

Overproduction of Indole-3-Acetic Acid in Free-Living Rhizobia Induces Transcriptional Changes Resembling Those Occurring in Nodule Bacteroids

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Free-living bacteria grown under aerobic conditions were used to investigate, by next-generation RNA sequencing analysis, the transcriptional profiles of *Sinorhizobium meliloti* wild-type 1021 and its derivative, RD64, overproducing the main auxin indole-3-acetic acid (IAA). Among the upregulated genes in RD64 cells, we detected the main nitrogen-fixation regulator *fixJ*, the two intermediate regulators *fixK* and *nifA*, and several other genes known to be FixJ targets. The gene coding for the sigma factor RpoH1 and other genes involved in stress response, regulated in a RpoH1-dependent manner in *S. meliloti*, were also induced in RD64 cells. Under microaerobic condition, quantitative real-time polymerase chain reaction analysis revealed that the genes *fixJL* and *nifA* were up-regulated in RD64 cells as compared with 1021 cells. This work provided evidence that the overexpression of IAA in *S. meliloti* free-living cells induced many of the transcriptional changes that normally occur in nitrogen-fixing root nodule.

The symbiotic relationships of soil bacteria and legume plants have been studied as mutualistic associations for over 100 years. Many legumes enter into this symbiosis with nitrogen-fixing bacteria, collectively called rhizobia.

The interaction begins by dual recognition. The host roots exude flavonoids or betaines, which stimulate the synthesis of so-called Nod factors (lipochitin oligosaccharides) by the bacterial partners. The host plant perceives the Nod factors and triggers the root nodule organogenesis required for the accommodation of rhizobia (Oldroyd and Downie 2008). Inside the nodule, rhizobia convert the inert nitrogen molecule into ammonia, which is exported to the plant in exchange for carbohydrates.

Rhizobial genes required for symbiotic nitrogen fixation include those involved in the biosynthesis of Nod factor, the assembly of the nitrogen-fixing apparatus, and in bacteroid metabolism. On the other hand, the transcription of plant genes (nodulin genes) is specifically induced in root tissue, as a consequence of the interaction with rhizobia. During the late stages of symbiosis, the expression of specific bacterial genes,

such as those for nitrogen fixation (*nif* and *fix* genes), is linked to the decrease of oxygen partial pressure to which infecting bacteria are exposed (Gibson et al. 2008). The *nif* and *fix* genes are organized in distinct clusters with structure and location species-specific. In *S. meliloti*, both cluster I (*nifHDKE*, *nifN*, *fixABCX*, *nifA*, *nifB*, *frdX*) and cluster II (*fixLJ*, *fixK*, *fixNOQP*, *fixGHIS*) genes are located on megaplasmid pSymA. The cluster II genes map at about 220 kb downstream of the *nifHDKE* operon and are transcribed in the opposite direction (Fischer 1994).

It is known that the transcription of the *nifA* gene is mainly controlled by promoter p_{nifA} , situated between *fixABCX* and *nifA*.

The expression of *nifA* from p_{nifA} and the synthesis of NifA protein are low under free-living aerobic conditions (Fischer 1994), but it increases in both bacteroids and free-living cells growing under microaerobic conditions.

In the latter conditions the transcriptional activation of *fixABCX* and other *nif* genes is highly dependent on *fixL* and *fixJ* genes and leads to a further enhancement of *nifA* expression. FixL autophosphorylates and transmits phosphate to the FixJ response regulator. Once phosphorylated, FixJ activates transcription of the *nifA* and *fixK* genes, encoding two intermediate regulators that, in turn, induce the expression of *nif* and *fix* structural genes involved in respiration and nitrogen fixation (Fischer 1994). More recently, two novel FixJ-regulated genes, *proB2* and *SMc03253*, both involved in the metabolism of proline, have also been identified (Ferrières et al. 2004).

In *S. meliloti*, the *nif* and *fix* genes promoters can be activated by both *ntrC* and *nifA* gene products; which regulatory gene products (NtrC or NifA) is operative depends on whether the *S. meliloti* organisms are in free-living or symbiotic conditions. In free-living *S. meliloti* cells, grown under ammonia-limiting conditions, the transcription of the *nif* and *fix* genes is activated by the *ntrC* gene product. By contrast, in the symbiotic state, *ntrC* is not required for *nifA* transcription, which is transcribed from a *nifA*-specific promoter (Fischer 1994).

The *ntrC* gene is also involved in the transcriptional activation of nitrogen-related genes. Under free-living ammonia-limiting conditions, the GlnD sensor protein modifies the PII proteins GlnB and GlnK, which, in turn, modulate the activity of many targets, including the AmtB ammonium transporter and the glutamine synthetases GSI and GSII. Modified PII proteins also control the activity of the two-component regulatory system NtrBC, resulting in the NtrC phosphorylation and activation of nitrogen catabolism and assimilation (Yurgel et al. 2010, 2013).

Several important steps in nitrogen fixation, as well as nodule formation, are affected by stress conditions, which might be considered limiting factors. In the soil, variations of temperature,

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

osmolarity, or pH, as well as nutrient starvation are the stress conditions most frequently faced by rhizobia (Zahran 1999).

Stress response in bacteria, as in rhizobia, is essential for effective adaptation to environmental changes or in the bacterial physiological state. It deals with the cellular ability to sense protein folding and other signals, leading to the activation of proteins, such as molecular chaperones, proteases, and regulatory factors, playing an important role in promoting homeostasis under stress conditions. This response is mediated by global regulatory mechanisms acting at the transcriptional level, with the participation of specialized RNA polymerase subunits, the alternative sigma factors (Barnett et al. 2012; de Lucena et al. 2010; Martínez-Salazar et al. 2009).

Among the rhizobia, the *S. meliloti* genome encodes 14 alternative sigma factors: RpoN, essential for the transcription of nitrogen-fixation genes; 11 extracytoplasmic function-type sigma factors (RpoE1 to RpoE10 and Fecl); and two RpoH/heat shock-type sigma factors (RpoH1 and RpoH2). Multiple RpoH sigma factors are common in alphaproteobacterial genomes (Barnett et al. 2012; de Lucena et al. 2010; Martínez-Salazar et al. 2009).

In *S. meliloti*, RpoH1 and RpoH2 share 44% sequence identity and are 38 and 40% identical, respectively, to the *Escherichia coli* RpoH heat shock sigma factor. In *E. coli*, the main role of RpoH is to maintain protein-folding homeostasis under high temperature and other conditions that denature proteins (Barnett et al. 2012).

S. meliloti rpoH1 mutants are severely impaired for growth at 37°C, fail to grow at 40°C, and are sensitive to acid pH, deoxycholate, sodium dodecyl sulfate, and crystal violet. These results suggest that the heat-shock response in *Rhizobium* spp. could overlap the response to other stimuli (Barnett et al. 2012). RpoH1 is also required for successful symbiosis. Indeed, in symbiosis with alfalfa, the *rpoH1* mutant exhibits a nitrogen-fixation defect (Fix⁻ phenotype), whereas the *rpoH1 rpoH2* double mutant exhibits a nodule formation defect (Nod⁻ phenotype). On the other hand, no apparent phenotype has been observed for the *rpoH2* single mutant, both in culture and symbiotic situations. The *rpoH1* gene is expressed within a symbiotic nodule, while *rpoH2* is not induced to any significant extent (Mitsui et al. 2004). To establish new root structures, the nodules, rhizobia use and manipulate the endogenous molecular and physiological pathways of their hosts.

It is widely reported that changes in auxin signaling are associated with many processes of nodule formation (Breakspear et al. 2014; Cooper 2007; Deinum et al. 2012; Desbrosses and Stougaard 2011; Glick 2012; Grunewald et al. 2009; Huo et al. 2006; Kazan 2013; Mathesius 2008; Spaepen and Vanderleyden 2011; Sukumar et al. 2013; van Noorden et al. 2007).

The most abundant form of auxin in plants is indole-3-acetic acid (IAA). Thimann (1936) showed that root nodules contain a higher IAA content compared with uninfected root tissue. Later studies confirmed that auxin accumulates in the nodule during its formation (Ghosh and Basu 2006; Mathesius 2008). Moreover, IAA-overproducing rhizobium led to the formation of nodules containing higher amounts of IAA (Pii et al. 2007).

Research on the role of IAA in microorganisms revealed that IAA can act as a signal molecule able to influence behavior of both IAA-producing and nonproducing species. Indeed, gene expression changes triggered by IAA have been reported for several microbes (Spaepen and Vanderleyden 2011; Spaepen et al. 2007).

We have previously shown that IAA overproduction in different rhizobium species led to beneficial effects, both in free-living bacteria and host plants. Indeed, an increase in N-fixation and plant yield was observed for plants nodulated by the specific IAA-overproducing rhizobia (Bianco and Defez 2009, 2010a; Bianco et al. 2014, 2010; Camerini et al. 2008; Imperlini et al. 2009). For the IAA-overproducing RD64 strain, a

S. meliloti 1021 derivative expressing the auxin-synthesis chimeric operon *p-iaaMtns2* (Defez and Spena 1998; Pandolfini et al. 2000), we have also reported that both free-living RD64 cells and Mt-RD64 plants are more able to overcome different stressful environmental conditions. These effects were consistent with the morphology observed for root nodules elicited by IAA-overproducing rhizobia. For these root nodules, a more extended nitrogen-fixation zone and a reduced senescent zone were observed as compared with plants nodulated by the wild-type strain. Furthermore, when meristematic activity of nodule cells was monitored, dividing plant cells could still be observed after 52 days of infection (Camerini et al. 2008).

The object of the present study was to gain new insight into global changes in gene expression related to the increased biosynthesis of IAA in free-living RD64 cells.

RNA-seq analysis was applied to compare the transcriptome of the IAA-overproducing *S. meliloti* RD64 strain with the wild-type *S. meliloti* 1021 strain. In addition, real-time quantitative polymerase chain reaction PCR (qPCR) analysis was used not only to validate RNA-seq experiments but, also, to evaluate, under microaerobic and nitrogen-limited conditions, the expression levels of genes directly involved in nitrogen fixation and metabolism.

Our results showed that, under free-living conditions, IAA overproduction by rhizobia directly or indirectly leads to the activation of key genes of the nitrogen-fixation process, including many FixJ-regulated genes and those involved in stress response in rhizobia.

RESULTS AND DISCUSSION

RNA-seq: gene expression overview.

The aim of the present study was to use the messenger (m)RNA of *S. meliloti* as a model system and Illumina RNA-sequencing technologies to gain insight into the mechanisms behind the different phenotypes observed for the IAA-overproducing rhizobium RD64.

We performed RNA-seq analysis on RD64 cells for two main reasons. The IAA is an unstable molecule (Nissen and Sutter 1990) and, thus, to maintain its concentration during the experiment, we would need to add additional IAA to the bacterial culture medium as the experiment progressed. The presence of the *p-iaaMtns2* construct in RD64 ensures the continuous production of IAA in free-living rhizobium, as previously observed (Bianco et al. 2014). Furthermore, our future aim will be the transcriptome analysis during symbiosis. For this study, bacteroids of nodules induced by 1021 and RD64 strains will be used.

The IAA biosynthetic genes introduced in RD64 cells encode for enzymes that use tryptophan (Trp) to produce IAA, leading to an increase in auxin synthesis of at least 100-fold ($56 \pm 13 \mu\text{M}$) as compared with wild-type 1021 cells ($0.60 \pm 0.01 \mu\text{M}$) (Bianco et al. 2014).

To check the possibility that this IAA overproduction could lead to Trp limitation, we had already treated the RD64 cells with 0.5 mM Trp and measured the expression of selected genes by quantitative reverse transcription (qRT)-PCR analyses. For Trp-treated RD64 cells, we did not find significant alteration in the expression of the selected genes as compared with untreated RD64 cells (Imperlini et al. 2009). The specificity of IAA effects was also previously verified by comparing the expression patterns of wild-type 1021 cells with those of RD64 cells and 1021 cells treated with IAA and other chemically (indole [IND], Trp, indole-3-carboxylic acid [ICA]) or functionally related (2,4-dichlorophenoxyacetic acid [2,4-D]) molecules. We found that the number of transcripts significantly up- or downregulated in ICA-, IND-, and Trp-treated cells were 324, 232, and 211, respectively. On the other hand, 112, 67, and 83 genes were differentially expressed in strain 1021 cells treated with IAA and 2,4-D

and in RD64 cells, respectively, as compared with 1021 wild-type cells. When we compared the lists of the differentially expressed genes obtained for RD64 and 1021 cells treated with IAA, we found that 26 genes (31% of RD64 genes) were present in both lists. The comparison between RD64 and 1021 cells treated with 2,4-D revealed 27 common genes (32% of RD64 genes). The percentages were far lower when such comparison was made between RD64 cells and the other three samples (ICA-, IND-, and Trp-treated cells) (Bianco and Defez 2010b; Imperlini et al. 2009).

In this work, total RNA was isolated from three biological replicates of the wild-type *S. meliloti* 1021 strain and its derivative, RD64, which is able to produce about 100 times more IAA. Total RNA was extracted from bacterial cultures in the late exponential growth phase. After depletion of ribosomal RNA by the use of a commercial capture and depletion system, the sequencing libraries were constructed and sequenced, using Illumina protocol as described below.

For both the control and modified strains, the raw sequence output consisted of 22 to 28 million reads, each with a length of 50 nucleotides, indicating that similar amounts of data were generated for the two analyzed strains. Alignments to the *S. meliloti* 1021 reference genome were generated and an average of 6,847,618 (\pm 741,549) reads could be unambiguously mapped for both strains (Table 1).

Table 1. Summary of *Sinorhizobium meliloti* strains cDNA samples sequenced using the Illumina HiSeq2000

Sample	Total reads	Mapped reads ^y	Unique reads ^z
1021 a	18,870,493	18,421,487 (97.6%)	3,746,387 (19.8%)
1021 b	27,157,374	26,453,615 (97.4%)	7,031,952 (25.9%)
1021 c	22,210,676	21,779,367 (98.0%)	5,837,201 (26.3%)
RD64 a	28,120,274	27,104,478 (96.4%)	6,358,460 (22.6%)
RD64 b	27,557,767	26,479,066 (96.0%)	7,647,455 (27.7%)
RD64 c	25,178,164	24,200,420 (96.1%)	7,363,023 (29.2%)

^y The percentage represents the proportion of the mapped reads to the total reads sequenced.

^z The percentage represents the proportion of unique reads to the mapped reads.

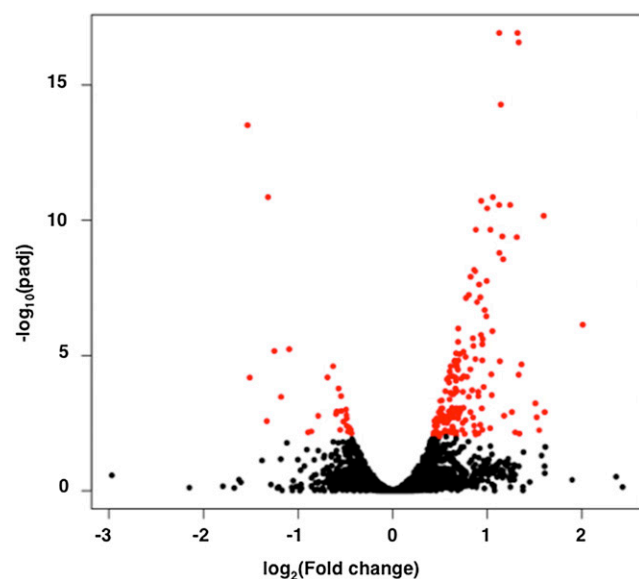


Fig. 1. Volcano plot of overall gene-based differential expression results for the RD64 cells as compared with the wild-type 1021 cells. Each point in the plot corresponds to a statistically tested gene, with red dots representing the differentially expressed genes according to an adjusted P value < 0.05 . The x axis is the base 2 logarithm of the fold change, and the y axis is the negative base 10 logarithm of the adjusted P value.

Quantitative analysis of the *S. meliloti* transcriptome.

Genes were considered as differentially expressed if the fold change (FC) was ≥ 1.3 and the adjusted P value ($padj$) was < 0.05 . The magnitude distribution of the significantly changed genes was illustrated by Volcano plot analysis (Fig. 1). Using these criteria, a total of 328 genes were significantly differentially expressed in RD64 cells as compared with the control strain, including 249 (76% of the total differentially expressed genes) upregulated genes and 79 (24% of total differentially expressed genes) downregulated genes. Hierarchical clustering analysis was performed on the top 50 differentially genes (Fig. 2). It is straightforward to identify highly expressed genes in RD64 cells (Fig. 2). A comprehensive list of differentially expressed genes is provided in Supplementary Table 1.

We analyzed the distribution of the differentially expressed genes across the different replicons and saw that 69% of these genes were located on the chromosome, 14% on pSymA and 17% on pSymB (Fig. 3). The ratio between up- and downregulated genes was 3.4 and 1.3 for genes located on the chromosome and pSymB, respectively. The ratio greatly increased (ratio = 8.4) when the pSymA symbiotic megaplasmid was considered, due to the fact that only five downregulated genes were on pSymA.

The sequence data identified more than double the number of differentially expressed genes when compared with those obtained for the RD64 cells in microarrays analysis (Imperlini et al. 2009) genes. About 20% of the RD64 genes were common to the two data sets. Furthermore, a more in-depth analysis showed that several genes, found as up- or downregulated in the microarray analysis, were now not classified as such because, despite having a FC greater or just below the fixed threshold, their P values were higher than 0.05 (e.g., *metF* [FC = 1.2, $padj$ = 0.14], *mexF1* [FC = 1.4, $padj$ = 0.17] and *tufB* [FC = 0.82, $padj$ = 0.15]).

This discrepancy could be due to the use of total RNA and enriched mRNA for the cDNA synthesis in microarray-based and sequencing-based transcriptome analysis, respectively. Moreover, since, in microarray analysis, the transcript abundance was inferred from hybridization intensity rather than direct measurement, as in sequencing analysis, the derived data presented a higher level of noise. This could explain why many genes selected as differentially expressed in this study had not previously been found in microarray analysis, as they had high P values.

Functional classification of differentially expressed genes.

In our analysis, we found that about 5% of *S. meliloti* 1021 protein-coding genes were significantly altered in RD64 cells as compared with the 1021 control cells (Table 2).

The 328 genes exhibiting a FC ≥ 1.3 could be classified into five broad functional categories, according to Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation. Based on this annotation, a significant fraction of all differentially expressed genes identified were genes annotated as metabolism proteins (25%) and proteins with hypothetical functions (43%) (Fig. 4). A smaller number of differentially expressed genes were in the functional classifications related to environmental information processing (13%), genetic information processing (10%), and cellular processes (9%) (Fig. 4).

For all functional classes, the upregulated genes exceeded the downregulated ones. This result was more evident for genes related to the metabolism and genetic information processing functional classes, for which the percentages of upregulated genes were 57 and 91%, respectively.

Metabolism.

Within this functional class, many genes involved in both the early steps of the nodulation process and nitrogen fixation were found. Indeed, two genes, *noeA* and *noeB*, involved in the

nodulation process of *S. meliloti* (Capela et al. 2005) were up-regulated in RD64 cells. These results indicate that RD64 cells were able to activate the nitrogen-fixation process from the very early stages.

Among the nitrogen-fixation genes, an induced expression was found for the major regulator *fixJ* and for the two intermediate regulators *nifA* and *fixK*, with the latter showing the highest expression levels among the total induced genes. Numerous targets known to be under FixJ control were identified as upregulated in RD64 cells. These genes included the *fixNOQP* operon encoding the *bb3*-type cytochrome c oxidase, which has high affinity for O₂ and, thus, is required to support bacteroid respiration under conditions of low oxygen present in root nodules (Dixon and Kahn 2004; Fischer 1994).

Among the differentially expressed genes, we also found the genes related to the denitrification process (*nosD*, *nosF*, *nosY*, and *nosZ*). Denitrification performed by bacteria plays a pivotal role in the prevention of early nodule senescence and, hence, the maintenance of efficient symbiosis (Bobik et al. 2006; Cabeza et al. 2014).

Our data show that the five known genes (*fixK1*, *fixK2*, *nifA*, *proB2*, and SMc03253) directly regulated by FixJ in *S. meliloti* were significantly induced in RD64 cells. Thus, our data are in agreement with the literature regarding the

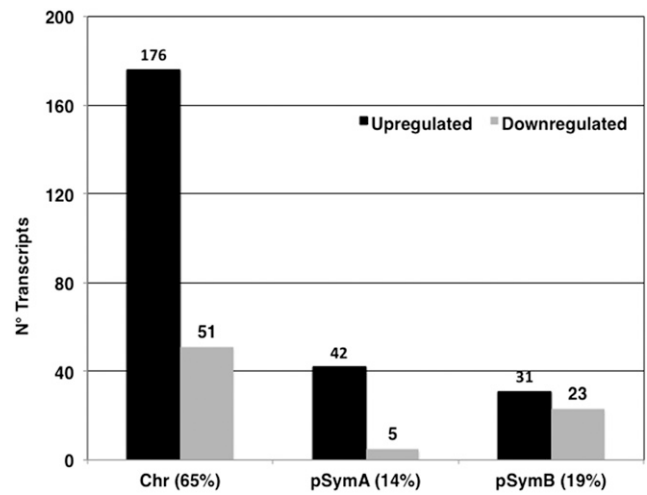


Fig. 3. Differentially expressed genes categorized by functional classification according to the Kyoto Encyclopedia of Genes and Genomes annotation. Bars represent the number of upregulated (black) and downregulated (gray) genes in the RD64 cells compared with the wild-type 1021 cells. The percentage of differentially expressed genes in each category is given in parentheses. The number close to each bar represents the up- or downregulated gene.

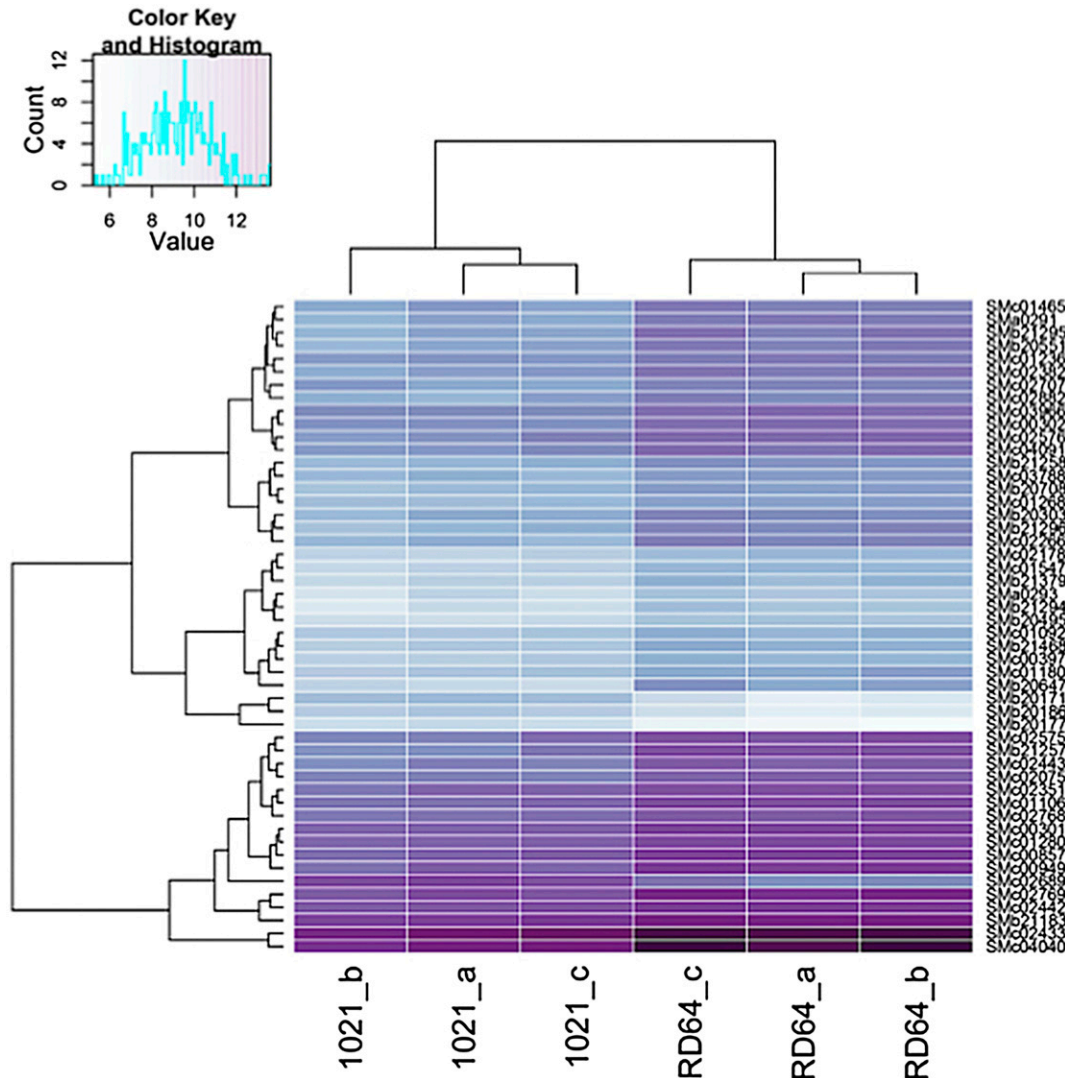


Fig. 2. Heatmap of the top 50 differentially expressed genes. Purple indicates high expression, violet indicates intermediate expression, and blue indicates low expression. The color key shown on the top left represents the normalized log₂-transformed counts.

structure of the FixJ regulon (Ferrières et al. 2004; Cabeza et al. 2014). SMc03253 and *proB2* genes share homology with genes involved in proline biosynthesis. The induced expression of these genes is consistent with the results we

have previously shown regarding the accumulation of higher intracellular trehalose levels in RD64 cells and the increased tolerance of these cells to several abiotic stresses (Bianco and Defez 2009).

Table 2. The 34 most highly induced genes (fold change >2.0) in RD64 cells when 1021 wild-type cells were used as reference

Gene ID	Gene name	Protein	Fold change	<i>padj</i> ^z
SMb20647	–	Hypothetical protein	4.022976497	7.27E-07
SMc03252	<i>proB2</i>	Putative glutamate 5-kinase	3.060659107	0.02457717
SMa0763	–	Hypothetical protein	3.05791676	0.024089027
SMa0814	<i>nifB</i>	NifB FeMo cofactor biosynthesis protein	3.045500785	0.001253723
SMc04040	<i>ibpA</i>	Heat shock protein	3.02444228	6.86E-11
SMa1910	–	Hypothetical protein	2.923919328	0.005857607
SMa0661	–	Hypothetical protein	2.868608184	0.001935564
SMa0762	<i>fixK</i>	Transcriptional regulator FixK	2.840304579	0.000597217
SMc01589	–	Hypothetical protein	2.605471889	0.037721839
SMa0815	<i>nifA</i>	NifA transcriptional activator	2.568192498	2.13E-05
SMa0767	<i>fixQ2</i>	FixQ2 nitrogen-fixation protein	2.528821202	0.00784321
SMc02266	–	Conserved hypothetical protein	2.516819005	2.58E-17
SMa0771	–	Hypothetical protein	2.516507033	5.19E-05
SMb21296	–	Hypothetical protein	2.494762603	1.16E-17
SMb21295	–	Putative small heat shock protein, hsp20 family	2.481613905	4.19E-10
SMa0766	<i>fixO2</i>	FixO2 cytochrome c oxidase subunit	2.454167769	0.00698866
SMa0811	<i>fdxN</i>	FdxN ferredoxin	2.395985709	0.001244498
SMb21257	–	Adenylate cyclase	2.364048062	2.69E-11
SMa0769	<i>fixP2</i>	FixP2 Diheme c-type cytochrome	2.258430265	0.0017073
SMb21294	–	Putative small heat shock protein, hsp20 family	2.243601401	2.74E-09
SMb20551	–	Hypothetical protein	2.230116797	3.91E-10
SMa0291	–	Hypothetical protein	2.205943988	5.17E-15
SMb21379	–	Conserved hypothetical protein	2.190843643	1.63E-05
SMc01547	–	Conserved hypothetical protein	2.182762035	1.61E-09
SMc00949	–	Hypothetical protein	2.180317208	1.16E-17
SMc01180	–	Conserved hypothetical transmembrane protein	2.179122903	2.69E-11
SMc02769	–	Conserved hypothetical transmembrane protein	2.081595724	1.38E-11
SMc02882	–	CysZ-like protein	2.07475901	1.25E-06
SMa1184	<i>nosF</i>	NosF ATPase	2.06794638	0.045220162
SMc00591	–	Hypothetical/unknown signal peptide protein	2.065392583	0.00029052
SMc03253	–	L-proline cis-4-hydroxylase	2.058212205	5.04E-05
SMc02382	–	Conserved hypothetical protein	2.045010205	2.22E-10
SMc03251	–	Conserved hypothetical protein	2.045005729	0.022773579
SMa0810	<i>fixU</i>	FixU nitrogen-fixation protein	2.041187517	0.004883599

^z *padj* = adjusted *P* value.

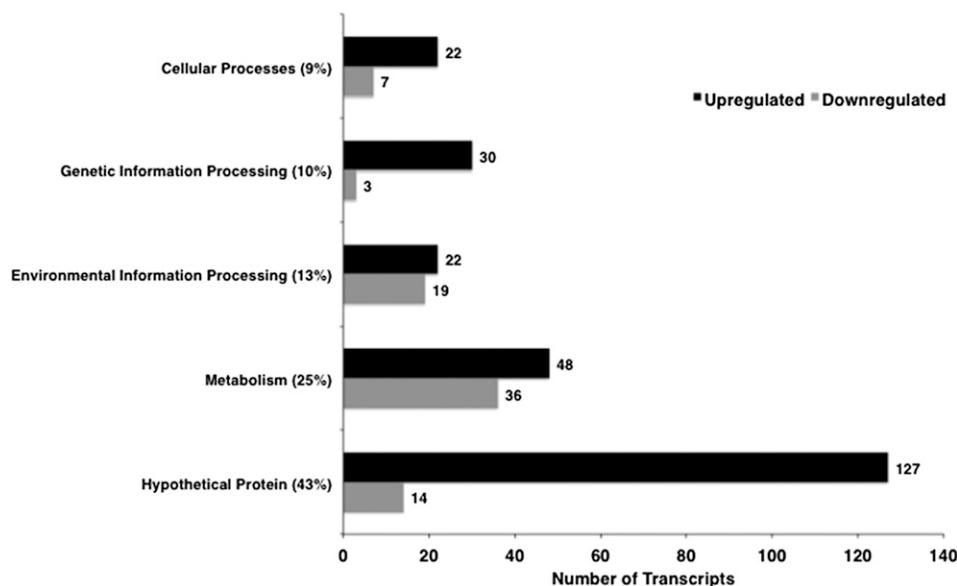


Fig. 4. Distribution of differentially expressed genes across the chromosome, pSymA and pSymB of *Sinorhizobium meliloti* 1021. The numbers in brackets represent the percentages of differentially expressed genes for each replicon.

Although it has not been shown that SMc03253 and *proB2* genes are essential for symbiosis, they might provide an advantage to the bacterium in a natural environment. Indeed, the role of proline as osmoprotectant has been described in many bacteria (Sleator and Hill 2002).

In addition, among the upregulated genes, we also found the *ocd* gene coding for ornithine cyclodeaminase, catalyzing the direct conversion of L-ornithine to L-proline with ammonia release (Soto et al. 1994).

The gene *pdhAa* coding for pyruvate dehydrogenase alpha2 subunit protein allowing the formation of acetyl-CoA, a common starting metabolite of tricarboxylic acid cycle (TCA) and poly- β -hydroxybutyrate (PHB) biosynthesis, was also induced in RD64 cells. These results were consistent with those obtained in previous studies. The intracellular content of acetyl-CoA in RD64 cells increased up to 40% as compared with 1021 cells (Imperlini et al. 2009). Moreover, for these cells, the activity of key enzymes in TCA cycle regulation (citrate synthase and α -ketoglutarate dehydrogenase) and PHB biosynthesis significantly increased (Imperlini et al. 2009).

PHB accumulation in RD64 cells might function as a sink for reducing equivalents, such as NAD(P)H, allowing the TCA cycle to operate in microaerobic conditions required for nitrogen fixation (Dunn 1998).

Of the 36 genes found to be downregulated in the functional class of metabolism, we verified that most of them were involved in the metabolism of small molecules, purine and pyrimidine biosynthesis, and ATP biosynthesis.

Environmental information processing.

Many genes related to stress response (*ibpA*, *htpG*, *clpB*, *groES5*, *groEL*, *dnaK*), known to belong to the *rpoH* regulon in different rhizobia (de Lucena et al. 2010; Martínez-Salazar 2009; Mitsui et al. 2004), were up-regulated in RD64 cells. In particular, considering the list of all differentially expressed genes, the *ibpA* gene coding for a heat shock protein is among those showing the highest expression level (Table 2).

The upregulation of SMb21295 and SMb21294 genes, coding for two members of hsp20 family heat shock proteins, and the SMc01106 gene, coding for a small heat shock protein, was also observed in these cells. Similar data were previously reported for *Escherichia coli* cells exogenously treated with IAA (Bianco et al. 2006a and b), thus showing it is a widespread effect.

In addition, several genes (*htpX*, *clpS*, *clpB*, *clpP1*, *clpA*) encoding proteases and protein modification enzymes responsible for disassembling protein aggregates that accumulate in the cytoplasm under stress conditions, showed increased expression levels in RD64 cells.

Overall, we found that the stress response genes differentially expressed (upregulated) in RD64 cells were common to those obtained in other transcriptional analyses regarding the stress response of *S. meliloti* and other rhizobia, thus indicating that they were not specific genes involved in IAA-response (Alexandre et al. 2014; Cytryn et al. 2007; de Lucena et al. 2010; Jeon et al. 2011; López-Leal et al. 2014; Yurgel et al. 2013).

The increased expression of all these genes is not due to growth defects, as we have previously shown (Defez 2006) that the growth curves of RD64 cells and 1021 cells treated with IAA concentrations from 0.05 to 2 mM did not differ from those of 1021 wild-type cells. On the contrary, this result is in agreement with the ability of these cells to better cope with different stress conditions (Bianco and Defez 2009, 2010a).

The genes most highly repressed mainly encode components involved in transport (i.e., ABC transport systems), glycolysis, purine and pyrimidine biosynthesis, motility, and flagellar biosynthesis (*mcpU*, *mcpT*, *mcpV*, *flhA* and *flaD*). Several studies have demonstrated that the repression of motility-

related genes in *S. meliloti* is essential for effective nodule invasion. Once nodulation has been initiated, motility is no longer required for bacteroid development or effective nitrogen fixation and might be detrimental to the invasion process (Barnett et al. 2004; Capela et al. 2006).

Consistent with this, the downregulation of flagellar and chemotaxis genes has been observed in expression analysis of nodules induced by *S. meliloti* as well as other rhizobia (Li et al. 2013; Pessi et al. 2007; Vercruysse et al. 2011). Relying on these studies, we could say that the physiology of RD64 cells is much more similar to those of symbiotic cells than to free-living cells.

Genetic information processing.

Within this class, we found that the *rpoH1* gene, coding for one of the two alternative σ^{32} factors present in the *S. meliloti* genome, showed an increased expression. This sigma factor operates under heat shock and oxidative stress and is required for effective nitrogen-fixing symbiosis with alfalfa. As above reported, we also observed the upregulation of many stress response genes known to be under the control of *rpoH1* in *S. meliloti*. These data are in agreement with those we already obtained by microarray analysis (Imperlini et al. 2009) and with those concerning the ability of RD64 cells to better overcome abiotic stress conditions, through the induction of different defense systems (Bianco and Defez 2009, 2010a).

In addition, genes coding for proteins known to maintain genomic integrity (mismatch repair, excision repair, and recombination) such as *ruvA*, *lexA*, *vsr*, *recA*, *uvrA*, and *uvrB* were up-regulated in RD64 cells. These results lead us to speculate that, in RD64 cells, a more active transcription-coupled repair mechanism might reduce the occurrence of genomic alterations. A lower mutation rate could allow the descendants of RD64 cells to retain their phenotype, i.e., their ability to better withstand stress conditions and to efficiently activate the main systems involved in both the early and later stages of the nitrogen-fixation process. This hypothesis could be verified by DNA-sequencing analysis of RD64 and 1021 cells.

qRT-PCR analysis.

qRT-PCR analysis was performed to evaluate expression levels of selected genes in RD64 and 1021 cells grown under different conditions. For each gene, the $2^{-\Delta\Delta C_t}$ values refer to relative fold differences in RD64 cells compared with the wild-type 1021 cells.

RNA-seq validation.

To validate the RNA-seq data, a qRT-PCR analysis was performed for 19 up- or downregulated genes. The list of selected genes (Table 3) included the *fixJ*, *fixK2*, *nifA*, and *rpoH1* genes.

qRT-PCR data were significantly correlated to the RNA-seq data (Pearson's correlation coefficient $r = 0.87$) (Fig. 5). For some genes, an induction level higher than the one obtained in RNA-seq experiment was measured. This difference was probably due to the fact that qRT-PCR is a highly quantitative and sensitive technique.

Expression of nitrogen-fixation genes under aerobic and microaerobic conditions.

In this work, we evaluated the expression level of the *fixJL* genes, coding for the two-component system that, in *S. meliloti*, turns on nitrogen-fixation genes in microaerobiosis as well as of the regulators *nifA* and *fixK*, transcriptionally activated by *fixJ* gene product.

We also measured the expression of the *fixT2* gene, coding for the repressor FixT acting as inhibitor of FixL-phosphate synthesis. The activation of this repressor prevents the transcription of the two regulator genes *nifA* and *fixK* (Garnerone et al. 1999).

Table 3. Primers used in quantitative polymerase chain reaction (PCR) analysis

Gene ID ^z	Gene ^z	Forward (5'→3')	Reverse (5'→3')
SMc04040	<i>ibpA</i>	GACCTATCCGCCCTACAACA	GAAGCGGACTTGATCTCGAC
SMa1183	<i>nosD</i>	CGAAGACCGACGAAGAGAAC	TGATGAAGGAGTTGCTGCTG
SMc03788	<i>dnaE2</i>	CCAATATCCGGATGAAAACG	CTTGTGGACGGTCAGGAAAT
SMc03966	<i>ruvA</i>	AAGCTGAAGGGCACGATAGA	CGCACATAGGTCTCGATGAA
SMc00646	<i>rpoH1</i>	GTGAGGAAGAGGTCGTCTCG	TCAGAACCTTCATGGCATTG
SMc01183	<i>lexA</i>	ATCCGTACCGGTTGAAATGA	CCGAATATGCGTGTTCGTA
SMc00760	<i>recA</i>	AATTCTTTGCGGCTCGTAGA	TTTCCGGACCATAGATCTCG
SMc01235	<i>uvrA</i>	CTTCCAGCGCGTAAAGGTTAG	GCGAGCGTAAGACAGGTTTC
SMc02163	<i>pgi</i>	GGCAAGAAGATCACCGATGT	GTCTCGATCGTGGTGAAGGT
SMc00480	<i>icd</i>	AACCTGGACGAATCGATCAC	TTCTCTGTCGAACACCTTCT
SMc00869	<i>atpF2</i>	GCTGCTTACGAGCAGGAGTT	GCAAGAGCCTTCGACTTGAT
SMa1227	<i>fixJ</i>	CTCGTGACGGACCTGAGAAT	GCAGCAACCAGATGTTTCTAG
SMa1229	<i>fixL</i>	AAAAGCGCATCATCGGTATC	TTTCGCCCTCTCATTTAGG
SMa0815	<i>nifA</i>	CCTTGCAAGAGCATTCTCTTC	TCTTTGACCTGGCGAGAGTT
SMa0814	<i>nifB</i>	CTGAAATCCCGACGAGATA	CGTGTCCAAAATGTTCTGTTG
SMa0825	<i>nifH</i>	TCCACGACCTCCCAAAATAC	CGCACTTGATGCCTCTGTAA
SMa1225	<i>fixK1</i>	CATTCTTTCTTTGCCGAAGC	CGCAAAGATCGACGAGAAAT
SMa0762	<i>fixK2</i>	AAGCCAAACCACAGTCCATC	CATCTGAAAGGAGGCGGTAG
SMa1220	<i>fixN1</i>	AATGGTACGTCGACCTCTGG	AGATTGTTGACGACGTGCAG
SMa1216	<i>fixO1</i>	ATCGAGAAGGTCGAGGGAAT	AAAGGGATGGTCGTACATGG
SMa1214	<i>fixQ1</i>	CTCGCAATGACGTTGTTCTT	CCTCCTTTAACGGGATGACA
SMa1213	<i>fixP1</i>	CAGCTTCTATGCCACGATCA	CGGATTTGCGATGATCTCTT
SMa0765	<i>fixN2</i>	GGCATGGTCTTCTCCATCAT	TGTAGTGGCTCAGCGAATTG
SMa0766	<i>fixO2</i>	GTGGAAATCGCACCACTCTT	CTGAAACGGGTGATCGTACA
SMa0767	<i>fixQ2</i>	GGAAACCTATACGGCCATGC	GCCTGAGCAGCAGACTTTTT
SMa0769	<i>fixP2</i>	ATATGCCGTCGCTTATCCAT	TTGTGCGCAATTTACCTTGA
SMa1211	<i>fixG</i>	CCAACCCAAAACCAAGCTA	GATCCACGGCGTCAGATAGT
SMa1210	<i>fixH</i>	CGGTCATGAGCCTCTTCTTC	CATTGCGGGTAAGGACGTAG
SMa1209	<i>fixI1</i>	GTCGTCTGTACCAATCCCTGT	GATCAGTGCCGAATCCAGT
SMa1208	<i>fixS1</i>	GAACACCCTTATCTATCTCATCCA	TGATTACCCCTCGCCATC
SMa0760	<i>fixT2</i>	TGTTGGATGGGAAAACCAIT	TCTCATCGTCGAGAATCGTG
SMa1170	<i>cycB2</i>	CTGGTGTAAGGAGTGTCACG	GGCACGTGTCAGGGATAGAT
SMc01317	<i>rpoB</i>	CGTCAACAAGTACGGCTTCA	CGTCCATCAGGTTGATGTTG
SMc00128	—	CTTCAGCATGAACGACCAGA	AAGAACCGCGTAACCTTCCT

^z Genes shown in bold were selected for quantitative reverse transcription-PCR analysis to validate the RNA-seq data.

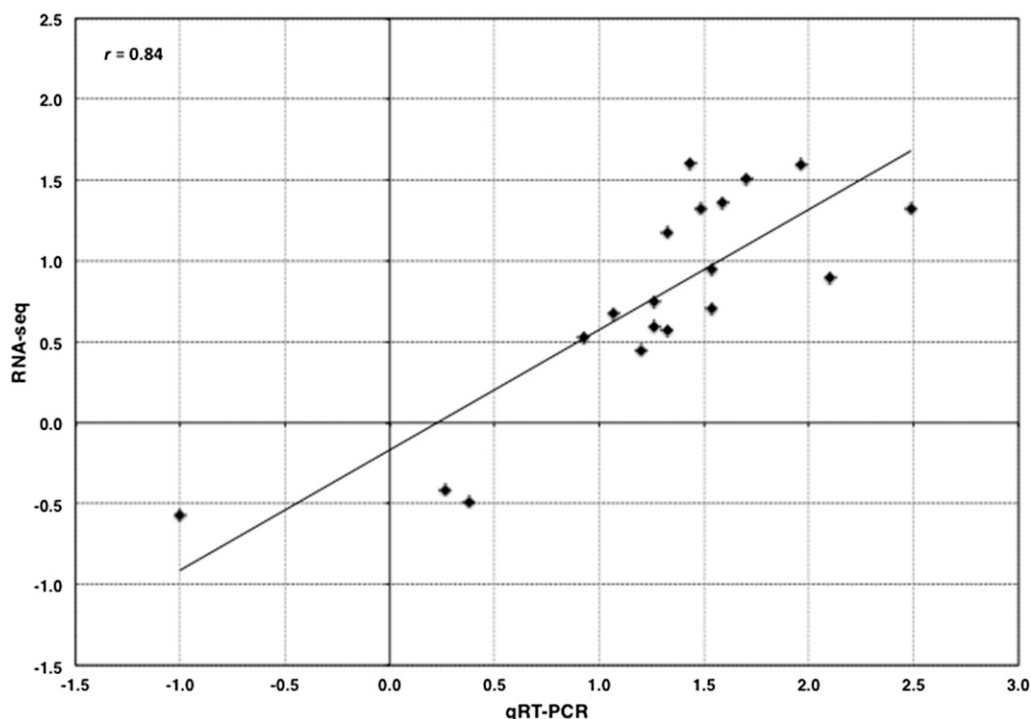


Fig. 5. Validation of RNA-seq results by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Expression of 19 representative genes, differentially expressed in *Sinorhizobium meliloti* RD64 cells as compared with those of strain 1021 cells, was determined using qRT-PCR. The log₂-transformed expression ratios from RNA-seq (vertical axis) and qRT-PCR (horizontal axis) are shown. Pearson's correlation coefficient (*r*) is indicated.

For this purpose, total RNA was extracted from RD64 and 1021 cells grown in rich medium under both aerobic and microaerobic conditions. No growth rate difference was observed between 1021 and RD64 cells, as the optical densities recorded in both experimental conditions were the same.

Aerobic conditions. The highest expression levels were observed for genes *nifA*, *fixK1*, and *fixT2*, coding for the intermediate regulators and for the repressor (Table 4). The simultaneous upregulation of these three genes had already been observed in the analysis of bacterial gene expression during symbiosis (Roux et al. 2014).

Transcription of nitrogen-fixation genes was also evaluated in *fixJ* and *nifA* *S. meliloti* mutant strains. The exogenous treatment of these strains with IAA at a final 0.5 mM concentration did not lead to any alteration in the growth rates, as no difference in the optical density was recorded for untreated and IAA-treated mutant cells. Furthermore, no significant induction in the transcript levels of the selected genes was observed when compared with the untreated mutant strains (Supplementary Table S2). This result lead us to suppose that IAA might act upstream of *fixJ*, possibly at the level of its phosphorylation.

Microaerobic conditions. The comparison of the qPCR data obtained for RD64 and 1021 strains under microaerobic and aerobic conditions revealed a moderate induction level of *fixL*, *fixK1*, and *fixT2* genes in RD64 cells; by contrast, *fixJ* gene showed an almost 10-fold upregulation than the one measured under aerobic condition (Table 4). As far as the *nifA* gene, directly regulated by FixJ, is concerned, a significant induction level was also observed (relative level = 11.4). Under this condition, we did not detect a further induction of the *fixK2* gene.

Under microaerobic conditions, we observed only a slight upregulation of the *fixL* gene as compared with the aerobic one. We hypothesized that the upregulation already observed in aerobic conditions was sufficient to trigger, in microaerobiosis, the phosphorylation of the FixJ N-terminal domain by FixL-phosphate. Preliminary qPCR data regarding the expression of the *fixL* gene in 1021 and RD64 bacteroids showed that the expression of this gene increased in RD64 cells (data not shown). The upregulation level observed was very similar to that measured under free-living conditions (this study).

Our data indicate that IAA is able to positively affect the expression of the two main components (*fixJ* and *nifA*) required for the expression of the nitrogen-fixation genes under microaerobic free-living conditions. It also occurs in conditions (aerobic free-living) very different from those lying within infected nodule tissue, in which a very low free-oxygen concentration is present. Inside root nodules, the presence of respiratory oxidases allows efficient respiration and ATP generation in low-oxygen environments, preventing nitrogenase inactivation (Pessi et al. 2007).

Table 4. Quantitative reverse transcription-polymerase chain reaction analysis of nitrogen-fixation genes in free-living *Sinorhizobium meliloti* cells grown under aerobic and microaerobic conditions

Gene	Relative level ^z	
	Aerobiosis	Microaerobiosis
<i>fixJ</i>	1.5 ± 0.2	28.3 ± 3.3
<i>fixL</i>	1.9 ± 0.1	3.3 ± 0.6
<i>nifA</i>	4.4 ± 0.7	11.4 ± 3.5
<i>fixK1</i>	4.5 ± 0.2	5.9 ± 0.5
<i>fixK2</i>	3.5 ± 0.2	3.2 ± 0.3
<i>fixT2</i>	4.1 ± 0.7	6.1 ± 0.4

^z Relative gene expression levels of *fixJ*, *fixL*, and *nifA* genes from comparative cycle threshold (CT) method; $2^{-\Delta\Delta CT} > 1$, gene more highly expressed in the modified RD64 strain; $2^{-\Delta\Delta CT} < 1$, gene more highly expressed in wild-type strain 1021. Values are the means ± standard deviation of four different biological replicates.

By using an indicator of cell redox potential, the 2,3,5-triphenyl-2H-tetrazoliumchloride (TTC), we found that exponentially grown RD64 cells produced up to 40% more of the insoluble formazan product as compared with 1021 cells (1021: 62 ± 7 mg of Formazan per gram of cells; RD64: 86 ± 7 mg of Formazan per gram of cells). This result indicated that RD64 cells generated an increased amount of reducing equivalents.

Reducing agents for tetrazolium are known to be NADH and NADPH, mainly produced by the TCA cycle (Dunigan et al. 1995). The data obtained in the TTC assay are in agreement with those previously reported regarding the activation of the TCA cycle rate controlling enzymes in RD64 cells (Imperlini et al. 2009).

In our study, the expression levels of the genes coding for terminal oxidases were measured under free-living aerobic conditions (Table 5). A significant induction of all tested genes was observed in RD64 cells. The highest expression levels were measured for copy two of the *fixNOQP* operon, which should be detectable only in bacteroids.

These results were similar to those reported by Yurgel et al. (1998). *S. meliloti* mutants with enhanced ability to reduce a tetrazolium salt had a higher expression level of the *fixNOQP* genes, as compared with the wild-type strain.

The higher expression of genes having high affinity for O₂ might lead to a lowering of O₂ concentration, thus allowing the transcription of nitrogen-fixation genes observed in RD64 cells.

Gene expression analysis under nitrogen-limited conditions.

It is widely reported that phosphorylated NtrC protein is able to activate the operons involved in ammonium assimilation and nitrogen metabolism as well as the *nifA* and *nifHDKE* genes (Fischer 1994; Szeto et al. 1987).

To study the regulation of these activities in RD64 and 1021 cells, we analyzed the expression level of the *ntrC* gene as well as of genes involved in nitrogen fixation and in the nitrogen stress response (NSR) in two different conditions: i) minimal medium supplemented with 18 mM ammonia (M1) and ii) minimal medium supplemented with 5 mM glutamate (M2) (Table 6).

For the M1 medium (very rich in nitrogen), we found that *ntrC* and almost all the NSR genes tested were repressed in RD64 cells compared with that in 1021 cells, with a very low level of expression observed for the *glnII* gene coding for the glutamine synthase GSII. No significant alteration in gene expression was observed for the *glnD* sensor gene.

Table 5. Quantitative reverse transcription-polymerase chain reaction analysis of FixJ-regulated genes coding for terminal oxidases in free-living *Sinorhizobium meliloti* cells grown under aerobic conditions

Gene	Relative level ^z
<i>fixN1</i>	3.9 ± 0.7
<i>fixO1</i>	2.5 ± 0.3
<i>fixQ1</i>	3.5 ± 0.9
<i>fixP1</i>	2.1 ± 0.1
<i>fixN2</i>	5.3 ± 1.3
<i>fixO2</i>	5.6 ± 1.2
<i>fixQ2</i>	2.8 ± 0.2
<i>fixP2</i>	3.9 ± 0.2
<i>fixG</i>	7.0 ± 0.5
<i>fixH</i>	3.8 ± 0.7
<i>fixI</i>	3.4 ± 0.6
<i>fixS</i>	4.0 ± 0.8
<i>cycB2</i>	3.8 ± 0.5

^z Relative gene expression levels of FixJ-regulated genes from comparative cycle threshold (CT) method; $2^{-\Delta\Delta CT} > 1$, gene more highly expressed in the modified RD64 strain; $2^{-\Delta\Delta CT} < 1$, gene more highly expressed in wild-type strain 1021. Values are the means ± standard deviation of four different biological replicates.

A moderate expression level was observed for *nifA* and *nifB* genes in both 1021 and RD64 cells, with a slight increase measured only for the *nifA* gene in RD64 cells compared with that for 1021 cells. A significant upregulation was also observed for the *nifN* gene, which is independent of the *nif* regulon.

In the M2 medium (nitrogen-limited conditions), all the NSR genes were up-regulated except for the *glnD* gene, whose expression, as in previous cases, was unchanged, probably due to the fact that, as a sensor, it is constitutively expressed irrespective of the nitrogen source (Yurgel et al. 2010, 2013). The highest expression levels were observed for *glnK*, *glnII*, and *amtB* genes.

Under these conditions, the expression levels of the *nifA* and *nifB* genes in both 1021 and RD64 cells were higher than those observed in nitrogen-rich medium, with a slight upregulation measured for RD64 cells. A moderate transcription level was also detected for the *nifH* gene. This result was similar to that reported by Szeto et al. (1987) and reinforces the fact that *S. meliloti* 1021, like other rhizobia, is capable of a low level of nitrogen fixation in its natural habitat.

We verified that, in nitrogen-limited medium, the *nifH* gene expression increased in RD64 cells (Table 6). Considering that, under nitrogen limited conditions, the transcription of *nifH* gene is dependent on *ntrC*, the higher expression level observed in RD64 cells could be due to the higher expression of *ntrB* and *ntrC* genes measured in these cells.

The upregulation of the *nifN* gene observed in M2 medium was very similar to the one measured in the M1 medium.

The analysis of the results obtained for the NSR and nitrogen-fixation genes in the two tested growth conditions leads us to speculate that RD64 cells efficiently perceive the extracellular levels of nitrogen and were more able to activate the transcription of *nif* genes under free-living nitrogen-limited conditions.

Conclusions.

In this study, RNA-seq and qRT-PCR were applied to analyze the transcriptome of the IAA-overproducing *S. meliloti* RD64 strain as compared with the wild-type 1021. Comparison showed that the greatest number of transcriptome changes was observed in genes involved in metabolism, environmental information processing, and genetic information processing.

Table 6. Quantitative reverse transcription-polymerase chain reaction analysis of nitrogen stress responsive genes and *nif* genes in free-living *Sinorhizobium meliloti* cells grown under different nitrogen conditions

Gene	Relative level ^x	
	M1 ^y	M2 ^z
<i>ntrB</i>	0.70 ± 0.11	1.9 ± 0.1
<i>ntrC</i>	0.53 ± 0.05	2.5 ± 0.4
<i>glnA</i>	0.57 ± 0.12	1.8 ± 0.2
<i>glnB</i>	0.68 ± 0.13	3.7 ± 0.4
<i>glnD</i>	1.21 ± 0.18	0.9 ± 0.1
<i>glnK</i>	0.45 ± 0.11	5.5 ± 1.0
<i>glnII</i>	0.06 ± 0.01	5.2 ± 0.8
<i>amtB</i>	0.28 ± 0.04	5.1 ± 0.8
<i>nifA</i>	1.4 ± 0.1	1.6 ± 0.1
<i>nifB</i>	1.2 ± 0.2	1.6 ± 0.1
<i>nifH</i>	n.d.	3.3 ± 0.4
<i>nifN</i>	2.1 ± 0.3	1.9 ± 0.1

^x Relative gene expression levels of NSR and *nifA* genes from comparative cycle threshold (CT) method; $2^{-\Delta\Delta CT} > 1$, gene more highly expressed in the modified RD64 strain; $2^{-\Delta\Delta CT} < 1$, gene more highly expressed in wild-type strain 1021. Values are the means ± standard deviation of four different biological replicates.

^y Nitrogen-rich medium = minimal medium supplemented with 18 mM ammonia. n.d. = not detected.

^z Nitrogen-limited medium = minimal medium supplemented with 5 mM glutamate.

In particular, the data presented here indicate that the overproduction of IAA in the rhizobium free-living RD64 positively affects the expression of the *nifA* and *fixK* genes, encoding the two main intermediate regulators, as well as many other genes involved in nitrogen fixation. This effect was observed under both aerobic and microaerobic conditions.

It is known that nitrogen-fixation genes are usually activated in microaerobiosis, which seems, therefore, to be a major physiological factor inducing the symbiotic nitrogen-fixation machinery in *S. meliloti*. Two main factors may have contributed to the induction of nitrogen-fixation genes in RD64 cells under the aerobic conditions used in our experiments.

i) The overproduction of IAA in RD64 cells might have stimulated the phosphorylation of FixJ and, thus, its transcriptional activity. Considering that, in RD64 cells, we observed the activation of two other systems, Ntr (this study) and Pho (previous study) (Bianco and Defez 2009), controlled by homologous signal transduction proteins, we hypothesize that the action of IAA in free-living rhizobia could be mediated by changes in protein phosphorylation. In particular, IAA might affect the activity of histidine kinases, acting as phospho donor in many molecular mechanisms, including nitrogen fixation in rhizobia. Further study in this direction could provide additional data to test our hypothesis. ii) The higher expression of high-affinity terminal oxidases encoding genes observed in RD64 cells might decrease the oxygen availability, leading to the formation of a microaerobic environment, promoting the expression of nitrogen-fixation genes. Additional investigation based on FixJ-dependent gene expression in *fixNOQP* mutants could be useful to examine this issue.

In the soil and rhizosphere, several stresses, including nutrient starvation, oxygen limitation, hyper-osmolarity, thermal shock, and more, negatively affect the survival of rhizobia and, thus, their ability to establish symbiotic relationships with legume plants. The persistence of rhizobia in the absence of their host plant is more dependent on their ability to deal with adverse environmental factors than during symbiosis, in which the nodule represents a protective environment.

In our study, we observed that, under low-nitrogen conditions, the RD64 free-living cells more effectively performed the switching-on of ammonium assimilation and the upregulation of genes involved in stress response. These results are related to their ability to better survive and persist under unfavorable conditions (Defez 2006).

Overall, our results lead us to hypothesize that the RD64 strain has good potential to establish an effective symbiosis with its host plant. A global transcriptional analysis during symbiosis could be a first step toward validating our hypothesis.

We speculate that auxin IAA could be a plant signal used to induce the expression of rhizobia nitrogen-fixation genes inside root nodules.

We also hypothesize that, despite the present study having been performed in free-living rhizobia, bacterial IAA overexpression might improve the nitrogen fixation in different plant-bacteria associations that could poorly establish a microaerobic environment.

Table 7. Strains and plasmid used in this work

Strain or plasmid	Description ^z	Reference or source
<i>Sinorhizobium meliloti</i>		
1021	SU 47 (2011), Str ^r	Galibert et al. 2001
RD64	1021 derivative, Spm ^r	Pandolfini et al. 2000
EK 101	1021 derivative, Nod ⁺	Bobik et al. 2006
	Fix ⁻ , <i>fixJ</i> ::Tn5, Str ^r Nm ^r	
GMI 292	1021 derivative, Nod ⁺	Bobik et al. 2006
	Fix ⁻ , <i>nifA</i> ::Tn5, Str ^r Nm ^r	
Plasmid	<i>p-iaaMtms2</i>	Pandolfini et al. 2000

^z Str^r, Spm^r, Nm^r = resistant to streptomycin, spectinomycin, and neomycin, respectively.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids.

The bacterial strains used in this study are listed in Table 7. The IAA-overproducing RD64 strain is a derivative of *S. meliloti* 1021 strain, containing the p-*iaaM*tms2 construct, in which the coding region of the *iaaM* gene of *Pseudomonas savastanoi* was positioned downstream from an 85-bp promoter sequence and the *tms2* coding region of *Agrobacterium tumefaciens* was placed at the 3' end of the *iaaM* gene, as described by Defez and Spena (1998) and Pandolfini et al. (2000). Bacterial strains were aerobically grown in TYR (RNA-seq and qPCR analyses) (Camerini et al. 2008), in minimal mannitol ammonium chloride medium (qPCR analysis) (Hooykaas et al. 1977) and in minimal medium containing glutamate 5 mM as nitrogen source (qPCR analysis). For growth under low-oxygen conditions, the oxygen was excluded by flushing the headspaces with N₂, and vials were sealed and incubated for 4 h at 30°C on a shaker at 200 rpm. The bacteria were grown for 4 h under N₂ for two main reasons: i) previous studies under aerobic conditions (Bianco and Defez 2009) have shown that 1.5 h of IAA-treatment were sufficient to induce one generation time in 1021 and RD64 cells and to trigger changes in gene expression, and ii) preliminary experiments have revealed that under microaerobic conditions, bacteria need at least 3 h to replicate.

For the IAA treatment, *S. meliloti* EK101 and GMI292 cultures (optical density at 600 nm [OD₆₀₀] = 0.7) were split into two cultures; an IAA solution was added to one culture to a final concentration of 500 µmol/ml and the second one was left untreated (control). After 3 h of incubation at 30°C (OD₆₀₀ = 1.5), independent cell batches of control and IAA-treated cells were collected and were stored at -80°C for use in experiments. The bacterial cells used in the RNA-seq and qPCR analyses were collected in the exponential growth phase (OD₆₀₀ = 0.7).

Streptomycin (200 mg/liter), spectinomycin (200 mg/liter), and neomycin (100 mg/liter) were included as required.

Quantification of the TTC reduction ability in liquid medium.

The TTC assay was performed as previously described (Tachon et al. 2009) with some modifications. Briefly, 1.5 ml of exponentially grown culture (OD₆₀₀ = 0.7) grown in TYR medium were harvested by centrifugation (8,000 × g for 5 min) and were washed with 1 ml of 50 mM sodium-phosphate buffer, pH 7.5. The cell pellets were then resuspended in 1 ml of 50 mM sodium-phosphate buffer, pH 7.5, containing 24 mM TTC and were immediately incubated for 1 h at 30°C. After incubation, the cells were harvested by centrifugation (8,000 × g for 5 min) and were resuspended in 1 ml of dimethyl sulfoxide (DMSO) to solubilize formazan. The absorbance of the supernatant was measured at 510 nm. For quantification, a calibration curve was established with formazan solution prepared with DMSO. Data are the mean ± standard deviation (SD) of at least five biological replicates, each conducted at different times.

RNA-seq analysis.

RNA extraction and mRNA enrichment. RNA was purified from cells up to exponential growth phase (OD₆₀₀ = 0.7) in TYR medium as previously described (Imperlini et al. 2009). Residual DNA present in the RNA preparations was removed, using the RNase-free TURBO DNase I kit (Ambion), according to the manufacturer's instructions. MICROExpress kit (Ambion) was then used to enrich bacterial mRNA from purified total RNA by removing the 16S and 23S ribosomal RNAs. RNA quality was evaluated on a BioAnalyzer (Agilent) chip, prior to cDNA library synthesis.

The sequencing libraries were constructed using the TruSeq RNA-seq sample prep kit from Illumina (Illumina, Inc.),

skipping the first part of the protocol, consisting of the purification of poly-A containing mRNA molecules, using poly-T oligo-attached magnetic beads. RNA-seq libraries were loaded in six-plex into a single lane of a flowcell V. 3 and were sequenced in single read 50-bp set-up with a HiSeq2000 sequencer by IGA Technology Services.

Sequence analyses.

As the first step, we have checked the quality of the 50-bp single-end reads (about 20 to 30 million per sample) using FastQC (version 0.11.2). No specific biases or problems were noticed; therefore, all the reads underwent the mapping step.

Sequencing reads from three biological replicates were aligned to the *S. meliloti* 1021 reference genome, using Bowtie2 (v 2.0.0) (Langmead and Salzberg 2012), an ultrafast and memory-efficient program for aligning short reads derived from transcribed RNA to the reference genome.

The reference genome of *S. meliloti* 1021 (ASM696v1) was downloaded from the National Center for Biotechnology Information (NCBI). Default alignment parameters were used, except for the reporting option -k 10. The Integrated Genomics Viewer (Robinson et al. 2011) was used to check how the reads aligned over the *S. meliloti* 1021 reference genome.

Unambiguous (i.e., unique reads) reported from Bowtie were used to estimate the gene expression levels. In particular, BEDTools (v 2.16.2) (Quinlan and Hall 2010) was used to count the number of reads that map to each gene in the *S. meliloti* genome (ASM696v1), using the annotation files in GFF format, downloaded from NCBI. Differential expression of identified genes was calculated with R (v 2.15.2) using DESeq (v 1.10.1) (Anders and Huber 2010).

DESeq utilizes a negative binomial distribution for modeling read counts per gene and implements a variance-stabilization approach for normalizing the counts. *P* values are adjusted for multiple testing using the method of Benjamini and Hochberg (1995). Genes with an adjusted *P* value smaller than 0.05 were considered differentially expressed; moreover, a cut of 1.3 FC was applied to select those genes with higher biological effects.

The KEGG database was used to cluster the differentially expressed genes into functional categories.

qRT-PCR analysis.

RNA purification and removal of DNA contamination was performed as described in the previous section. After purification and quality checking by agarose gel electrophoresis, the RNA concentration was determined by absorbance at 260 nm and the RNA was stored at -20°C until further use. First-strand cDNA was synthesized from 1 µg of total RNA with the RETROscript kit (Applied Biosystems) and random decamers, according to the manufacturer's instructions.

qRT-PCR was performed as previously described (Bianco and Defez 2009), except that the iQ SYBR Green supermix (Bio-Rad) was used.

Specific primer pairs, designed using Primer3 software for the selected genes, are reported in Table 3.

Primers for *rpoB* and Smc00128 (constitutively expressed genes) were included in all the qRT-PCR analyses for the purpose of data normalization. During the reactions, the fluorescence signal due to SYBR Green intercalation was monitored to quantify the double-stranded DNA product formed in each PCR cycle. Results were recorded as relative gene expression changes after normalizing for *rpoB* genes expression and were computed using the comparative cycle threshold method ($2^{-\Delta\Delta CT}$) as previously described (Bianco et al. 2006b).

The $2^{-\Delta\Delta CT}$ value was >1 for genes more highly expressed in the RD64 cells and <1 for genes more highly expressed in the 1021 wild-type strain.

qRT-PCR data are the mean \pm SD of at least four biological replicates each conducted at different times.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- KEGG *S. meliloti* 1021 database:
http://www.genome.jp/kegg-bin/show_organism?menu_type=pathway_maps&org=sme
- National Center for Biotechnology Information (NCBI) Gene Expression Omnibus series GSE69880:
<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69880>
- NCBI *Sinorhizobium meliloti* database:
<http://www.ncbi.nlm.nih.gov/genome/?term=Sinorhizobium+meliloti>