic conditions.

Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to verify the induction of

Fig. 2. K-means clustering of the microarray dataset. Each box shows a transcriptional response of genes partitioned into a separate cluster. Cluster number and gene count in the cluster are shown within cluster box. The six pairwise comparisons obtained among different growth conditions are indicated on the x axis. The signal log ratios of gene expression are indicated on the y axis. The gray lines in each cluster represent the data for individual genes; the black line designates a centroid.

sulfonate monooxygenases (bll6451 and bll7010) observed in DNA microarray analysis. The expression values were normalized to the mRNA levels of the housekeeping gene *parA*, which was previously shown to be a good candidate for normalizing gene expression data (Cytryn et al. 2007). Consistent with the microarray analysis, expression levels of bll6451 or bll7010 were the lowest in cells growing with cysteine and were induced approximately three- or 11-fold, respectively, in cells growing with sulfate. Growth with sulfonates led to a further 64- and 343-fold increase in expression of bll6451 and bll7010, respectively (Table 2). These results suggest that sulfonate utilization genes in *B. japonicum* USDA 110 are repressed by cysteine, partially derepressed by sulfate, and fully induced only in the absence of both cysteine and sulfate. Thus, the metabolic regulation of sulfonate utilization gene in *B. japonicum* appears similar to that found in *E. coli* and *P. putida* (Kertesz 1999; van der Ploeg et al. 2001).

Analysis of the recently reported transcriptomics and proteomics data revealed that both bll6451 and bll7011 were expressed in *B. japonicum* bacterioids isolated from soybean nodules (Mesa et al. 2008; Pessi et al. 2007). Consistent with these reports, qRT-PCR analysis showed that both bll6451 and bll7010 were significantly expressed in *B. japonicum* bacterioids in soybean nodules compared with constitutively expressed gene *parA*. These results suggest that bacterioids are likely utilizing sulfonates or related organic S compounds for their sulfur nutrition in the root nodules.

Regulation of sulfonate metabolism in *B. japonicum*.

Previously reported transcriptomic and proteomic analysis suggested that the expression of sulfonate utilization genes might be regulated by the FixLJ-FixK2-FixK1 cascade or through RpoN1+2 (Delmonte et al. 2010; Mesa et al. 2008). However, *B. japonicum* mutants defective in these genes (Narberhaus et al. 1996; Nellen-Anthamatten et al. 1998) did not show any defect in sulfonate utilization (data not shown), indicating that the genetic regulation of sulfur assimilation in *B. japonicum* might be through a novel, as-yet-undefined mechanism.

Functional analysis of bll6451 or bll7010 for sulfonate utilization and symbiosis.

The role of bll6451 or bll7010 in sulfonate utilization was determined by both functional complementation of an *E. coli* mutant defective in sulfonate utilization (*AssuD AtauD*) as well as by generating and analyzing *B. japonicum* mutants with disrupted bll6451 (strain 6451) or bll7010 (strain 7010). The plasmid bll7010 could complement the *E. coli* (*AssuD AtauD*) mutant for growth on HEPES but not on taurine. In contrast, bll6451 failed to complement the mutant growth with HEPES or taurine (data not shown). Consistent with these observations, *B. japonicum* sp. strain 7010 failed to grow with HEPES but grew similarly to the wild type on taurine, whereas strain 6451 could grow well with both HEPES or taurine but could not utilize dibenzothiophene (Table 3). These results

Table 1. Genes of sulfate or sulfonate metabolism part of cluster 8 and their induction ratios in various S sources

Gene no.	Gene name ^b	Gene description	log ₂ R/G ratios ^a		
			Sulfate	HEPES	Taurine
blr1233	...	Putative sulfonate-binding protein	1.5	2.5	1.0
bll1475	<i>nodQ</i>	NodQ bifunctional enzyme	4.5	3.2	4.3
bll1476	<i>cysD</i>	Sulfate adenylate transferase	4.1	3.6	3.1
blr1479	<i>cysI</i> *	Sulfite reductase*	3.6	3.1	1.8
blr1481	<i>cysH</i>	Phosphoadenosine phosphosulfate reductase	3.2	2.6	2.1
blr1482	<i>cysP</i> *	ABC transporter sulfate-binding protein	2.9	3.4	2.3
blr1483	<i>cysT</i> *	Sulfate ABC transporter permease protein	1.8	2.0	2.2
blr1485	<i>cysA</i> *	Sulfate ABC transporter ATP-binding protein	1.9	1.9	1.5
bll6449	...	Hypothetical protein bll6449	4.5	5.2	4.6
bll6450	...	Probable substrate-binding protein	-0.2	1.9	1.7
bll6451	...	Probable alkanesulfonate monooxygenase	0.6	2.8	3.4
bll6453	...	ABC transporter ATP-binding protein	0.7	1.8	1.2
bll6455	...	ABC transporter substrate-binding protein	0.5	2.9	0.9
bll7007	<i>ssuE</i> *	Putative oxidoreductase	0.3	2.2	1.4
bll7008	<i>ssuC</i> *	Aliphatic sulfonate ABC transporter	0.8	3.0	2.6
bll7009	<i>ssuB</i> *	Aliphatic sulfonate transporter permease protein	2.1	2.7	1.4
bll7010	<i>ssuD</i>	Sulfonate monooxygenase	2.7	4.1	2.2
bll7011	<i>ssuA</i> *	Aliphatic sulfonate-binding protein	2.6	4.7	2.0

^a Ratios compared with growth on cysteine. HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

^b An asterisk (*) indicates suggested gene name and description based on this study.

Table 2. Quantitative reverse-transcription polymerase chain reaction analysis of sulfonate oxygenase genes in *Bradyrhizobium japonicum* USDA 110 cells

S source or condition	Gene					
	bll6451			bll7010		
	Expression ^a	Expression ratio		Expression ^a	Expression ratio	
		Na ₂ SO ₄	Cysteine		Na ₂ SO ₄	Cysteine
Culture						
Na ₂ SO ₄	0.31 ± 0.22	1.0	2.8	0.52 ± 0.35	1.0	11.4
HEPES ^b	1.88 ± 0.93	6.1	17.1	9.74 ± 1.42	18.9	214.5
Taurine	7.05 ± 2.62	23.0	63.9	15.6 ± 2.78	30.3	343.7
Cysteine	0.11 ± 0.01	0.4	1.0	0.045 ± 0.006	0.1	1.0
In planta						
Bacteroid	0.80 ± 0.10	2.6	7.3	0.53 ± 0.16	1.0	11.8

^a Absolute expression values were normalized to the housekeeping gene *parA*. Values are means ± standard deviation of three biological replicates.

^b HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

suggest that, in *B. japonicum*, the bll7007-to-bll7011 operon is mainly involved in the utilization of alkane sulfonates and the bll6449-to-bll6454 operon may be important for utilization of heterocyclic sulfur compounds. The utilization of taurine, however, is independent of these two sulfonate utilization clusters. However, both these mutants were not significantly affected in their symbiotic interaction with soybean and formed nodules that were similar to the wild type strain (data not shown). The lack of effect on the nodulation could reflect the functional redundancy of the sulfonate utilization system in *B. japonicum* or availability of other S sources such as sulfate-esters (discussed below) or inorganic S.

Sulfatase activity of *B. japonicum* USDA 110 in symbiosis.

Apart from sulfonates, sulfate-esters are major organic sulfur compounds found in soils. In addition, plants contain sulfate-ester compounds such as choline-sulfate in high amounts (Nissen and Benson 1961). *B. japonicum* USDA 110 contains six ORF annotated as sulfatases, indicating that such compounds could be important for its S nutrition in soil as well as during symbiosis. The sulfatase activity of *B. japonicum* USDA 110 in free-living or symbiotic conditions was determined using chromogenic substrate 5-bromo-4-chloro-3-indoxyl sulfate (X-sulfate). *B. japonicum* cells growing on arabinose-gluconate (AG) medium showed intense blue color after 3 days of incubation (not shown) and the soybean nodules containing *B. japonicum* bacteroids cleaved X-sulfate and were stained blue (Fig. 3). No color was observed when soybean roots were incubated with X-sulfate. This indicates that the detected sulfatase activity was due to the bacteria present in the nodules and not from the soybean plant. These results are in contrast to an earlier study that did not detect *B. japonicum* sulfatase activity in soybean nodules (O'Hara et al. 1987) but are in agreement with recent transcriptomic and proteomic studies showing expression of an arylsulfatase encoded by bll2665 (Delmonte et al. 2010; Mesa et al. 2009). These results suggest that S-ester compounds might be utilized by *B. japonicum* for its sulfur nutrition in the plant host.

In summary, *Bradyrhizobium* spp. strains are metabolically diverse and have the ability to utilize a wide variety of sulfur compounds, including alkane sulfonates and sulfate-esters, to support growth and metabolism. Genomic, transcriptomic, and genetic analyses indicates that *B. japonicum* USDA 110 can utilize sulfonate and heterocyclic sulfur compounds via two operons, bll7007 to bll7011 and bll6449 to bll6455. Additionally, *B. japonicum* USDA 110 also expresses the sulfonate monooxygenases and sulfatases in nodules formed on soybean. The expression of genes involved in organic sulfur utilization in root nodules suggests that *B. japonicum* USDA 110 in the bacteroid state may obtain sulfur from sulfonates or sulfur-ester compounds. Interestingly, genes comprising the sulfonate monooxygenases operons were recently shown to be involved in host-specific adaptation of *B. japonicum* USDA 110 (Koch et al. 2010). It is possible that *B. japonicum* undergoes different levels of sulfur starvation or encounters different sources of sulfonates in various legume hosts. Because sulfur-esters and sulfonates may constitute up to 95% of total sulfur in aro-

bic soils, our results suggest that these sulfur sources likely play an important role in the soil survival strategy of *Bradyrhizobium* spp. and in their symbiotic association with their cognate legume host.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions.

Bradyrhizobium cells were grown at 30°C, with shaking at 200 rpm, in AG medium (Sadowsky et al. 1987), or in sulfur-free minimal medium (MHM) containing 3.3 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 3.8 g of Na_2HPO_4 , 6.7 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.15 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 13 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.202 g of NaCl, 0.32 g of NH_4Cl , 1 g of L-arabinose, and 1 g of sodium gluconate, pH 7.0, per liter. The *E. coli* strains were grown on Luria-Bertani medium. Ampicillin and kanamycin (100 µg/ml) were added as required.

Utilization of sulfur sources by *Bradyrhizobium* strains.

Bradyrhizobium strains were grown in 100 ml of AG medium at 30°C until stationary phase. Cells were collected by centrifugation at $10,000 \times g$ for 10 min, washed twice in sulfur-free MHM, resuspended in 100 ml of the same medium, and incubated at 30°C for 4 days to induce sulfur starvation. Aliquots of the cell suspensions (25 µl) were individually inoculated into 5 ml of MHM supplemented with 2 mM Na_2SO_4 or different sulfur sources, including the sulfonate organic buffers HEPES, MES, MOPS, N-cyclohexyl-2-aminoethanesulfonic acid, 3-(cyclohexylamino)-1-propanesulfonic acid, and TES. EMS, butane sulfonate, octanesulfonate, a substituted sulfonate (taurine), and the aromatic sulfonates 2-anthraquinone sulfonate and 4,5-dihydroxy-2-7-naphthalene sulfonate were also tested as S sources for the bradyrhizobia. Cultures were incubated at 30°C, with shaking, and the optical density at 600 nm (OD_{600}) was measured after 7 days of growth.

Sample preparation for microarray analyses.

Sulfur-starved *B. japonicum* USDA 110 cells were inoculated into 15 ml of MHM medium individually supplemented with 2 mM each cysteine, sodium sulfate, HEPES, or taurine to an initial $\text{OD}_{600} = 0.02$. Two biological replicates were used for each analysis. Cultures were incubated at 30°C until OD_{600} reached approximately 0.5 to 0.6, and 1.5 ml of cold stop solution (5% water-saturated phenol in 95 % ethanol) was added to

Fig. 3. Sulfatase activity of *Bradyrhizobium japonicum* USDA 110 bacteroids in soybean nodules. Nodules were collected and incubated in 100 mM potassium phosphate buffer, pH 7.0, containing 5-bromo-4-chloro-3-indoxyl sulfate (X-sulfate). Highly stained bacteroids can be seen in the nodule. No color was detected in plant roots incubated with X-sulfate (not shown).

Table 3. Growth (optical density at 600 nm) of *Bradyrhizobium japonicum* and putative sulfonate monooxygenase mutants with different organic sulfur sources

Strain	Sulfur sources		
	HEPES	Dibenzothiophene	Taurine
USDA 110	0.52 ± 0.04	0.45 ± 0.05	0.72 ± 0.03
7010	0.05 ± 0.01	0.52 ± 0.04	0.62 ± 0.05
6451	0.49 ± 0.06	0.04 ± 0.01	0.59 ± 0.07

each culture. The cultures were centrifuged at $10,000 \times g$ for 10 min at 4°C , the collected cells were immediately frozen by immersion in liquid nitrogen, and cell pellets were stored at -80°C until used for RNA extraction. Total RNA was extracted using the hot phenol method (Bittner et al. 2003), digested with DNaseI, and purified as described previously (Chang et al. 2007). RNA quality was determined by electrophoresis on 0.8% agarose gels.

Microarray expression analysis.

Transcriptional profiling of *B. japonicum* USDA 110 was performed using whole-genome 70-mer oligonucleotide arrays that contain probes for 8,453 ORF printed on glass slides (Chang et al. 2007). These arrays were previously used for characterizing responses of *B. japonicum* to chemoautotrophic growth (Franck et al. 2008) or to desiccation stress (Cytryn et al. 2007). The cDNA synthesis, fluorescent dye labeling, microarray hybridization, and image acquisition procedures used were essentially as previously published (Chang et al. 2007; Cytryn et al. 2007). Spot intensities were obtained from each slide using GenePix Pro 6.0 software (Molecular Devices Corp., Sunnyvale, CA, U.S.A.). All GenePix results and data files produced by the scanning software were uploaded into CARMAweb (Rainer et al. 2006) for normalization and data analysis. Median F635 and B635 values were used for each spot, and flagged spots were ignored. The algorithms used were NORMEXP (correction of background signal), LOESS (within-array normalization), and AQUANTILE for (between-array standardization) (Smyth and Speed 2003). For each comparison of culture growth in two different media, two replicate arrays were produced with swap-dye patterns; these were averaged as biological replicates (arithmetic mean). To generate lists of differentially expressed genes for each comparison, the genes with $|M| \geq 1$ and $|A| \geq 10\%$ percentile range in that comparison were considered [$M = \log_2(R/G)$; $A = \log_2(R \times G)$]. The unions and intersections of the gene lists among comparisons were visualized as Venn diagrams.

Data clustering.

The normalized data set was imported into GENESIS microarray analysis software (version 1.7) (Sturm et al. 2002) as described (Gyaneshwar et al. 2005). Euclidean distance was used as a measure of gene profile similarity. K-means clustering ($K = 16$) and principal components analysis were done to analyze the microarray data, essentially as described earlier (Gunasekera et al. 2008). Gene expression changes were determined for the following comparisons: growth in cysteine (Cys) versus sulfate; Cys versus HEPES; Cys versus taurine; sulfate versus HEPES, sulfate versus taurine, and HEPES versus taurine.

Construction of blI7010 and blI6451 mutants of *B. japonicum*.

Genes blI7010 and blI6451 were amplified by PCR using primers targeting the upstream and downstream sequences of these genes (Supplementary Table S7). PCR products were ligated into pBlueScript SK(+) vector and a kanamycin resistance cassette was inserted into each fragment. The disrupted genes were PCR amplified, cloned into suicide vector pSUP202, and transferred into *B. japonicum* USDA 110 by conjugation, and transconjugants were selected for kanamycin resistance. The correct genomic structure of the disrupted genes containing the kanamycin-resistance cassette was verified by PCR. The resulting mutants were named 6451 and 7010.

Soybean planting and bacteroid isolation from nodules.

Wild-type *B. japonicum* USDA 110 was grown in AG medium and diluted to 10^7 cells/ml in sterile water, and 1-ml ali-

quots of the dilutions were inoculated onto surface-sterilized *Glycine max* 'Lambert' seed. Plant assays were done in sterile Leonard jar assemblies containing a 3:1 mixture of vermiculite and perlite as previously described (Sadowsky et al. 1987). Soybean seed were surface sterilized by immersion in 100% ethanol for 5 s, followed by treatment with 1.0% sodium hypochlorite for 5 min. Seed were washed 10 times with sterile distilled water prior to planting. After inoculation, seed were covered with a 1-cm layer of sterilized paraffin-coated sand. Plants were watered with nitrogen-free plant nutrient solution (Keyser and Cregan 1987), and incubated for 30 days in a plant growth chamber at 25°C , with a photoperiod of 16 h.

To isolate bacteroids from soybean nodules, approximately 1 g of fresh nodules was sampled from 4-week-old soybean plants. The nodules were immediately ground in liquid nitrogen, and the resulting powders were suspended with 1.5 ml of cold phosphate-buffered saline buffer. The homogenate was filtered through three layers of Miracloth (Calbiochem Inc., La Jolla, CA, U.S.A.), and centrifuged at $200 \times g$ for 5 min to remove debris. The pellet was discarded, and the supernatant was transferred to a clean centrifuge tube and centrifuged at $8,000 \times g$ for 10 min to pellet the bacteroids. RNA was isolated from the bacteroids by using the hot-phenol method (Bittner et al. 2003).

Real-time qRT-PCR.

cDNA for qRT-PCR was synthesized from 1 μg of total RNA template, either from cells grown with different sulfur sources or from bacteroids isolated from nodules. A 1- μl aliquot of random hexamers at 250 ng/ μl (Invitrogen Co., Carlsbad, CA, U.S.A.) and 1 μl of 10 mM dNTP mix were added to 11 μl of RNA solution. The mixture was heated at 65°C for 5 min, chilled on ice for 1 min, and 4 μl of 5 $\times$ first-strand buffer, 1 μl of 0.1 M dithiothreitol, 1 μl of RNaseOUT (Invitrogen), and 200 units of Superscript III reverse transcriptase (Invitrogen Co.) were added to the solution. The solution was incubated at 50°C for 1 h, then incubated at 70°C for 15 min. The resultant sample was kept at -20°C as cDNA samples until qRT-PCR was performed. Reaction mixtures (25 μl) contained cDNA synthesized from 50 ng of RNA, 12.5 μl of iTaq SYBR Green Supermix with ROX (Bio-Rad, Hercules, CA, U.S.A.), 11.25 μl of nuclease-free water, and 0.5- μM concentrations each of forward and reverse gene-specific primers. The primer sets used in these PCR tests were as follows: blI6451-F2 and blI6451-R2 (for blI6451), blI7010-F2 and blI7010-R2 (for blI7010), and blI0631-F and blI0631-R (for *parA*). The qRT-PCRs were run on an Applied Biosystems (Foster City, CA, U.S.A.) 7500 real-time PCR system using Sequence Detection System software, version 1.3. The PCR program consisted of an initial denaturation of 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Expression values for three biological replicates for each treatment were normalized to the expression level of *parA* (blI0631), which is a housekeeping gene in the *B. japonicum* USDA 110 genome (Cytryn et al. 2007).

Histochemical staining for sulfatase expression.

At least three nodules from three different soybean plants were assessed for the expression of sulfatase, using the chromogenic substrate X-sulfate. The nodules were bisected using a sterile razor blade and incubated in 0.1 M phosphate buffer, pH 7.0, containing X-sulfate at $50 \mu\text{g ml}^{-1}$ and 0.01% Triton-X-100. To avoid the induction of sulfatases during the incubation, chloramphenicol was added to the assay at $100 \mu\text{g ml}^{-1}$. The activity of sulfatases was monitored by development of blue color, indicating hydrolysis of X-sulfate.

ACKNOWLEDGMENTS

This work was supported, in part, by a grant from the College of Letters and Sciences and Research Growth Initiative, University of Wisconsin, Milwaukee (to P. Gyaneshwar) and by the Minnesota Agricultural Experiment Station (to M. J. Sadowsky). We thank H.-M. Fisher and F. Narberhaus for *B. japonicum* mutants and D. Saffarini and M. McBride for critical reading of the manuscript.

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