JOURNAL OF BACTERIOLOGY, Oct. 2008, p. 6697–6705 Vol. 190, No. 20
0021-9193/08/$08.00+0 doi:10.1128/JB.00543-08

Whole-Genome Transcriptional Profiling of *Bradyrhizobium japonicum* during Chemoautotrophic Growth▽†

William L. Franck,[1] Woo-Suk Chang,[1] Jing Qiu,[2] Masayuki Sugawara,[3] Michael J. Sadowsky,[3] Stephanie A. Smith,[4]‡ and Gary Stacey[1,5,6]*

National Center for Soybean Biotechnology, Division of Plant Sciences, University of Missouri, Columbia, Missouri 65211[1]; Department of Statistics, University of Missouri, Columbia, Missouri 65211[2]; Department of Soil, Water, and Climate, University of Minnesota, St. Paul, Minnesota 55108[3]; Department of Biological Sciences, Wright State University, Dayton, Ohio[4]; and Division of Biochemistry[5] and Department of Molecular Microbiology and Immunology,[6] University of Missouri, Columbia, Missouri 65211

Received 21 April 2008/Accepted 19 July 2008

Bradyrhizobium japonicum **is a facultative chemoautotroph capable of utilizing hydrogen gas as an electron donor in a respiratory chain terminated by oxygen to provide energy for cellular processes and carbon dioxide assimilation via a reductive pentose phosphate pathway. A transcriptomic analysis of *B. japonicum* cultured chemoautotrophically identified 1,485 transcripts, representing 17.5% of the genome, as differentially expressed when compared to heterotrophic cultures. Genetic determinants required for hydrogen utilization and carbon fixation, including the uptake hydrogenase system and components of the Calvin-Benson-Bassham cycle, were strongly induced in chemoautotrophically cultured cells. A putative isocitrate lyase (*aceA*; blr2455) was among the most strongly upregulated genes, suggesting a role for the glyoxylate cycle during chemoautotrophic growth. Addition of arabinose to chemoautotrophic cultures of *B. japonicum* did not significantly alter transcript profiles. Furthermore, a subset of nitrogen fixation genes was moderately induced during chemoautotrophic growth. In order to specifically address the role of isocitrate lyase and nitrogenase in chemoautotrophic growth, we cultured *aceA*, *nifD*, and *nifH* mutants under chemoautotrophic conditions. Growth of each mutant was similar to that of the wild type, indicating that the glyoxylate bypass and nitrogenase activity are not essential components of chemoautotrophy in *B. japonicum*.**

Bradyrhizobium japonicum, the nitrogen-fixing endosymbiont of soybeans, colonizes soil environments that vary widely in organic carbon composition, oxygen concentration, and carbon dioxide levels. *B. japonicum* retains considerable metabolic and energetic diversity to facilitate survival in these environments. *B. japonicum* respires aerobically in the presence of oxygen during heterotrophic growth, and most isolates can respire anaerobically as denitrifiers when nitrate is provided as a terminal electron acceptor in the absence of oxygen (9, 48). Furthermore, under free-living microaerobic conditions supplemented with hydrogen and carbon dioxide, many *B. japonicum* strains, including USDA122 and USDA110, will grow chemoautotrophically, coupling hydrogen oxidation to aerobic respiration and carbon dioxide fixation (24, 33).

Hydrogen oxidation in *B. japonicum* was extensively studied in strains USDA122 and USDA110 due to its potential for enhancing symbiotic nitrogen fixation efficiency. As a symbiont, *B. japonicum* USDA110 expresses an uptake hydrogenase that serves to recapture electrons lost via hydrogen evolution resulting from nitrogenase activity (15). Expression of the uptake hydrogenase system of *B. japonicum* also occurs during heterotrophic growth when an atmosphere containing 2% oxygen and 2% hydrogen is supplied (47). Under these conditions no ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity is detected (47). In addition, the uptake hydrogenase is expressed when *B. japonicum* is cultured chemoautotrophically under microaerobic conditions (1% oxygen) when supplied with hydrogen (10%) and carbon dioxide (5%) in a minimal medium lacking an organic carbon source (33). RuBisCO activity is present under these conditions at levels sufficient to account for all assimilated carbon (33).

The recent availability of Affymetrix (25) and oligonucleotide microarray (5) platforms for *B. japonicum* USDA110 provides useful tools to analyze the environmental responses of the bacterium on a global scale. Transcriptomic studies of *B. japonicum* cultured in minimal and rich media (5), in the bacteroid state (5), and responding to osmotic stress (5) and desiccation (8) as well as iron limitation (41, 51) have all been reported. In addition, analysis of the NifA and σ^{54} regulons from anaerobically cultured cells was recently completed (25). Here we extend the list of transcriptomic studies by generating mRNA profiles of *B. japonicum* USDA110 grown chemoautotrophically to better characterize the transcriptional and physiological responses to an alternative mode of growth based upon hydrogen oxidation and carbon dioxide fixation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains used in this study are listed in Table 1. *B. japonicum* strain USDA110 was routinely maintained on HM salts medium (6) supplemented with 0.5% yeast extract or on peptone-yeast extract (PYE) medium (49) supplemented with 0.04% arabinose at 30°C with shaking

* Corresponding author. Mailing address: 271E Christopher S. Bond Life Sciences Center, University of Missouri, Columbia, MO 65211. Phone: (573) 884-4752. Fax: (573) 884-9676. E-mail: staceyg@missouri.edu.

† Supplemental material for this article may be found at http://jb.asm.org/.

‡ Present address: Applied Biology and Aerosol Technology, Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201.

▽ Published ahead of print on 8 August 2008.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristic	Source or reference
B. japonicum strains		
USDA110	Wild type	USDA, Beltsville, MD
A3	*nifD*::Tn*5*	23
H1	*nifH*::Tn*5*	23
aceA mutant	*aceA*::Km	This study
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
Plasmids		
pRK2073	RK2, Tra^+ Sm^r	32
pKnockout-Ω (pKOΩ)	Suicide vector	50
pHP45Ω-Km	Km cassette	16
pKOΩ-ICL	pKOΩ containing 2.7-kb fragment including entire *aceA* gene	This study
pKOΩ-ICL-Km	Km cassette inserted into AgeI site of pKOΩ-ICL	This study

(200 rpm). Antibiotic concentrations of 150 μg ml^{-1} kanamycin and 30 μg ml^{-1} chloramphenicol were used for *B. japonicum* when necessary. *Escherichia coli* strains were cultured on Luria-Bertani (42) medium at 37°C with antibiotic concentrations as follows, when necessary: 50 μg ml^{-1} kanamycin, 50 μg ml^{-1} ampicillin, and 50 μg ml^{-1} streptomycin.

B. japonicum was grown chemoautotrophically in a medium containing Ormerod's minimal salts (35) supplemented with 0.1 μg ml^{-1} biotin, 1 μg ml^{-1} each of nicotinic acid and thiamine-HCl, 0.25 μg ml^{-1} pyridoxal-HCl, 0.12 μg ml^{-1} inositol, 136 mg $liter^{-1}$ $KH_2PO_4 \cdot H_2O$, 2 μM $CoSO_4 \cdot 7H_2O$, and 0.2% $(NH_4)_2SO_4$. In experiments to test the effect of an exogenous carbon source on growth and gene expression, arabinose was added to 0.04%.

Cells were grown for RNA extraction in 2-liter Fernbach flasks containing 500 ml of minimal medium. Inoculum was grown in 10 ml of PYE to late logarithmic phase and collected by centrifugation at 24°C and ~9,000 × *g*. Cell pellets were gently resuspended with the same medium into which they would be inoculated (minimal medium with or without arabinose supplementation) to remove any residual PYE and centrifuged again. The cells were resuspended in 1 ml of growth medium and used to inoculate 500-ml cultures. Starting culture optical densities at 595 nm were typically 0.01 to 0.03. After inoculation, the cultures were sealed and flushed for 20 min with a gas mixture of 1% O_2, 5% CO_2, 10% H_2, and 84% N_2. Control flasks were sealed under an ambient air atmosphere. The flasks were statically incubated at 30°C and gently mixed twice daily until they were harvested after 7 days of incubation.

RNA isolation. Cells from 500-ml cultures were harvested by centrifugation for 10 min at 4°C and 6,000 × *g* in a fixed-angle rotor. The cell pellets were resuspended in 10 ml ice-cold medium, transferred to cold 15-ml conical tubes and centrifuged for 10 min at 4°C and 8,000 × *g* in a swinging bucket rotor. After discarding the supernatant, the cell pellets were immediately stored at −80°C. Total RNA was extracted using a hot phenol method (1). DNase treatment and RNA purification were carried out as previously described (5). RNA samples were electrophoresed in 0.8% agarose gels prior to use to ensure RNA integrity.

Microarray expression analysis. Transcriptional profiling of *B. japonicum* was performed using whole-genome 70-mer oligonucleotide microarrays containing probes specific to 8,453 predicted open reading frames printed by the Washington University Genome Sequencing Center (St. Louis, MO) and described by Chang et al. in 2007 (5). Synthesis of cDNA, labeling, and hybridization were completed as previously published (5). All hybridizations were performed with 2 μg of labeled cDNAs. Three independent biological replicates of cells grown heterotrophically, chemoautotrophically, and chemoautotrophically supplemented with arabinose were generated. Three independent comparisons were performed such that each culture condition was compared to the other two culture conditions. Three replicate hybridizations, including a dye swap for each replication, were performed for each comparison with the exception of the chemoautotrophy and heterotrophy comparison, for which two replications were performed.

Spot intensities were obtained from each slide using GenePix Pro 6.0 software (Molecular Devices Corp.). The base 2 logarithms of the mean foreground intensities from GenePix software were normalized by the joint lowess normalization method (7) using R/maanova software (0.98_7 version; Jackson Laboratory, Bar Harbor, ME; http://www.jax.org/staff/churchill/labsite/software/Rmaanova/index.html). No background subtraction was used since more than 20% of the spots would have negative values if background correction was applied. After normalization of each array, all 32 channels (2 channels per array) were median centered so that the median log-scale expression levels were all zero. The 1,280 empty and control spots were removed from the downstream analysis to reduce the dimension. A linear mixed effects model was applied to the normalized log-scale expression measures separately for each gene using R/maanova software. Each linear mixed effects model included array effect, dye effect, spot effect, biological replicate effect, and culture condition effect, where the array, spot, and biological replicate effects were treated as random effects. Since the spots are printed in duplicate on each array, spot effects were included to account for the technical replicates. *t* tests for three pairwise comparisons were conducted to identify significantly differentially expressed genes across any two culture conditions. *P* values from the *t* statistics were obtained via the Matest statement in the R/mmanova software, and a *q*-value (45) was computed for each *P* value to produce lists of differentially expressed genes with estimated false discovery rates of 5%.

qRT-PCR analysis. Microarray expression data were confirmed for a selected set of genes by quantitative reverse transcription-PCR (qRT-PCR). RNA was isolated, DNase treated, and purified as described above from cultures of two independent biological replicates. Three micrograms of RNA was reverse transcribed with 200 U of Moloney's murine leukemia virus reverse transcriptase (Promega, Madison, WI). Quantitative RT-PCR was performed with an ABI 7500 real-time PCR system with sequence detection system software version 1.3 (Applied Biosystems, Foster City, CA) utilizing Sybr green PCR master mix (Applied Biosystems, Foster City, CA). Gene-specific primers are listed in Table 2. PCR conditions were as follows: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; stage 3, 45 cycles of 95°C for 15 s and 60°C for 1 min; stage 4 (for dissociation), 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Data obtained for two technical replicates of each biological replicate were normalized to the elongation factor *fusA* (bll5403), which is constitutively expressed under the given culture conditions.

Construction of a putative isocitrate lyase mutant. To generate a mutant in blr2455 (encoding a putative isocitrate lyase, *aceA*), a 2,705-bp DNA fragment containing the entire predicted blr2455 open reading frame and an additional 530-bp 5′ and 396-bp 3′ portions of the sequence were PCR amplified using primers *aceA* F (5′-ACCGGATTGGCCTCATAG-3′) and *aceA* R (5′-GATAC TTACGGCACCCACAG-3′). The PCR product was inserted into the XcmI site of the pKnockout Ω suicide vector (50), creating pKOΩ-ICL. Insert identity was confirmed by DNA sequence analysis. A kanamycin (Km) cassette from pHP45Ω-Km (16) was PCR amplified with the primers 5′-GCTGAACCGGTCCTAGGCCA CTAACTAA-3′ and 5′-CAGTAACCGGTTGATTGATTGAGCAAGC-3′, containing AgeI sites (underlined) and subsequently inserted into an AgeI site in blr2455 to generate pKOΩ-ICL-Km. pKOΩ-ICL-Km was transferred from *E. coli* DH5α to *B. japonicum* USDA110 by triparental mating with the helper plasmid pRK2073 (32). Transconjugants were selected based upon kanamycin resistance and streptomycin sensitivity. Mutants were confirmed by colony PCR and Southern blot analysis (data not shown).

Growth assays were performed with the mutant in minimal medium (5) buffered with 1.1 g $liter^{-1}$ 4-morpholinoethanesulfonic acid and 1.3 g $liter^{-1}$ 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid and supplied with 20 mM acetate as a carbon source. The growth phenotype of the *B. japonicum* USDA110

TABLE 2. Gene-specific primers for quantitative RT-PCR

Gene ID (gene name)	Forward primer	Reverse primer
bll5403 (*fusA*)	ACAAGTACAAGGTTCCGCGCAT	TCAACGATGTCGGCAAGACACT
blr0305	CGACGAGCGCGCCATTCTAT	AAGGCACGTTGTTGCCGGT
blr1743 (*nifD*)	ACCAACAGCGTCGCAGAAATCA	TTGGTGCACGTTGAGGTGCTT
blr1769 (*nifH*)	TATTCTGCACGCCAAGGCTCAA	TCAACGCAGCGAATGTCCTGGTA
blr2455 (*aceA*)	GTCAGCGAAAGGATCAGGCACAT	TTCGCTGCATCGATCTCGCTCT
blr2585 (*cbbL*)	TTCGCGGCAAGGAGCGTTAT	TCGGTGTCCTTTGGCGTGTAGT
blr2588	ATCGGAAGGCGCGATCGAA	AAGCTGGGACTCGAGGAAGGA
bll6941 (*hupL*)	AGAACGCCAATTCGATCCGCAA	TCCAGCGCGTGAAGGTGATAGAA
bll7911	CGGCAGCATCAGTTCTACCGA	TTGCCTTGCTTCTTGCCCTTCT

aceA mutant, relative to the wild-type strain, was assessed using Biolog GN plates (Biolog, Inc., Hayward, CA). Wild-type and mutant strains were grown for 3 days at 30°C on R2A agar medium (Difco, Detroit, MI), and cells were suspended in Biolog GN/GP inoculation broth (Biolog, Inc.) to a final density of approximately 10^9 cells/ml. Cells (150 μl) were inoculated into the wells of Biolog GN plates and incubated for 24 h at 30°C, and color development in each well was determined at 540 nm using a microplate reader.

Microarray data accession numbers. All microarray data are available at the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) via GEO series accession numbers GSE10295, GSE10296, and GSE10298.

RESULTS AND DISCUSSION

To characterize global gene expression profiles of *B. japonicum* USDA110 during chemoautotrophic growth, we employed a whole-genome oligonucleotide DNA microarray, which was previously described and validated (5). Three growth conditions were used to compare chemoautotrophic and heterotrophic growth. *B. japonicum* was cultured heterotrophically in a defined medium (as described in Materials and Methods) supplemented with arabinose at 0.04% under ambient atmospheric conditions. Chemoautotrophic cultures were grown in the same defined medium under an artificial atmosphere consisting of 1% O_2, 5% CO_2, 10% H_2, and 84% N_2 in which energy is derived from the oxidation of hydrogen and carbon is assimilated via carbon dioxide fixation. Alternatively, *B. japonicum* was cultured under chemoautotrophic conditions in medium supplemented with arabinose at 0.04% to address potential effects of mixotrophic growth. Transcriptional profiles of cells grown under these three conditions were compared in a pairwise manner such that each individual condition was compared to the remaining two conditions.

Comparison of the chemoautotrophic condition to the heterotrophic condition identified 1,485 genes as differentially expressed at a 1.5-fold cutoff (856 upregulated and 629 downregulated) (see Tables S1 and S2 in the supplemental material). Likewise, when the arabinose-supplemented chemoautotrophic condition was compared to the heterotrophic condition, 1,227 genes were identified as differentially expressed (641 upregulated and 586 downregulated) (see Tables S3 and S4 in the supplemental material). Substantial overlap was observed between the two comparisons; 601 upregulated genes and 528 downregulated genes were identical in the two data sets, suggesting that the chemoautotrophic and the arabinose-supplemented chemoautotrophic conditions are highly similar at the transcriptional level. In a direct comparison of the two chemoautotrophic conditions (i.e., the presence and absence of arabinose), no genes were identified as differentially expressed. These results indicate that the addition of arabinose to chemoautotrophic conditions does not influence gene expression under our growth conditions, and a mixotrophic mode of growth under microaerobic conditions in the presence of a carbon source was not observed.

Classification by functional category of the differentially expressed genes is summarized in Fig. 1. Genes annotated as hypothetical comprise about 50% of the differentially expressed genes in each data set. Differentially expressed genes belonging to the categories of cell envelope, cellular processes, central intermediary metabolism, and energy metabolism are more highly represented in cells cultured under chemoautotrophic conditions. Included in these categories are genes involved in expression of the uptake hydrogenase, the Calvin-Benson-Bassham (CBB) cycle, nitrogen fixation, ATP synthesis, and components of the respiratory chain and biosynthesis of pili and flagella, outer membrane proteins, and a subset of chaperones.

Uptake hydrogenase expression and respiration. *B. japonicum* USDA110 possesses a membrane-bound uptake hydrogenase capable of oxidation of molecular hydrogen and passage of electrons into an aerobic respiratory chain. The genome of *B. japonicum* encodes the hydrogenase's large and small subunits, numerous accessory and maturation proteins, and regulatory proteins organized in a large cluster of 23 genes (bll6924 to bll6944, bll6947, and bll6949) spanning 29 kb (27). This system was characterized in detail previously (34), and mutations in the core hydrogenase subunits abolished the ability to grow chemoautotrophically under hydrogen-oxidizing conditions (26, 28). In addition, the *B. japonicum* genome also encodes a second uncharacterized, partial uptake hydrogenase system located on the symbiosis island (27). This cluster begins with the large and small subunits of the uptake hydrogenase and includes 15 additional genes or gene remnants encoding accessory and maturation proteins (27). It is unclear if this cluster on the symbiotic island encodes a functional hydrogenase system, when it is functional, or if it is subject to similar regulation as the characterized hydrogenase system. Our expression data indicate that only a *hupL* homolog (blr1721) from this cluster of hydrogenase genes is more than 1.5-fold upregulated under hydrogen-oxidizing conditions. Alternatively, DNA microarray analysis of *B. japonicum* bacteroids identified five genes from this symbiotic cluster as upregulated in bacteroids (5). Therefore, the symbiotic uptake hydrogenase cluster is unlikely to play an important role in chemoautotrophic growth but may contribute to hydrogen uptake in planta.

Transcriptional data from chemoautotrophically cultured cells identified 18 of the 23 genes on the characterized uptake

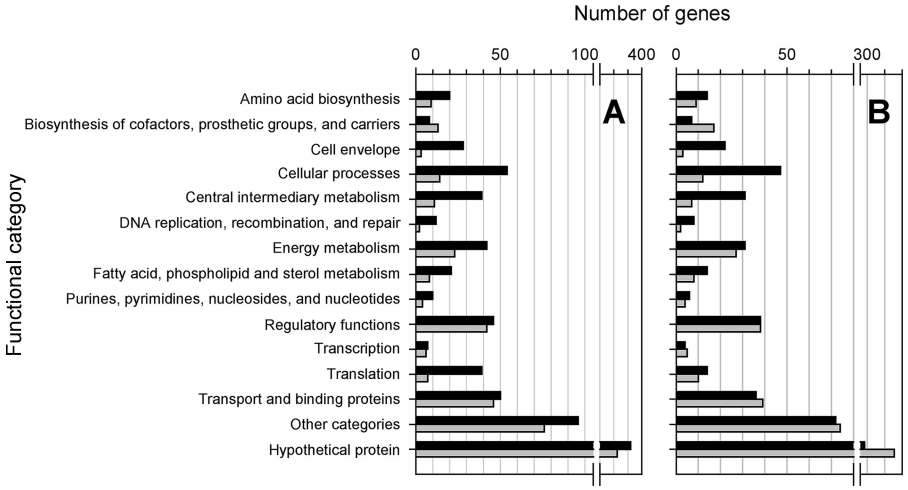

FIG. 1. Functional classification of differentially expressed genes with transcript changes greater than 1.5-fold. (A) Chemoautotrophy and heterotrophy comparison. (B) Arabinose-supplemented chemoautotrophy and heterotrophy comparison. In both panels, black bars represent the chemoautotrophic condition and gray bars represent the heterotrophic condition. Functional classifications were derived from *B. japonicum* genome annotations available via Rhizobase (http://bacteria.kazusa.or.jp/rhizobase/) and the JCVI Comprehensive Microbial Resource (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi).

hydrogenase cluster as being upregulated (Table 3). Included are four of the most strongly upregulated genes, *hupL* and *hupS*, encoding the subunits of the uptake hydrogenase, *hupC*, a probable *b*-type cytochrome involved in electron transfer, and *hupF*, a hydrogenase accessory protein (20, 43). HoxA, a transcriptional activator of the NtrC-like family of two-component response regulators (13), was also upregulated greater than twofold. A deletion mutant of *hoxA* was demonstrated to have no free-living hydrogenase activity; however, bacteroids retained full hydrogenase activity, indicating differential regulation of free-living and symbiotic hydrogenase expression (13). Recently, Hauser et al. (25) identified a subset of hydrogenase genes, including *hupK* (bll6933), *hupD* (bll6939), *hupC* (bll6940), *hupL* (bll6941), and *hupS* (bll6942), as being NifA regulated under anaerobic conditions. Expression of the *B. japonicum* hydrogenase system is dependent on RpoN activity (2), and an RpoN binding site was identified upstream of *hupS* (2, 25). Both HoxA and NifA contain RpoN-interacting domains as identified by InterProScan (39). A possible interpretation of these results is that HoxA directly or indirectly induces expression of the *B. japonicum* uptake hydrogenase under free-living microaerobic conditions, while NifA may regulate the uptake hydrogenase under symbiotic conditions as previously suggested (13). Regulation of the uptake hydrogenase in *Rhizobium leguminosarum* bv. viciae was demonstrated to be under the control of NifA (3). *R. leguminosarum* bv. viciae does not contain a functional HoxA, and unlike *B. japonicum*, it does not express the uptake hydrogenase under free-living microaerobic conditions (3, 36). However, in *B. japonicum* no traditional NifA binding site was identified upstream of *hupS* (25), and binding of NifA to the uptake hydrogenase promoter region was not detected (12). Durmowicz and Maier provided evidence for a role of $FixK_2$ in symbiotic hydrogenase expression (12). Mutants of *fixJ*, part of the FixLJ oxygen-responsive two-component system, and *fixK*$_2$ lack hydrogenase activity under symbiotic conditions, but it is not clear if $FixK_2$ acts directly to regulate hydrogenase expression (12). NifA and $FixK_2$ are components of separate regulatory cascades (11), and it remains unclear how much cross talk exists between the two. At the present time, it is clear that hydrogenase regulation in free-living *B. japonicum* is subject to control by HoxA, although the exact means of symbiotic hydrogenase regulation remain unresolved.

The membrane-bound uptake hydrogenase of *B. japonicum* passes electrons into an electron transport chain terminated by oxygen. *B. japonicum* contains four heme-copper cytochrome-type terminal oxidases reported to have different affinities for oxygen and functioning under different oxygen concentrations (46). The *coxBA* genes, encoding an *aa*$_3$-type cytochrome, are expressed at high oxygen concentrations (21). Dot blot analysis of *coxA* mRNA levels revealed a sixfold reduction in transcript abundance when cultured microaerobically (1% oxygen) compared to results with fully aerated cultures (21). We report 3.5- and 2.6-fold upregulation of *coxB* and *coxA* when cultured in 1% oxygen (Table 3). Furthermore, terminal oxidases encoded by *coxMNOP* and *coxWXYZ* are required for microaerobic chemoautotrophic growth (46). Either oxidase was sufficient for growth, and only a double mutant displayed a chemoautotrophic growth deficiency (46). Under our growth conditions two genes, *coxN* and *coxP*, were downregulated under microaerobic conditions (see Tables S2 and S4 in the supplemental material).

B. japonicum cultured heterotrophically derives energy from both substrate-level phosphorylation and oxidative phosphorylation. When grown chemoautotrophically, the primary means of energy generation is by oxidative phosphorylation requiring ATP synthase activity. Two ATP synthase complexes encoded by five genes each are found in the *B. japonicum* genome (bll0439 to bll0443 and bll1185 to bsl1189). As shown in Table 3, 8 of the 10 genes encoding ATP synthase components are induced during chemoautotrophic growth, demonstrating increased ATP generation by oxidative phosphorylation.

CO_2 fixation and carbon metabolism. Facultative chemoautotrophs such as *B. japonicum* utilize the CBB reductive pen-

TABLE 3. *B. japonicum* genes significantly upregulated during chemoautotrophic growth[a]

Protein function and gene ID	Gene product	Fold change	
		C/H[b]	CA/H[c]
Uptake hydrogenase			
hupT (bll6924)	Two-component hybrid sensor and regulator	2.77	2.70
hoxA (bll6925)	Two-component response regulator	2.43	2.60
hoxX (bll6926)	Probable HoxX protein	2.39	2.31
hypE (bll6927)	HypE protein	2.33	1.96
hypC (bsl6929)	Hydrogenase expression/formation protein	4.53	3.37
hypF (bll6930)	Hydrogenase maturation protein	1.73	1.63
hypB (bll6931)	HypB protein	3.96	3.13
hypA (bll6932)	HypA protein	5.50	3.89
hupK (bll6933)	HupK protein	1.99	1.59
hupI (bsl6935)	HupI protein	5.85	3.46
hupH (bll6936)	HupH protein	3.12	2.40
hupG (bll6937)	HupG protein	3.24	2.59
hupF (bsl6938)	Hydrogenase expression/formation protein	13.09	5.95
hupD (bll6939)	HupD protein	3.07	2.15
hupC (bll6940)	HupC protein	39.62	20.31
hupL (bll6941)	Uptake hydrogenase large subunit	11.61	7.56
hupS (bll6942)	Uptake hydrogenase precursor	29.38	19.05
hupC (bll6947)	Probable Ni/Fe-hydrogenase B-type cytochrome subunit	2.48	2.46
Respiration			
atpB (bll1185)	FoF1 ATP synthase B chain	4.29	3.03
atpB (bll1186)	FoF1 ATP synthase B′ chain	5.06	3.48
atpC (bsl1187)	FoF1 ATP synthase C chain	4.26	3.15
atpA (bll1188)	FoF1 ATP synthase A chain	2.93	2.29
atpI (bsl1189)	FoF1 ATP synthase subunit I	1.57	ND[d]
atpD (bll0440)	ATP synthase beta chain	1.93	1.60
atpG (bll0441)	ATP synthase gamma chain	1.72	1.52
atpA (bll0442)	ATP synthase alpha chain	2.17	1.72
coxB (blr1170)	Cytochrome *c* oxidase subunit II	3.50	3.26
coxA (blr1171)	Cytochrome *c* oxidase subunit I	2.58	2.45
coxG (blr1174)	Putative cytochrome *c* oxidase assembly protein	2.43	2.17
cycA (blr7544)	Cytochrome c_{550}	2.74	2.65
cycM (blr1423)	Cytochrome *c*	1.76	ND
napC (blr7040)	Cytochrome *c*-type protein	2.40	ND
CO_2 fixation and carbon metabolism			
cbbF (blr2581)	Putative D-fructose-1,6-bisphosphatase protein	2.37	1.80
cbbP (blr2582)	Putative phosphoribulokinase protein	6.98	4.71
cbbT (blr2583)	Transketolase	3.82	2.36
cbbA (blr2584)	Putative fructose-1,6-bisphosphate aldolase protein	9.98	5.56
cbbL (blr2585)	Ribulose 1,5-bisphosphate carboxylase/oxygenase large subunit	14.41	7.00
cbbS (blr2586)	Ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit	5.46	3.82
cbbX (blr2587)	Probable CbbX protein	7.49	4.14
cbbE (blr2588)	Ribulose-phosphate 3-epimerase	3.44	2.60
bll1137	Probable carbonic anhydrase	2.05	2.25
bll4865	Putative carbonic anhydrase	1.60	1.69
aceA (blr2455)	Putative isocitrate lyase	28.58	31.03
ppc (blr2955)	Phosphoenolpyruvate carboxylase	1.58	1.70
Nitrogen fixation and nitrogen metabolism			
fdxN (bsr1739)	Ferredoxin	2.11	1.77
nifD (blr1743)	Nitrogenase molybdenum-iron protein alpha chain	4.69	3.09
nifK (blr1744)	Nitrogenase molybdenum-iron protein beta chain	3.46	2.60
nifE (blr1745)	Nitrogenase molybdenum cofactor synthesis protein	1.98	1.76
nixN (blr1746)	Nitrogenase molybdenum cofactor synthesis protein	2.62	1.92
nifX (blr1747)	Iron-molybdenum cofactor processing protein	3.14	2.54
fdxB (bsr1750)	Ferredoxin	1.84	1.57
nifH (blr1769)	Dinitrogenase reductase protein	1.85	ND
nifQ (blr1770)	Molybdenum processing protein	1.59	ND
fixC (blr1774)	Flavoprotein	1.93	1.64
Chaperone			
$groEL_3$ (bll2059)	GroEL3 chaperonin	3.25	ND
$groES_4$ (blr5625)	10-kDa chaperonin	6.53	5.87
$groEL_4$ (blr5626)	60-kDa chaperonin	6.83	5.39

Continued on following page

TABLE 3—*Continued*

Protein function and gene ID	Gene product	Fold change	
		C/H[b]	CA/H[c]
groES$_2$ (blr6978)	Chaperonin	4.31	ND
groES$_5$ (bsr7532)	10-kDa chaperonin (protein CPN10)	4.13	3.78
groEL$_5$ (blr7533)	60-kDa chaperonin	3.08	2.50
Cell envelope			
bll1204	Outer membrane immunogenic protein precursor	7.57	7.43
bll1766	Outer membrane protein	5.42	4.33
blr4700	Putative outer membrane immunogenic protein precursor	10.67	8.76
blr4701	Putative outer membrane immunogenic protein precursor	7.94	8.56
bll6888	Putative porin	7.52	9.13
bll7469	Putative outer membrane immunogenic protein precursor	6.92	7.21
Chemotaxis			
ctpI (bll1434)	Pilus assembly protein	1.74	1.69
ctpG (bll1436)	Pilus assembly protein	1.63	1.67
ctpE (bll1438)	Pilus assembly protein	2.73	2.72
ctpD (bll1439)	Pilus assembly protein	1.79	1.89
ctpC (bll1440)	Pilus assembly protein	2.45	2.71
ctpA (bsl1442)	Pilus assembly protein pilin subunit	6.56	8.04
cheA (blr2192)	Chemotaxis two-component hybrid sensor and regulator	2.24	2.43
cheW (blr2193)	Probable purine-binding chemotaxis protein	2.19	2.18
cheY (blr2194)	Chemotaxis two-component response regulator	4.28	4.52
cheY (blr2342)	Chemotaxis two-component response regulator	1.98	2.44
pilA (bsl6587)	Components of type IV pilus pilin subunit	5.48	6.16
ctpA (bsl7141)	Components of type IV pilus pilin subunit	6.60	7.68
bll6865	Flagellin	2.38	2.31
bll6866	Flagellin	2.62	2.68
fliE (bll5812)	Flagellar hook basal body complex protein	2.15	2.31
flgC (bll5813)	Flagellar basal body rod protein	2.04	2.28
flgD (bll5814)	Probable flagellar basal body rod protein	1.97	2.10
fliL (bll5826)	Flagellar synthesis protein	2.81	2.52
fliX (bll5837)	Probable flagellar assembly protein	1.56	ND
fliN (blr7002)	Flagellar motor switch protein	2.11	2.28

[a] Differentially expressed genes were selected based on a 1.5-fold cutoff.
[b] C/H, chemoautotrophy (C) and heterotrophy (H) comparisons.
[c] CA/H, a comparison of arabinose-supplemented chemoautotrophy (CA) and heterotrophy (H).
[d] ND, not determined.

tose phosphate cycle as the primary means of carbon assimilation during autotrophic growth (30). The *B. japonicum* genome contains a cluster of nine genes encoding the key components of the CBB cycle (bll2580 to blr2588) (27). Eight of the nine genes of this cluster are strongly upregulated during chemoautotrophic growth, as shown in Table 3. Included are two enzymes unique to this cycle (37, 44), RuBisCO and phosphoribulokinase, which are both strongly upregulated. The only gene of the cluster not upregulated is the divergently transcribed *cbbR*, encoding a LysR-type transcriptional regulator that is weakly constitutively expressed in another facultative autotroph, *Ralstonia eutropha* (44).

Carbon dioxide (or bicarbonate) is required not only for autotrophic growth but also heterotrophic growth of many bacteria (31). An enzymatic activity of importance to carbon dioxide metabolism is the interconversion of carbon dioxide and bicarbonate catalyzed by carbonate dehydratase/carbonic anhydrase. *B. japonicum* contains four putative carbonic anhydrases (bll1137, bll2065, bll4863, and bll4865) and one putative carbonate dehydratase (blr0500), of which two, bll1137 and bll4865, are induced under chemoautotrophic conditions (Table 3). The first, bll1137, is a putative α-type carbonic anhydrase with strong similarity (67% amino acid identity) to a characterized periplasmic α-type carbonic anhydrase designated *acaP* from *Rhodopseudomonas palustris* (38). *R. palustris acaP* is expressed in response to anaerobic conditions and functions to enhance the extracellular conversion of HCO_3^- to CO_2 at neutral pH, where spontaneous conversion is slow (38). The second gene, bll4865, is a putative β-type carbonic anhydrase with similarity (44% amino acid identity) to a β-type carbonic anhydrase of *Chlamydomonas reinhardtii*. The β-type carbonic anhydrase of *C. reinhardtii* is expressed in mitochondria of cells grown under low CO_2 and is believed to function in stabilization of the mitochondrial pH during photorespiration (14). Carbonic anhydrases also play a role in cyanobacteria by concentrating CO_2 in carboxysomes to enhance RuBisCO activity (4). The role of carbonic anhydrases in chemoautotrophically grown *B. japonicum* is not known but it will be interesting to determine if they play a role in enhancing RuBisCO activity or CO_2 incorporation.

Is isoctrate lyase essential for chemoautotrophic growth? Among the most highly upregulated genes found in chemoautotrophically cultured cells is a putative isocitrate lyase (blr2455), which is induced approximately 30-fold (Table 3). The putative *B. japonicum* isocitrate lyase has 75% amino acid identity to the characterized *aceA* gene of *Pseudomonas aerugi-*

nosa PAO1 (10). Isocitrate lyase and malate synthase together comprise the glyoxylate cycle of plants and bacteria. The glyoxylate cycle serves as a bypass of the tricarboxylic acid (TCA) cycle and is used during growth on hexose sugars and glycolytic intermediates when an anaplerotic function is required to regenerate four-carbon compounds of the TCA cycle lost for biosynthetic purposes (29). It is also utilized by many bacteria for growth on acetate and other two-carbon substrates when avoidance of the two decarboxylation reactions of the TCA cycle is warranted to prevent a futile cycle (22). In *B. japonicum*, isocitrate lyase activity is readily detected when cells are grown on carbon sources that are metabolized via acetyl coenzyme A, including acetate and poly-β-hydroxybutyrate (22). Less activity is detected when grown on galactose or pyruvate, and no activity is supported by arabinose, malate, or glycerol (22).

Based upon the strong induction of isocitrate lyase, it was hypothesized that the isocitrate lyase activity of the glyoxylate cycle in *B. japonicum* would play an important role in partitioning carbon flow between energy generation and biosynthetic processes when cultured chemoautotrophically. To further address the role of isocitrate lyase in chemoautotrophic growth of *B. japonicum*, a deletion mutant in *aceA* (the putative isocitrate lyase, blr2455) was constructed. As indicated above, isocitrate lyase activity is most readily detected in *B. japonicum* when acetate is provided as a carbon source. The mutant and wild type were cultured aerobically in minimal medium with acetate provided as the sole carbon source. Consistent with isocitrate lyase mutations in other organisms, acetate supported growth of the wild-type strain but not the mutant (10, 40).

The metabolism of the *aceA* mutant was further characterized by using Biolog GN2 microplates. Results of this analysis indicated that the mutant failed to utilize leucine, Tween 40, Tween 80, or propionic acid, whereas growth on these substrates was observed in the wild-type strain. It was previously reported that *aceA* is also involved in the assimilation of acetate and leucine in *P. aeruginosa* (10). Similar to acetate, all of these compounds are metabolized through acetyl coenzyme A and enter metabolism via the TCA cycle or the glyoxylate cycle. These results support the identification of blr2455 as isocitrate lyase (*aceA*).

Chemoautotrophic growth of the isocitrate lyase mutant and wild type were similar, and no differences in growth rate or final culture density were observed (data not shown), indicating a nonessential role for this gene in chemoautotrophy. Therefore, irrespective of the strong expression of isocitrate lyase under chemoautotrophic growth and contrary to the initial hypothesis, the data indicate that a functioning TCA cycle is sufficient to meet both the energetic and biosynthetic needs of the organism in the absence of an isocitrate lyase-mediated glyoxylate bypass.

The second enzyme of the glyoxylate cycle, malate synthase, was not identified as differentially expressed under chemoautotrophic conditions. However, Green et al. (22) reported malate synthase activity in arabinose-grown *B. japonicum*, indicating transcripts may be present under all our growth conditions and as such the activity was not detected as differentially expressed. Another enzyme serving an anaplerotic function is phosphoenolpyruvate (PEP) carboxylase, which produces oxaloacetate via carboxylation of phosphoenolpyruvate. Transcripts for PEP carboxylase (blr2955) were upregulated 1.6- to 1.7-fold under chemoautotrophic conditions. Therefore, our data suggest that *B. japonicum* cultured chemoautotrophically employs the anaplerotic reactions of the glyoxylate cycle and PEP carboxylase to generate four-carbon intermediates for biosynthetic processes, while simultaneously limiting the two decarboxylation reactions of the TCA cycle.

Nitrogen metabolism. *B. japonicum* was previously reported to utilize nitrate and ammonia as nitrogen sources during chemoautotrophic growth (33). However, N_2 as a sole nitrogen source would not support chemoautotrophic growth (33). Interestingly, when grown chemoautotrophically in the presence of ammonia, *B. japonicum* expresses the core components of the nitrogenase: *nifH*, *nifD*, and *nifK*, two ferrodoxins involved in nitrogen fixation, and *fixC*, and all were induced at levels 1.5- to 4.5-fold greater than under the heterotrophic condition (Table 3). *B. japonicum* nitrogenase expression in symbiotic bacteroids is primarily regulated by oxygen status, in contrast to free-living diazotrophs, in which oxygen and nitrogen status play a combined role (11, 17). We hypothesized that the unnecessary expression and synthesis of the nitrogenase complex under chemoautrophic conditions in the presence of adequate inorganic nitrogen would reduce fitness of the bacterium and as a result nitrogenase mutants would display enhanced chemoautotrophic growth. However, when tested for growth under chemoautotrophic conditions, the *B. japonicum nifH* and *nifD* mutants (23) grew the same as wild-type *B. japonicum* (data not shown), indicating there is no obvious fitness penalty associated with expression of the nitrogenase genes. Based upon the inability of *B. japonicum* to grow chemoautotrophically utilizing N as a sole nitrogen source, we concluded that the low-level expression of the nitrogenase components does not contribute significantly to nitrogen acquisition in chemoautotrophically grown cells but rather likely reflects a transcriptional artifact of growth under microaerobic conditions.

Chaperones. Molecular chaperones are required for proper folding and assembly of proteins and higher-order protein structures, often in response to environmental stresses. Fifteen chaperones were identified as upregulated and six downregulated in the two chemoautotrophic conditions, respectively (see Tables S1 and S2 in the supplemental material). Included are $GroEL_3$, $GroES_4$, $GroEL_4$, $GroES_5$, and $GroEL_5$ (Table 3). Nitrogenase activity in *B. japonicum* bacteroids requires either $GroEL_3$ or $GroEL_4$ for full activity (19). $GroEL_3$ was identified as a member of the NifA regulon and is expressed in bacteroids and anaerobic cultures (18, 25). *groEL*$_4$ was previously reported to be expressed in aerobic and anaerobic cultures at similar levels and to a lesser extent in bacteroids (18). Moreover, our studies indicate that *groEL*$_4$ was upregulated six- to sevenfold in chemoautotrophic cultures (Table 3). *groEL*$_5$ was also reported to be expressed at low levels in anaerobic cultures (18), and we observed three- to fourfold-higher expression in chemoautotrophic cultures relative to heterotrophically grown cells. The apparent change in chaperone transcript profiles suggests that the microaerobic environment required for induction of chemoautotrophic growth also leads to expression of a different subset of chaperones that are optimized to function under low oxygen tension.

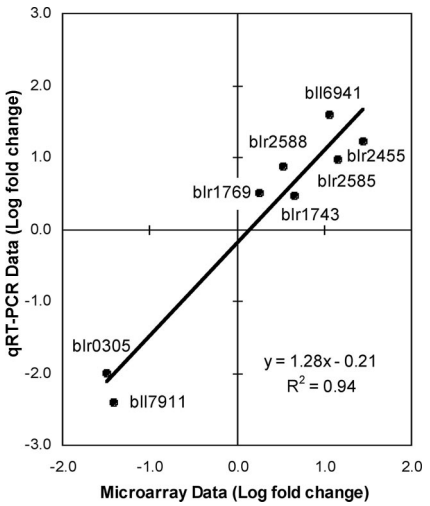

FIG. 2. Comparison of log-transformed qRT-PCR data and microarray data of eight representative genes selected from Tables S1 to S4 in the supplemental material.

Quantitative RT-PCR. To validate and provide an indication of the sensitivity of the microarray data, we performed qRT-PCR for eight genes listed in Table 2. The genes were selected to represent the range of induction levels obtained for genes up- or downregulated in chemoautotrophically grown cells. The qRT-PCR analysis was performed on two biological replicates of chemoautotrophic and heterotrophic cells that were generated independently of those used in the microarray analysis. The comparison of qRT-PCR data to that obtained from the microarray analysis is presented in Fig. 2. The results demonstrate good agreement of expression values ($R^2 = 0.94$) between the two methodologies with different RNA samples used for each approach, indicating that the transcriptional profiles are accurate and reproducible.

Conclusions. The presented transcriptomic data highlight the major physiological and metabolic changes of *B. japonicum* required for chemoautotrophic growth utilizing molecular hydrogen and carbon dioxide. The primary changes in transcript profiles reflect different means of energy generation and the need to assimilate carbon through the CBB cycle. While central carbon metabolism of the cells remains unchanged at a transcriptional level, there is a bias toward anaplerotic functions, as is evident by the observed induction of the glyoxylate cycle. Chemoautotrophically grown cultures supplemented with arabinose did not utilize a mixotrophic mode of growth based on transcript profiles. However, utilization of arabinose in these cultures cannot be determined solely based upon transcript data alone. Based upon previously published results indicating a requirement of low oxygen levels for induction of the uptake hydrogenase and enzymes of the CBB cycle, as well as our observation of induction of nitrogenase, it appears that the oxygen status of the cell is a key signal for the transition to a chemoautotrophic mode of growth. It is of interest that oxygen is also a primary signal for transition to the nitrogen-fixing bacteroid state, highlighting its importance in environmental adaptations of *B. japonicum*. The transcriptomic data presented here provide a foundation for future work in studying the physiology and genetic regulation underlying facultative chemoautotrophy in *B. japonicum*.

ACKNOWLEDGMENTS

We thank Hans-Martin Fischer for providing the *B. japonicum nifH* and *nifD* mutant strains.

This research was supported by grant 2004-35064-14708 from the USDA/CSREES/NRI.

REFERENCES

1. **Bittner, M., R. Butow, J. DeRisi, M. Diehn, J. Eberwine, C. B. Epstein, R. Glynne, S. Grimmond, T. Ideker, J. E. Kacharmina, S. Katsabanis, J. Khan, J. Lee, C. L. Liu, P. Marciano, F. M. Marincola, T. McIntosh, D. Monte, J. R. Pollack, V. Rhodius, S. Somerville, E. Tom, E. Wang, J. S. Wei, D. Willhite, and S. Ybarra.** 2003. Expression analysis of RNA, p. 101–288. *In* D. Bowtell and J. Sambrook (ed.), DNA microarrays: a molecular cloning manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
2. **Black, L. K., and R. J. Maier.** 1995. IHF- and RpoN-dependent regulation of hydrogenase expression in *Bradyrhizobium japonicum*. Mol. Microbiol. **16:**405–413.
3. **Brito, B., M. Martinez, D. Fernandez, L. Rey, E. Cabrera, J. M. Palacios, J. Imperial, and T. Ruiz-Argueso.** 1997. Hydrogenase genes from *Rhizobium leguminosarum* bv. viciae are controlled by the nitrogen fixation regulatory protein NifA. Proc. Natl. Acad. Sci. USA **94:**6019–6024.
4. **Cannon, G. C., C. E. Bradburne, H. C. Aldrich, S. H. Baker, S. Heinhorst, and J. M. Shively.** 2001. Microcompartments in prokaryotes: carboxysomes and related polyhedra. Appl. Environ. Microbiol. **67:**5351–5361.
5. **Chang, W.-S., W. L. Franck, E. Cytryn, S. Jeong, T. Joshi, D. W. Emerich, M. J. Sadowsky, D. Xu, and G. Stacey.** 2007. An oligonucleotide microarray resource for transcriptional profiling of *Bradyrhizobium japonicum*. Mol. Plant-Microbe Interact. **20:**1298–1307.
6. **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.
7. **Cui, X., M. K. Kerr, and G. A. Churchill.** 2003. Transformations for cDNA microarray data. Stat. Appl. Genet. Mol. Biol. **2:**4.
8. **Cytryn, E. J., D. P. Sangurdekar, J. G. Streeter, W. L. Franck, W. S. Chang, G. Stacey, D. W. Emerich, T. Joshi, D. Xu, and M. J. Sadowsky.** 2007. Transcriptional and physiological responses of *Bradyrhizobium japonicum* to desiccation-induced stress. J. Bacteriol. **189:**6751–6762.
9. **Delgado, M. J., N. Bonnard, A. Tresierra-Ayala, E. J. Bedmar, and P. Muller.** 2003. The *Bradyrhizobium japonicum napEDABC* genes encoding the periplasmic nitrate reductase are essential for nitrate respiration. Microbiology **149:**3395–3403.
10. **Diaz-Perez, A. L., C. Roman-Doval, C. Diaz-Perez, C. Cervantes, C. R. Sosa-Aguirre, J. E. Lopez-Meza, and J. Campos-Garcia.** 2007. Identification of the aceA gene encoding isocitrate lyase required for the growth of Pseudomonas aeruginosa on acetate, acyclic terpenes and leucine. FEMS Microbiol. Lett. **269:**309–316.
11. **Dixon, R., and D. Kahn.** 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. **2:**621–631.
12. **Durmowicz, M. C., and R. J. Maier.** 1998. The $FixK_2$ protein is involved in regulation of symbiotic hydrogenase expression in *Bradyrhizobium japonicum*. J. Bacteriol. **180:**3253–3256.
13. **Durmowicz, M. C., and R. J. Maier.** 1997. Roles of HoxX and HoxA in biosynthesis of hydrogenase in *Bradyrhizobium japonicum*. J. Bacteriol. **179:**3676–3682.
14. **Eriksson, M., J. Karlsson, Z. Ramazanov, P. Gardestrom, and G. Samuelsson.** 1996. Discovery of an algal mitochondrial carbonic anhydrase: molecular cloning and characterization of a low-CO_2-induced polypeptide in *Chlamydomonas reinhardtii*. Proc. Natl. Acad. Sci. USA **93:**12031–12034.
15. **Evans, H. J., A. R. Harker, H. Papen, S. A. Russell, F. J. Hanus, and M. Zuber.** 1987. Physiology, biochemistry, and genetics of the uptake hydrogenase in rhizobia. Annu. Rev. Microbiol. **41:**335–361.
16. **Fellay, R., J. Frey, and H. Krisch.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of gram-negative bacteria. Gene **52:**147–154.
17. **Fischer, H. M.** 1994. Genetic regulation of nitrogen fixation in rhizobia. Microbiol. Rev. **58:**352–386.
18. **Fischer, H. M., M. Babst, T. Kaspar, G. Acuna, F. Arigoni, and H. Hennecke.** 1993. One member of a gro-ESL-like chaperonin multigene family in *Bradyrhizobium japonicum* is co-regulated with symbiotic nitrogen fixation genes. EMBO J. **12:**2901–2912.
19. **Fischer, H. M., K. Schneider, M. Babst, and H. Hennecke.** 1999. GroEL chaperonins are required for the formation of a functional nitrogenase in *Bradyrhizobium japonicum*. Arch. Microbiol. **171:**279–289.
20. **Fu, C., and R. J. Maier.** 1994. Sequence and characterization of three genes within the hydrogenase gene cluster of *Bradyrhizobium japonicum*. Gene **141:**47–52.

21. **Gabel, C., and R. J. Maier.** 1993. Oxygen-dependent transcriptional regulation of cytochrome *aa3* in *Bradyrhizobium japonicum*. J. Bacteriol. **175:**128–132.
22. **Green, L. S., D. B. Karr, and D. W. Emerich.** 1998. Isocitrate dehydrogenase and glyoxylate cycle enzyme activities in *Bradyrhizobium japonicum* under various growth conditions. Arch. Microbiol. **169:**445–451.
23. **Hahn, M., L. Meyer, D. Studer, B. Regensburger, and H. Hennecke.** 1984. Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. Plant Mol. Biol. **3:**159–168.
24. **Hanus, F. J., R. J. Maier, and H. J. Evans.** 1979. Autotrophic growth of H_2-uptake-positive strains of *Rhizobium japonicum* in an atmosphere supplied with hydrogen gas. Proc. Natl. Acad. Sci. USA **76:**1788–1792.
25. **Hauser, F., G. Pessi, M. Friberg, C. Weber, N. Rusca, A. Lindemann, H. M. Fischer, and H. Hennecke.** 2007. Dissection of the *Bradyrhizobium japonicum* $NifA^+$ σ^{54} regulon, and identification of a ferredoxin gene (*fdxN*) for symbiotic nitrogen fixation. Mol. Genet. Genomics **278:**255–271.
26. **Hom, S. S., P. D. Novak, and R. J. Maier.** 1988. Transposon Tn*5*-generated *Bradyrhizobium japonicum* mutants unable to grow chemoautotrophically with H_2. Appl. Environ. Microbiol. **54:**358–363.
27. **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.
28. **Kim, H., and R. J. Maier.** 1990. Transcriptional regulation of hydrogenase synthesis by nickel in *Bradyrhizobium japonicum*. J. Biol. Chem. **265:**18729–18732.
29. **Kornberg, H. L.** 1966. The role and control of the glyoxylate cycle in *Escherichia coli*. Biochem. J. **99:**1–11.
30. **Kusian, B., and B. Bowien.** 1997. Organization and regulation of *cbb* CO_2 assimilation genes in autotrophic bacteria. FEMS Microbiol. Rev. **21:**135–155.
31. **Kusian, B., D. Sultemeyer, and B. Bowien.** 2002. Carbonic anhydrase is essential for growth of *Ralstonia eutropha* at ambient CO_2 concentrations. J. Bacteriol. **184:**5018–5026.
32. **Leong, S. A., G. S. Ditta, and D. R. Helinski.** 1982. Heme biosynthesis in *Rhizobium*. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from *Rhizobium meliloti*. J. Biol. Chem. **257:**8724–8730.
33. **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.
34. **Maier, R. J., and E. W. Triplett.** 1996. Toward more productive, efficient, and competitive nitrogen-fixing symbiotic bacteria. Crit. Rev. Plant Sci. **15:**191–234.
35. **Ormerod, J. G., K. S. Ormerod, and H. Gest.** 1961. Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. Arch. Biochem. Biophys. **94:**449–463.
36. **Palacios, J. M., J. Murillo, A. Leyva, G. Ditta, and T. Ruiz-Argueso.** 1990. Differential expression of hydrogen uptake (*hup*) genes in vegetative and symbiotic cells of *Rhizobium leguminosarum*. Mol. Gen. Genet. **221:**363–370.
37. **Pereto, J. G., A. M. Velasco, A. Becerra, and A. Lazcano.** 1999. Comparative biochemistry of CO_2 fixation and the evolution of autotrophy. Int. Microbiol. **2:**3–10.
38. **Puskas, L. G., M. Inui, K. Zahn, and H. Yukawa.** 2000. A periplasmic, alpha-type carbonic anhydrase from *Rhodopseudomonas palustris* is essential for bicarbonate uptake. Microbiology **146:**2957–2966.
39. **Quevillon, E., V. Silventoinen, S. Pillai, N. Harte, N. Mulder, R. Apweiler, and R. Lopez.** 2005. InterProScan: protein domains identifier. Nucleic Acids Res. **33:**W116–W120.
40. **Reinscheid, D. J., B. J. Eikmanns, and H. Sahm.** 1994. Characterization of the isocitrate lyase gene from *Corynebacterium glutamicum* and biochemical analysis of the enzyme. J. Bacteriol. **176:**3474–3483.
41. **Rudolph, G., G. Semini, F. Hauser, A. Lindemann, M. Friberg, H. Hennecke, and H. M. Fischer.** 2006. The iron control element, acting in positive and negative control of iron-regulated *Bradyrhizobium japonicum* genes, is a target for the Irr protein. J. Bacteriol. **188:**733–744.
42. **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.
43. **Sayavedra-Soto, L. A., G. K. Powell, H. J. Evans, and R. O. Morris.** 1988. Nucleotide sequence of the genetic loci encoding subunits of *Bradyrhizobium japonicum* uptake hydrogenase. Proc. Natl. Acad. Sci. USA **85:**8395–8399.
44. **Shively, J. M., G. van Keulen, and W. G. Meijer.** 1998. Something from almost nothing: carbon dioxide fixation in chemoautotrophs. Annu. Rev. Microbiol. **52:**191–230.
45. **Storey, J. D., and R. Tibshirani.** 2003. Statistical significance for genomewide studies. Proc. Natl. Acad. Sci. USA **100:**9440–9445.
46. **Surpin, M. A., and R. J. Maier.** 1998. Roles of the *Bradyrhizobium japonicum* terminal oxidase complexes in microaerobic H_2-dependent growth. Biochim. Biophys. Acta **1364:**37–45.
47. **van Berkum, P.** 1987. Expression of uptake hydrogenase and hydrogen oxidation during heterotrophic growth of *Bradyrhizobium japonicum*. J. Bacteriol. **169:**4565–4569.
48. **van Berkum, P., and H. H. Keyser.** 1985. Anaerobic growth and denitrification among different serogroups of soybean rhizobia. Appl. Environ. Microbiol. **49:**772–777.
49. **Weaver, K. E., and F. R. Tabita.** 1983. Isolation and partial characterization of *Rhodopseudomonas sphaeroides* mutants defective in the regulation of ribulose bisphosphate carboxylase/oxygenase. J. Bacteriol. **156:**507–515.
50. **Windgassen, M., A. Urban, and K. E. Jaeger.** 2000. Rapid gene inactivation in *Pseudomonas aeruginosa*. FEMS Microbiol. Lett. **193:**201–205.
51. **Yang, J., I. Sangwan, A. Lindemann, F. Hauser, H. Hennecke, H. M. Fischer, and M. R. O'Brian.** 2006. *Bradyrhizobium japonicum* senses iron through the status of haem to regulate iron homeostasis and metabolism. Mol. Microbiol. **60:**427–437.