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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Genes commonly involved in acid tolerance are not overexpressed in the plant microsymbiont *Mesorhizobium loti* MAFF303099 upon acidic shock

Marta Laranjo · Ana Alexandre · Solange Oliveira

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Abstract Rhizobia are legume bacterial symbionts that fix nitrogen in the root nodules of plants. The aim of the present study was to investigate the global transcriptional response of rhizobia upon an acidic shock. Changes in the transcriptome of cells of *Mesorhizobium loti* strain MAFF303099 upon an acidic shock at pH 3 for 30 min were analysed. From a total of 7,231 protein-coding genes, 433 were found to be differentially expressed upon acidic shock, of which 322 were overexpressed. Although most of the overexpressed genes encode hypothetical proteins, the two most represented Cluster of Orthologous Group (COG) categories are 'defence mechanisms' and 'transcription'. Differentially expressed genes are dispersed throughout the chromosome, with the exception of the symbiosis island, where most genes remain unchanged. A significant number of transcriptional regulators and ABC transporter genes are overexpressed. No overexpression of genes typically associated to acid tolerance in rhizobia, such as *act* and *exo* genes, was detected. Overall, this study suggests a transcriptional response to acidic shock of *M. loti* distinct from other rhizobia. Additional studies are in course to explore the role of some of the highly overexpressed genes and to further elucidate the molecular bases of acid stress response.

Keywords Rhizobia · Microarray · Transcriptome · Stress · pH · Acid

Introduction

Rhizobia are soil bacteria that can live as saprophytes or form nitrogen-fixing symbiosis with legumes. Rapid adaptation of bacteria to diverse environmental changes is carried out by a series of global regulatory networks, which are actually stress response systems that control the simultaneous expression of a large number of genes and respond to changes of temperature, pH, nutrients, salts and oxidation (Alexandre and Oliveira 2013; Ron 2006).

The productivity of leguminous crops is strongly affected by soil acidity, which may be natural or result from agricultural practices and industrial pollution. The problem with acidic soils is the limited availability of some essential plant nutrients (e.g. calcium and molybdenum) and the toxic levels of heavy metals, such as aluminium or manganese (Graham and Vance 2000). Soil acidity problems may be overcome by developing legume-rhizobia associations able to tolerate acidic soil conditions.

Environmental pH affects rhizobia survival and saprophytic growth in soil. Furthermore, rhizobia are usually more sensitive to low pH than legumes, which affects the establishment of symbiosis (Zahran 1999). Knowledge of pH-stress survival mechanisms is important for the development of rhizobia strains that will better survive soil acidity thus improving crop yields.

Mesorhizobium species nodulate a very diverse set of legume species, including the model legume, *Lotus japonicus,* and an important legume in human diet, *Cicer arietinum*

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M. Laranjo · A. Alexandre · S. Oliveira
ICAAM-Instituto de Ciências Agrárias e Ambientais Mediterrânicas, Universidade de Évora, Pólo da Mitra, Ap. 94, 7002-554 Évora, Portugal

M. Laranjo · A. Alexandre
IIFA-Instituto de Investigação e Formação Avançada, Universidade de Évora, Ap. 94, 7002-554 Évora, Portugal

S. Oliveira (✉)
Departamento de Biologia, Universidade de Évora, Pólo da Mitra, Ap. 94, 7002-554 Évora, Portugal
e-mail: ismo@uevora.pt

(Laranjo et al. 2014). The optimal pH described for mesorhizobia is between 6 and 8 and the pH range is 4 to 10 (Chen et al. 2005). In general, low pH seems stressful for mesorhizobia; however, *Mesorhizobium loti* MAFF303099 is particularly tolerant to pH 5 (Laranjo and Oliveira 2011). Studies on the biogeography of chickpea mesorhizobia suggest that, in this genus, acid tolerance may be related to the pH of the isolate origin soil (Brígido and Oliveira 2013).

A mixture of constitutive and inducible strategies can contribute to bacterial survival in an acidic environment, namely, the removal of protons, alkalisation of the external environment, changes in the composition of the cell envelope, production of general stress proteins and chaperones, expression of transcriptional regulators, and responses to changes in cell density (Cotter and Hill 2003; Kanjee and Houry 2013).

Cell envelopes are the first barriers that protect bacteria from the surrounding environment and the concomitant stresses. Thus, the first line of defence against adverse environmental pH should minimize cell envelope permeability, either to prevent ingress of protons or their loss (Hall et al. 1995).

ATP-binding cassette (ABC) transporters have been shown to be involved in acid tolerance, e. g. in *Streptococcus pneumoniae* (Martín-Galiano et al. 2005), playing an important role in cytoplasmic pH regulation. This extremely diverse class of transporters couple the energy of ATP hydrolysis to the translocation of solutes (small molecules as well as ions) across biological membranes. The basic unit of an ABC transporter consists of four core domains: two transmembrane domains and two ATP-binding domains (Higgins 2001).

In general, chaperones and proteases have been implicated in the response of bacteria to environmental changes. However, the role of important chaperone systems, such as GroEL and DnaK, in acid stress does not seem to be as crucial as described for the heat-shock response in many bacteria. For example, in *Escherichia coli*, the chaperones so far implicated in acid response are Hsp31, HdeA and HdeB (Kanjee and Houry 2013).

Most pH stress protection systems include a mechanism for sustaining cytoplasmic pH, and some of these systems offer cross-protection to other stresses. In *E. coli*, there are three main independent acid resistance (AR) systems: the oxidative or glucose-repressed, the glutamate-dependent and the arginine-dependent AR system (Audia et al. 2001; Foster 2004).

Some bacterial species show an increased survival rate after challenge at lethal acid pH if they are first subjected to a period of mild sub-lethal acidic conditions, a process known as adaptive acid tolerance or acid tolerance response (ATR), which has been described in *E. coli* (Goodson and Rowbury 1989). The stationary phase alternative sigma factor, σ^S (also known as RpoS or sigma 38), is required for ATR in *Salmonella typhimurium* (Lee et al. 1995). The RpoS alternative sigma factor is also regulated under acidic shock at the transcriptional level in *E. coli* (Audia et al. 2001).

In *E. coli*, other genes found to be involved in acid stress response include *gad* (glutamic acid decarboxylase) and *omp* (outer membrane protein) genes (Foster 2001), as well as genes encoding components of the electron transport chain (*cyo*, *ndh*, *sdh* and *nuo* genes) (Kanjee and Houry 2013).

Studies on rhizobia acid stress response have been conducted in *Ensifer medicae* (Tiwari et al. 2004), *Ensifer meliloti* (de Lucena et al. 2010; Hellweg et al. 2009), *Rhizobium tropici* (Graham et al. 1994) and *M. loti* (Correa and Barneix 1997; Correa et al. 1999). Overall, these studies suggest that *act* (for acid tolerance) and *exo* (exopolysaccharide I biosynthesis) genes are commonly involved in acid response.

In rhizobia, acid tolerance mechanisms seem to involve regulation of cytoplasmic pH, proton exclusion and/or extrusion (Graham et al. 1994) and exopolysaccharide (EPS) production (Correa et al. 1999). The quantity of EPS produced by rhizobia isolates from *Cicer*, *Phaseolus*, *Leucaena* and *Melilotus* species is positively correlated with acid tolerance, and it is postulated that EPS could modify the rhizobia microenvironment and so decrease the stresses induced by an acid soil (Cunningham and Munns 1984). However, in *M. loti* strains, the EPS amount seems to have no correlation with acid tolerance, as tolerant strains showed lower ability to produce EPS under acidic conditions (Correa and Barneix 1997).

Acid response in rhizobia involves a range of genes that are essential for growth at low pH, including some that are specific to acid stress response, the *act* genes, such as *actA*, *actP*, *actR*, and *actS*, and *exoR* (exopolysaccharide regulatory protein) gene. At least three regulatory systems exist in rhizobia: the two-component sensor-regulator system, *actSR*, is essential for induction of the adaptive ATR; another system involves the low pH-induced transcriptional regulator gene, *phrR* (pH regulated), which may control other low pH-regulated genes; the third circuit controls the expression of a pH-regulated structural gene, *lpiA* (low pH inducible) (Glenn et al. 1999). In addition, a number of orphan proteins of unknown function whose concentration changes at low pH was described in rhizobia (Dilworth et al. 2001).

E. medicae WSM419 has at least two systems responding to low pH: the *phrR* system and the system regulating *lpiA*, which is specific to low pH (Dilworth et al. 2001). In addition, the *actS-actR* sensor-regulator system, where *actS* is a sensor histidine kinase transmembrane protein and *actR* a transcription regulator protein, is essential for acid tolerance and expressed constitutively in *E. medicae*, regardless of the medium pH (Tiwari et al. 1996). Nevertheless, there are other genes whose expression is modulated by low pH, such as for example *nodA* and *nodF* in *Rhizobium leguminosarum* (Richardson et al. 1988).

Acid tolerance in *M. loti* strains seems to involve constitutive mechanisms, like outer membrane permeability, as well as adaptive responses to the medium pH, such as the stage of

bacterial growth and differential protein expression (Correa and Barneix 1997). However, little is known about the genes involved in the response to acidic stress.

Global transcriptional analysis can contribute to our knowledge on the mechanisms involved in rhizobia response to acidity. Only two microarray studies involving acid stress have been performed, both in *E. meliloti* 1021 (de Lucena et al. 2010; Hellweg et al. 2009), an acid-sensitive strain (Laranjo and Oliveira 2011). These studies indicate that the response of *E. meliloti* to low pH is characterised by the upregulation of *exo* genes, involved in exopolysaccharide I biosynthesis, and downregulation of flagellar and chemotaxis genes.

The aim of the present study was to analyse the global transcriptional response of *M. loti* MAFF303099 following an acidic shock. To our knowledge, this is the first study on the global transcriptional response to acidic stress in *Mesorhizobium*.

Materials and methods

Bacterial strains and growth conditions

The bacterial strain used in this study was *M. loti* strain MAFF303099. For gene expression profiling, three independent cultures were grown overnight at 28 °C in YMB (Vincent 1970) medium to an optical density of 0.3 (540 nm). A 10-ml volume of cells was used for each treatment: cells subjected to a pH of 3 for 30 min (acidic shock with a 1-M HCl solution) and cells exposed to no pH change (control).

RNA isolation and processing

After the acidic shock, cells were harvested immediately and total RNA was extracted using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) with DNase (Roche Applied Science, Penzberg, Germany) treatment following the manufacturer's instructions. Once absence of residual DNA was confirmed, concentration and purity were determined using a Nanodrop ND-1000 UV-visible spectrophotometer (Thermo Scientific, Waltham, USA). RNA integrity was checked with an Agilent 2100 Bioanalyser using a RNA Nano assay (Agilent Technologies, Santa Clara, USA).

Microarray experiments

Microarray experiments were performed at BIOCANT-Genomics Unit (Cantanhede, Portugal) as a service.

Each microarray experiment was conducted in three biological replicates. The messenger RNA (mRNA) microarrays (*Mesorhizobium loti* MAFF303099 40 K) were carried out as described by the manufacturer (MYcroarray, Ann Arbor, USA). The array has 20,450 probes, of which 7,231 are unique, covering 99.3 % of all genes. Slide images were acquired using the DNA Microarray B Scanner (Agilent Technologies, Santa Clara, USA) and scanned with an intensity of 100 % PTM in the green channel.

Microarray analysis

Data were extracted using the QuantArray software (Packard BioScience, Meriden, USA). The arrays were analysed using the BRB data analysis tools for Excel (Simon et al. 2007) and normalized using the median. These data were then used to identify the genes with differential expression using the MeV4.0 software package (Saeed et al. 2006). A statistical Student's *t* test with a *p* value threshold of 0.01 was applied. The data were deposited in the NCBI Gene Expression Omnibus (GEO) under the record number GSE43527.

DNAPlotter (Carver et al. 2009) was used to generate circular DNA maps showing transcriptomics data.

Validation of microarray data by quantitative real-time RT-PCR

DNA microarray data were validated by quantitative real-time reverse transcription PCR (RT-PCR). For reverse transcription, 1 μg of total RNA from *M. loti* MAFF303099 was used. Complementary DNA (cDNA) was synthesized using Maxima® First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA) according to the manufacturer's instructions. Primers used to amplify selected *M. loti* MAFF303099 genes (Table 1) were designed using Primer Express 3.0 software (Applied Biosystems, Carlsbad, USA). RT-PCR amplification mixtures used 2.5 ng of template cDNA, 2× SYBR Green PCR Master Mix and 0.3 mM of reverse and forward primers for each gene in a total volume of 25 μl. Reactions were performed using a model 7500 thermocycler (Applied Biosystems, Carlsbad, USA). The expression ratio of the target genes was determined relative to reference genes *hisC*, *rpoA* and *sigA*, which showed no variation in the transcript abundance under the experimental conditions used here. Relative quantification of gene expression by real-time RT-PCR was determined by applying the ΔΔCt method (Pfaffl 2001).

Gene re-annotation

Since the full-genome annotation available for *M. loti* strain MAFF303099 was published in 2000 (Kaneko et al. 2000), most of the genes found to be overexpressed encode unknown or hypothetical proteins. We used Blast2GO (Götz et al. 2008) to annotate all differentially expressed genes encoding unknown or hypothetical proteins and STRING (http://string-db.org) to assign Clusters of Orthologous Groups (COGs) (http://www.ncbi.nlm.nih.gov/COG/) to these newly

Table 1 Primers list used for validation of microarray data by real-time RT-PCR

Locus tag	Gene	Primer sequence (5′-3′)
mlr5786	*hisC*	fwd: GGATAGCGTGGCGATGATG
		rev: TTGAGCACCCTGCAACGTT
mlr0325	*rpoA*	fwd: CCTCTATTCGCCCGTCAAGA
		rev: CGTCATGGTCAGCTTGTCATAGTC
mll2386	*sigA*	fwd: GCCCTCTGCTCGACCTTTCC
		rev: AGCATCGCCATCGTGTCCTC
mll3842	*citZ*	fwd: AAAAGCGCTCGACACCTATCTG
		rev: CGAAGGTCGAGGCGTTGA
mll6578	*fixK*	fwd: TCGTTGCCGTCGCATTCT
		rev: GTTGCGTTTCCGAGTCGAA
mll6630	*fixN*	fwd: GAGCCTTTCCGACAGCATATGT
		rev: ACTTGTGTTGCGCAGAAGAACA
mll1528	–	fwd: TCACCAGGATCGCCAATTG
		rev: AGCAGCCGGCGAATGTC
mlr2394	*groEL*	fwd:GTCGTAGAGGGCATGCAATTC
		rev: GACGCGCATCTTGTCCTGAT
mll3429	*clpB*	fwd:GGAGCTTGTCGGCCTTGA
		rev: AAGCCCGAGCTTCTGCTTCT

annotated genes (Szklarczyk et al. 2011). MicrobesOnline Operon Predictions (www.microbesonline.org/operons/) was used for operon prediction (Price et al. 2005).

Results

Global changes in gene expression induced by acidic shock

From a total of 7,231 protein-coding genes present in the *M. loti* MAFF303099 genome, 433 genes (6 %) were found to be differentially expressed after the acidic shock with an *M* value distribution between 3.9 and −6.1 (Fig. 1 and Supplementary Fig. S1). Furthermore, upregulation dominates over downregulation: 322 genes were found to be overexpressed whereas only 111 were underexpressed. Genes were considered as differentially expressed if $p \leq 0.01$, considering an average false discovery rate (FDR) of 0.11. Low *M* values were considered, as long as $p \leq 0.01$, since low level changes in gene transcription, commonly disregarded, may be an important part of cells response, as pointed out by Wren and Conway (2006).

From the 7,231 genes annotated in the *M. loti* MAFF303099 genome, 6,702 are located in the chromosome, 320 in pMLa and 209 in pMLb (Kaneko et al. 2000). Replicon distribution analysis of the 433 differentially expressed genes showed that 400 (92 %) are chromosomal, 13 (3 %) are in pMLa and 20 (5 %) are in pMLb. Most differentially expressed genes are upregulated in all three replicons: 295 genes in the chromosome, 9 in pMLa and 18 in pMLb (Table 2 and Fig. 2). From the 111 downregulated genes upon the acidic shock, 105 are in the chromosome, 4 in pMLa and 2 in pMLb (Table 2 and Fig. 2). It is noteworthy that 10 % of the pMLb genes are differentially expressed (and 90 % of these are overexpressed), while the percentage is smaller for the other replicons (chromosome, 6 %; pMLa, 4 %).

Differentially expressed genes are dispersed throughout the chromosome, with the exception of the symbiosis island. The expression level of genes located in the symbiosis island remains essentially unaltered (Fig. 2a), with only eight genes (approximately 1 %) being differentially expressed, namely, three overexpressed and five underexpressed, among a total of 580 genes (Kaneko et al. 2000).

It was possible to assign COGs to 355 of the 433 differentially expressed genes according to predicted gene functions (Table 3 and Supplementary Table S1). Genes were distributed by all 21 functional (COG) categories included in the genome. Figure 3 summarizes the percentage of differentially expressed genes in each COG category represented on the microarray. About 5 % of the genes not assigned to a COG were differentially expressed upon the acidic shock.

The COG categories with the largest percentage of overexpressed genes are ‘Defence Mechanisms’ (V) (6 out of 67 genes) and ‘Transcription’ (K) (53 out of 594 genes) (Fig. 3).

The COG category with the largest number of genes with a significantly decreased expression is ‘general function prediction only’ (R) (14 genes) followed by ‘signal transduction mechanisms’ (T), ‘energy production and conversion’ (C) and ‘carbohydrate transport and metabolism’ (G) (10 genes in each category).

To confirm data obtained by microarray analysis, we examined the expression of six genes (*citZ*, *fixK*, *fixN*, mll1528, *groEL* and *clpB*) by real-time RT-PCR (Table 4). Based on the results obtained by microarray analysis, the genes selected for real-time RT-PCR were chosen among those overexpressed, underexpressed and not differentially expressed. Results obtained by quantitative real-time PCR are globally in agreement with microarray data (Table 4).

Transcriptional regulators/sigma factors

From the 433 genes found to be differentially expressed upon the acidic shock, 48 genes are annotated as transcriptional regulators, of which 42 were overexpressed. These correspond to approximately 12 % of the transcriptional regulators encoded in the MAFF303099 genome.

The overexpressed regulator genes with the highest fold induction (*M* values between 3.2 and 2.6) belong to different families of transcriptional regulators, namely, mll5152 and mlr7736 from the MarR family, mll3694 encoding a FixK

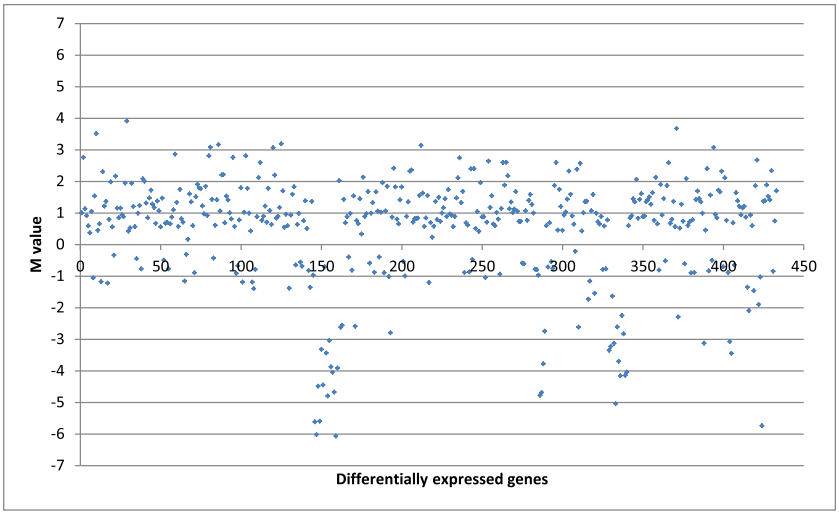

Fig. 1 Microarrays analysis of *M. loti* MAFF303099 upon the acidic shock. *M* value distribution for the differentially expressed genes ($p \leq 0.01$). Overexpressed genes (322) have positive *M* values, while underexpressed genes (111) have negative *M* values

homologue, mll4997 from the LacI family, mll0250 from the IclR family and mll4816 from the TetR family.

Genes involved in cell envelope/ABC transporters

Gene mll1528 was found to be the most highly overexpressed gene (M=3.9) upon the acidic shock. It encodes a small integral membrane protein with an iron permease conserved domain, which is probably part of an ABC transporter system. Several genes with an *M* value higher than 2 upon the acidic shock (Table 3) are involved in 'cell wall/membrane/envelope biogenesis' and 'carbohydrate transport and metabolism'. For example, gene mll0693 (M=3.5) encodes a sugar transferase involved in the cell envelope (outer membrane) biogenesis.

We have found at least 24 differentially expressed ABC transporter genes, of which 18 are upregulated (*M* values between 3.9 and 0.2). These 18 upregulated genes, likely to belong to 14 different ABC transporter systems, include five permeases and five ATP-binding proteins. Gene mll3590 (M=3.1) encodes a periplasmic component of an ABC-type sugar transport system similar to the *E. coli ugpB* gene. It is in the same operon as gene mll3591, which is also overexpressed (M=1.4). Gene mll4997 (M=3.1) encodes a periplasmic component of an ABC-type sugar transport system similar to the *E. coli rbsB* gene. It is located upstream of the operon containing genes mll4996, mll4993 and mll4992, encoding an ABC-type sugar transport system. Gene mlr3639 (M=2.6) encodes a trehalose-/maltose-binding protein, which is a periplasmic component of an ABC-type sugar transport system similar to the *E. coli ugpB* gene and is part of an operon containing genes mlr3640, mlr3641, mlr3643, mlr3644 and mlr3645. The different induction levels found among the genes of some predicted operons may be due to internal regulation (promoters or terminators), premature termination or differential mRNA degradation (Laing et al. 2006).

Table 2 Number of genes overexpressed and underexpressed upon acidic shock, in each replicon

	Number of overexpressed genes	Number of underexpressed genes
Chromosome	295	105
pMLa	9	4
pMLb	18	2

Genes involved in exopolysaccharide biosynthesis

M. loti MAFF303099 genome has 32 genes annotated as involved in EPS synthesis. Among these, only one was found to be overexpressed, namely, *exsG* (mlr3704), a sensory transduction histidine kinase (M=1.9).

Acid tolerance (*act*) genes

A cation transporting P-type ATPase (acid-induced copper pump) (mlr5325) with homology to *actP* is reported as overexpressed (M=2.4). Other important *act* genes, such as the genes mlr5307 and mlr5308 coding for the two-component regulatory system ActS/ActR, respectively, or the apolipoprotein n-acetyltransferase gene *actA* (mlr5543), were found to be not differentially expressed.

Similarly, the transcription levels of the genes *lpiA* (mll8344) and *phrR* (mlr5544) remained unchanged.

Nitrogen fixation genes

fix genes are involved in nitrogen fixation and can code electron transport chains to nitrogenase, cytochrome oxidase and transcriptional regulators (Terpolilli et al. 2012). Nine *fix*

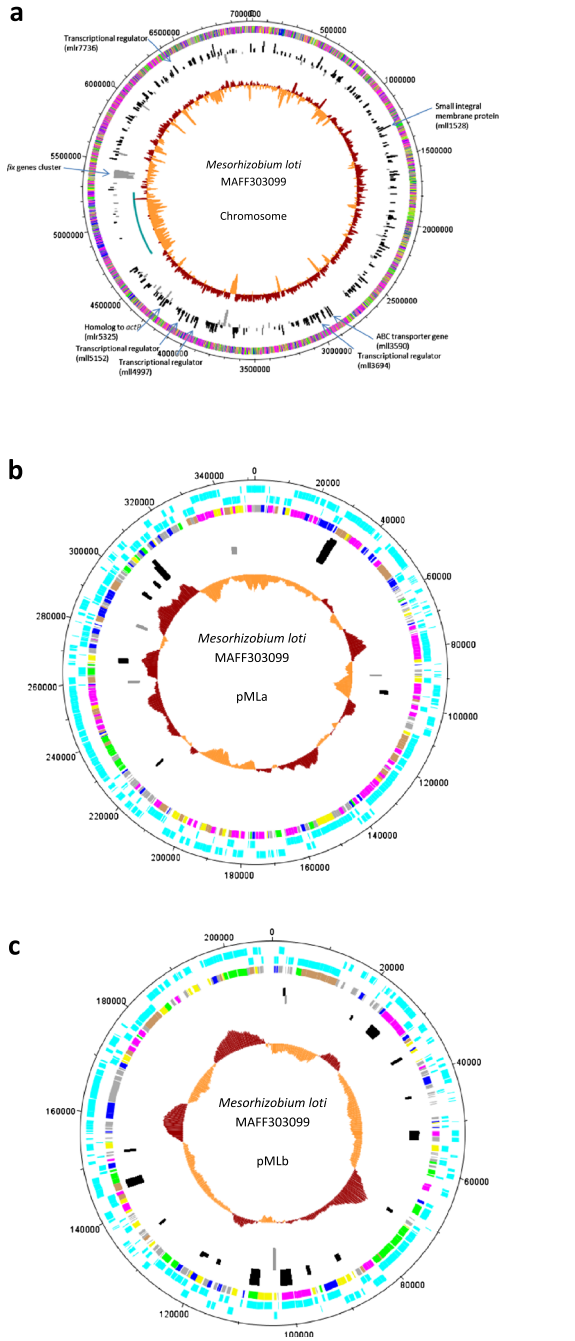

Fig. 2 Circular plots of the chromosome (**a**) and the two plasmids (**b**, **c**) from *M. loti* MAFF303099: *outer ring*, COG group for each gene; *middle ring*, acid shock transcriptome data (*M* values); *inner ring*, %GC. Plasmid plots include two additional outer rings displaying genes encoded in the plus strand (*outermost ring*) and minus strand. COG colours: information storage and processing, *blue*; cellular processes and signalling, *green*; metabolism, *magenta*; poorly characterized, *yellow*; more than one COG category, *brown*; no COG, *light grey*. Transcriptome data: overexpressed (*black*) and underexpressed genes (*grey*). %GC data: above average (*red*) and below average (*orange*). The symbiosis island is marked in *blue* in the chromosome plot (coordinates 4644792 to 5255766) (Kaneko et al. 2000). Some of the most relevant genes in the acid shock response are mapped in the chromosome plot (Color figure online)

genes are found to be severely underexpressed: *fixN* (mll6630), *fixK* (mll6578), *fixI* (mll6624), *fixO* (mll6629), *fixP* (mll6628), *fixG* (mll6626), *fixQ* (msl6627), *fixS* (msl6623) and *fixH* (mll6625). All these genes are located in a chromosomal cluster outside the symbiosis island (coordinates 4644792 to 5255766) (Fig. 2a). However, some of these genes have multiple copies, such as *fixH* and *fixNOPQ*, which have one homologue within the symbiosis island, whose expression remained unaltered.

On the other hand, a second *fixK* homologue, mll3694, located in the chromosome but outside the symbiosis island, is upregulated (M=3.2). FixK is a transcriptional regulator required for low pH induction of *fix* genes, which is regulated through ActR (Fenner et al. 2004).

Proteases and chaperones genes

No significant upregulation of proteases and chaperones genes was observed in the present microarray analysis, except for one serine protease gene (mlr7692) with homology to *degP1* that was found to be overexpressed (M=1.8).

However, the expression values obtained with real-time PCR for *groEL* indicates its overexpression (M=1.6), while for *clpB*, a slight underexpression (M=−0.5) was detected upon acidic shock.

Discussion

When subjected to the acidic shock, *M. loti* strain MAFF303099 showed 322 genes overexpressed and 111 underexpressed. All three replicons show a higher number of induced over repressed genes.

Similarly to the observed response of MAFF303099 following the acidic shock, also in *E. meliloti* strain 1021 (de Lucena et al. 2010; Hellweg et al. 2009), upregulation dominates over downregulation. The MAFF303099 transcriptional response included the overexpression of a significant number of genes encoding transcriptional regulators and ABC transporters.

Contrary to the response of *E. meliloti* to low pH, in which a large number of exopolysaccharide biosynthesis genes are upregulated and motility and chemotaxis genes are downregulated (Hellweg et al. 2009), in strain MAFF303099, these genes were found to remain mostly unchanged. Only 1 out of 32 genes involved in EPS biosynthesis was found to be overexpressed. Our results suggest that the response of *M. loti* MAFF303099 to acidic stress does not include an increase in the synthesis of EPS. This hypothesis is supported by previous studies reporting that *M. loti* acid-tolerant strains showed a decreased production of EPS in acidic medium (Correa and Barneix 1997). Other authors had already reported a lack of

Table 3 List of genes with higher increased expression ($M \geq 2$) upon the acidic shock

Locus tag	Location	Gene description	COG category[a]	M value
mll1528	Chr	Small integral membrane protein	S	3.9
mlr8209	Chr	Transcriptional family with acetyltransferase activity	E/K	3.7
mll0693	Chr	Sugar transferase	M	3.5
mll5152	Chr	Transcriptional regulator	K	3.2
mll3694	Chr	Transcriptional regulator	T	3.2
mlr0132	Chr	Type 12 methyltransferase	H	3.1
mll3590	Chr	Sugar transporter sugar binding protein	G	3.1
msl1808	Chr	Hypothetical protein	-	3.1
mll4997	Chr	Family transcriptional regulator	G	3.1
mll2749	Chr	DMT superfamily	R	2.9
mll3568	Chr	Esterase lipase-like protein	I	2.8
mll4607	Chr	Ku protein	S	2.8
mll0250	Chr	Transcription regulator	K	2.8
mll4093	Chr	Transglutaminase domain protein	E	2.8
mlr1995	Chr	Hypothetical protein	not in COG	2.7
msr4805	Chr	Hypothetical protein	-	2.7
mlr3136	Chr	Hypothetical protein	not in COG	2.6
mlr3707	Chr	Hypothetical protein	not in COG	2.6
mlr3639	Chr	Trehalose/maltose binding protein	G	2.6
mll4816	Chr	Tetr family transcriptional regulator	K	2. 6
mlr4831	Chr	Phenylalanine 4-monooxygenase (*ppha*)	E	2.6
mlr7736	Chr	Transcriptional regulator	K	2.6
mlr5354	Chr	Haloalkane dehalogenase	R	2.6
mll8569	Chr	Transcriptional regulator	G/K	2.4
mlr9679	pMLb	Circadian oscillation regulator kaic-like protein	L/T	2.4
mlr2526	Chr	Methylated-DNA-protein-cystein methyltransferase	L	2.4
mlr2436	Chr	NADH-dependent dyhydrogenase	R	2.4
mlr5325	Chr	Cation transporting P-type atpase	P/S	2.4
mll9638	pMLb	Serine protein	T	2.4
msr8675	Chr	Hypothetical protein	S	2.3
mlr5096	Chr	Phosphoenolpyruvate carboxykinase	C	2.3
mll9633	pMLb	Formate dehydrogenase	C	2.3
msl3831	Chr	Conserved hypothetical transmembrane protein	not in COG	2.3
mll0857	Chr	Transcriptional regulator	K	2.3
mll3842	Chr	Citrate synthase 2	C	2.2
mll3776	Chr	Streptothricin-acteyl-transferase	R	2.2
mll5048	Chr	Family protein	S	2.2
mlr3777	Chr	Family transcriptional regulator	K	2.2
mll1046	Chr	Cytochrome C oxidase subunit I	S	2.2
mll7529	Chr	Glyoxalase bleomycin resistance protein dioxygenase	R	2.1
mlr7359	Chr	Transcriptional regulator	K	2.1
mll4785	Chr	NADPH:ferredoxin reductase	C	2.1
msl5548	Chr	Hypothetical protein	-	2.1
mlr1797	Chr	Conserved domain protein	S	2.1
mlr9030	pMLa	Hydantoinase	E/Q	2.1
mll1959	Chr	BA14K family protein	not in COG	2.1
mlr7106	Chr	Dehydrogenase	I/Q/R	2.1
mll6735	Chr	Arginine/ornithine antiporter	E	2.0
mll1989	Chr	Family transcriptional regulator	K/T	2.0

NCBI gene annotation was used, except for grey-shaded gene descriptions, which are the result of Blast2GO analysis

Chr chromosome, *pMLa* plasmid, *pMLb* plasmid, − COG not determined

[a] COG category letters according to NCBI functional categories (http://www.ncbi.nlm.nih.gov/COG/grace/fiew.cgi)

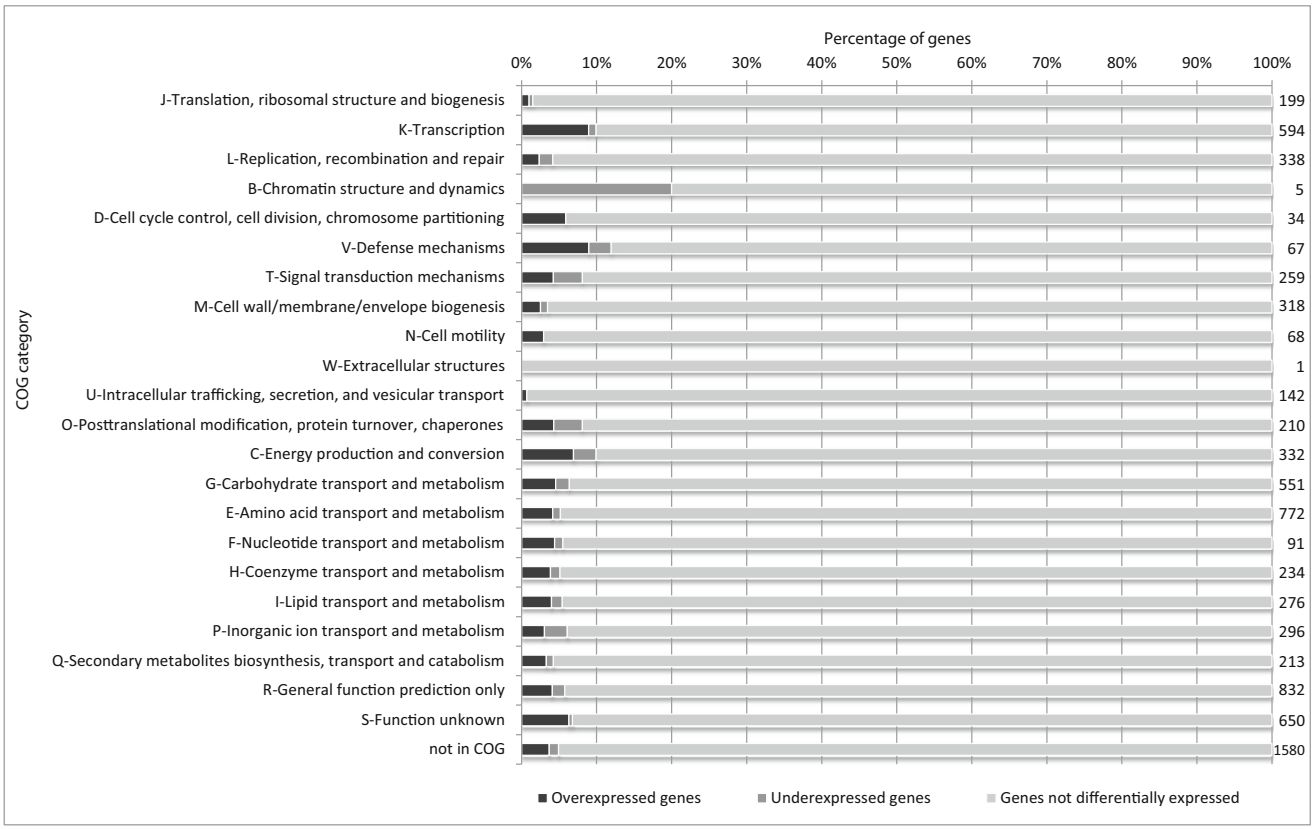

Fig. 3 Percentage of differentially expressed genes by COG category

correlation between exopolysaccharide synthesis and acid tolerance (Chen et al. 1993).

The different tolerance levels to low pH of *M. loti* MAFF303099 (tolerant) and *E. meliloti* 1021 (sensitive) results not only from their different gene content and regulation but also from their distinct transcriptional response to acidity. Since *M. loti* is acid-tolerant and shows no significant upregulation of genes previously described to be involved in acid tolerance, we may hypothesise a constitutive expression of these genes and/or an alternative mechanism of acid tolerance. Furthermore, comparisons should be carefully considered as different stresses were applied in each study: *E. meliloti* 1021 was subjected to a pH shift from 7 to 5.75 for 60 min (Hellweg et al. 2009), while *M. loti* MAFF303099 suffered an acidic shock from pH 7 to pH 3, for 30 min. However, Hellweg et al. (2009) have shown that genes are rapidly induced (or repressed) after an acidic shock, which suggests that the shock period per se cannot explain the different results obtained with these two rhizobia strains.

Table 4 Quantitative real-time RT-PCR analysis for validation of microarray data

Locus tag	Gene	*M* value	
		Microarrays	Real-time PCR
mll3842	*citZ*	2.2	3.4
mll6578	*fixK*	−6.0	−6.0
mll6630	*fixN*	−6.1	−6.7
mll1528	–	3.9	1.4
mlr2394	*groEL*	n.d.e.	1.6
mll3429	*clpB*	n.d.e.	−0.5

n.d.e. not differentially expressed

In our study, the transcriptional levels of most *act* genes, commonly involved in acid response in rhizobia, remained unaltered. Interestingly, in *E. medicae* WSM419, which is also an acid-tolerant strain, *actR* and *actS* are constitutively expressed irrespectively of the external pH (Tiwari et al. 1996).

Of the three known acidity sensor systems in root nodule bacteria (Dilworth et al. 2001), ActS-ActR, PhrR and LpiA, none of the encoding genes was found to be differentially expressed in our study, which suggests that the acid tolerance mechanisms in *Mesorhizobium* are probably different and rely on other genes.

In the present study, the unaltered expression of genes found to be involved in acid response in other rhizobia together with the high tolerance of MAFF303099 to acidity may suggest that these genes are constitutively expressed in *M. loti* MAFF303099 and/or other resistance mechanisms are involved. These might essentially comprise genes coding for

transcriptional regulators and ABC transporter systems, since many of these genes were found to be upregulated in the present study.

All bacterial genomes encode an essential housekeeping sigma factor and most have at least one alternative sigma factor. Housekeeping sigma factors recognize a large set of promoters, while alternative sigma factors recognize specific groups of promoters for genes with a shared function (Gruber and Gross 2003). Since alternative sigma factors compete with the housekeeping sigma factor, changes in gene expression can occur by controlling expression, activity and availability of alternative sigma factors (Österberg et al. 2011). The presence of a large number of alternative sigma factors in a species appears to correlate with a diverse lifestyle (Gruber and Gross 2003). *M. loti* MAFF303099 has 25 putatively annotated sigma factors. Among these, at least eight are alternative sigma factors: three *rpoN* (nitrogen limitation), three *rpoE* (extracytoplasmic-ECF type) and two *rpoH* (heat shock) sigma factors. RpoH2 is an alternative sigma factor involved in the regulation of EPS synthesis in rhizobia, acting as a transcriptional regulator of the *exo* genes (Kaufusi et al. 2004). In *M. loti* MAFF303099, the *rpoH2* gene (mlr3862) was not found to be differentially expressed upon the acidic shock, which could probably explain the unaltered transcription level of almost all genes involved in EPS biosynthesis.

Bacterial survival in a medium with high proton concentration is dependent upon its ability to maintain a constant cytoplasmic pH. This can partly be achieved by avoiding the diffusion of protons from the medium into the cell, so changes in envelope composition (phospholipid, fatty acid, and protein composition) may be an adaptation to survive at low pH, as suggested for *M. loti* (Correa et al. 1999). In the present study, several genes coding for outer membrane and other cell envelope proteins, namely, ABC transporters, were reported as overexpressed, thus probably contributing to maintenance of the cell internal pH.

Our results have shown a number of genes encoding transcriptional regulators of the MarR and TetR families to be overexpressed upon the acidic shock. These are repressors that control expression of genes coding for efflux pumps (Grkovic et al. 2002). *marR* regulator genes have been found to be induced by acidic stress in other bacteria such as *Dickeya dadantii* (Reverchon et al. 2010). Regulators from the MarR and TetR families have also been reported to be associated with oxidative stress response in *Bradyrhizobium japonicum* (Masloboeva et al. 2012).

Although some chaperones and proteases have been described to be involved in the acid response in bacteria, no significant upregulation of these genes was observed in the present microarray analysis. However, a previous study with several mesorhizobia species indicated that genes encoding important chaperones, such as DnaK and GroESL, are induced upon acidic shock in most acid-tolerant strains (Brígido and Oliveira 2013). In the present study, the real-time RT-PCR analysis shows an induction of the *groEL* gene (mlr2394), although the microarray data indicated no differential expression. We cannot exclude the possibility that the inherent variability of microarray data may lead to some biologically important changes in gene expression being statistically excluded from the analysis, as described before in other bacteria (Martín-Galiano et al. 2005). Another chaperone, known to interact with the DnaKJ system, is ClpB. This chaperone gene was found to be not differentially expressed in the present study, and this is consistent with previous results in *Mesorhizobium ciceri* that showed that the *clpB* knockout did not affect the ability of the mutant strain to tolerate an acidic shock (Brígido et al. 2012). Agreeing with previous studies that had shown the protease gene *degP* as the most strongly induced gene in *E. meliloti* upon acidic shift (de Lucena et al. 2010), our results have shown overexpression of a serine protease gene with homology to *degP*, a secondary response mechanism, which breaks down misfolded proteins that could not be recovered by the chaperones, when the external pH is very low.

A recent study reported the transcriptome analysis of *M. loti* MAFF303099 when subjected to heat shock (Alexandre et al. 2014). A global comparison of these two responses to stress shows that the number of genes differentially expressed is much smaller in the case of acidic shock, and moreover, the response to acid conditions is mostly driven by gene upregulation, while the heat shock led to an extensive gene downregulation.

Comparing this study in *M. loti* MAFF303099 with two previous studies in *E. meliloti* 1021 (de Lucena et al. 2010; Hellweg et al. 2009), distinct genes were found to be overexpressed. Our results suggest that the response mechanisms to an acidic shock are not identical among rhizobia species and may account for the acid tolerance of the strain, involving the induction of genes associated with different functional groups. Furthermore, our results have shown that a large number (approximately 16 %) of the responsive genes belong to the group of hypothetical or unknown genes. The present study can contribute for a better understanding of the molecular bases of response to acidic pH, with the acid-induced genes representing promising targets for future investigations.

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