Sinorhizobium meliloti Differentiation During Symbiosis with Alfalfa: A Transcriptomic Dissection

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Sinorhizobium meliloti is a soil bacterium able to induce the formation of nodules on the root of specific legumes, including alfalfa (*Medicago sativa*). Bacteria colonize nodules through infection threads, invade the plant intracellularly, and ultimately differentiate into bacteroids capable of reducing atmospheric nitrogen to ammonia, which is directly assimilated by the plant. As a first step to describe global changes in gene expression of *S. meliloti* during the symbiotic process, we used whole genome microarrays to establish the transcriptome profile of bacteria from nodules induced by a bacterial mutant blocked at the infection stage and from wild-type nodules harvested at various timepoints after inoculation. Comparison of these profiles to those of cultured bacteria grown either to log or stationary phase as well as examination of a number of genes with known symbiotic transcription patterns allowed us to correlate global gene-expression patterns to three known steps of symbiotic bacteria bacteroid differentiation, i.e., invading bacteria inside infection threads, young differentiating bacteroids, and fully differentiated, nitrogen-fixing bacteroids. Finally, analysis of individual gene transcription profiles revealed a number of new potential symbiotic genes.

The ability to measure simultaneously the expression level of thousands of genes at the transcription level has made microarrays a tool of choice for studying host-microbe interactions. Several studies have thus reported changes of gene expression occurring both in the host and the infecting agent in various host-microbe systems (Bryant et al. 2004; Conway and Schoolnik 2003; Hinton et al. 2004; Wan et al. 2002). A major difficulty arising from these reports is that RNA samples are often complex mixtures, originating either from both host and microbe or from microbe cells at various stages of the infection process with potentially different gene-expression profiles, thus complicating the interpretation of the results. To clearly describe the temporal variation of gene expression during an infection process, each stage of the process needs to be studied independently.

The symbiosis between the gram-negative soil bacterium *Sinorhizobium meliloti* and its host legume *Medicago sativa* is

one example of these complex host-microbe systems. Symbiosis starts with a sophisticated developmental process during which both partners undergo differentiation in a concerted way. Flavonoids released in the rhizosphere by the plant induce in the bacteria the synthesis and export of lipochitooligosaccharides, the Nod factors. Nod factors, in turn, trigger the formation of new organs on the plant root, called nodules (Long 2001; Spaink 2000). Bacteria infect the developing nodules through tubules of plant origin, called infection threads, in which they divide and thus colonize the host tissues (Gage 2004). Bacteria at the tip of the infection thread are eventually released in the cytoplasm of plant cells through an endocytosis-like process. Once released, the bacteria, called bacteroids at this stage, start to differentiate (Oke and Long 1999a). Ultrastructural studies have described five discrete steps in bacteroid differentiation, each restricted to a defined histological zone of the nodule (Vasse et al. 1990). Type 1 bacteroids are dividing bacteria recently released from infection threads and are found in the distal part of zone II (infection zone); type 2 bacteroids elongate without dividing and are found in the proximal part of zone II; type 3 bacteroids have stopped elongation and are found in interzone II-III, where they fill most of the host cells; type 4 bacteroids are the same size as type 3 bacteroids but are found in cells of distal zone III (fixation zone) and are characterized by their ability to fix nitrogen; and finally, type 5 bacteroids, which no longer fix nitrogen, are found in the proximal part of zone III.

Thanks to the availability of the whole *S. meliloti* genome sequence (Galibert et al. 2001), dedicated macro- and microarrays carrying a limited number of *S. meliloti* genes have been constructed and used to determine the partial transcriptome of bacteria grown in various symbiotic and nonsymbiotic conditions (Ampe et al. 2003; Barloy-Hubler et al. 2004; Bergès et al. 2003). However, these reports did not give a comprehensive view of the global changes in gene expressions, in particular, those experienced by bacteria in the nodules vs. free-living conditions. Whole-genome macro- and microarrays were more recently constructed and were used to determine the complete transcriptome of *S. meliloti* in a number of free-living conditions, sometimes directly relevant to symbiosis (for example, addition of luteolin or oxygen limitation; Barnett et al. 2004; Becker et al. 2004; Capela et al. 2005; Chao et al. 2004; Davalos et al. 2004; Hoang et al. 2004; Krol and Becker 2004; Puskas et al. 2004; Rüberg et al. 2003), but only two of them addressed this question directly on symbiotic bacteria isolated from *Medicago* nodules (Barnett et al. 2004; Becker et al. 2004). Among other conclusions, the latter studies revealed a profound modification of bacterial gene expression in planta as compared with in vitro conditions. However, these data were limited due to the fact that entire mature nodules were used, and therefore,

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the RNA preparations contained a mixture of bacterial RNAs from different stages of nodule invasion (discussed above). Obviously, more refined studies are needed to characterize the separate transcription profiles of invading, differentiating, and nitrogen-fixing bacteria, in order to reveal specific changes in individual gene expression during the symbiotic process.

In this work, as a first step toward this goal, we have investigated the global transcription pattern of *S. meliloti* at different stages of the symbiosis, using whole-genome microarrays. For this purpose, we have used bacterial RNA preparations from wild-type nodules, harvested at various timepoints after inoculation, or bacterial RNA from nodules induced by a bacterial mutant blocked at an early stage of the symbiotic process. Comparison of these transcriptomes to those of cultured bacteria grown either to log or stationary phase allowed us to correlate global gene-expression patterns to known steps of symbiotic bacteria bacteroid differentiation. Examination of individual gene-expression patterns confirmed the known expression patterns of selected genes, thus further validating this approach, and revealed a number of new putative symbiotic genes.

RESULTS

Overall bacterial gene expression is strongly down-regulated inside infection threads.

Before internalization in plant cells and differentiation into bacteroids, *S. meliloti* enters and invades the developing nodule through tubules of plant origin called infection threads. Bacterial mutants have been isolated in which bacteria colonize plants through normal infection threads and are released in plant cells but then rapidly degenerate without further differentiating. Thus, all bacteria present in nodules induced by the *S. meliloti bacA* mutant on alfalfa are contained inside infection threads (Glazebrook et al. 1993). We took advantage of this mutant to determine the transcriptome profile of bacteria contained inside infection threads. RNA was prepared from alfalfa (*M. sativa* Europe) nodules induced by a *S. meliloti bacA* mutant and was compared with RNA from wild-type bacteria cultivated to mid-log phase in Vincent minimal medium (Table 1, experiment 1). As RNA material extracted from nodules is a mixture of bacterial and plant RNAs, the proportion of RNA from both origins was systematically measured, using Agilent RNA chips in order to label equivalent amounts of bacterial RNA from nodules and from cultured bacteria. The average of four hybridization experiments is shown in Figure 1A.

The global shape of the scatter plot appeared unusual, showing a large number of down-regulated genes in *bacA*-elicited nodules as compared with bacteria cultivated to mid-log phase. This particular shape is partly due to the fact that many genes in nodules produce a hybridization signal below the detection threshold and, thus, scatter around the horizontal line corresponding to the background value. Indeed, only approximately

50% of the genes showed a signal significantly (i.e., three times) higher than the background in nodules, whereas more than 80% displayed a significant signal in culture (Table 1, experiment 8). Moreover, most of the genes expressed above background in nodules are expressed at a lower level than in culture. This transcriptome profile was not due to the plant used, as a similar pattern was observed with another ecotype of alfalfa (*M. sativa* Sitel, $n = 1$), as well as with *M. truncatula* ($n = 1$, data not shown). It seems not to be a secondary effect of the *bacA* mutation either, as the global transcription profiles of the *bacA* mutant in culture, in both log and stationary phases, were similar to those of the wild-type strain in the same growth phases ($n = 2$, data not shown).

The strong bias in the distribution toward the low values made it impossible to perform a valid normalization using a global method, and we were not able to precisely establish the number of differentially expressed genes. Nevertheless, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) performed on three randomly chosen down-regulated genes (*hemA*, *rplM*, *rpsA*) confirmed a strong repression (7- to 11-fold) in nodules as compared with cultured cells (Table 2). We therefore conclude from these experiments that the overall gene expression in bacteria contained in infection threads is strongly down-regulated.

The bacterial transcriptome profile changes during nodule development.

After the initial invasion of nodule tissues through infection threads, bacteria are released into plant cells of the inner root cortex. The bacteria, surrounded by a membrane of plant origin and now called bacteroids, then differentiate into nitrogen-fixing organelles. To determine the transcriptomic pattern of bacteroids at various stages of their differentiation, we harvested alfalfa nodules at various timepoints after inoculation by wild-type *S. meliloti* cells.

We first harvested nodules as soon as they appeared on the roots, i.e., 5 days postinoculation (dpi) under our conditions. These nodules were small and round-shaped and did not fix nitrogen, as evidenced by their white color and their inability to reduce acetylene (data not shown). Nevertheless, *fix* and *nif* genes were more strongly transcribed in these ineffective nodules than in the control culture, showing that bacteroid differentiation had already been initiated in these nodules, as previously described by Vasse and associates (1990). Induction of *nifA*, *nifH*, and *fixK* in these nodules was confirmed by qRT-PCR (Table 2). Interestingly, overall gene expression was found to be higher in 5-day-old nodules than in infection-thread bacteria, and the transcription profile of bacteria from 5-day-old nodules closely resembled that of mid-log phase bacteria (Fig. 1B, Table 1, experiment 2).

We subsequently harvested nodules at 8 and 14 to 18 dpi. At these stages, the nodules were elongated, pink, and displayed

Table 1. Hybridization experiments

Experiment ^a	<i>n</i> ^b	Bacterial strain	Condition	Genes detected ($\pm$ SD) ^c	Genes with $M > 3$, $P < 0.01$, FDR < 0.01
1	4	VO2119($\Delta bacA$)	<i>Medicago sativa</i> nodules 11 dpi	3,119 ($\pm$ 430)	133
2	5	Rm1021 (wt)	<i>M. sativa</i> nodules 5 dpi	4,336 ($\pm$ 542)	59
3	4	Rm1021 (wt)	<i>M. sativa</i> nodules 8 dpi	2,409 ($\pm$ 1865)	108
4	4	Rm1021 (wt)	<i>M. sativa</i> nodules 14-18 dpi	3,303 ($\pm$ 985)	91
5	4	Rm1021 (wt)	Late log phase (T2)	5,574 ($\pm$ 288)	0
6	4	Rm1021 (wt)	Early stationary phase (T4)	4,821 ($\pm$ 435)	50
7	4	Rm1021 (wt)	Late stationary phase (T6)	3,233 ($\pm$ 858)	1
8	29	Rm1021 (wt)	Mid log phase	5,056 ($\pm$ 859)	—

^a Experiment # 8 corresponds to the control used in all hybridizations.

^b *n* = number of hybridizations per experiment.

^c Statistics for the different hybridization slides. A gene was considered as detected on a slide when the corresponding signal was more than three times the local background signal. SD = standard deviation.

nitrogenase activity (not shown). Bacteria from 14- to 18-day-old nodules displayed transcriptome profiles indistinguishable from those of 8-day-old nodules (Figs. 1 and 2 and data not shown). In contrast to 5 dpi nodules, bacteria from older nodules displayed a lot of repressed genes as compared with mid-log phase bacteria, with only approximately 40 to 50% of genes showing expression above background levels (Table 1, experiments 3 and 4; Fig. 1C and D). The same observation was made with 18-dpi nodules from another ecotype of *M. sativa* (*M. sativa* Sitel; *n* = 1) and from *M. truncatula* (*n* = 1; not shown).

Taken together, these data reveal that harvesting nodules at two different timepoints (5 and 8 dpi or more) makes it possible to isolate bacteria with different transcriptome profiles that, we postulate, reflect distinct stages of bacteroid differentiation.

Downregulation of *S. meliloti* genes in nodules could result from growth arrest.

To determine whether the downregulation of bacterial genes described above was due to the growth status of bacteria in the nodules, we tested how the global gene-expression pattern of free-living *S. meliloti* varied according to the growth phase of the culture. For this purpose, wild-type bacterial cells were cultivated in Vincent minimal medium and samples were collected at various timepoints of the growth curve (Fig. 3A). Total RNA was prepared and samples were harvested in late log phase (T₂), early stationary phase (T₄), and late stationary phase (T₆) were subjected to hybridization using, as a reference, the sample harvested in mid-log phase (T₀). The average of four hybridization experiments is shown in Figure 3B through D.

Fig. 1. Comparison of *Sinorhizobium meliloti* transcriptomes in culture and in planta. The scatter plots show the log₂ of the signals obtained for the experimental condition (y axis) vs. wild-type bacteria grown to mid-log phase in Vincent minimal medium (x axis) taken as reference. Experimental conditions were as follows. **A**, *bacA* mutant bacteria from 11-day-old *Medicago sativa* nodules. **B**, Wild-type bacteria (Rm1021) from 5-day-old *M. sativa* nodules. **C**, Wild-type bacteria (Rm1021) from 8-day-old *M. sativa* nodules. **D**, Wild-type bacteria (Rm1021) from mature *M. sativa* nodules.

Table 2. Validation of microarray results by quantitative reverse transcription-polymerase chain reaction (RT-PCR)^a

Gene	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	M _A	M _B	M _A	M _B	M _A	M _B	M _A	M _B
<i>hemA</i>	-0.94	-3.10	0.57	-0.16	-0.94	-1.53	-1.57	-1.07
<i>rplM</i>	-3.67	-2.76	-2.12	-0.37	-2.92	-3.74	-3.96	-3.63
<i>rpsA</i>	-3.61	-3.45	-1.97	-0.13	-2.66	-2.85	-3.36	-3.56
<i>smc01774</i>	6.83	8.73	3.17	5.38	3.00	1.77	2.04	1.28
<i>smc02156</i>	2.62	2.55	0.60	1.71	-1.38	-1.79	-2.68	-2.01
<i>smc00885</i>	5.46	7.22	2.87	5.03	1.88	2.38	1.70	1.77
<i>fixK</i>	4.41	9.13	2.42	7.40	7.34	10.27	6.39	9.55
<i>nifA</i>	2.10	3.71	2.34	4.32	8.27	9.48	7.37	9.37
<i>nifH</i>	-0.21	6.01	3.35	8.56	8.52	16.48	8.75	15.02
<i>sma0806</i>	-1.85	3.41	-1.24	4.78	4.96	5.56	2.98	3.33
<i>smb20014</i>	6.59	0.25	7.51	1.31	8.74	0.28	8.56	1.03
<i>smb20139</i>	1.78	-0.32	0.51	1.26	4.05	-1.40	3.72	-1.15
<i>smb21139</i>	4.72	-2.30	7.10	0.84	8.14	-1.57	6.39	-1.31

^a M values (log₂ ratios experiment/control) from microarrays (M_A) and from quantitative RT-PCR (M_B) for the indicated genes and experiments. Values in bold type represent strong discrepancies.

As long as bacteria grew exponentially (T_0 , T_2), approximately 90% of the *S. meliloti* genes yielded a significant hybridization signal (Table 1, experiment 5; Fig. 3B). As soon as growth slowed down, the global hybridization pattern changed dramatically and resembled those of *bacA* and wild-type old nodules (discussed above), with a large number of repressed genes; the expression of only 78% of genes was still detectable in the very early stationary phase (T_4), and this number dropped to 52% in late stationary phase (T_6) (Table 1, experiments 6 and 7; Fig. 3C and D).

Fig. 2. Hierarchical clustering of conditions and changes in gene expression. Cluster analysis of the 235 genes induced ($M > 3$; $P < 0.01$, FDR < 0.01) in at least one of the four following conditions. *bacA*-induced *Medicago sativa* Europe nodules, wild-type *M. sativa* Europe nodules at 5, 8, and 14 to 18 days postinoculation (dpi). A total of 21 individual hybridizations were analyzed, including single experiments performed on *M. sativa* Sittel and *M. truncatula*, and are indicated at the bottom as BMSE (*bacA-M. sativa* Europe), BMSS (*bacA-M. sativa* Sittel), BMT (*bacA-M. truncatula*), W5MSE, W8MSE, W18MSE (wild type-*M. sativa* Europe at 5, 8, and 14 to 18 dpi, respectively), W18MSS (wild-type-*M. sativa* Sittel), W18MT (wild type-*M. truncatula*) followed by the hybridization number, when applicable. The color coding of $\log_2$ ratios is shown on the top left, and the cluster numbers are indicated on the tree.

Among the genes most highly down-regulated upon entry into the stationary phase are those encoding proteins of the translation machinery (ribosomal proteins, translation factors) and the transcription machinery (subunits of the RNA polymerase, transcription factors), which is consistent with global downregulation of gene expression.

Two proteomic studies have previously reported changes in stationary phase (Chen et al. 2003; Guerreiro et al. 1999), and both report around 50 proteins up-regulated in this condition. Among the 50 proteins identified by Chen and associates (2003), 16 are encoded by genes up-regulated more than two-fold during the log to stationary phase transition in the present study. In addition, we confirm the induction, in early stationary phase, of genes known to be up-regulated under starvation conditions (*hmgA*, *ndiA*) (Davey and de Bruijn 2000; Milcamps and de Bruijn 1999).

These data reveal that the transcriptome of *S. meliloti* is strongly affected during the log to stationary phase transition, a large number of genes being down-regulated upon growth arrest. We therefore assume that the strong reduction of gene expression observed in some bacteria extracted from nodules results from their growth arrest.

Hierarchical cluster analysis of individual conditions and gene-expression patterns.

As part of our systematic comparison of the expression patterns of bacteria from nodules with those of cultured bacteria, we attempted to identify the genes differentially expressed (induced or repressed) under these conditions. However, the strong difference in gene-expression levels between mid-log phase bacteria and bacteria from infection threads or from mature nodules made normalization difficult and rendered the precise identification and quantification of the genes induced or repressed in cultured cells versus nodules impossible. Therefore, we focused on the genes unambiguously induced in nodules in comparison with cultured bacteria instead ($M > 3$, $P < 0.01$, false discovery rate [FDR] < 0.01) and examined the variations of individual gene expression during the symbiotic process. We identified a set of 235 genes induced under at least one of four conditions, i.e., *bacA*-induced *M. sativa* Europe nodules and wild-type *M. sativa* Europe nodules at 5, 8, and 14 to 18 dpi.

A double hierarchical clustering was performed on these 235 genes and on 21 individual hybridizations, including single experiments performed using *M. sativa* Sittel and *M. truncatula* (Fig. 2). Independent hybridization experiments of the same condition clustered together, revealing a good reproducibility of the experiments. Hybridizations of wild-type nodules at 8 and 14 to 18 dpi were part of the same cluster, confirming the close relationship between these two conditions (discussed above). Two additional clusters were distinguished, one with 5-dpi wild-type nodules confirming the difference with 8-dpi wild-type nodules (discussed above) and one with *bacA*-induced nodules. Principal component analysis of these data led to the same conclusions. We assume that these three clusters of conditions represent three stages of the symbiosis that we will hereafter refer to as early (*bacA*), intermediate (5 dpi), and late (8 to 18 dpi).

Cluster analysis of the 235 genes revealed seven clusters of gene-expression profiles, three of them containing only one or two genes (clusters 5, 6, and 7). Therefore, most of the genes fall in one of four clusters. Subsequently, a K-means cluster analysis allowed us to refine the classification of 11 genes in one of four classes. Results of the hierarchical clustering are discussed below.

Two genes (cluster 7) were highly expressed under all conditions. However, the validity of this group is questionable,

since we could not confirm the strong induction of these two genes (*smb20014* and *smb21139*) by qRT-PCR (Table 2). This discrepancy may be due to cross-hybridization with nodule-specific plant cDNAs. In contrast, 233 genes were found to be more expressed in one or two particular stages of the symbiosis.

A total of 36 genes (cluster 2) were specifically induced in early stages of the symbiosis (Fig. 4A). The *nodAB* genes, involved in the synthesis of Nod factors, were found in this group. These genes were already known to be expressed to a higher level in the early steps of the symbiosis (Sharma and Signer 1990), thus supporting our data. qRT-PCR also con-

firmed the microarray results for two other genes of this group (*smc01774* and *smc00885*) (Table 2). As *nodAB* genes are required in early stages of the symbiosis (Jacobs et al. 1985), genes that cocluster with them are potential candidates for classification as new preinfection and infection genes. One of them is *ccmA*, encoding a protein involved in the maturation of cytochrome *c*. We constructed a *S. meliloti* *ccmA* mutant and observed that it has a *Fix*⁻ phenotype on *M. sativa*. Interestingly, a *Rhizobium phaseoli* cytochrome *c*-deficient mutant induced empty nodules on *Phaseolus vulgaris* (Soberón et al. 1993), supporting a role for *c*-type cytochrome in nodule in-

Fig. 3. Comparison of cultivated *Sinorhizobium meliloti* transcriptomes at various timepoints of the growth curve. **A.** Growth curve of one typical experiment, showing evolution of the optical density at 600 nm with time. Samples were harvested at the indicated timepoints (circled) named T_0 (mid-log phase), T_2 (late log phase), T_4 (early stationary phase), T_6 (late stationary phase). **B** through **D**, Scatter plots of the comparison of bacteria from samples T_2 , T_4 , and T_6 , respectively, with sample T_0 taken as reference.

Fig. 4. Hierarchical clustering of changes in gene expression. **A**, Cluster 2 and **B**, cluster 4. Gene names are indicated on the right. **B**, genes known to be under the control of *FixK* or *FixJ* or under the control of *NifA* (C. Bobik, E. Meilhoc and J. Batut, *unpublished data*) are indicated by * or #, respectively.

fection. Another class of 96 genes (cluster 1), although displaying a less-clear expression pattern, still had a tendency to be expressed to a higher level in early and intermediate stages than in late stages of the symbiosis.

A total of 101 genes appeared to be more highly expressed in late stages than in early stages of the symbiosis (clusters 3, 4, 5, and 6) (Fig. 4B). As expected, genes involved in the synthesis of the nitrogenase and in microoxic respiration, known to be under the control of the oxygen-sensing two-component system FixLJ and to be induced in nodules belong to these clusters (C. Bobik, E. Meilhac, and J. Batut, *unpublished data*). Interestingly, genes under the control of the FixJ-induced FixK regulator or under the direct control of FixJ clustered separately from genes known or supposed to be controlled by the other FixJ-induced regulator NifA (except *smc03253* and *nifE*, which clustered separately, and *fixU*, which was erroneously classified). This almost-exact correlation further validates our cluster analysis (Fig. 4B). It is likely that genes co-clustering with known NifA- or FixK-regulated genes also play an important role in late symbiotic stages.

DISCUSSION

In this paper, we report on a whole genome transcriptomic study of *S. meliloti* in planta, the objective of which was to identify the bacterial gene-expression profile at different stages of the symbiotic interaction with the lucerne *M. sativa*. For this purpose, we used bacteria from nodules harvested at various timepoints after inoculation and a bacterial mutant blocked at an early stage of the symbiosis.

A number of bacterial genes were found to be down-regulated in nodules. Interestingly, this strong downregulation was also observed for cultured bacteria upon entry into the stationary phase, suggesting that a strong, global reduction of gene expression is a hallmark of growth arrest. A similar observation has been recently reported for *Streptococcus pyogenes* (Beyer-Sehlmeyer 2005). The transcriptome profile of most nodule bacteria is in agreement with the nongrowing status of nitrogen-fixing bacteroids. In contrast, among the genes found induced upon entry into stationary phase, very few were found to be induced in nodules as well. This is consistent with the previous observation that the proteomic patterns of *S. meliloti* in stationary phase and in nodules share poor similarity (Djordjevic 2004; Djordjevic et al. 2003; Guerreiro et al. 1999).

Downregulation of gene expression in *bacA*-induced nodules suggests that bacterial growth is blocked inside infection threads. This observation is not related to the age of the nodules, as preliminary experiments using *bacA*-elicited nodules harvested only five days after inoculation gave similar results (data not shown). This growth arrest is surprising at first sight, given the expected dividing status of these invading bacteria. However, the growth dynamics of bacteria inside infection threads has been previously studied using bacteria tagged with fluorescent proteins (Gage 2002). That study clearly showed that only the bacteria near the tip of the infection thread grew actively while more distal bacteria did not grow. Our results are thus in complete agreement with those presented in that study. It should be noted at this point that the transcriptome of infection-thread bacteria has been determined using a bacterial mutant and that we cannot exclude that some of our observations are the direct consequence of the mutation and are not specific to infection-thread bacteria. Nevertheless, preliminary experiments performed with a plant mutant blocked at the infection stage (TE7; Bénaben et al. 1995) suggest that this is not the case.

In contrast to bacteria from infection threads, bacteria from young nodules (5 dpi) showed a transcriptome profile

typical of growing bacteria whereas bacteria from older nodules (8 dpi and more) displayed a transcriptome profile characteristic of nongrowing bacteria. From the ultrastructural study of nodules and differentiating bacteroids (Vasse et al. 1990), we know that 5-day-old nodules contain a mixture of infection-thread bacteria and bacteroids of types 1, 2, and 3, while older nodules differ by the appearance or accumulation or both of nitrogen-fixing type 4 bacteroids. We thus interpret the transcriptome profile of 5-day-old nodules as resulting mainly from type 1 and 2 bacteroids, which actively divide and elongate, respectively, and thus display a high level of gene expression similar to that of growing cells. The detectable expression of *fix* and *nif* genes in these nodules would result from type 3 bacteroids, as it is known that the transcription of these highly expressed genes starts at interzone II-III of mature nodules (Soupène et al. 1995), which only contains type 3 bacteroids, and is present in young nodules (Vasse et al. 1990). Hence, young nodules may represent a relatively complex stage in which up to three types of bacteroids may coexist. The reduction of overall gene expression in 8-day-old and older nodules would result from the relative accumulation of nongrowing type 4 bacteroids, which become the major type of bacteroids at this developmental stage. We therefore propose that we were able to determine the global transcriptome pattern of bacteroids at two successive stages of their differentiation.

We show here that a number of bacterial genes are down-regulated in mature nodules. In a previous study, we and others reached a different conclusion using the same type of samples (Becker et al. 2004). In that study, all genes gave detectable signals in the two channels, presumably because of a higher sensitivity of the microarrays used (based on PCR fragments instead of 70mer oligonucleotides in the present study). Moreover, the culture used as control by Becker and associates (2004) was harvested in the late-log phase, which could also explain the discrepancy with the present data. Nevertheless, in a recent study, Barnett and collaborators (2004) also reported a lower number of *S. meliloti* genes expressed in mature *M. truncatula* nodules than in cultured bacteria (Supplementary Table 6 in Barnett et al. 2004), in good agreement with our observations.

To validate our results, we performed qRT-PCR experiments on 13 genes having different expression profiles (Table 2; Fig. 5). Whereas expression of nine genes was confirmed by qRT-PCR, we observed some discrepancies between array and RT-PCR results for some genes or conditions or both. The reasons for this may be diverse. First, the number of RNA samples was not equivalent, only one for RT-PCR and four to five for microarrays. Also, the normalization method used in the two kinds of experiments was different (normalization by the mean in one case and by 16S rRNA in the other case) and thus could lead to different ratios. In addition, qRT-PCR is much more sensitive than array experiments. For some genes expressed below the background in some conditions, the array ratio may be underestimated. This was probably the case for *nifH* in *bacA* nodules, for which we found an induction as compared with cultured bacteria by qRT-PCR but not by microarray analysis. Some cross-hybridizations with other genes or spot contaminations may also occur in microarrays and can explain other discrepancies. This may be the case for *smb20139*, which was found to be expressed in late stages of symbiosis but was not validated by qRT-PCR. For two genes that are strongly induced during the symbiosis, *smb20014* and *smb21139*, we suspect the microarray signal to result from cross-hybridization with nodule-specific plant cDNAs. These results, therefore, highlight the importance of validating microarray results with alternative methods.

Interestingly, cluster analysis of our data is fully consistent with the known coregulation pattern of FixJ-dependent genes belonging to the FixK and NifA regulons, which validates our approach. From previous studies of the transcription profiles of FixJ-dependent genes in planta using *lacZ*-gene fusions, it was known that these genes were induced in interzone II-III and in zone III (Soupène et al. 1995), suggesting that they were not expressed in infection threads nor in bacteroids present in zone II. However, our results suggest an earlier activation of many FixK-dependent genes, which could result from a reduction of oxygen tension from the beginning of the infection process. This discrepancy could be due to a higher sensitivity of our detection system or to the fact that we detect transcripts whereas previous experiments had been carried out with translational fusions. Nevertheless, qRT-PCR confirmed the early induction of *fixK* (Table 2). In contrast, NifA-dependent genes appeared to be expressed to a higher level in late stages than in early stages of the symbiosis, which is in agreement with the known oxygen sensitivity of the NifA regulator (Fig. 4B). Induction of *nifA* and *nifH* was confirmed by qRT-PCR to be higher in late stages (Table 2).

Several reports have described the transcriptome and proteome of *S. meliloti* in various free-living conditions, including carbon, nitrogen, or phosphate limitation, osmotic or oxidative stresses, microaerobiosis, as well as transition to the stationary phase (Barloy-Hubler et al. 2004; Becker et al. 2004; Chen et al. 2003; Davalos et al. 2004; Djordjevic 2004; Djordjevic et al., 2003; Krol and Becker 2004; Rüberg et al. 2003). We compared all these data to genes found induced in nodules in the present paper, with the aim of identifying environmental conditions encountered by the bacteria in the different nodule compartments. Although expression of several genes identified in this study has also been observed in other conditions, we were not able to establish a good correlation between a given signal and a particular compartment of the nodule, apart from oxygen limitation that is clearly a signal for inducing gene expression during the symbiotic process. We assume that the addition of several signals, including conditions not yet tested in transcriptome experiments on free-living bacteria, is responsible for gene regula-

tion in nodules. Alternatively, the transcriptome profiles that we determined here could still be the superimposition of the transcription patterns of several other bacterial states that we were not able to isolate using our approach, as we suggest above for 5-day-old nodules, thus complicating the interpretation of the data. It should be noted that among the 233 genes upregulated in at least one nodule compartment, only 14 gene products have been identified in nodules by a proteomic approach and only six were found in nodules only. This poor overlap between the transcriptome and the proteome was already reported by Becker and associates (2004).

In conclusion, our study not only made possible the description at the global transcriptome level of various steps of bacterial life inside the nodule but also revealed genes induced in early and late stages of symbiosis. In each class, genes already known to be required for efficient symbiosis were identified. We assume that several genes identified here will turn out to be involved in the symbiotic process. Among them, 109 encode proteins with unknown functions, including 15 putative transcription regulators. We are presently testing the symbiotic phenotype of mutants of these candidate genes.

MATERIALS AND METHODS

Bacterial strains and in vitro growth conditions.

The bacterial strains used in this study were Rm1021 (Sm^R derivative of SU47; Meade et al. 1982) and its *ΔbacA::aadA* derivative VO2119 (Oke and Long 1999b). Cultures were grown in Vincent minimal medium, modified as described (Becker et al. 2004) at 30°C. For experiments shown in Figure 1, bacteria were grown until the optical density at 600 nm (OD₆₀₀) was =0.5 in a 1.5-liter bioreactor, as described by Becker and associates (2004). For timecourse experiments (Fig. 3), bacteria were grown to OD₆₀₀ = 0.5 (T₀) in a 1-liter flask containing 200 to 250 ml of medium at 30°C under agitation. Samples were collected at OD₆₀₀ = 0.4 to 0.5 (T₀), and at 3 (T₂), 6 (T₄), and 24 h (T₆) thereafter. In these conditions, the doubling time was approximately 160 min. Bacteria were rapidly harvested by filtration on 0.2-μm membranes and were frozen in liquid nitrogen and stored at -80°C until RNA extraction. Three independent timecourse experiments were performed (Table 3).

Plants and growth conditions.

Seeds of *Medicago sativa* cv. Europe or Sitel or of *M. truncatula* J6 were surface-sterilized and grown in aeroponic tanks as previously described (Ampe et al. 2003; Gallusci et al. 1991). Wild-type nodules were harvested 5, 8, 14, or 18 days after inoculation, as indicated in the text. For each time-point, all nodules (visibly at the same developmental stage) from all plants of the aeroponic tank were collected. *bacA*-induced nodules were harvested 11 days after inoculation. Nodules were frozen in liquid nitrogen and stored at -80°C until RNA extraction. A 1-g nodule sample was derived from approximately 15 to 20 plants for wild-type mature nodules

Fig. 5. Comparison of microarray and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) results. M values calculated from microarray and qRT-PCR experiments were plotted. Black points correspond to concordant results, whereas empty points correspond to discrepancies.

Table 3. Experimental design

Experiment	Cultures or tanks	RNA extractions ^a	Hybridizations
1	4	4	4
2	2	3	5
3	2	3	4
4	3	4	4
5	3	4	4
6	3	4	4
7	3	4	4

^a Each RNA extraction was performed from a single culture, or from a pool of nodules from a single tank.

or from approximately 30 to 40 plants for young wild-type or *bacA* nodules. Unless otherwise indicated, two to four independent aeroponic tanks were used for each condition (Table 3). Acetylene reduction tests were performed on isolated roots from aeroponic tanks, as described by Turner and Gibson (1980).

For plant assays of the symbiotic phenotype of the *S. meliloti ccmA* mutant, seeds of *M. sativa* cv. Europe were surface-sterilized, germinated and allowed to grow on nitrogen-free Fahreus medium in test tubes for three days. A total of 10 plants were inoculated with 2.10^3 cells of the *ccmA* mutant or the wild-type Rm1021 strain per plant. The nodulation kinetics were followed for 25 days, and then, the dry weight of plants was measured and compared to that of the wild-type strain. The whole test was performed twice.

RNA preparations.

Each RNA extraction was performed from a single culture or from a pool of nodules from a single tank as previously described (Cabanes et al. 2000 and Becker et al. 2004, respectively), followed by DNaseI treatment (Qiagen clean-up procedure, Qiagen, Hilden, Germany). For nodule bacteria, RNA samples were prepared after the bacteria were enriched by differential centrifugation as described (Cabanes et al. 2000), except for wild-type mature or 8-day-old nodules, for which RNA was sometimes prepared without bacterial enrichment. Bacterial enrichment changed the bacterial to plant RNA ratio but did not affect the quality of RNA, as determined using Agilent RNA nanochips and by comparing total vs. enriched RNA by microarray analysis (data not shown). Typical yields of RNA per gram of nodules were 300 to 600 µg without enrichment and 30 to 60 µg with enrichment. RNAs prepared from wild-type mature nodules contained an average of approximately 20% of bacterial RNA or 35% after bacterial enrichment. Young wild-type nodules or Fix^- nodules contained approximately 50% of bacterial RNA after enrichment. RNA was prepared from uninfected *M. truncatula* roots as described (El Yahyaoui et al. 2004).

Labeling, hybridizations, and analyses.

The amount of RNA from nodules to be labeled was adjusted in order to label the same amount of RNA as from the control culture (i.e., 10 to 20 µg of RNA from a mid-log phase culture). Cy3- and Cy5-labeled cDNAs were prepared according to DeRisi and associates (1997). For each experiment, unless indicated in the text, at least three independent RNA preparations, coming from three independent cultures or at least two independent aeroponic tanks were used, as described in Table 3.

The microarrays used for this study were described in Krol and Becker (2004) and were purchased from A. Becker (University of Bielefeld, Germany). They consist of glass slides, carrying mainly 70mer oligonucleotides representative of the 6,208 predicted open reading frames of *S. meliloti* spotted in triplicate, as well as a number of control spots (Krol and Becker 2004). Hybridizations were performed as described previously (Becker et al. 2004). Data were acquired on a GenePix 4000 scanner (Axon Instruments, Foster City, CA, U.S.A.), and quantifications of median signal and median local background intensities for each spot were performed using GenePix Pro 3.0 (Axon Instruments). Data analyses were carried out using Genesight 3.5 (Biodiscovery, Inc., El Segundo, CA, U.S.A.). In Table 1, signals were considered as significant when they exceeded the local background value by three times. All experiments were normalized using the mean of the signals. M ($\log_2$ ratio experiment/control) and P (t -test) values were calculated with Genesight. The FDR criterion of <0.01 to correct P values from the t -test was veri-

fied when $P(k) < 0.01 \times k/m$, with k being the rank of P values and m the total number of genes. A list of 57 genes was discarded from all analyses, as they were found to cross-hybridize with plant root cDNAs. As many genes were expressed at a level close to or lower than the background, hierarchical clustering and K means clustering of M values were performed with Genesight on the genes with $M > 3$, $P < 0.01$, and $\text{FDR} < 0.01$. For cluster analyses, missing M values were replaced with the average M value of all genes in the corresponding conditions.

qRT-PCR analyses.

Reverse transcription was performed on nodule RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and random hexamers as primers. A single RNA sample was tested for each condition. cDNAs were used for running real-time PCR on a LightCycler system (Roche, Mannheim, Germany), using the FastStart DNA Master^{PLUS} SYBRGreen I kit (Roche) according to the manufacturer's instructions. Every reaction was carried out in triplicate. 16S RNA was used as a reference for normalization. Genes and primers used were:

rrn16S, GATAAGCCGAGAGGAAGGTG and GTGTAGCCCAGCCCGTAAG,
hemA, CGGCGCAATGACCTATCTGGACGAA and CGGAAGCGAAGGAGCGGATGAAGT,
rplM, GCCGTCGCCAGATGAAGAACC and GAGGACGGCCGGCTGCTGTGC,
rpsA, ATCGGCCAGCAGGTCAAGGTTTCAG and CGAGCGAGATGCGGTGGGTTTC,
smc01774, AGCGGCGAGCGTCGAAATGAAGT and CGCTGCCCCGGTGTTGCCTTGCTCT,
smc02156, ACGGGTGTGTGGGAGGATA and ACGATGTTGCTGCGGAAG,
smc00885, GCAGGCGGAGAATCAAGTTT and GCTGGCATTGTAGACGGACT,
fixK, AGCAGGATCCAACCATCTTTCTTT and CACATTGCCTGCCGATTACCA,
nifA, CAGCAGGCGTGAGCGGACATTTG and GCTGGCGGGCGGAACCTCTGG,
nifH, GGACGTGCTCAAAGTGGGTTACAGA and GCATCGCAAAGCCCGCCGATACATA,
smc0806, ATGGTTGACGAAAGTAATGCAGGGC and TGCTTTCACTTCTGCATCCGCC,
smb20014, CGGCAATCCCTTCTACCTC and TGCTGAAATACTCGGTCTCG,
smb20139, CCTGGAAAACCTGAGACTGAATAC and CAGCACGACGAAAACGTATG,
smb21139, CGAAGATGTGGTGCCTGTGC and CCGTTGAAGGTCCAGAAGTC.

Plasmid insertion mutagenesis of *ccmA*.

The suicide plasmid pVO155 (Oke and Long 1999b) was digested with *Bam*HI and *Xba*I and was purified using the PCR clean-up system (Promega, Madison, WI, U.S.A.). A 387-bp DNA fragment of the *ccmA* gene was produced by PCR with primers CGGGATCCATGGCTGAAGGTTTGAAGTGC and GCTCTAGAAGATAGCCGAAGGGCAGAT containing a *Bam*HI and an *Xba*I restriction site, respectively (underlined). The PCR product was digested with *Bam*HI and *Xba*I and was ligated into the pVO155 plasmid and transformed into DH5α *Escherichia coli* cells. The resulting plasmid construct was checked by sequencing and was conjugated into *S. meliloti* Rm1021 using *E. coli* K12 pRK2013 as helper strain in a triparental mating. Transconjugants were selected three times on Luria Bertani plates containing 2.5 mM CaCl_2 , 2.5 mM MgSO_4 , and 200 µg of streptomycin per

milliter, and 1 µg of neomycin per milliliter. A single colony was selected, and the correct integration of the plasmid was confirmed by Southern blot analysis.

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LITERATURE CITED

- Ampe, F., Kiss, E., Sabourdy, F., and Batut, J. 2003. Transcriptome analysis of *Sinorhizobium meliloti* during symbiosis. *Genome Biol.* 4:R15.
- Barloy-Hubler, F., Chéron, A., Hellégouarch, A., and Galibert, F. 2004. Smc01944, a secreted peroxidase induced by oxidative stresses in *Sinorhizobium meliloti* 1021. *Microbiology* 150:657-664.
- Barnett, M. J., Toman, C. J., Fisher, R. F., and Long, S. R. 2004. A dual-genome symbiosis chip for coordinate study of signal exchange and development in a prokaryote-host interaction. *Proc. Natl. Acad. Sci. U.S.A.* 101:16636-16641.
- Becker, A., Bergès, H., Krol, E., Bruand, C., Rüberg, S., Capela, D., Lauber, E., Meilhoc, E., Ampe, F., de Bruijn, F. J., Fourment, J., Francez-Charlot, A., Kahn, D., Küster, H., Liebe, C., Pühler, A., Weidner, S., and Batut, J. 2004. Global changes in gene expression in *Sinorhizobium meliloti* 1021 under microoxic and symbiotic conditions. *Mol. Plant-Microbe Interact.* 17:292-303.
- Bénaben, V., Duc, G., Lefebvre, V., and Huguet, T. 1995. TE7, An inefficient symbiotic mutant of *Medicago truncatula* Gaertn. cv. Jemalong. *Plant Physiol.* 107:53-62.
- Bergès, H., Lauber, E., Liebe, C., Batut, J., Kahn, D., de Bruijn, F. J., and Ampe, F. 2003. Development of *Sinorhizobium meliloti* pilot microarrays for transcriptome analysis. *Appl. Environ. Microbiol.* 69:1214-1219.
- Beyer-Sehlmeyer, G., Kreikemeyer, B., Horster, A., and Podbielski, A. 2005. Analysis of the growth phase-associated transcriptome of *Streptococcus pyogenes*. *Int. J. Med. Microbiol.* 295:161-177.
- Bryant, P. A., Venter, D., Robins-Browne, R., and Curtis, N. 2004. Chips with everything: DNA microarrays in infectious diseases. *Lancet Infect. Dis.* 4:100-111.
- Cabanes, D., Boistard, P., and Batut, J. 2000. Identification of *Sinorhizobium meliloti* genes regulated during symbiosis. *J. Bacteriol.* 182:3632-3637.
- Capela, D., Carrere, S., and Batut, J. 2005. Transcriptome-based identification of the *Sinorhizobium meliloti* NodD1 regulon. *Appl. Environ. Microbiol.* 71:4910-4913.
- Chao, T. C., Becker, A., Buhrmester, J., Pühler, A., and Weidner, S. 2004. The *Sinorhizobium meliloti* *fur* gene regulates, with dependence on Mn(II), transcription of the *sitABCD* operon, encoding a metal-type transporter. *J. Bacteriol.* 186:3609-3620.
- Chen, H., Teplitski, M., Robinson, J. B., Rolfe, B. G., and Bauer, W. D. 2003. Proteomic analysis of wild-type *Sinorhizobium meliloti* responses to *N*-acyl homoserine lactone quorum-sensing signals and the transition to stationary phase. *J. Bacteriol.* 185:5029-5036.
- Conway, T., and Schoolnik, G. K. 2003. Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. *Mol. Microbiol.* 47:879-889.
- Davalos, M., Fourment, J., Lucas, A., Bergès, H., and Kahn, D. 2004. Nitrogen regulation in *Sinorhizobium meliloti* probed with whole genome arrays. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 241:33-40.
- Davey, M. E., and de Bruijn, F. J. 2000. A homologue of the tryptophan-rich sensory protein TspO and FixL regulate a novel nutrient deprivation-induced *Sinorhizobium meliloti* locus. *Appl. Environ. Microbiol.* 66:5353-5359.
- DeRisi, J. L., Iyer, V. R., and Brown, P. O. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* 278:680-686.
- Djordjevic, M. A. 2004. *Sinorhizobium meliloti* metabolism in the root nodule: A proteomic perspective. *Proteomics* 4:1859-1872.
- Djordjevic, M. A., Chen, H. C., Natera, S., Van Noorden, G., Menzel, C., Taylor, S., Renard, C., Geiger, O., the *Sinorhizobium* DNA Sequencing Consortium, and Weiller, G. F. 2003. A global analysis of protein expression profiles in *Sinorhizobium meliloti*: Discovery of new genes for nodule occupancy and stress adaptation. *Mol. Plant Microbe Interact.* 16:508-524.
- El Yahyaoui, F., Küster, H., Ben Amor, B., Hohnjec, N., Pühler, A., Becker, A., Gouzy, J., Vernie, T., Gough, C., Niebel, A., Godiard, L., and Gamas, P. 2004. Expression profiling in *Medicago truncatula* identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. *Plant Physiol.* 136:3159-3176.
- Gage, D. J. 2002. Analysis of infection thread development using Gfp- and DsRed-expressing *Sinorhizobium meliloti*. *J. Bacteriol.* 184:7042-7046.
- Gage, D. J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol. Mol. Biol. Rev.* 68:280-300.
- Galibert, F., Finan, T. M., Long, S. R., Pühler, A., Abola, P., Ampe, F., Barloy-Hubler, F., Barnett, M. J., Becker, A., Boistard, P., Bothe, G., Boutry, M., Bowser, L., Buhrmester, J., Cadieu, E., Capela, D., Chain, P., Cowie, A., Davis, R. W., Dreano, S., Federspiel, N. A., Fisher, R. F., Gloux, S., Godrie, T., Goffeau, A., Golding, B., Gouzy, J., Gurjal, M., Hernandez-Lucas, I., Hong, A., Huizar, L., Hyman, R. W., Jones, T., Kahn, D., Kahn, M. L., Kalman, S., Keating, D. H., Kiss, E., Komp, C., Lelaure, V., Masuy, D., Palm, C., Peck, M. C., Pohl, T. M., Portetelle, D., Purnelle, B., Ramsperger, U., Surzycki, R., Thebault, P., Vandenbol, M., Vorholter, F. J., Weidner, S., Wells, D. H., Wong, K., Yeh, K. C., and Batut, J. 2001. The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* 293:668-672.
- Gallusci, P., Dedieu, A., Journet, E. P., Huguet, T., and Barker, D. G. 1991. Synchronous expression of leghaemoglobin genes in *Medicago truncatula* during nitrogen-fixing root nodule development and response to exogenously supplied nitrate. *Plant Mol. Biol.* 17:335-349.
- Glazebrook, J., Ichige, A., and Walker, G. C. 1993. A *Rhizobium meliloti* homolog of the *Escherichia coli* peptide-antibiotic transport protein SbmA is essential for bacteroid development. *Genes Dev.* 7:1485-1497.
- Guerreiro, N., Djordjevic, M. A., and Rolfe, B. G. 1999. Proteome analysis of the model microsymbiont *Sinorhizobium meliloti*: Isolation and characterisation of novel proteins. *Electrophoresis* 20:818-825.
- Hinton, J. C., Hautefort, I., Eriksson, S., Thompson, A., and Rhen, M. 2004. Benefits and pitfalls of using microarrays to monitor bacterial gene expression during infection. *Curr. Opin. Microbiol.* 7:277-282.
- Hoang, H. H., Becker, A., and Gonzalez, J. E. 2004. The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. *J. Bacteriol.* 186:5460-5472.
- Jacobs, T. W., Egelhoff, T. T., and Long, S. R. 1985. Physical and genetic map of a *Rhizobium meliloti* nodulation gene region and nucleotide sequence of *nodC*. *J. Bacteriol.* 162:469-476.
- Krol, E., and Becker, A. 2004. Global transcriptional analysis of the phosphate starvation response in *Sinorhizobium meliloti* strains 1021 and 2011. *Mol. Genet. Genomics* 272:1-17.
- Long, S. R. 2001. Genes and signals in the rhizobium-legume symbiosis. *Plant Physiol.* 125:69-72.
- Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *J. Bacteriol.* 149:114-122.
- Milcamps, A., and de Bruijn, F. J. 1999. Identification of a novel nutrient-deprivation-induced *Sinorhizobium meliloti* gene (*hmgA*) involved in the degradation of tyrosine. *Microbiology* 145:935-947.
- Oke, V., and Long, S. R. 1999a. Bacteroid formation in the *Rhizobium-legume* symbiosis. *Curr. Opin. Microbiol.* 2:641-646.
- Oke, V., and Long, S. R. 1999b. Bacterial genes induced within the nodule during the *Rhizobium-legume* symbiosis. *Mol. Microbiol.* 32:837-849.
- Puskas, L. G., Nagy, Z. B., Kelemen, J. Z., Rüberg, S., Bodogai, M., Becker, A., and Dusha, I. 2004. Wide-range transcriptional modulating effect of *ntrR* under microaerobiosis in *Sinorhizobium meliloti*. *Mol. Genet. Genomics* 272:275-289.
- Rüberg, S., Tian, Z. X., Krol, E., Linke, B., Meyer, F., Wang, Y., Pühler, A., Weidner, S., and Becker, A. 2003. Construction and validation of a *Sinorhizobium meliloti* whole genome DNA microarray: Genome-wide profiling of osmoadaptive gene expression. *J. Biotechnol.* 106:255-268.
- Sharma, S. B., and Signer, E. R. 1990. Temporal and spatial regulation of the symbiotic genes of *Rhizobium meliloti* in planta revealed by transposon Tn5-*gusA*. *Genes Dev.* 4:344-356.
- Soberón, M., Aguilar, G. R., and Sanchez, F. 1993. *Rhizobium phaseoli* cytochrome *c*-deficient mutant induces empty nodules on *Phaseolus vulgaris* L. *Mol. Microbiol.* 8:159-166.
- Souppene, E., Foussard, M., Boistard, P., Truchet, G., and Batut, J. 1995.

- Oxygen as a key developmental regulator of *Rhizobium meliloti* N₂-fixation gene expression within the alfalfa root nodule. Proc. Natl. Acad. Sci. U.S.A. 92:3759-3763.
- Spaink, H. P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. Annu. Rev. Microbiol. 54:257-288.
- Turner, G. L., and Gibson, A. H. 1980. Measurement of nitrogen fixation by indirect means. Pages 111-138 in: Methods for Evaluating Biological Nitrogen Fixation. F. J. Bergersen, ed. Wiley, Chichester, U.K.
- Vasse, J., de Billy, F., Camut, S., and Truchet, G. 1990. Correlation between ultrastructural differentiation of bacteroids and nitrogen fixation in alfalfa nodules. J. Bacteriol. 172:4295-4306.
- Wan, J., Dunning, F. M., and Bent, A. F. 2002. Probing plant-pathogen interactions and downstream defense signaling using DNA microarrays. Funct. Integr. Genomics 2:259-273.

AUTHOR-RECOMMENDED INTERNET RESOURCES

ArrayExpress database: www.ebi.ac.uk/arrayexpress
 Bioinformatique Genopole Toulouse, *Sinorhizobium meliloti* 1021 genome project webpage:
bioinfo.genopole-toulouse.prd.fr/annotation/iANT/bacteria/rhime/