Genome-Wide Transcript Analysis of *Bradyrhizobium japonicum* Bacteroids in Soybean Root Nodules

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The transcriptome of endosymbiotic *Bradyrhizobium japonicum* bacteroids was assessed, using RNA extracted from determinate soybean root nodules. Results were compared with the transcript profiles of *B. japonicum* cells grown in either aerobic or microaerobic culture. Microoxia is a known trigger for the induction of symbiotically relevant genes. In fact, one third of the genes induced in bacteroids at day 21 after inoculation are congruent with those up-regulated in culture by a decreased oxygen concentration. The other induced genes, however, may be regulated by cues other than oxygen limitation. Both groups of genes provide a rich source for the possible discovery of novel functions related to symbiosis. Samples taken at different timepoints in nodule development have led to the distinction of genes expressed early and late in bacteroids. The experimental approach applied here is also useful for *B. japonicum* mutant analyses. As an example, we compared the transcriptome of wild-type bacteroids with that of bacteroids formed by a mutant defective in the RNA polymerase transcription factor σ^{54} . This led to a collection of hitherto unrecognized *B. japonicum* genes potentially transcribed in planta in a σ^{54} -dependent manner.

Additional keywords: GeneChip, nitrogen fixation, RpoN.

Rhizobia have two different life-styles, either as free-living soil bacteria or as nitrogen-fixing endosymbionts (also called bacteroids) within root nodules of legume host plants. In a well-balanced physiological interaction, the microsymbiont fixes atmospheric nitrogen and provides ammonia as a nitrogen source to the plant in exchange for a carbon and energy source generated by photosynthesis (Long 2001; Prell and Poole 2006). Nitrogen-fixing bacteroids heavily depend on C4-dicarboxylic acids, which are further respired through the tricarboxylic acid cycle (Lodwig and Poole 2003; Poole and Allaway 2000; Yurgel and Kahn 2004).

Nodule development is accompanied by a dramatic decline of the free oxygen level in the infected nodule parenchyma, which is a prerequisite for the activity of the oxygen-labile nitrogenase in bacteroids. At first sight, an oxygen-limiting condition appears to oppose the bacteroids' need for ATP production by oxidative phosphorylation in order to fuel the energy-demanding nitrogen fixation process (Dixon and Kahn 2004; Downie 2005). Bacteroids solve this dilemma, however, by inducing a symbiosis-specific *cbb₃*-type cytochrome oxidase having a very high affinity for oxygen (Preisig et al. 1996) and by coordinating the expression of genes for respiration and nitrogen fixation via sophisticated genetic regulatory networks. In fact, a decreased oxygen tension induces the expression of many rhizobial genes and is believed to be an important signal for their differentiation into bacteroids (Batut and Boistard 1994; Fischer 1994).

The aforementioned are only a few of the massive physiological changes that occur when free-living rhizobia convert to bacteroids. The availability of several complete rhizobial genome sequences (RhizoBase and RhizoDB websites) now provides an opportunity to study the genetic basis underlying physiological adaptations at a genome-wide scale. Such global views on rhizobial gene expression in symbiosis have been reported for the *Sinorhizobium meliloti*–*Medicago sativa* symbiosis (Barnett et al. 2004; Becker et al. 2004; Capela et al. 2006), which is characterized by indeterminate type of nodules (Gage 2004). Much less data are available for the rhizobial gene expression profile in the so-called determinate nodules (Gage 2004), as in the case of the *Mesorhizobium loti*–*Lotus japonicus* symbiosis (Uchiumi et al. 2004). In the present study, we report on the first global transcription profiling of *B. japonicum* from determinate soybean nodules. Based on the genome sequence of *B. japonicum* 110 (9.1 Mb) (Kaneko et al. 2002), a custom-made Affymetrix GeneChip has been designed representing 8,266 annotated *B. japonicum* genes plus intergenic regions (Hauser et al. 2007). Using this array, we monitored global *B. japonicum* transcript levels under free-living aerobic and microaerobic conditions and in symbiosis. It was of interest not only to arrive at a comprehensive list of genes specifically expressed in symbiosis but also to compare the results with the protein expression pattern of *B. japonicum* isolated from nodules, which has recently been reported (Sarma and Emerich 2005, 2006). Moreover, we assessed how the transcription profile of the *B. japonicum*–*Glycine max* symbiosis compares with that of the two other symbioses so far studied (*S. meliloti*–*M. sativa*, *M. loti*–*L. japonicus*).

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The microarray data is available in the National Center for Biotechnology Information Gene Expression Omnibus database and are accessible through GEO Series accession number GSE8478.

*The e-Xtra logo stands for “electronic extra” and indicates additional information is available online. Seven supplemental tables and one supplemental figure are published online.

RESULTS AND DISCUSSION

Table 1 presents a survey of all microarray-based transcriptome analyses performed in this study, involving two different *B. japonicum* strains (wild type and *rpoN₁₊₂* mutant), three different physiologic conditions (aerobic, microaerobic, and symbiotic growth), and four different timepoints in nodule and bacteroid development (10, 13, 21, and 31 days postinoculation [dpi]). Great care was taken to avoid false results by applying rigorous criteria for normalization procedures and statistical analyses based on numerous biological replicates (discussed below). Furthermore, our previous validations of microarray-based data with reporter gene fusion, quantitative reverse transcription-polymerase chain reaction, and primer-extension techniques have shown that gene expression data, once they have passed these restrictive filters, are trustworthy with a high probability (Hauser et al. 2006; Rudolph et al. 2006; Yang et al. 2006a and b).

Precaution was particularly necessary in comparisons between free-living cells grown in synthetic medium and bacteroids within the highly complex environmental conditions of a nodule (Table 1). Therefore, stringency for nodule data was further tightened by the following means: i) although all *B. japonicum* probe sets on the GeneChip had been pruned against soybean expressed sequence tags (EST), we carried out hybridizations with cDNA derived from nonnodulated soybean roots as control, and only those bacteroid genes were considered as being expressed whose signal value was at least twice as high as the value obtained after hybridization with root cDNA; and ii) genes that were not called “present”, i.e., found to be expressed in at least 80% of the replicates, were excluded. While such stringent criteria helped minimize false positives, the likely loss of several differentially expressed genes with moderate fold-change values had to be acquiesced.

Before taking a global view on genes expressed in symbiosis, two reference data sets were established that list all genes expressed in *B. japonicum* cells grown in peptone salts–yeast extract (PSY) medium under either aerobic or microaerobic conditions. Of the 8,266 genes represented on the array, a total of 5,439 genes in aerobic and 5,568 genes in microaerobic cultures were found to be expressed (Table 1; Fig. 1). A total of 1,007 genes responded to microoxia with either an increased or a decreased gene expression activity (620 vs. 387 genes, respectively; Table 1). These 1,007 genes are compiled in Supplemental Table 1; they are of potential relevance for symbiosis because microoxia is known to mimic, at least partly, the environmental conditions bacteroids face in root nodules. In fact, we encountered here many of the already known target genes controlled by members of the low-oxygen responsive

regulatory cascades FixLJ-FixK₂ (Nellen-Anthamatten et al. 1998) and RegSR-NifA (Bauer et al. 1998; Hauser et al. 2007), such as *fixNOQP*, *fixGHIS*, *napEDABC*, *nnrR*, *nirK*, *rpoN₁*, and *nifDKEN*, *nifH*, *frxA*, *groESL₃*, respectively.

Global transcript expression pattern in soybean bacteroids as compared with culture-grown *B. japonicum*.

A much lower number of genes (2,780) were detected as being expressed in soybean bacteroids as compared with cells grown in aerobic culture (Table 1; Fig. 1). This suggests that many genes expressed during growth in synthetic medium may not be needed in symbiosis. Out of these 2,780 genes, a remarkably high number (2,369) are expressed both in symbiosis and in aerobic culture. This leaves 411 genes whose expression appears to occur solely in bacteroids (Fig. 1) (Supplemental Table 2).

To identify genes that are differentially expressed in symbiosis by more than a factor of either ± 2 , we performed a

Fig. 1. Venn diagram of genes considered to be “present” (i.e., expressed) in bacteroids and in aerobic cultures. While 5,439 genes were expressed in aerobiosis, only 2,780 are expressed in bacteroids and 411 are expressed solely in bacteroids. Only genes that were present in 80% of all replicates were considered.

Table 1. Survey of transcriptome analyses performed in this study

Experimental comparison ^a	Genes “present” ^b	Differential ^c	Upregulated ^d	Downregulated ^e
WT free-living aerobic	5,439	...	...	...
WT free-living microaerobic	5,568	...	...	...
WT bacteroids (21 dpi)	2,780	...	...	...
WT free-living microaerobic vs. aerobic	...	1,007	620	387
WT bacteroids (21 dpi) vs. free-living aerobic	...	2,778	692	2,086
WT bacteroids 13 dpi vs. 10, 21, and 31 dpi	...	...	59	nd
WT bacteroids 10 and 13 dpi vs. 21 and 31 dpi	...	...	57	75
<i>rpoN₁₊₂</i> vs. WT, both free-living aerobic	...	241	187	54
<i>rpoN₁₊₂</i> vs. WT, both free-living microaerobic	...	341	136	205
<i>rpoN₁₊₂</i> bacteroids vs. WT bacteroids (both at 21 dpi)	...	789	339	450

^a WT = wild type; dpi = days postinoculation.

^b Number of expressed genes detected in 80% of the replicates on the array (detection *P*-value ≤ 0.06).

^c Total number of differentially expressed genes (up- and downregulated) using genes called “present” in bacteroids or in aerobiosis as gene list.

^d Number of genes with increased expression in the first condition.

^e Number of genes with decreased expression in the first condition; nd = not determined.

gene-by-gene comparison of the expression profiles in aerobically grown cells and in bacteroids from nodules (21 dpi). 2,778 genes showed a differential expression (Supplemental Table 3), with 692 genes being induced in bacteroids and 2,086 being down-regulated (Table 1). These 2,778 genes were classified according to the 15 categories issued by the Kazusa DNA Research Institute (Kaneko et al. 2002) (Fig. 2). Within the class of 692 bacteroid-induced genes, there was a statistically significant overrepresentation of genes belonging to two of the 15 categories (Fig. 2, on the right), whereas we found an overrepresentation of genes belonging to six of the 15 categories (Fig. 2, on the left) among the 2,086 downregulated genes. This latter observation might reflect the resting stage of mature bacteroids in which a global deceleration of housekeeping functions occurs in favor of symbiosis-specific processes. In fact, decreased expression was observed for genes that are normally active in exponentially growing cells, such as cell-division and motility genes, genes involved in translation (aminoacyl-tRNA synthetases, ribosomal proteins, chain elongation, and peptide release factors), and certain sugar transporters.

Among the 692 genes induced in bacteroids, there are 102 genes with fold-change values >30. Their fold-change values were compared with those observed after microaerobic induction in culture (Table 2). While readers may browse through the list and draw their own conclusions, we offer a few comments here. i) The previously known *nif* and *fix* genes gather in the overrepresented category “central intermediary metabolism,” which nicely confirms the utility of the transcriptomics ap-

proach. ii) From the genes belonging to the overrepresented category “transport and binding proteins,” the *modAB* genes (blr6951 to blr6952) and a *modB* homolog (blr1719) encode putative molybdate transporters. It seems plausible that bacteroids induce molybdate uptake systems to meet the molybdenum demand for nitrogenase biosynthesis. The importance of efficient molybdate uptake systems for symbiotic nitrogen fixation in *B. japonicum* has already been pointed out previously (Delgado et al. 2006; Maier et al. 1987). iii) Several transcriptional regulators (“regulatory functions” category) showed increased expression in bacteroids (Table 3), and the majority of them (about 75%) have, so far, not been implicated or studied in the context of symbiosis. High induction factors are generally not typical for regulatory genes. It comes as a surprise, therefore, that a new LuxR family member (encoded by blr1880) shows a fold-change value of 74.8 (Tables 2 and 3). Also highly expressed are two nodulation genes involved in repression (*nolA*, *nodD2*), which is consistent with an overall negative control of nodulation (*nod*) genes in bacteroids (Loh and Stacey 2003). iv) In the so-called “other categories,” we found genes involved in oxidative stress response (e.g., the *ahpCD*-encoded alkylhydroperoxide reductase subunits blr1776 and blr1777; Table 2). This observation nurtures the debate whether or not endosymbiotic rhizobia are oxygen-stressed despite living in conditions of microoxia. Yet, the high cytochrome content in bacteroids and the high concentration of oxygenated leghemoglobin in the infected nodule cells are factors that may contribute to the formation of reactive oxygen species (Pauly et al. 2006).

Fig. 2. Differentially expressed genes categorized by functional classification according to the Kazusa annotation (RhizoBase website). Percentages of induced and repressed genes are given for the comparison of wild-type bacteroids (21 dpi) vs. wild-type cells grown aerobically. Percentages are calculated by dividing the number of significantly induced or repressed genes in each category by the total number of retained genes in the corresponding category. Asterisks indicate statistical significance for overrepresented genes in a given category (P -value ≤ 0.01).

Since a very low oxygen concentration is a signal for activation of several symbiotic genes in *B. japonicum* (Sciotti et al. 2003), we compiled all genes that are induced both in microoxia (discussed above) and in symbiosis (Supplemental Table 4). This criterion applies to 233 (34%) of the 692 genes induced in symbiosis, corroborating again the importance of the low-oxygen signal as a trigger of gene expression in bacteroids. One example, a succinate-semialdehyde dehydrogenase-encoding gene (blr3998), which belongs to the functional category “energy metabolism,” is singled out here. This enzyme bypasses the 2-oxoglutarate dehydrogenase reaction in the trichloroacetic acid (TCA) cycle and has been proposed to enhance TCA-cycle function during symbiotic nitrogen fixation (Green et al. 2000). The other 66% of the genes in-

duced in bacteroids, not overlapping with the microaerobic stimulon, suggest the existence of signals other than oxygen limitation for gene activation. Interestingly, this group of genes contains *pmtA*, the gene for phosphatidylethanolamine (PE) *N*-methyltransferase, which converts PE into phosphatidylcholine and has been shown to be important for an efficient symbiotic interaction of *B. japonicum* with its host plant (Minder et al. 2001).

The chromosomal distribution of genes that are up-regulated in bacteroids was evaluated (Fig. 3; discussed below). The data showed that 27% of the bacteroid-induced genes are contained within the 410-kb “symbiotic region” previously defined by Göttfert and associates (2001) and partially overlap with the extended 681-kb “symbiotic island” proposed by Kaneko and

Table 2. The 102 most highly induced genes (fold change >30) in wild-type bacteroids when aerobically grown wild-type cells were used as reference

Gene number ^a	Description ^b	Gene name ^c	Fold change	
			Bacteroids vs. aerobic ^d	Microaerobic vs. aerobic ^e
blr2106	L-ectoine synthase	<i>ectC</i>	1201.0	19.7
blr2143	Similar to cytochrome P450-family protein	—	1008.0	—
bsr2010	—	—	358.7	4.7
blr8234	—	—	316.8	—
blr2131	Probable oxygenase	—	298.2	15.9
blr2146	Dehydrogenase	—	243.3	—
blI1767	—	—	242.0	3.8
bsI1637	—	—	215.9	—
blI2047	—	—	208.7	—
blI1858	—	—	207.9	—
blr2011	—	—	203.5	—
blr3719	—	—	182.4	—
blr1850	—	—	177.7	4.0
blr2071	Similar to inosamine-phosphate amidinotransferase	—	170.8	4.5
blI1777	Alkyl hydroperoxide reductase	<i>ahpC</i>	169.8	7.8
blI2065	Carbonic anhydrase	<i>icfA</i>	156.5	—
blr1638	—	—	155.0	—
blr2132	—	—	147.7	7.1
blI2004	—	—	146.8	—
blr1964	Putative sugar hydrolase	—	145.0	—
blr1755	<i>Rhizobium etli</i> <i>iscN</i> homolog	—	138.7	7.0
blr1726	—	—	137.6	—
blr6951	Molybdenum ABC transporter molybdate-binding protein	<i>modA</i>	128.0	18.7
blI1981	—	—	125.8	—
bsr1758	—	—	125.5	—
blr1765	Ferredoxin	<i>fer2</i>	124.9	—
blI1754	—	—	120.9	5.5
blI1906	<i>N</i> -acetyltransferase homolog NrgA	<i>nrgA</i>	120.0	8.3
blr1719	Molybdenum transport system permease protein	<i>modB</i>	119.5	—
blI1872	—	—	115.4	4.2
blr1743	Nitrogenase molybdenum-iron protein alpha chain	<i>nifD</i>	107.3	13.8
blr2144	Cytochrome P-450 B-1	<i>CYP112</i>	107.0	7.6
blr1744	Nitrogenase molybdenum-iron protein beta chain	<i>nifK</i>	106.1	12.4
bsr1757	Nitrogen fixation protein	<i>fixU</i>	105.4	4.8
bsr1760	Ferredoxin-like protein	<i>frxA</i>	105.3	4.0
blI2063	Phenolhydroxylase homolog	<i>nrgC</i>	105.0	4.4
blr1769	Dinitrogenase reductase protein	<i>nifH</i>	96.2	7.0
blr1771	Nitrogenase stabilizing/protective protein	<i>nifW</i>	95.2	2.2
blI1944	—	—	89.7	3.8
blI1875	—	—	85.7	—
blr1770	Molybdenum processing protein	<i>nifQ</i>	85.0	3.2
blr1853	Cytochrome P450 family protein	—	84.6	—
blI2060	GroES3 chaperonin	<i>groES</i>	83.0	4.4
blI2066	—	—	81.4	—
blr1756	Nitrogenase metaloclusters biosynthesis protein	<i>nifS</i>	78.7	4.1
blI1776	Alkyl hydroperoxide reductase	<i>ahpD</i>	78.6	4.1
bsr1739	Ferredoxin	—	76.0	8.8
blI2125	Probable dioxygenase	—	75.0	—

Continued on following page

^a Nomenclature according to Kaneko and associates (2002).

^b Gene description according to Kaneko and associates (2002). — indicates unknown or hypothetical proteins.

^c Gene name according to the EMBL-EBI database.

^d Comparing bacteroids and aerobically grown cells.

^e Comparing microaerobically and aerobically grown cells; — indicates the gene was not differentially expressed.

associates (2002). Two other regions also had an above-average content of genes that are up-regulated in symbiosis (Fig. 3). Region I carries genes for several monooxygenases (blr3677 through blr3679), a dicarboxylate transporter DctA (blr3723), and the poly- β -hydroxybutyrate biosynthetic enzymes (blr3724 and blr3725); region II encodes the hydrogen uptake system (bll6927 to bll6949), the molybdate transport complex (blr6951 through blr6953), and the periplasmic nitrate reductase (bsr7036 through blr7040). The accumulation of symbiotically relevant genes in the “symbiotic island” is correlated with a significantly lower G+C content, whereas the correlation between a lower G+C content and the high density of up-regulated genes is less compelling in regions I and II (Fig. 3).

Genes expressed early and late in bacteroid development.

The in-nodule gene expression inventory reported so far concerned bacteroids at 21 dpi, a timepoint when maximal nitrogen-fixation activity had been routinely observed under the plant-cultivation conditions used in our laboratory. In order to possibly identify genes expressed early and late in bacteroid development, we analyzed the global transcription profile in a nodulation timecourse experiment. Soybean nodules were harvested at 10, 13, 21, and 31 dpi, having relative symbiotic nitrogen fixation activities of 9, 95, 100, and 61%, respectively, and RNA was extracted from all samples for microarray analysis. A clustering analysis (Fig. 4) led to the following classification: i) a cluster of genes maximally expressed at 10 and 13 dpi, ii) genes maximally induced at 13 dpi, and iii) a cluster of

Table 2. Continued from preceding page

Gene number ^a	Description ^b	Gene name ^c	Fold change	
			Bacteroids vs. aerobic ^d	Microaerobic vs. aerobic ^e
blr1880	Transcriptional regulatory protein LuxR family	—	74.8	—
blr1851	—	—	74.2	—
blr1759	FeMo cofactor biosynthesis protein	<i>nifB</i>	74.1	2.9
blr1879	—	—	72.0	—
bll3193	—	—	65.1	—
blr1745	Nitrogenase molybdenum-cofactor synthesis protein	<i>nifE</i>	62.9	4.7
bll2009	—	—	61.1	—
blr6952	Molybdenum ABC transporter permease protein	<i>modB</i>	59.7	6.0
blr2133	—	—	58.7	5.3
bll6634	Probable phosphatidylethanolamine <i>N</i> -methyltransferase	<i>pmtA</i>	58.1	—
blr3677	Putative monooxygenase component	—	56.9	—
blr2145	Cytochrome P-450 BJ-3	<i>CYP114</i>	55.0	3.3
blr3675	Putative alcohol dehydrogenase	—	54.4	—
blr1901	—	—	52.2	—
bsl1857	—	—	50.6	—
blr1686	Putative aminotransferase protein	—	48.2	—
blr2134	—	—	47.9	3.3
blr1746	Nitrogenase molybdenum-cofactor synthesis protein	<i>nifN</i>	47.8	2.9
blr1773	Electron transfer flavoprotein alpha chain	<i>fixB</i>	47.6	2.2
bll2059	GroEL3 chaperonin	<i>groEL</i>	47.4	2.9
bll2067	Nodule formation efficiency protein	<i>nfeC</i>	47.4	—
bll1634	—	—	45.1	—
blr3679	Putative monooxygenase component	—	44.8	—
blr1954	—	—	43.6	—
bll2049	Anthranilate phosphoribosyltransferase	<i>trpD</i>	43.3	—
bll1979	—	—	42.1	—
bsr2005	—	—	41.7	—
bll2085	—	—	41.3	—
blr1852	Similar to pantoate-beta-alanine ligase	—	39.1	—
blr2108	Probable peptide synthetase	—	39.0	—
bsr2765	<i>cbb3</i> oxidase, subunit IV	<i>fixQ</i>	38.3	59.6
bll2018	Similar to ABC transporter, amino acid binding protein	—	38.3	—
blr1774	Flavoprotein	<i>fixC</i>	38.1	—
blr4988	—	—	37.7	—
bll6950	Putative pyrophosphorylase	<i>modD</i>	36.3	—
bll2017	Similar to ABC transporter, amino acid binding protein	—	36.2	—
bll6942	Uptake hydrogenase precursor	<i>hupS</i>	35.1	—
bsl2048	—	—	35.1	—
bll5734	ABC transporter nitrate-binding protein	<i>nrtA</i>	34.4	—
blr1747	Iron-molybdenum cofactor processing protein	<i>nifX</i>	33.5	—
blr1761	Iron-sulfur cofactor synthesis protein	<i>nifZ</i>	33.2	2.1
blr3680	—	—	33.1	—
bll2515	Similar to pyruvate phosphate dikinase	—	32.9	—
blr2611	—	—	32.8	27.5
bll2046	—	—	32.7	—
bsl2020	—	—	32.6	—
bll6941	Uptake hydrogenase large subunit	<i>hupL</i>	32.2	—
blr2038	Electron transfer flavoprotein beta chain	<i>fixA</i>	31.9	—
bsr1907	—	—	31.6	—
bsr1764	—	—	30.9	—
blr2763	Cytochrome <i>c</i> oxidase (<i>cbb3</i> type)	<i>fixN</i>	30.6	52.2
blr1748	—	—	30.4	2.7
bll2019	Transcriptional regulatory protein MerR family	<i>nolA</i>	30.2	−2.0
blr1854	—	—	30.0	—

genes expressed more highly at 21 and 31 dpi. The complete list of genes falling into these classes is provided in Supplemental Table 5. These genes might be useful reference markers for nodule and bacteroid development.

Genes for a type III secretion system (T3SS; blr1812 to blr1822) were among those that are maximally induced at 10

and 13 dpi. Although T3SS genes are not essential for soybean nodulation, they are known to be coregulated to a large extent with early *nod* genes (Krause et al. 2002). The class of 59 genes whose expression was maximal at 13 dpi contained a number of transporter genes, including several putative phosphate transport operons such as blr1092 to blr1097, suggesting

Table 3. The 39 transcriptional regulators induced in wild-type bacteroids compared with wild-type cells grown in aerobic culture

Gene number ^a	Description ^b	Gene name ^c	Fold change ^d
blr1880	Transcriptional regulatory protein LuxR family	–	74.8
blI2019	Transcriptional regulatory protein MerR family	<i>nolA</i>	30.2
blI2021	Transcriptional regulatory protein LysR family	<i>nodD2</i>	19.5
blr5805	Transcriptional regulatory protein Crp family	–	19.4
blr1216	Transcriptional regulatory protein Ferric uptake regulator family	–	10.7
blr0612	Nitrogen regulatory protein PII	<i>glnK</i>	8.9
blr4466	Transcriptional regulatory protein AraC family	–	8.4
blr1847	VirK protein homolog	–	7.5
blI2604	Transcriptional regulatory protein MarR family	–	5.8
blr6408	Transcriptional regulatory protein AraC family	–	5.8
blI3386	Transcriptional regulatory protein AraC family	–	5.6
blI6414	Transcriptional regulatory protein LuxR family	–	5.5
blI0966	Two-component hybrid sensor and regulator	–	4.6
blr7666	Transcriptional regulatory protein AraC family	–	3.8
blI2465	MoxR family protein	–	3.4
blr1096	Phosphate regulon, two-component response regulator	<i>phoB</i>	3.3
blr2037	Nif-specific regulatory protein	<i>nifA</i>	3.2
blI5291	Transcriptional regulatory protein ArsR family	–	3.1
blI2109	Transcriptional regulatory protein Crp family	<i>fixK</i>	3
blr2285	Two-component response regulator	–	3
blr3685	Transcriptional regulatory protein Fis family	–	3
blr5345	Transcriptional regulatory protein ArsR family	–	2.9
blr5735	Transcriptional regulatory protein Fis family	–	2.9
blI1688	Probable suppressor protein	–	2.7
blI6061	Transcriptional regulatory protein Crp family	<i>fixK</i>	2.7
blI2023	Transcriptional regulatory protein LysR family	<i>nodD1</i>	2.6
blr0347	Transcriptional regulatory protein MarR family	–	2.5
bsr6672	Transcriptional regulatory protein Fis family	–	2.5
blI7016	Two-component hybrid sensor and regulator	–	2.5
blI7588	Transcriptional regulatory protein AsnC family	–	2.5
blI2758	Two-component response regulator	–	2.4
blr5860	Transcriptional regulatory protein GntR family	–	2.4
blI1179	Two-component sensor histidine kinase	–	2.3
blr3443	Transcriptional regulatory protein TetR family	–	2.3
blr4773	Two-component hybrid sensor and regulator	<i>nwsA</i>	2.3
blI1150	Transcriptional regulatory protein LysR family	–	2.2
blI2786	Transcriptional regulatory protein LysR family	–	2.2
blI6060	Putative catabolite gene activator	–	2.2
blI7017	Transcriptional regulatory protein LuxR family	–	2.1

^a Nomenclature according to Kaneko and associates (2002).

^b Gene description according to Kaneko and associates (2002).

^c Gene name according to the EMBL–EBI database.

^d Fold change of expression, comparing bacteroids and aerobically grown cells.

Fig. 3. Plot of the linearized *Bradyrhizobium japonicum* genome and a summary of differential gene expression at all regions in symbiotic bacteroids relative to aerobic cultures. Across the entire genome, the track in red marks the percentage of upregulated genes and in blue, the percentage of downregulated genes. The G+C content is shown in cyan. All percentages were computed with a sliding window algorithm that considered 50 genes at a time. Note the most prominent upregulation in the symbiosis island and in regions I and II.

that young bacteroids require an adequate phosphate supply. A previous global study on the Pho regulon in *S. meliloti* (Krol and Becker 2004) showed that phosphate starvation causes oxidative stress; hence, a high-affinity phosphate transporter might help to overcome such an adverse condition. The class of 75 genes specifically induced in mature nodules (21 and 31 dpi) again contained many transporter genes. Among genes preferentially expressed late, there are, for example, some that appear to be involved in uptake, activation, and utilization of sulfate or sulfonate, suggesting that fully developed bacteroids may run into sulfur-limiting conditions. In fact, the *Escherichia coli* *ssuD*-encoded alkanesulfonate monooxygenase, for which there are two homologs in *B. japonicum*, is expressed only under conditions of sulfate or cysteine starvation (Eichhorn et al. 2000).

Comparing transcriptomics with proteomics.

Sarma and Emerich (2005, 2006) have compared the proteomes of culture-grown *B. japonicum* and soybean bacteroids by two-dimensional gel electrophoresis. Of the 289 proteins they detected under aerobic growth conditions, 277 (96%) were found to be expressed in our study. Another 87 proteins were detectable exclusively in the bacteroid state (Sarma and Emerich 2005). It was gratifying to see, with our transcriptomics approach, that 69 out of those 87 proteins were encoded by genes whose expression was found in bacteroids and 29 were among those that we found to be transcriptionally up-regulated (Supplemental Table 6). Among the 69 bacteroid-specific genes or gene products that were congruent in both studies, we found not only nitrogenase—as expected—but also three cytochromes P450, two chaperonins (GroES₃ and GroEL₃), and the *phbC*-encoded poly-β-hydroxybutyrate polymerase. The additional 18 proteins identified by Sarma and Emerich (2005, 2006) are encoded by genes that were expressed but failed to pass our stringent filtering criteria for expression. Despite a remarkable degree of overlap between the transcriptomics and proteomics data, only the combination of both techniques may yield the information needed to ever reveal the full complement of genes or proteins expressed in bacteroids. In particular, the ability of proteomics technologies to accurately measure the expression level of proteins and detect post-translational modifications that effect protein function, activity state, and localization provide insights that are not amenable by transcriptomics studies. On the other hand, global transcriptomics studies have the benefit to detect expression levels for genes encoding proteins of low abundance and thus provide a more complete list of the elements.

Comparison of transcriptomics data from three rhizobial systems.

We compared our global transcription study on *B. japonicum* with similar studies on bacteroids of *S. meliloti* (Barnett et al. 2004; Becker et al. 2004; Capela et al. 2006) and *M. loti* (Uchiumi et al. 2004) and found a high concordance. Out of the 2,778 *B. japonicum* genes that are differentially expressed in symbiosis, 1,229 have orthologous genes in *S. meliloti* (National Center for Biotechnology Information [NCBI] genome comparison website). While the three *S. meliloti* studies reported varying numbers of regulated genes, the percentage of genes found to be regulated alike in *B. japonicum* ranged between 80 and 92%. We observed that concordance of our study was most pronounced with Barnett and associates (2004) who, interestingly, used a similar type of Affymetrix array. Examples of genes that were induced in the *B. japonicum*–*Glycine max* symbiosis but repressed in the *S. meliloti*–*M. sativa* nodules include phosphate/phosphonate transport systems (*phoCDE*, *pstABC*), an ABC-type transporter (*bll4544*), and a cold-shock

protein (*bsr7468*). Conversely, genes that were repressed in *B. japonicum* bacteroids but induced in at least two of the three *S. meliloti* studies include the citrate synthase (*gltA*; *blr4839*) and a putative outer-membrane protein (*blr4700*). Of the 2,778 genes that are differentially expressed in bacteroids of *B. japonicum*, 1,261 have orthologs in *M. loti*. A total of 84% of the genes differentially expressed in *M. loti*–*L. japonicus* nodules and having a *B. japonicum* ortholog were also found to be similarly regulated in our study. The differences uncovered in the three symbiotic systems might be trivial consequences of the different experimental conditions applied (e.g., medium composition, nodule age) or due to disparate normalization and analysis criteria or both. On the other hand, they might reflect the different environmental conditions *B. japonicum* meets in determinate nodules vs. *S. meliloti* in the indeterminate nodules. While the regulation of a large percentage of orthologs is conserved in the three symbiosis systems, we find a large proportion of the remaining genes have no detectable homolog and are specific to *B. japonicum*.

Fig. 4. Hierarchical clustering of changes in gene expression during nodule development. Gene expression profiles were determined from *Bradyrhizobium japonicum*–*Glycine max* nodules at 10 and 13 or 21 and 31 days postinoculation (dpi). The color code for expression levels is shown at the bottom.

In-nodule transcriptome analysis of a Nod⁺ Fix⁻ mutant.

Research in our own laboratory as well as in other laboratories has generated a large collection of *B. japonicum* 110 mutant derivatives that are still able to elicit soybean nodules but are unable to carry out symbiotic nitrogen fixation (Nod⁺ Fix⁻ phenotype). We were interested in exploring whether our transcriptomics approach could be extended to nodules infected by such mutants. The transcription factor σ^{54} -deficient mutant (strain N50-97; genotype *rpoN*₁⁻, *rpoN*₂⁻) was chosen for this purpose because i) it still forms a discernible number of bacteroids in spite of its pleiotropic phenotype (Kullik et al. 1991), ii) σ^{54} is the key sigma factor for transcription of nitrogen fixation genes, so the mutant can be used to define the σ^{54} regulon, iii) all genes transcribed with the help of σ^{54} depend on an activator protein, and iv) the genes belonging to the σ^{54} regulon in anaerobically grown *B. japonicum* have already been studied extensively (Hauser et al. 2007). Here, we compared the transcriptome in *rpoN*₁₊₂ mutant bacteroids with that of wild-type bacteroids, both at 21 dpi. For reference, similar mutant vs. wild type comparisons were also done in free-living cells grown aerobically and microaerobically (Table 1).

A total of 789 genes were found to be differentially expressed in *rpoN* mutant-infected nodules as compared with nodules infected with the *B. japonicum* wild type. A complete list of these genes is provided in Supplemental Table 7. As a consequence of the missing σ^{54} , the expression of 450 genes is decreased, whereas 339 genes respond to this change with an increased expression, and—remarkably—the σ^{54} regulon in symbiosis (789 genes) is larger than the σ^{54} regulons in microaerobic and aerobic cultures (341 and 241 genes, respectively; Table 1). This underscores the symbiotic importance of σ^{54} . The remarkably high number of genes with increased expression in nodules infected by a *rpoN*₁₊₂ mutant could be due to indirect cryptic effects.

Of the 450 σ^{54} -dependent genes, 96 are potentially transcribed by the RNA polymerase- σ^{54} complex, because we identified σ^{54} promoters (−24/−12 consensus sequences) in the 5′-flanking DNA to the first gene of each transcription unit (not shown). Expression of 35 of these 96 genes also depended on σ^{54} in microaerobic culture. Almost all of them have recently been shown to be activated by the low oxygen-responsive enhancer-binding protein NifA (Hauser et al. 2007). The symbiotic induction of the other 61 potential RpoN targets (Table 4) is new and different. Interestingly, several of the newly identified potential RpoN targets lack a NifA binding site in the promoter region (e.g., the blr3719 to blr3722 genes encoding a putative 2-oxo-acid dehydrogenase complex), suggesting that they might be activated by enhancer-binding proteins (EBP) other than NifA. An in silico search for proteins containing a σ^{54} -interacting domain allowed us to identify 12 potential EBP encoded in the *B. japonicum* genome (blr0890, blr1896, blr2037, blr3685, blr3731, blr4488, blr4490, blr5735, blr6925, blr7003, blr7130, blr7291). In conclusion, the in-nodule *rpoN* mutant analysis has led to a substantial expansion of the previously known σ^{54} regulon by possible new targets transcribed in planta.

Concluding remarks.

Our global transcript profile analysis showed i) a massive difference between gene expression under aerobic and symbiotic conditions (66 vs. 34% of all genes) and ii) a dramatic change (33%) in expression when comparing bacteroids with free-living cultures.

1-Aminocyclopropane-1-carboxylate (ACC) is the precursor of ethylene biosynthesis in plants. Rhizobia can degrade ACC by means of ACC deaminase, whereby they overcome the ethylene-mediated inhibition of nodulation (Ma et al. 2003; Uchiumi et al. 2004). In the present work, quite fittingly, the *B. japonicum* ACC deaminase gene (blr0241; *acdS*) was found to

be well-induced in bacteroids (fold change 20.1) and strongly dependent on σ^{54} (fold change −73.0 in *rpoN*⁻ bacteroids), and its gene product belonged to those bacteroid proteins detected by proteomics. The ferredoxin encoded by *fdxN* (bsr1739), which is highly induced in bacteroids (fold change 76) and strongly dependent on σ^{54} (fold change −47.8 in *rpoN*⁻ bacteroids), was previously shown to be required for efficient symbiotic nitrogen fixation (Hauser et al. 2007). These are just two of the many examples of the convergence of symbiosis-related genetic and functional information. To provide similar details for every relevant gene goes beyond the scope of this paper. Even more numerous are those genes—several hundred, in fact—for which functional information is either only predictable, at best, or not available at all.

The gene with the highest fold change of expression (*ectC*; Table 2) has recently been shown to be a member of the NifA+ σ^{54} regulon. Yet, an *ectC* knock-out mutation did not cause a symbiotic defect (Hauser et al. 2007). Hence, highly induced transcription in bacteroids does not necessarily guarantee symbiotic essentiality of that gene. Nevertheless, while it cannot be expected that all of the newly discovered bacteroid-expressed genes are important or even essential for symbiosis, we are optimistic that our approach will lead to hitherto unexplored facets of bacteroid physiology and biochemistry. In order to track down new symbiotic functions, mutagenesis of all candidate genes followed by adequate phenotypic screens would be the method of choice. Importantly, this would allow an unbiased testing of all candidates, including the many genes implied in symbiosis for the first time here but that lack any functional annotation. Unfortunately, such a high-throughput method for creating and screening rhizobial mutants is not readily available. Instead, we may have to carefully select a smaller number of candidates that promise to be relevant for symbiosis. The integration of results from our global transcriptome comparisons and the phenotypes of specific knock-out mutants together with comparative genomics and data mining by various bioinformatics approaches and assistance from educated guesses on the basis of available literature will hopefully allow us to make such a prioritization. Most promising targets in this respect are probably those genes that have no recognizable, compensatory homologs in the genome and whose functions cannot be by-passed in cells by other means. While we have deliberately focused our initial data analysis on the annotated genes, the tiling design of our custom Affymetrix array, despite its limited resolution, will allow an identification not only of novel genes missed in the original annotation (Hauser et al. 2007) but also of potential noncoding RNAs.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Cells of the *B. japonicum* wild-type 110*spc*4 (Regensburger and Hennecke 1983) and the *rpoN*₁₊₂ double mutant strain N50-97 (Kullik et al. 1991) were grown under aerobic and microaerobic conditions in PSY medium (Regensburger and Hennecke 1983) containing 0.1% L-arabinose and spectinomycin (100 μ g ml⁻¹). Aerobic cultures were grown with rigorous shaking (180 rpm) in 5-liter Erlenmeyer flasks containing 200 ml of medium. Microaerobic cultures were grown under a nitrogen gas atmosphere that contained 0.5% oxygen with moderate shaking (80 rpm) in 500-ml rubber-stoppered serum bottles containing 25 ml of medium. The gas phase (0.5% O₂, 99.5% N₂) was exchanged every 8 to 14 h.

Plant growth conditions and inoculation.

Infection of soybean seedlings (*Glycine max* L. Merr., cv. Williams; provided by D. N. Navarro and F. Temprano, CIFA-

Las Torres Tomejil, Spain) and determination of nitrogenase activity in bacteroids were performed as described previously (Göttfert et al. 1990). Nodules from five plants were collected for each GeneChip hybridization experiment. For studies on the kinetics of bacteroid development, nodules were harvested at 10, 13, 21, and 31 dpi and immediately frozen in liquid nitrogen.

RNA purification, cDNA synthesis, labeling, and hybridization.

Cells were grown to mid-exponential phase (optical density at 600 nm of 0.4 to 0.5). For harvesting, cultures were rapidly transferred to cold tubes containing one tenth of the culture volume of “stop solution” (10% Tris-HCl-buffered phenol [pH

Table 4. The 61 genes that showed decreased expression in nodules infected by an *rpoN*₁₊₂ mutant strain and which have a predicted RpoN binding site in the promoter region

Gene number ^a	Description ^b	Gene name ^c	Fold change ^d
blr0612*	Nitrogen regulatory protein PII	<i>glnK</i>	–16.8
blr0613*	Ammonium transporter	<i>amtB</i>	–11.0
bll0889*	Putative transport protein	–	–7.4
blr1036*	ABC transporter substrate-binding protein	–	–5.3
blr1719*	Molybdenum transport system permease protein	<i>modB</i>	–20.4
blr1726*	Unknown protein	–	–120.0
blr1747*	Iron-molybdenum cofactor processing protein	<i>nifX</i>	–43.1
blr1765*	Ferredoxin	<i>fer2</i>	–41.0
blr1879*	Hypothetical protein	–	–35.1
blr1954*	Unknown protein	–	–155.0
blr1971*	Putative peptidase	–	–22.1
blr1992*	Unknown protein	–	–90.1
blr2011*	Unknown protein	–	–82.0
blr2036*	Oxidoreductase	<i>fixR</i>	–3.4
bll2085*	Hypothetical protein	–	–27.5
blr2143*	Similar to cytochrome P450-family protein	–	–82.6
bsl2575*	Hypothetical protein	–	–32.6
blr2725*	Hypothetical protein	–	–11.6
bll3193*	Unknown protein	–	–74.6
bll3316	ABC transporter substrate-binding protein	–	–5.6
blr3337	ABC transporter substrate-binding protein	–	–2.6
blr3338	ABC transporter permease protein	–	–2.2
blr3341*	ABC transporter substrate-binding protein	–	–4.6
blr3342	ABC transporter permease protein	–	–2.2
bll3363	Unknown protein	–	–9.7
blr3677*	Putative monooxygenase component	–	–57.8
blr3719	Hypothetical protein	–	–358.4
blr3720	Probable pyruvate dehydrogenase	–	–7.5
blr3721	Dihydrolipoamide S-acetyltransferase	<i>aceF</i>	–11.0
blr3722	Dihydrolipoamide dehydrogenase	<i>lpd</i>	–20.0
blr4169*	Glutamine synthetase II	<i>glnII</i>	–5.9
bll4384*	Probable substrate-binding protein	–	–20.1
blr4624	Hypothetical protein	–	–3.7
bll4798	Putative glutaminase	–	–2.8
bll4943	ATP-dependent Clp protease, ATP-binding subunit	<i>clpX</i>	–2.2
blr5374*	Hypothetical protein	–	–7.9
blr5573	ABC transporter ATP-binding protein	–	–2.7
blr5574	ABC transporter substrate-binding protein	–	–8.9
blr5598	ABC transporter permease protein	–	–4.0
blr5599	ABC transporter permease protein	–	–2.2
bll5733*	Nitrate ABC transporter permease protein	<i>nrtB</i>	–3.9
bll5734*	ABC transporter nitrate-binding protein	<i>nrtA</i>	–23.5
bll6091	ABC transporter ATP-binding protein	–	–2.6
bsl6119	Unknown protein	–	–2.4
blr6145*	C4-dicarboxylate transport protein	<i>dctA</i>	–2.4
bll6414	Transcriptional regulatory protein LuxR family	–	–3.1
bll6702	Glutathione S-transferase	–	–6.3
bsl6929	Hydrogenase expression/formation protein	<i>hypC</i>	–3.4
bll6936	HupH protein	<i>hupH</i>	–6.7
bll6937	HupG protein	<i>hupG</i>	–3.4
bll6939*	HupD protein	<i>hupD</i>	–48.5
bll6940*	HupC protein	<i>hupC</i>	–61.7
bll6941*	Uptake hydrogenase large subunit	<i>hupL</i>	–33.8
bll6942*	Uptake hydrogenase precursor	<i>hupS</i>	–178.3
blr6953*	Molybdenum ABC transporter ATP-binding protein	<i>modC</i>	–5.0
blr6955*	Unknown protein	–	–2.8
bll7176*	Hypothetical protein	–	–3.0
bll7178*	Hypothetical protein	–	–5.7
bll7179*	Putative N-methylhydantoinase B	–	–3.2
bll7180*	Hydantoin utilization protein	–	–4.3
blr7289*	Putative alkanal monooxygenase	<i>luxA2</i>	–14.7

^a Asterisks (*) indicate the RpoN-dependent genes activated by NifA in anaerobiosis. Nomenclature according to Kaneko and associates (2002).

^b Gene description according to Kaneko and associates (2002).

^c Gene name according to the EMBL–EBI database.

^d Fold change of expression, comparing *rpoN*₁₊₂ mutant bacteroids with wild-type bacteroids.

8] in ethanol), were centrifuged for 5 min ($10,800 \times g$, 4°C), the supernatant was decanted, and cells were frozen in liquid nitrogen and stored at -80°C . For RNA isolation from symbiotically grown bacteria, approximately 10 frozen nodules were placed in a 2-ml Eppendorf tube and were disrupted and homogenized using a TissueLyzer (Qiagen, Valencia, CA, U.S.A.) (three times for 50 s; 30 Hertz) with a tungsten carbide bead (3 mm; Qiagen). Total RNA was isolated from the disrupted nodule material and from free-living bacterial cultures with the hot phenol-extraction procedure described previously (Babst et al. 1996). RNA purification, first-strand cDNA synthesis, fragmentation, and terminal labeling were done as previously described (Hauser et al. 2007). Fragmented cDNA generated from bacteroids were end-labeled in a final volume of 50 μl , using double amounts of labeling reagent and terminal deoxynucleotide transferase as compared with the previous protocol. Hybridization and washing procedures were carried out as described (Hauser et al. 2007). Between 2 and 2.5 μg of cDNA generated from culture-grown bacteria and 6 and 7.5 μg (for wild-type and *rpoN*₁₊₂-deficient bacteroids, respectively) of total bacteroid cDNA were hybridized to the GeneChips. The amount of bacteroid cDNA was estimated from the proportion of bacterial/plant RNA in nodules, which was roughly 1:2 and 1:3 for wild-type and *rpoN*₁₊₂-deficient bacteroids, respectively (Bioanalyzer, Agilent technologies, Palo Alto, CA, U.S.A.). As control, an equal amount of bacterial cDNA was loaded on the GeneChip, and we assessed the constitutive expression of the primary sigma factor *sigA*. In order to determine the extent of cross-hybridization of the GeneChip probes with plant RNA, RNA was isolated from the roots of soybean plants as described above, and cDNA was fragmented and labeled according to the protocol for free-living bacteria. A custom-made Affymetrix GeneChip representing 8,266 annotated *B. japonicum* genes and 5,261 intergenic sequences on both strands was used in this study (Hauser et al. 2007). Importantly, the whole set of *B. japonicum* oligonucleotide probes had been pruned against soybean EST sequences to minimize cross-hybridization with plant RNA. At least six biological replicates were analyzed for the wild type and the *rpoN*₁₊₂ mutant N50-97 grown under different free-living conditions. For the timecourse experiments with nodulated soybean plants (discussed above), at least two biological replicates were examined.

Data processing, normalization, and further analyses.

Hybridization signals were processed using the Gene Chip Operating System software from Affymetrix. Signals were scaled to a target signal intensity of 500 by using all probe sets. Data were further analyzed using the GeneSpring gene expression analysis software version 7.3 (Agilent). To minimize false positives, very stringent criteria were applied. For culture-grown bacteria or bacteroids, only those genes that are “present” (i.e., expressed, based on the detection *P*-value ≤ 0.06) (Hauser et al. 2007) in 80% of all replicates were further considered. Moreover, we excluded all genes in analyses with bacteroids for which the expression was not at least two times higher than in the control hybridizations with root material only. Thereby, we excluded those signals that might have originated from cross-hybridization with plant RNA. For these cross-hybridization control experiments, the amount of plant RNA was normalized to the signal of the *Glycine max* 18S rRNA. For experiments comparing different conditions among culture-grown bacteria or bacteroids, each chip was normalized to the median. Values below 0.01 were set to 0.01. Importantly, for the comparison of the expression profiles of culture-grown bacteria with bacteroids, no median normalization was applied, since the majority of genes are down-regulated in the bacteroids, and the signal distribution in these two conditions was

very different (Supplemental Figure 1). Therefore the assumptions underlying a median normalization do not apply. For statistical comparisons, the Wilcoxon rank-sum test was applied with a *P*-value threshold of 0.01. If not possible due to fewer numbers of biological replicates (<5), the student *t*-test with a *P*-value threshold of 0.01 was applied. Genes were considered as differentially expressed if the fold change was >2 when comparing different conditions among culture-grown bacteria or symbiotic bacteria.

For each gene, we computed the percentage of up- and downregulated neighboring genes on the chromosome (Fig. 3). The neighboring genes were defined by a sliding window containing 50 successive genes on the chromosome. The G+C content of a chromosomal position was computed as the percentage of G and C nucleotides within a window of 1,000 nucleotides surrounding the chromosomal position.

For a functional classification of the differentially expressed genes, we used the Kazusa functional gene classification for *B. japonicum* (RhizoBase website). All entries were categorized according to 14 functional categories and an additional category containing genes encoding hypothetical proteins. Hypergeometric tests (Fisher's exact test) were used to identify functional groups that are significantly over- or underrepresented among the differentially expressed genes relative to all genes. Only categories with a *P*-value ≤ 0.01 were considered as being significantly over- or underrepresented. The hierarchical clustering of genes (Fig. 4) was performed in GeneSpring 7.3.1. As a similarity measure, the Euclidean distance was used, and clusters were aggregated using the complete linkage algorithm. NifA and RpoN (σ^{54}) consensus DNA-binding sequences in the promoter regions of selected genes were identified using position-specific frequency matrices (Hauser et al. 2007). EBP with a σ^{54} interacting domain in *B. japonicum* genome were predicted using the Interpro database.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Affymetrix GeneChip website: <http://www.affymetrix.com>
 EMBL-EBI database: www.ebi.ac.uk/embl
 NCBI Gene Expression Omnibus (GEO) database: www.ncbi.nlm.nih.gov/geo
 NCBI pairwise genome comparison webpage: www.ncbi.nlm.nih.gov/sutils/geneplot.cgi
 RhizoBase database: bacteria.kazusa.or.jp/rhizobase
 RhizoDB website: rhizo.bham.ac.uk