An Oligonucleotide Microarray Resource for Transcriptional Profiling of *Bradyrhizobium japonicum*

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A DNA microarray, comprising 70-mer oligonucleotides, representing 8,453 open reading frames (ORFs), was constructed based on the *Bradyrhizobium japonicum* strain USDA110 genomic sequence. New annotation predicted 199 additional genes, which were added to the microarray and were shown to be transcribed. These arrays were used to profile transcription in cells under a variety of conditions, including growth in minimal versus rich medium, osmotic stress, and free-living cells versus bacteroids. Increased expression was seen for genes involved in translation, motility, and cell envelope synthesis in rich medium whereas expression increased in minimal medium for genes involved in vitamin biosynthesis and stress responses. Treatment with 50 mM NaCl activated stress-inducible genes but repressed genes involved in chemotaxis and motility. Strikingly, no known transport systems for accumulation of compatible solutes or osmoprotectants were induced in response to osmotic stress. A number of *nif*, *fix*, and *hup* genes, but not all, were upregulated in bacteroids. The *B. japonicum* type III secretion system, known to be important in early nodulation, was downregulated in bacteroids. The availability of a reliable, low-cost *B. japonicum* microarray provides a useful tool for functional genomic studies of one of the most agriculturally important bacteria.

Additional keywords: transcriptomics.

Transcriptional profiling allows for a comprehensive view of changes in gene expression that occur as a result of a cellular response to environmental perturbations. Rhizobia are of interest due to their ability to symbiotically fix nitrogen with leguminous plants. The culmination of this symbiotic relationship is the formation of root nodules, in which the bacteria convert atmospheric dinitrogen into ammonia to be used by the host plant in exchange for host-derived carbohydrates and nutrients. Nodule initiation and development is a complex process involving signal exchange between both symbiotic partners and differentiation of the rhizobia into bacteroids inside nodules. Soybean is one of the major crops produced worldwide and, therefore, knowledge of the *Bradyrhizobium*

japonicum–soybean symbiosis has great agricultural and ecological benefits.

Transcriptomics in rhizobia has been enabled by the availability of the complete genome sequences for *Mesorhizobium loti* (Kaneko et al. 2000), *Rhizobium etli* (Gonzalez et al. 2006), *Sinorhizobium meliloti* (Galibert et al. 2001), *R. leguminosarum* (Young et al. 2006), and *B. japonicum* (Kaneko et al. 2002). Indeed, Affymetrix GeneChips and polymerase chain reaction (PCR) fragment- and oligonucleotide-based microarrays are available for *S. meliloti* (Ampe et al. 2003; Barloy-Hubler et al. 2004; Barnett et al. 2004; Becker et al. 2004; Berges et al. 2003; Davalos et al. 2004; Puskas et al. 2004; Ruberg et al. 2003) and *M. loti* (Uchiumi et al. 2004). Recently, an Affymetrix oligonucleotide array was developed for *B. japonicum* (Hauser et al. 2006; Rudolph et al. 2006; Yang et al. 2006). Relatively few studies have been done with *B. japonicum* microarrays, perhaps due to the high cost of the Affymetrix platform. However, gene expression in *S. meliloti* has been well-studied under a variety of conditions, including growth in minimal versus rich medium (Barnett et al. 2004; Becker et al. 2004), osmotic (Ruberg et al. 2003) and oxidative stress (Barloy-Hubler et al. 2004) under microoxic versus oxic conditions (Becker et al. 2004; Hauser et al. 2006; Uchiumi et al. 2004), as free-living cells versus bacteroids (Ampe et al. 2003; Barnett et al. 2004; Becker et al. 2004; Uchiumi et al. 2004), and under conditions influencing nitrogen regulation (Davalos et al. 2004; Puskas et al. 2004).

A key aspect of bacterial survival in soil is successful adaptation to environmental fluctuations, such as changes in temperature, water availability, pH, nutrients, and salinity. Rhizobia in fallow soil must persist until they encounter the nutrient-rich rhizosphere and initiate infection of their compatible host. In order to enhance symbiotic nitrogen-fixation, legume seeds often are inoculated with rhizobial cultures prior to sowing. A preferred method for delivery is seed coating; however, this approach often is unsuccessful for *B. japonicum* because they do not survive the drying process. Roughley and associates (1993) reported that 95% of *Bradyrhizobium* spp. died within 4 h of inoculation of the seed and planting in which the bacteria were likely exposed to desiccation-mediated osmotic stress. Therefore, the ability of *B. japonicum* to survive osmotic stress could have practical importance. A number of studies have shown that *B. japonicum* fails to grow at concentrations exceeding 100 mM NaCl (Boncompagni et al. 1999) whereas some rhizobia, including *S. meliloti* and *M. loti*, are tolerant to 300 to 700 mM NaCl (Boncompagni et al. 1999; Elsheikh 1998; Elsheikh and Wood 1990; Zahran 1999). Once free-living

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bacteria develop into bacteroids within the nodule, they also become more osmotically sensitive. Moreover, within the nodule, a transition occurs from an oxygen-rich to an oxygen-poor environment. Indeed, oxygen availability has been shown to be a major regulator of gene expression in bacteroids (Ampe et al. 2003; Barnett and Fisher 2006; Becker et al. 2004; Fischer 1994; Hauser et al. 2006; Layzell et al. 1990).

In order to improve inoculant performance and to optimize the benefits of biological nitrogen fixation, it is important to fully understand how *B. japonicum* responds to alterations in its environment and to the physiological changes that occur during bacteroid differentiation. To aid in this understanding, we constructed a low-cost, oligonucleotide-based DNA microarray for *B. japonicum*. These arrays, which contain 8,453 predicted open reading frames (ORFs) from *B. japonicum* and 36 control genes, were used to profile *B. japonicum* gene expression in cells grown in rich versus minimal medium, cells treated with 50 mM NaCl, and free-living cells versus bacteroids. Results of these studies show that the constructed arrays are useful for transcriptional profiling of *B. japonicum* grown under a variety of conditions.

RESULTS AND DISCUSSION

Genome annotation.

The *B. japonicum* strain USDA110 genome was sequenced and machine-annotated by the Kazusa DNA Research Institute (KDRI) (Kaneko et al. 2002). Although 8,317 ORFs were identified by this group, an examination of this initial annotation suggested that some ORFs were missed. Our analysis suggested the presence of an additional 199 ORFs (Supplemental Table 1) that were overlooked in the original annotation (Kaneko et al. 2002), giving a total of 8,516 ORFs. Whereas oligonucleotides for 8,516 ORFs were designed, 63 ORFs derived from the original annotation were excluded before printing the DNA microarray slides due to the lack of suitable unique probes. Consequently, the final arrays contained 8,453 predicted ORFs from the *B. japonicum* and 36 control genes.

Three different microarray experiments (discussed below) were used to determine whether these additional ORFs hybridized with a given cDNA. Because the array was constructed on a two-color (cy3 and cy5) basis, a higher than background intensity in either the cy3 or cy5 channel indicated the presence of a transcript. Thus, the sum of cy3 and cy5 intensities for each ORF was plotted in the order of intensity strength (Fig. 1). As a threshold, the sum of mean background intensities for cy3 and cy5 across the array was used. Most of the 199 newly annotated ORFs showed higher intensity than the background, with a few exceptions in the minimal versus rich medium comparison (Fig. 1A). However, those few ORFs not expressed appeared to be more greatly expressed in the osmotic and bacteroid microarray experiments (Fig. 1B and C), suggesting that all of the 199 ORFs added to the annotation are most likely transcribed genes.

Validation of microarray experiments.

In order to validate the DNA microarray results, the expression of a number of genes found to be up- or downregulated in bacteroids also was measured by using quantitative reverse-transcription polymerase chain reaction (qRT-PCR). The genes

Fig. 1. Distribution of intensity values of 199 additional open reading frames (ORFs). Intensity was calculated from the sum of cy3 (532-nm channel) and cy5 (635-nm channel) intensity for each ORF. White lines indicate the sum of mean background intensities for cy3 and cy5 across the array. Background intensity values (mean $\pm$ standard deviation) are 497 ± 30 , 473 ± 63 , and 222 ± 23 for **A**, minimal versus rich medium; **B**, osmotic stress; and **C**, free-living cells versus bacteroids, respectively.

Table 1. Gene-specific primers used for quantitative reverse-transcription polymerase chain reaction and corresponding amplicon size

Locus	Forward primer	Reverse primer	Amplicon size (bp)
blr1769	ACCACTTCGAGAACACGCT	TTGGCGTGCAGATAAGGCGA	107
blI6939	ACAATCGCATCCTGGTGCTCG	ATGGTGACGTTGTGACGGCAGC	115
blr1746	ACTCACAAAGTTGCCGCTCGT	CAACTGCCTTCTCCACCCAT	80
blr1744	TTTGTCGCGGCACTTCAAGGA	AGCCCGTCGGTCATGTTGTT	99
blI0735	TCAAGCTCACACGCGCGAA	ATCGCGCGGATGAAGCAGG	103
blI5813	GATCAAGCCGGACCAAGTCGAA	TATTTGACGTTGCCGGTTCGCA	81
blr1812	TGGCGCAAGTAGCTCCCAAT	CGGACTTGCCAGCACATTCGT	107
blr1813	AAATTCAGGAGCGCGAGGCT	TTCCGTCCTTGCGGACAACA	87
blr1819	CTTGGTCTCCTGGCCTTTGCT	AAAGGACGATGTTCCGGCGGT	118
blI2757	ACACCCAAATCGGTGGCAAGA	AGATCTCCTCGTCCTTGCGGT	120

listed in Table 1 were selected based on fold expression changes and functional categories. Overall qRT-PCR and microarray data were very consistent ($R^2 = 0.96$) (Fig. 2), although, in general, higher expression was seen using qRT-PCR, reflecting the greater sensitivity of the method (slope = 1.14) (Barnett et al. 2004; Becker et al. 2004).

Minimal versus rich medium.

As the first test of the quality of the *B. japonicum* DNA microarrays, we profiled transcription of cells grown in minimal versus rich medium. These contrasting conditions were chosen for two reasons: i) they were likely to show a significant difference in gene expression (Tao et al. 1999) and ii) changes in nutritional status is one of the major environmental cues that *B. japonicum* often faces in soils and the rhizosphere.

One measure of array quality is spot hybridization efficiency. In this initial experiment, 15,580 (92.2%) of 16,906 spots (8,453 genes with two technical replicates) showed intensity values above background in at least four of six replicates (three biological replicates with dye swap). This high sensitivity of the *B. japonicum* DNA microarrays indicates that they are a reliable tool for high-throughput functional genomic studies of *B. japonicum*. Similar results were obtained for *S. meliloti* microarrays (91.3 and 97.3%) (Ruberg et al. 2003).

The similarity of the technical replicates on the arrays also was evaluated. To rule out possible dye effects and biological variation, we examined each array separately (Supplemental Fig.1). Ratios of duplicate spots were very consistent within the same array, indicating that these technical replicates provide more statistical confidence and, hence, a reliable gene expression profile is expected from the array.

A total of 656 genes were differentially expressed at a two-fold cut-off: 351 genes (4.1% of the genome) and 305 genes (3.6% of the genome) were significantly induced in minimal and rich medium, respectively (Table 2). Similarly, expression analysis of *Escherichia coli* showed that 5.2 and 2.8% of the total genome were induced in minimal and rich medium (Tao et al. 1999), respectively. These results indicate that more genes are upregulated in nutrient-limited environments. In *S. meliloti*, more genes were differentially regulated when the carbon source was glucose (13% of the 6,207 *S. meliloti*

genes) compared with succinate (8% of the genome) (Barnett et al. 2004), and Affymetrix chip analysis of succinate-grown *S. meliloti* revealed five times more changes in gene expression than a PCR-based microarray of the cells grown in the same carbon source, although a different minimal medium was used in these studies (M9 and Vincent's minimal medium, respectively) (Barnett et al. 2004; Becker et al. 2004).

One obvious expectation in a gene expression comparison between cells growing in minimal and rich medium would be an increased demand for protein translation machinery (e.g., ribosomal proteins) required for the faster growth in rich medium (Tao et al. 1999). As expected, 29 translation-related genes were expressed higher in rich medium than in minimal medium, the majority of which were ribosomal proteins. In contrast, although eight translation-related genes were more highly expressed in cells growing in minimal medium, none of them were ribosomal proteins (Table 2; Supplemental Table 2). This result is consistent with a previous study showing that approximately 80% of the total genes encoding ribosomal proteins are induced in *E. coli* cells grown in rich medium (Tao et al. 1999). However, higher transcript levels of the genes encoding ribosomal proteins were not observed in a transcriptional profile of *S. meliloti* growing in rich medium compared with minimal medium (Barnett et al. 2004; Becker et al. 2004), suggesting that regulation of the translation apparatus is different among rhizobia (at least between *B. japonicum* and *S. meliloti*) when they encounter fluctuations of nutrient availability.

B. japonicum grown in minimal medium is likely to suffer from a lack of cofactors and prosthetic groups due to nutrient limitation. To overcome this stressful situation, cells need to biosynthesize these molecules when grown in minimal medium. Twenty genes encoding cofactor biosyntheses were expressed higher in minimal medium, whereas none were expressed higher in rich medium (Table 2). Among these were genes related to vitamin biosyntheses: blr2095 (*bioB*), blr2097 (*bioF*), and blr2098 (*bioD*) for biotin synthesis; blt5475 to blt5478 for folic acid synthesis; and bsr6656 to bsr6659 (*thiS*GE) involved in thiamine synthesis (Table 2). In contrast to our findings, comparisons of *S. meliloti* grown in minimal versus rich medium (Barnett et al. 2004) did not show a comparable upregulation of vitamin biosynthetic genes.

It is well documented that vitamin limitation influences rhizobial growth in the rhizosphere (Streit et al. 1996), suggesting that the rhizosphere may represent a nutrient-limiting environment. Several vitamin auxotrophs (i.e., biotin, thiamine, and cobalamin) unable to grow in minimal medium either failed to establish a symbiosis with their hosts or formed ineffective nodules. Exogenous application of vitamins to these mutants restored effective nodule formation (Campbell et al. 2006; Schwinghamer 1970).

In addition to cofactor biosynthesis, a variety of other known stress-inducible genes were upregulated in minimal medium. Four genes were induced in minimal medium: bsr3154 (*cspA*) and bsl8249 (*cspA*), both encoding cold-shock proteins; blt0729 (*hspH*), encoding a small heat-shock protein; and blr7337 (*rpoH2*), encoding a σ^{32} -like transcription factor. However, no stress-inducible genes were observed in rich medium. This is consistent with gene expression in *E. coli* that revealed increased expression of the sigma factors *rpoS* and *rpoE*, and the global regulator *dps* in minimal medium (Tao et al. 1999). However, there were almost equal numbers of two-component regulatory genes differentially expressed in both culture conditions (Table 2). Similarly, the expression of genes encoding transporter proteins showed no bias toward growth in either minimal or rich medium (Table 2).

Interestingly, the expression of 27 flagella and chemotaxis biosynthetic genes significantly increased in rich medium, indi-

Fig. 2. Correlation between microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) data of the free-living versus bacteroid experiment for 10 genes. These genes were chosen based on fold induction and functional categories. Expression differences were log₂ transformed.

cating that motility functions of the faster-growing cells were activated (Table 2). Because energy for flagellar rotation is known to be derived from a proton motive force (Larsen et al. 1974; Manson et al. 1977), nutrient-rich conditions are conducive to the expression of motility. It has been suggested that cells shut off their motility function and thereby save energy in nutrient-poor conditions (White 2000a). Recently, transcriptional profiling of *S. meliloti* revealed that motility-related genes represented 25% of the 250 genes whose expression decreased in minimal medium (Barnett et al. 2004), which is in agreement with our findings. Unlike *B. japonicum* and *S. meliloti*, the flagellar motility apparatus of *E. coli* is not repressed in minimal medium (Tao et al. 1999).

Taken together, the overall transcriptional profile of *B. japonicum* grown in minimal versus rich medium differed significantly from the published studies of *S. meliloti*, except for genes involved in flagella and chemotaxis biosynthesis. Therefore, these two rhizobia may differ significantly in the way in which they respond to nutrient limitation.

Osmotic stress.

It is known that *B. japonicum* is very sensitive to osmotic stress, which is encountered in the soil, rhizosphere, and in planta environment (Boncompagni et al. 1999; Elsheikh and

Wood 1990). To characterize the osmotic stress response in *B. japonicum*, we analyzed the transcriptome upon exposure to 50 mM NaCl. *B. japonicum* did not grow in medium supplemented with 100 mM NaCl (Boncompagni et al. 1999), but grew at 50 mM NaCl with a reduced growth rate and final cell density (data not shown), suggesting that the osmotic conditions caused significant stress to growing *B. japonicum* cells.

Among 441 differentially expressed genes (1.5-fold cut-off), 269 were induced and 172 were repressed by NaCl treatment (Table. 3). A 2-fold cut-off was applied initially, but numerous genes whose expression level increased between 1.5- and 1.9-fold were located in operons or gene clusters with other genes induced 2-fold or higher (e.g., flagella and chemotaxis) (Supplemental Table 3), suggesting that all these genes should be considered as being differentially expressed.

One of the well-known bacterial responses to increased osmolarity is the accumulation of intracellular compatible solutes, such as K⁺, amino acids (e.g., glutamate), carbohydrates (e.g., trehalose), and osmoprotectants such as ectoine, glycine betaine, and choline. The latter compounds act as nontoxic cytoplasmic co-solvents whose level can be modulated by de novo synthesis or transport from the environment (Boncompagni et al. 1999; Csonka 1989; Wood 1999). No genes involved in

Table 2. Functional group assignment of a subset of *Bradyrhizobium japonicum* genes differentially expressed in response to rich medium^a

Locus (gene)	Fold change ^c	Functional groups and annotation ^d	Number of genes ^b	
			Up	Down
		Biosynthesis of cofactors, prosthetic groups, and carriers	0	20
blr3954 (<i>mmsA</i>)	-11.1	Methylmalonate-semialdehyde dehydrogenase	...	...
bsr6656 (<i>thiS</i>)	-4.4	Thiamine biosynthesis protein homolog	...	...
blr6658 (<i>thiE</i>)	-2.4	Thiamine-phosphate pyrophosphorylase	...	...
blr2095 (<i>bioB</i>)	-2.2	Biotin synthetase	...	...
bl15476	-7.3	Formate dehydrogenase iron-sulfur subunit	...	...
bl10751 (<i>trxA</i>)	-2.1	Thioredoxin C-1	...	...
		Cellular processes	37	6
bl14326	3.2	Putative methyl-accepting chemotaxis protein	...	...
bl11438 (<i>ctpE</i>)	3.0	Pilus assembly protein	...	...
blr5830 (<i>flgH</i>)	4.1	Flagellar L-ring protein precursor	...	...
bl15812 (<i>flhE</i>)	2.7	Flagellar hook-basal body complex protein	...	...
bl12207 (<i>flhA</i>)	3.2	Flagellar biosynthesis protein	...	...
blr5828 (<i>flgG</i>)	5.0	Flagellar basal-body rod protein	...	...
blr2506	2.8	Putative chemotaxis protein	...	...
bl10667 (<i>ctpC</i>)	2.3	CtpC protein	...	...
blr2192 (<i>cheA</i>)	2.2	Chemotaxis two-component hybrid sensor and regulator	...	...
		Translation	29	8
blr0740 (<i>rpsA</i>)	2.0	30S ribosomal protein S1	...	...
blr0482 (<i>rpsP</i>)	3.0	30S ribosomal protein S16	...	...
bsl0832 (<i>rpsT</i>)	2.1	30S ribosomal protein S20	...	...
bl14963 (<i>rplM</i>)	3.3	50S ribosomal protein L13	...	...
bl15397 (<i>rplB</i>)	2.2	50S ribosomal protein L2	...	...
bsl0708 (<i>rplM</i>)	2.2	50S ribosomal protein L35	...	...
bl14386 (<i>efp</i>)	3.2	Elongation factor P	...	...
	...	Amino acid biosynthesis	5	8
	...	Cell envelope (lipoproteins, porins, polysaccharides, etc.)	11	2
	...	Central intermediary metabolism	4	8
	...	DNA replication, recombination, and repair	3	1
	...	Energy metabolism	18	19
	...	Fatty acid, phospholipid and sterol metabolism	2	12
	...	Purines, pyrimidines, nucleosides, and nucleotides	3	2
	...	Regulatory functions	11	10
	...	Transcription	3	1
	...	Transport and binding proteins;	26	26
	...	Other categories	21	58
	...	Hypothetical protein	132	170
		Total	305	351

^a Differentially expressed genes were selected based on twofold cut off with *q* value ≤0.05.

^b Genes upregulated in rich medium are the same as those downregulated in minimal medium, and vice versa.

^c Positive and negative values indicate up- and downregulation in response to rich medium compared with minimal medium, respectively.

^d Functional groups and annotations (gene description) represent the first and third levels, respectively, among three different levels based on the RhizoBase annotation.

de novo synthesis of glutamate and trehalose were found to be upregulated in response to growth in 50 mM NaCl (Table 3). This suggests that minimally salt-stressed bradyrhizobial cells might preferentially utilize transporter systems under these conditions to accumulate compatible solutes or osmoprotectants. Although 14 genes encoding transport proteins were induced in these conditions, none belonged to known potassium and osmoprotectant uptake systems (Table 3). However, we cannot rule out the possibility that a novel transport system among the 14 upregulated genes may play a role in the osmotic response.

Analysis of *B. japonicum* USDA110 genome revealed no similarity to the ectoine, glycine betaine, and betaine choline carnitine transporter (BCCT) systems (Boscari et al. 2004; Kaneko et al. 2002), the latter of which was characterized in *S. meliloti*. The lack of such systems may explain the increased salt sensitivity of *B. japonicum* compared with other rhizobia. Interestingly, functional expression of the *S. meliloti* BCCT system in *B. japonicum* improved cell viability up to 80 mM NaCl in the presence of glycine betaine, but could not overcome

the deleterious effects of 100 mM NaCl, despite an accumulation of glycine betaine inside cells (Boscari et al. 2004).

In contrast to the lack of evidence supporting the accumulation of compatible solutes, 13 stress-related genes, including blr4635 (*mopA*), blr4637 (*hsp*), blr4653 (*dnaJ*), blr7740, blr7961, bsl8249 (*cpsA*), bll1167 (*sipS*), blr7337 (*rpoH2*), blr2591 (*dop*), blr3130 (*htrA*), blr7484, blr7485, and bll1168 (*tldD*), were induced up to sevenfold in response to osmotic stress (Table 3), indicating the involvement of a number of stress-related genes in osmoadaptation. Interestingly, four genes encode heat- or cold-shock proteins, suggesting an overlapping adaptation in response to different environmental stressors. In addition *rpoH2*, encoding a σ^{32} -like transcription factor, was induced in conditions of osmotic stress and growth in minimal medium (Table 3). This further suggests that *rpoH2* may play a key role in the global stress responses of *B. japonicum*. The majority of these genes, except *rpoH2*, previously were unidentified in microarray analyses of the osmotic stress response in *S. meliloti* (Dominguez-Ferreras et al. 2006), although some osmotic stress-inducible genes encoding heat- and cold-shock pro-

Table 3. Functional group assignments of a subset of genes differentially expressed in response to NaCl treatment^a

Locus (gene)	Fold change ^b	Functional groups and annotation ^c	Number of genes	
			Up	Down
		Cellular processes	7	34
blr4635 (<i>mopA</i>)	2.1	Chaperonin GroEL	...	...
blr4637 (<i>hsp</i>)	6.5	Probable HspC2 heat-shock protein	...	...
blr4653 (<i>dnaJ</i>)	2.2	Molecular chaperone DnaJ family	...	...
blr7740	1.5	Small heat-shock protein	...	...
blr7961	1.9	Probable HspC2 heat shock protein	...	...
bsl8249 (<i>cpsA</i>)	1.8	Cold-shock protein	...	...
bll0096	-1.6	Putative methyl accepting chemotaxis	...	...
bll1438 (<i>ctpE</i>)	-2.3	Pilus assemble protein	...	...
bll5813 (<i>flgC</i>)	-2.0	Flagellar basal-body rod protein	...	...
bll6865 (<i>fla</i>)	-2.7	Flagellin	...	...
blr2344 (<i>cheWII</i>)	-1.6	Probable chemotaxis protein	...	...
		Central intermediary metabolism	12	2
bll1167 (<i>sipS</i>)	1.6	Signal peptidase	...	...
		Transcription	1	1
blr7337 (<i>rpoH2</i>)	2.1	<i>sigma32-like transcription factor</i>	...	...
		Translation	4	10
blr2591 (<i>dop</i>)	3.1	Serine protease DO-like protease	...	...
blr3130 (<i>htrA</i>)	2.8	Serine protease DO-like precursor	...	...
blr7484	2.0	Hypothetical zinc protease	...	...
blr7485	1.8	Hypothetical zinc protease	...	...
		Transport and binding proteins	14	12
bll7103	1.8	ABC transporter permease protein	...	...
bll7988	1.9	Probable ATP-binding protein	...	...
blr2622 (<i>corA</i>)	2.8	Magnesium/cobalt transport protein	...	...
blr7037 (<i>napD</i>)	4.5	Periplasmic nitrate reductase	...	...
blr6149	-1.6	ABC transporter permease protein	...	...
blr7827	-1.5	ABC transporter substrate-binding protein	...	...
blr7824	-1.5	ABC-transporter ATP-binding protein	...	...
blr0607	-1.8	Probable ammonium transporter	...	...
	...	Amino acid biosynthesis	1	3
	...	Biosynthesis of cofactors, prosthetic groups, and carriers	2	0
	...	Cell envelope	5	8
	...	DNA replication, recombination, and repair	0	1
	...	Energy metabolism	12	7
	...	Fatty acid, phospholipid and sterol metabolism	4	0
	...	Purines, pyrimidines, nucleosides, and nucleotides	1	1
	...	Regulatory functions	17	6
	...	Other categories	20	8
bll1168 (<i>tldD</i>)	1.7	<i>TldD protein</i>	...	...
		Hypothetical protein	169	79
		Total	269	172

^a Differentially expressed genes were selected based on 1.5-fold cut-off with *q* value ≤ 0.05 .

^b Positive and negative values indicate up- and downregulation, respectively, in response to osmotic stress.

^c Functional groups and annotations (gene description) represent the first and third levels, respectively, among three different levels based on the RhizoBase annotation. Stress-related genes are indicated in italics.

teins were observed for *Bacillus subtilis* (Budde et al. 2006) and *Pseudomonas aeruginosa* (Aspedon et al. 2006). These results indicate that *Bradyrhizobium japonicum* may use these stress-responsive genes to compensate for the lack of compatible solute or osmoprotectant accumulation. However, these genes seem more likely to function only in a mild osmotic stress response due to the fact that *B. japonicum* did not grow at a NaCl concen-

tration above 100 mM (Boncompagni et al. 1999). In fact, the lack of glycine betaine and chlorine uptake systems suggests that *B. japonicum* behaves in a different manner when encountering osmotic stress compared with other osmotolerant rhizobia. From an inoculant perspective, it will be important to identify and characterize genes whose expression can improve cell survival during osmotic stress.

Table 4. Functional group assignments of genes differentially expressed in bacteroids^a

Locus (gene)	Fold change ^b	Functional groups and annotation ^c	Number of genes	
			Up	Down
		Central intermediary metabolism	31	23
blr1744 (<i>nifK</i>)	4.5	Nitrogenase molybdenum-iron protein β chain	...	...
blr1745 (<i>nifE</i>)	7.0	Nitrogenase molybdenum-cofactor synthesis protein	...	...
blr1746 (<i>nifN</i>)	5.5	Nitrogenase molybdenum-cofactor synthesis protein	...	...
blr1747 (<i>nifX</i>)	5.1	Iron-molibdenum cofactor processing protein	...	...
blr1761 (<i>nifZ</i>)	4.3	Iron-sulfur cofactor synthesis protein	...	...
blr1773 (<i>fixB</i>)	2.6	Electron transfer flavoprotein α chain	...	...
blr1774 (<i>fixC</i>)	4.3	Flavoprotein	...	...
bsr1757 (<i>fixU</i>)	3.0	Nitrogen fixation protein (<i>fixU</i>)	...	...
bsr1775 (<i>fixX</i>)	5.1	Probable ferredoxin	...	...
blr1724 (<i>hupD</i>)	2.8	HupD protein homolog, hupD C-terminal	...	...
blr1727 (<i>hupH</i>)	5.4	HupH protein homolog	...	...
blr1728 (<i>hupK</i>)	4.7	HupK protein homolog, hupK N-terminal	...	...
bsl6935 (<i>hupI</i>)	2.0	HupI protein	...	...
bll6939 (<i>hupD</i>)	2.2	HupD protein	...	...
bll2757 (<i>fixK2</i>)	-23.7	Transcriptional regulatory protein	...	...
blr2763 (<i>fixN</i>)	-4.2	Cytochrome c oxidase	...	...
blr2764 (<i>fixO</i>)	-2.3	Cytochrome c oxidase	...	...
bsr2765 (<i>fixQ</i>)	-2.4	cbb3 oxidase subunit IV	...	...
		Cellular processes	16	31
blr2144	2.5	Cytochrome P-450 BJ-1	...	...
blr2145	3.9	Cytochrome P-450 BJ-3	...	...
blr2147	6.3	Cytochrome P-450 BJ-4	...	...
blr1853	21.0	Cytochrome P450 family protein	...	...
bll6537	2.8	Putative cytochrome P-450	...	...
bll1800 (<i>rhcV</i>)	-2.1	RhcV protein	...	...
blr1813 (<i>rhcJ</i>)	-10.9	RhcJ protein	...	...
blr1816 (<i>rhcN</i>)	-2.0	RhcN protein	...	...
blr1818 (<i>rhcQ</i>)	-4.0	RhcQ protein	...	...
blr1819 (<i>rhcR</i>)	-3.3	RhcR protein	...	...
		Energy metabolism	15	35
blr1171 (<i>coxA</i>)	-2.1	Cytochrome C oxidase subunit I	...	...
blr1378 (<i>etfL</i>)	-5.9	Electron transfer flavoprotein large subunit	...	...
blr7544 (<i>cycA</i>)	-2.9	Cytochrome c550	...	...
bll5913 (<i>cycC</i>)	-2.7	Cytochrome C	...	...
blr1656	-10.2	<i>Endoglucanase</i>	...	...
	...	Amino acid biosynthesis	5	14
	...	Biosynthesis of cofactors, prosthetic groups, and carriers	10	6
	...	Cell envelope	7	18
	...	DNA replication, recombination, and repair	8	3
	...	Fatty acid, phospholipid and sterol metabolism	8	9
	...	Purines, pyrimidines, nucleosides, and nucleotides	4	8
	...	Regulatory functions	55	20
	...	Transcription	8	0
	...	Translation	7	23
	...	Transport and binding proteins	22	40
	...	Other categories (e.g., symbiosis, transposon, etc.)	84	75
bsl2015 (<i>nolZ</i>)	8.4	NolZ protein	...	...
bll2016 (<i>nolY</i>)	2.8	NolY protein	...	...
blr2062 (<i>noeI</i>)	3.3	Nodulation protein	...	...
blr1752	-6.3	<i>similar to NopP</i>	...	...
		Hypothetical protein	381	268
blr1649	-7.3	<i>Unknown</i>	...	...
blr1806	-10.6	<i>Similar to blr1806 (77% identity)</i>	...	...
blr1862	-5.7	<i>Unknown</i>	...	...
		Total	661	573

^a Differentially expressed genes were selected based on twofold cut-off with *q* value ≤ 0.05 .

^b Positive and negative values indicate up- and downregulation, respectively, in bacteroids.

^c Functional groups and annotations (gene description) represent the first and third levels, respectively, among three different levels based on the RhizoBase annotation. Italic letters indicate proteins that are likely to be secreted through a *Bradyrhizobium japonicum* type III secretion system (TTSS) induced by genistein. Annotation in italic letters was adopted from the previous study (Suss et al. 2006). Rhc proteins belong to *B. japonicum* TTSS machinery.

Consistent with downregulation of chemotaxis- and flagella-related genes by salt stress in *Shewanella oneidensis* MR-1 (Liu et al. 2005), *Bacillus subtilis* (Budde et al. 2006), and *Sinorhizobium meliloti* (Ruberg et al. 2003), the expression of 29 genes encoding motility proteins decreased in the presence of 50 mM NaCl (Table 3). Furthermore, functional analyses indicated that *Bradyrhizobium japonicum* had reduced motility in the presence of 50 mM NaCl (data not shown). This appears to be a general bacterial response to NaCl. *E. coli*, *Shewanella oneidensis*, and *Bacillus subtilis* all showed decreased motility when grown under conditions of high salinity (Li et al. 1993; Liu et al. 2005; Steil et al. 2003). The repression of motility may represent a strategy to survive a stressful condition by allowing for energy conservation to possibly produce stress-related proteins.

Free-living cells grown in rich medium versus bacteroids.

To address gene expression changes in the symbiotic state, we compared transcript levels between cultured cells and bacteroids. A total of 1,234 genes, representing approximately 15% of the genome, were differentially expressed at a twofold cut-off. In bacteroids, expression of 661 and 573 genes were induced and repressed, respectively, relative to those in free-living conditions (Table 4). Recent transcriptional profiling studies of *Sinorhizobium meliloti* showed that 63% more genes were downregulated in bacteroids than upregulated (up, 345 genes; down, 943 genes) (Barnett et al. 2004). In contrast, there were 74% more upregulated genes in the transcriptome of *M. loti* bacteroids than in free-living cells (up, 99; down, 26), although a more stringent fold cut-off (20-fold) was used compared with that used for *Bradyrhizobium japonicum* and *S. meliloti* (Uchiumi et al. 2004).

As expected, 22 genes involved in nitrogen fixation were highly induced in the bacteroid state (Table 4; Supplemental Table 4). Numerous *nif* and *fix* genes, but not all, appeared to be upregulated in bacteroids. This result is consistent with microarray studies done using *S. meliloti* and *M. loti* bacteroids (Barnett et al. 2004; Becker et al. 2004; Uchiumi et al. 2004). Genes involved in hydrogen uptake also were upregulated (Table 4), supporting the notion that *B. japonicum* recycles hydrogen molecules generated by nitrogenase activity to increase nitrogen fixation efficiency (White 2000b). Unexpectedly, several nodulation genes—*bsl2015* (*nolZ*), *bln2016* (*nolY*), *blr2027* (*nodC*), *blr2062* (*noeI*), and *blr2073* (*noeE*)—also were induced up to eightfold in bacteroids. Perhaps this result is not surprising, because expression of 10 nodulation genes also was found to be induced in *S. meliloti* bacteroids (Becker et al. 2004). It will be interesting to ascertain if these genes possess a secondary function involved in bacteroid differentiation or metabolism.

Given the sensitivity of all nitrogenases to oxygen, the nodule environment is relatively microaerobic. Despite oxygen limitation in bacteroids, four genes encoding for cytochrome P-450 enzymes, which function to bind and activate oxygen for transfer (Appleby et al. 1975; Keister et al. 1999), appeared to be induced up to 21-fold in bacteroids (Table 4). *B. japonicum* bacteroids may utilize these enzymes for respiration under the oxygen-limited conditions. These results paradoxically suggest that, although free oxygen in nodules is low (18 nM) (Layzell et al. 1990), cytochromes P-450 facilitate sufficient electron transfer through their high O₂ affinity for respiration to generate energy, which eventually is consumed for nitrogen fixation. A mutant study of cytochromes P-450, however, showed no defect in symbiosis phenotype (Tully and Keister 1993), suggesting that the P-450s are not essential in symbiotic function, but rather play a role in microaerobic environments (Daniel and Appleby 1972). Alternately, the cytochrome P-450s may be using an alternate electron acceptor in the bacteroid state.

Interestingly, a number of transcriptional regulatory genes, including two-component response regulators, were differentially expressed in bacteroids and, of these, 55 genes were upregulated and 20 genes were downregulated. The expression of more regulatory genes in nodules suggests that the plant environment is more complex than that found in laboratory culture medium. More interestingly, the greatest decrease (−23.7 fold) in gene expression was observed for *bln2757* (*fixK₂*) whose product is known to play a crucial role in the FixLJ-FixK₂ regulatory network by positively regulating a large group of genes, including *fixK₁*, *nnrR*, *hemABN₁N₂*, *hupSL*, *fixNOQP*, and *fixGHIS*, under anaerobic or microaerobic conditions (Fischer 1994; Hauser et al. 2006). Concomitant with the decreased expression of *fixK₂*, there was a corresponding decrease in gene expression of *fixNOQ* in bacteroids (Table 4). Conversely, a partial genome expression analysis of the *B. japonicum* symbiotic gene region revealed induction of *fixK₂* (9.5-fold) and its associated genes under low oxygen conditions (Hauser et al. 2006). These contrasting results suggest that the nodule environment is quite different from the microoxic condition, although oxygen limitation is a characteristic of the former in the context of symbiosis. Thus, what is known about the regulation of genes under microaerobic conditions might not apply directly for genes expressed in bacteroids.

Transcriptional analyses revealed that 22 and 40 transport-related genes were up- and downregulated, respectively, in bacteroids. Although many ABC transporter genes were identified, the decrease in expression of genes *bln1800* (*rhcV*), *blr1813* (*rhcJ*), *blr1816* (*rhcN*), *blr1818* (*rhcQ*), and *blr1819* (*rhcR*), whose products belong to the type III secretion system (TTSS), was of interest due to their importance for the establishment of the symbiosis with soybean. Mutational studies on the type III gene clusters (*tts*) of *B. japonicum* (Krause et al. 2002), *Rhizobium* sp. strain NGR234 (Vipey et al. 1998), and *S. fredii* (Bellato et al. 1997; Meinhardt et al. 1993) revealed that TTSS mutants are differentially affected in their ability to form nodules, depending on their host plants. Additionally, Krause and associates (2002) reported that a *B. japonicum* *ttsI* encoding the TtsI protein is essential for gene expression in the *tts* cluster and is activated by NodD1 and NodW in the presence of genistein, a soybean isoflavonoid which can induce *nod* gene expression. This suggests that a coordinated regulatory linkage between *nod* genes and the TTSS gene cluster may be important not only for Nod factor production but also for the secretion of extracellular proteins via the TTSS, leading to initiation of the reciprocal interaction between both partners. Indeed, Suss and associates (2006), using a proteomic approach, identified eight genistein-inducible extracellular proteins in *B. japonicum* that likely are secreted by the TTSS. Interestingly, five of the genes (*blr1649*, *blr1656*, *blr1752*, *blr1806*, and *bln1862*) encoding these extracellular proteins also were shown to be downregulated up to 10-fold in bacteroids (Table 4). This suggests that the secretion of extracellular proteins via the TTSS may play a role only during the initial phases of nodule initiation. In *Rhizobium* sp. strain NGR234, however, it is unclear whether TTSS genes are repressed in nodules, although both *nod* and TTSS genes are induced by flavonoids (Vipey et al. 1998).

Conclusions.

The purpose of this study was to develop and evaluate a relatively low-cost, whole-genome DNA microarray system for *B. japonicum*. The arrays are available to the public at cost and can be purchased by accessing our project website (Author-Recommended Internet Resources). The documented gene expression changes in *B. japonicum* under conditions of nutrient limitation and osmotic stress, and in the bacteroid state, illustrate that *B. japonicum* apparently responds differently from *S. meliloti*. This

may be due, in part, to the fact that the *B. japonicum*–soybean symbiosis leads to the formation of determinate root nodules, whereas the host plants of *S. meliloti* develop indeterminate nodules. In addition, overall genome organization and physiology of the two microsymbionts differ greatly. Although it has been demonstrated that plant factors provoke bacteroid differentiation in cool-season legumes (e.g., *Medicago* spp.) (Mergaert et al. 2006), our findings suggest that the different fate of nodule development could be due to the bacterial genetic ability and regulatory network in response to different environmental conditions. For example, unlike *S. meliloti*, *B. japonicum* responds to nutrient limitation by reducing expression of the translational apparatus and increasing expression of vitamin biosynthesis in minimal medium. Analysis of the transcriptional response of *B. japonicum* to treatment with NaCl revealed a lack of expression of pathways for accumulation or synthesis of compatible solutes or osmoprotectants, as well as a lack of apparent transporters for the uptake of such compounds. Gene expression analysis of *B. japonicum* bacteroids revealed that, as expected, the majority of the *nif* genes were highly expressed. Curiously, regardless of the low-oxygen environment, cytochrome P-450 genes are expressed and presumably play a role in metabolism, although they are not essential based on previously published mutant studies (Tully and Keister 1993). The *B. japonicum* TTSS system is clearly repressed in bacteroids, suggesting that this system likely functions only during initial establishment of the symbiosis. Taken together, our results show that the developed microarrays are a useful tool for functional genomic studies, and likely will prove to be a valuable community resource for those who study this agriculturally important symbiotic bacterium.

MATERIALS AND METHODS

Construction of the *B. japonicum* DNA microarray.

A whole-genome DNA microarray for *B. japonicum* strain USDA110 was constructed starting from the genome sequence published by Kaneko and associates (2002). Before microarray construction, we reexamined the available sequence data to predict all possible ORFs. Gene models from GeneMark and Glimmer2 (Author-Recommended Internet Resources) were used to generate a nonoverlapping unique list of all predicted ORFs, and unique 70-mer oligonucleotides were designed and synthesized for each of the predicted ORFs by Operon Biotechnologies, Inc. (Huntsville, AL, U.S.A.). Oligonucleotides were printed onto Corning epoxy-coated slides (Corning Inc., Lowell, MA, U.S.A.) at the Washington University Genome Sequencing Center (St. Louis). Each slide contained 8,453 predicted ORFs from the *B. japonicum* genome annotation and 36 control genes, printed in duplicate. More detailed information about the array is available at our project website (Author-Recommended Internet Resources). Slides also can be ordered, for a modest cost, via this website.

Bacterial growth and media.

B. japonicum strain USDA110 was used in all microarray experiments. Cells were grown at 30°C with shaking at 200 rpm, in minimal medium containing 0.3 g of K₂HPO₄, 0.3 g of KH₂PO₄, 0.5 g of NH₄NO₃, 0.1 g of MgSO₄ · 7H₂O, 0.05 g of NaCl, 4 ml of glycerol, and 1 ml of trace elements (Bishop et al. 1976) per liter of deionized water. Rich medium consisted of minimal medium amended with 0.5 g of yeast extract and 1 ml of vitamin stock (Bishop et al. 1976) per liter. For osmotic stress treatments, 50 mM NaCl was added to the rich medium. Cells grown in rich medium were used for free-living conditions, whereas bacteroids were harvested from nodulated plants as described below.

Plant growth and bacteroid isolation.

Soybean plants (*Glycine max* L. cv. Williams) were inoculated with *B. japonicum* USDA110, and grown in autoclaved Leonard jar assemblies containing a nitrogen-free nutrient solution (Karr et al. 1984). Seed were sown in equal parts of sand and vermiculite, both were washed thoroughly prior to planting, and plants were grown in the greenhouse with day and night temperatures of 28°C for 16 h and 20°C for 8 h, respectively, and 48 to 90 Klux of light for day. Nodules from 4-week-old plants were removed from the roots and immediately placed in ice water, and bacteroids were isolated and purified from nodules by using a sequential sucrose gradient method as previously described (Green and Emerich 1997). The gradient consisted of 42 and 57% (wt/wt) sucrose in MEP buffer (5 mM MgCl₂, 1 mM EDTA, and 50 mM potassium phosphate buffer, pH 7.0). RNA was isolated as described for free-living *B. japonicum*.

RNA isolation.

A 250-ml culture of *B. japonicum* USDA110 was grown to mid-log phase, optical density at 600 nm = 0.4 to 0.6, and immediately transferred to centrifuge tubes containing 25 ml of cold stop solution. The suspension was centrifuged for 10 min at 4°C at 9,700 × g, and cell pellets were frozen immediately in liquid nitrogen and stored at –80°C until RNA isolation. RNA was isolated using a hot phenol method as described previously (Bittner et al. 2003). The RNA samples were treated with DNase I (10 units, Promega Corp., Madison, WI, U.S.A.) and RNase inhibitor (40 units, Promega Corp.) for 30 min at 37°C, followed by phenol-chloroform extraction and column purification using the RNeasy Mini Kit (Qiagen, Germantown, MD, U.S.A.). The integrity of all RNA samples was confirmed on 0.8% agarose gels.

cDNA synthesis and hybridization.

Experiments to test microarray performance were done at the Genome Sequencing Center at Washington University. Initial studies compared transcription profiles of *B. japonicum* cells grown in minimal and rich medium. cDNA synthesis, hybridization, and scanning were performed as described on the center website (Author-Recommended Internet Resources). Raw data were obtained and analyzed as indicated below. Subsequent experiments were done to compare transcriptomes of cells grown with and without osmotic stress, or free-living cells versus bacteroids. These studies were done at the University of Minnesota and the University of Missouri, respectively. cDNA was synthesized and labeled as described previously (Bittner et al. 2003). Briefly, 20 µg of total RNA was used for cDNA synthesis with superscript III reverse transcriptase and random hexamers (Invitrogen Corp., Carlsbad, CA, U.S.A.). After digesting the remaining RNA by RNase H (Fermentas, Glen Burnie, MD, U.S.A.), cDNA was purified using a Microcon YM-30 column (Millipore, Bedford, MA, U.S.A.) and concentration was determined using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.). cDNA (4 µg) was used for labeling with either cy3 or cy5 (Amersham, Buckinghamshire, U.K.) and unincorporated dyes were removed using a Qiaquick PCR purification Kit (Qiagen).

Both cy3- and cy5-labeled cDNAs to be compared were mixed, dried to completion, and then resuspended in 70 µl of preheated hybridization buffer (nuclease-free water/formamide/20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]/1% sodium dodecyl sulfate [SDS] = 4:2.5:2.5:1) at 42°C. After adding 0.7 µl of salmon sperm DNA (10 mg/ml) to prevent nonspecific binding to the array, the mixture was hybridized at 42°C for 16 to 18 h. Hybridized arrays were washed with 1× SSC and 0.2% SDS at 42°C for 6 min and, subsequently, with

0.1× SSC and 0.2% SDS at room temperature for 6 min, and twice with 0.1× SSC at room temperature for 3 min. The arrays were scanned using an Axon GenePix 4000B scanner (Molecular Devices Corp., Sunnyvale, CA, U.S.A.).

Data analysis.

Signal intensities from six slides were analyzed using GenePix Pro 6.0 software (Molecular Devices Corp.) for minimal versus rich medium and free-living cells versus bacteroid comparisons, whereas those from four slides were analyzed for osmotic stress responses. Signal intensities were normalized for spot and slide abnormalities using spatial lowess, and then lowess-adjusted data subsequently were analyzed by mixed effect microarray analysis of variance (MAANOVA) (Kerr et al. 2000). The resulting variety–gene interaction values were combined with the residual noise from each spot to obtain the filtered and adjusted expression values (Kerr and Churchill 2001; Kerr et al. 2000). Both lowess and MAANOVA are part of the R/maanova microarray statistical analysis package (Author-Recommended Internet Resources). Expression values from two technical replicates in each array were combined, averaged, and subsequently subjected to significance analysis of microarray (SAM) using the SAM package (Tusher et al. 2001). Significant genes were selected based on a 1.5- or 2-fold cut off threshold with a false discovery rate lower than 5%.

qRT-PCR analysis.

To validate the microarray data, the expression of several genes was measured by qRT-PCR using the same RNA samples as used in the microarray experiments. Total RNA was reverse transcribed to generate cDNA, followed by quantitative PCR amplification using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) and gene-specific primers (Table 1) whose amplicon sizes were 80 to 120 bp. Genomic DNA contamination was checked with a control reaction mixture not containing reverse transcriptase. PCR reactions were monitored using an ABI 7500 Real Time PCR System with Sequence Detection System software (version 1.3; Applied Biosystems). PCR conditions were: 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. All expression values from two to three technical replicates of each biological replicate were normalized to the expression of *blt7457* (*hisS*) encoding histidyl t-RNA synthetase, a gene constitutively expressed under all conditions used.

Microarray data availability.

All of the microarray data have been deposited in Gene Expression Omnibus (GEO) (Author-Recommended Internet Resources) and are accessible through GEO series accession numbers GSE8033, GSE8036, and GSE8042 for rich versus minimal medium, osmotic stress, and free-living cells versus bacteroids, respectively.

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AUTHOR RECOMMENDED INTERNET RESOURCES

Bradyrhizobium Microarray Initiative website: www.bradygenome.org
 Gene Expression Omnibus (GEO) website: www.ncbi.nlm.nih.gov/geo/
 GeneMark website: exon.gatech.edu/GeneMark
 The Genome Sequencing Center at Washington University: genome.wustl.edu/services/microarray.cgi
 Glimmer2 website: www.cbcb.umd.edu/software/glimmer
 The Jackson Laboratory statistical analysis website: www.jax.org/staff/churchill/labsite/
 Kazusa DNA Research Institute RhizoBase website: www.kazusa.or.jp/rhizobase