A metabolomic approach to characterize the acid-tolerance response in Sinorhizobium meliloti

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Received: 3 February 2017 / Accepted: 17 April 2017
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Abstract

Introduction Sinorhizobium meliloti establishes a symbiosis with Medicago species where the bacterium fixes atmospheric nitrogen for plant nutrition. To achieve a successful symbiosis, however, both partners need to withstand biotic and abiotic stresses within the soil, especially that of excess acid, to which the Medicago-Sinorhizobium symbiotic system is widely recognized as being highly sensitive. Objective To cope with low pH, S. meliloti can undergo an acid-tolerance response (ATR(+)) that not only enables a better survival but also constitutes a more competitive phenotype for Medicago sativa nodulation under acid and neutral conditions. To characterize this phenotype, we employed metabolomics to investigate the biochemical changes operating in ATR(+) cells. Methods A gas chromatography/mass spectrometry approach was used on S. meliloti 2011 cultures showing ATR(+) and ATR(−) phenotypes. After an univariate and multivariate statistical analysis, enzymatic activities and/or reserve carbohydrates characterizing ATR(+) phenotypes were determined.

Results Two distinctive populations were clearly defined in cultures grown in acid and neutral pH based on the metabolites present. A shift occurred in the carbon-catabolic pathways, potentially supplying NAD(P)H equivalents for use in other metabolic reactions and/or for maintaining intracellular-pH homeostasis. Furthermore, among the mechanisms related to acid resistance, the ATR(+) phenotype was also characterized by lactate production, envelope modification, and carbon-overflow metabolism.

Conclusions Acid-challenged S. meliloti exhibited several changes in different metabolic pathways that, in specific instances, could be identified and related to responses observed in other bacteria under various abiotic stresses. Some of the observed changes included modifications in the pentose-phosphate pathway (PPP), the exopolysaccharide biosynthesis, and in the myo-inositol degradation intermediates. Such modifications are part of a metabolic adaptation in the rhizobia that, as previously reported, is associated to improved phenotypes of acid tolerance and nodulation competitiveness.

Keywords Metabolomics · Sinorhizobium meliloti · Acid-tolerance response · Acid stress

1 Introduction

Sinorhizobium meliloti is an α-proteobacterium able to establish a nitrogen-fixing symbiosis in indeterminate root nodules with legumes of the genera Medicago, Melilotus, and Trigonella. The capacity to fix atmospheric nitrogen for plant nutrition gives these associations a fundamental relevance to the nitrogen cycle, thus making rhizobia a subject of interest in agricultural-production systems as well.
Environmental conditions play an essential role in the control of legume–Rhizobium interactions. The establishment of a successful symbiotic interaction is coordinated by both partners and involves a complex system of intercommunication (Gage 2004; Gibson et al. 2008; Jones et al. 2007). This interaction will be successful if the symbiotic partners are able to overcome different biotic and abiotic conditions of the environment that, upon being present, could interfere with bacterial persistence, infection, nodulation, and nitrogen-fixation activity. High soil temperature, salinity, and moisture; metal toxicity; nutrient deficiency; and acid or alkaline pH are some of the environmental features that impose stress on both symbiotic partners (Zahran 1999).

The Sinorhizobium-Medicago symbiosis has been known for years to be extremely sensitive to acid stress (O’Hara et al. 1989; Glenn et al. 1999; Glenn and Dilworth 1994). A low pH in soil affects plant development, bacterial viability, and also several steps in bacterium-plant recognition and nodule formation (Glenn et al. 1999; Kochian et al. 2004; Caetano-Anollés et al. 1989; Ferguson et al. 2013). Accordingly, diverse approaches have been used to characterize the biological mechanisms that govern the resistance to acid stress of Sinorhizobium, as a model bacterium, and that could be targets for the biotechnologic manipulation of inoculants in order to improve their symbiosis performance in acid environments (Dilworth et al. 2001). Several mutagenesis studies have been carried out in Sinorhizobium strains to discover the genes involved in acid tolerance. These approaches have detected several loci whose expression varies according to the ambient pH (Reeve et al. 1998; Riccillo et al. 2000; Tiwari et al. 1996a, b; Vinuesa et al. 2003). None of these genes alone, however, is able to generate a phenotype completely tolerant or sensitive to acidity, thus pointing to the multigenic nature of the bacterial response to acid stress. On the basis of these observations, several omics approaches have been used to characterize the biological systems acting on acid-stressed cells (Reeve et al. 2004; Tiwari et al. 2004; Hellweg et al. 2009). Our laboratory recently contributed to an elucidation of the molecular mechanisms underlying the regulation of the crucial physiologic changes that occur in acid-stressed bacteria. In particular, Draghi et al. (2016) reported an integrated analysis entailing a combination of transcriptomic, metabolomic, and proteomic analyses of the responses of Sinorhizobium meliloti under controlled growth rates and pH in chemostat culture. The observed response to acidic pH was not based on a few specific genes, but rather involved whole sets of RNAs, proteins, and metabolites associated with various cellular functions. Under the low-pH conditions imposed in the chemostat, we demonstrated that the pentose-phosphate pathway (PPP) in those cultures exhibited increases in various transcripts, enzymes, and metabolites compared to Sinorhizobium meliloti cells grown at neutral pH (Draghi et al. 2016).

In addition, as with other bacteria, Sinorhizobium meliloti manifested an acid-tolerance response (ATR(+)) when faced with otherwise lethal acidic conditions (Dilworth et al. 1999; O’Hara and Glenn 1994). This class of response—defined as a cellular resistance to acid shock after growth for a determined length of time at a moderately low pH—exemplifies the variation in the phenotype of the cells relative to that under the previous growth conditions. That Sinorhizobium meliloti from acid-adapted cultures (of phenotype ATR(+)) exhibited an improved competitiveness for nodulation of the host plant Medicago sativa under growth conditions involving both acid and neutral pH is furthermore highly relevant (Draghi et al. 2010). In this regard, the ATR(+) phenotype not only becomes essential when bacteria must withstand acid stress, but also represents a desirable trait for rhizobial selection in acid environments in order to achieve a successful symbiosis under otherwise nonoptimal circumstances.

The availability of Sinorhizobium meliloti ATR(+) strains enabled the possibility to biotechnologically manipulate the bacterial culture conditions of strains that possessed recognized nitrogen-fixation abilities but exhibited poor performance in acidic soils. In order to obtain rhizobial inoculants with improved biological capabilities in such environments, further work will be required to increase our knowledge concerning both the molecular details responsible for acid tolerance and the characteristics of the rhizobial physiology during the processes leading to the acid-tolerant state. Within this context, one essential aspect that has not yet been studied is the metabolic alteration that occurs in rhizobia during the adaptive response to sublethal acidity—i.e., the ATR(+) phenotype. In the present work, this point was investigated through the application of a metabolomic approach to characterize the Sinorhizobium meliloti 2011. The experimental approach presented here served to highlight the metabolic changes associated to the process of transient acid adaptation in a model rhizobia.

2 Materials and methods

2.1 Bacterial strains and culture conditions

Sinorhizobium meliloti 2011 batch cultures were established in Evans minimal medium (Evans et al. 1970) containing 10 g L⁻¹ glucose as carbon source and 0.7 g L⁻¹ ammonium chloride as nitrogen source. The pH was controlled by the addition of 20 mM 2-(N-morpholino) ethanesulfonic acid or 20 mM 1,4-piperazine-diethanesulfonic acid piperezine to keep the pH close to 6.1 or
7.0, respectively. The rhizobia were grown at 28 °C and 250 r.p.m. in a rotary shaker up to the early log phase of growth (OD600 = 0.2 ± 0.05). Each primary culture was inoculated at a density insuring at least two generations of growth before harvesting.

2.2 Metabolomic profiling

From 200 ml of early log-phase cultures, cells were harvested by entrapment upon filtration through a 0.45-μm nitrocellulose filter membrane. The retained bacteria were scraped off the membrane and directly frozen in liquid nitrogen before lyophilization. The procedures for sample derivatization and metabolite identification and quantification by gas-chromatography–mass-spectrometry (GC–MS) were performed without modifications as described by Barsch et al. (2004). The relative peak area of each compound was determined by normalization to the ribitol internal standard (10 µM). Six replicates (10–30 mg cells dry weight) under each pH condition were analyzed.

2.3 Determination of enzymatic activities

Early log-phase cells from 100 ml cultures were centrifuged at 10,000×g for 30 min at 4 °C and washed once with phosphate buffer saline and the resulting pellets lyophilized for storage at −20 °C until use. The dry cells were resuspended in 2 ml of Tris–HCl 0.08 M, pH 7.5 and sonicated three times for 30 s at 50% power (Vibracell, Sonics & Materials Inc, Newtown, CT, USA). After cell disruption, the suspension was centrifuged at 30,000×g for 30 min at 4 °C to precipitate cell debris. The total protein content was measured in the supernatant with a Bradford Protein Assay (Bio-Rad Lab, Hercules, CA, USA). The pellet was dissolved in water, dialyzed against distilled water, lyophilized, and stored at −80 °C until use. The amount of EPS in these samples was determined by the anthrone method as previously described (Trevelyan et al. 1952).

Lactate dehydrogenase activity was measured by the phenazine-methosulfate (PMS-) coupled reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described by Futai (1973). One ml of reaction mixture contained 0.08 M Tris HCl pH 7.5, 120 µg PMS, 60 µg MTT, 2 mM D,L-lactic acid, and 10 mM NAD. The absorbance was measured at 570 nm after 30 min of incubation at 37 °C.

Urease activity was measured by quantitating the rate of ammonia release from urea to form indophenol, the generation of which reaction product was measured at 640 nm by the phenolphthalein method (Solorzano 1969). One unit of urease activity was defined as the amount of enzyme required for the hydrolysis of 1 µmol of urea per min at 37 °C. The assay contained 25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid pH 7.75, 0.5 mM ethylenediaminetetraacetic acid, and 50 mM of the urea substrate.

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Myo-inositol dehydrogenase activity was assayed by a modified procedure described by Poole et al. (1994) and determined at 570 nm by the PMS-coupled reduction of MMT, as cited above. The reaction buffer contained 50 mM NH4Cl, 0.4 mM NAD+, 50 mM Na2CO3 pH 10, and 100 mM myo-inositol. One unit of enzyme was defined as the amount of enzyme required to reduce of 1 µmol of MTT per min at 24 °C, at an ε value of 17 mM −1 cm−1 (Kistler and Lin 1971).

The absorbance obtained from enzyme-catalysed reactions was adjusted by subtracting background values from a reaction containing no substrate. All the experiments were performed in triplicate and the differences in activities analysed statistically by the Student t test.

2.4 Quantification of the content of polyhydroxybutyrate (PHB) and exopolysaccharides (EPSs)

Sinorhizobium meliloti pellets (10–20 mg of cells dry weight) were homogenized with sodium hypochlorite overnight at room temperature, washed with double-distilled water, precipitated with 1:1 alcohol:acetone, and resuspended in chloroform (Quelas et al. 2013). PHB was then determined as crotonic acid in H2SO4 (Law and Slepecky 1961). To isolate the EPSs, the supernatant from 200 ml of culture was first lyophilized to reduce the volume tenfold, then the EPSs were precipitated with three volumes of cold ethanol and the suspension incubated at −20 °C overnight, and finally the supernatant was centrifuged 10,000×g for 15 min. The pellet was dissolved in water, lyophilized, and stored at −80 °C until use. The amount of EPS in these samples was determined by the anthrone method as previously described (Trevelyan et al. 1952).

2.5 Determination of NADH and NAD+ concentrations

From 10 to 15 mg of cells dry weight, the NADH and NAD+ pools were extracted in acid or alkaline buffer according to the protocol described by Leonardo et al. (1996). The supernatants obtained were assayed for NADH and NAD+ by measuring the continuous spectrophotometric rate. For NADH, the reaction contained 100 mM Na2HPO4 pH 7.5, 2.3 mM sodium pyruvate, 0.033% (w/v) bovine-serum albumin, and 2–4 units of lactate dehydrogenase (Sigma). NADH standards from 0 to 0.2 mM were used for cofactor quantification. For NAD+, the reaction contained 100 mM Na2HPO4 pH 7.5, 3 mM sodium lactate, 0.033% (w/v) bovine-serum albumin, and 2–4 units of lactate dehydrogenase (Sigma). NAD+ standards from 0 to 0.10 mM were used. The reactions were carried out...
at 37 °C and the increase in absorbance at 340 nm read at 5 min after enzyme addition.

2.6 Statistical data analysis

Data matrix obtained was used for univariate and multivariate analysis. All metabolite concentrations were log-transformed and standardized (mean: 0 and standard deviation: 1) before the statistical analysis. The MetaboAnalyst 2.0 web server was used for data normalization and univariate data analysis (t test and metabolites Fold change) (Xia et al. 2012). For multivariate analysis, transformed data matrix was exported into SIMCA-P 14.0 (Umetrics, Umeå, Sweden) and the supervised orthogonal partial least square discriminant analysis (OPLS-DA) was applied in order to reveal significant differences between the metabolic fingerprints associated with the pH treatments. The efficiency and reliability of the PLS-DA models was assessed by R2X, R2Y and Q2 parameters, permutation tests (n = 999), and performing a sevenfold internal cross validation-analysis of variance (CV-ANOVA, p value ≤ 0.01). The discriminating metabolites were selected when Variable Importance in the Projection (VIP) values where higher than 1 (VIP > 1.0).

3 Results

3.1 Metabolite profiling of S. meliloti 2011 growing at different pHs in batch cultures

In order to obtain a snapshot of the metabolome of ATR(+) rhizobia compared to that of non-adapted cells to low pH, we performed a metabolome analysis on rhizobia from cultures grown at pH 6.1 and 7.0 respectively, which were harvested by rapid filtration and then frozen in liquid nitrogen as previously described by Barsch et al. (2004). The cell metabolites were extracted with methanol and then analysed by GC–MS after derivatization. A total of 61 metabolites, mainly involved in the metabolism of carbon and nitrogen compounds, could be identified and quantified (Supplementary Table 1). The univariate statistical analysis applied to the set of normalized data indicated that 21 metabolites (34.4%) became significantly varied in their intracellular concentrations (Student t test, p ≤ 0.05) when the extracellular pH was either pH 7.0 or pH 6.1 (Table 1). The orthogonal partial least squares discriminant multivariate analysis (OPLS-DA) demonstrated that bacteria derived from the neutral and acid cultures could be differentiated on the basis of their corresponding metabolome profiles (Fig. 1). The validation of the OPLS-DA model showed good predictability (Q2 = 0.818) and goodness of fit values (R2X = 0.542, R2Y = 0.954), and the cross validation analysis of variance showed lower values than the threshold (p = 0.00096). In addition, permutation plots comparing the goodness of fit (R2 and Q2) of the PLS-DA models with the goodness of fit of Y-permutated models (Supplementary Fig. 1) fulfilled the requirements to validate the model, as Q2 values from permutated models are lower than Q2 values from the original model and the regression line of the Q2-points intersects the vertical axis at, or below zero (Graham et al. 2016). Inspection of those components whose variable importance in projection (VIP) values were higher than one in the OPLS-DA revealed that 26 metabolites were informative for a discrimination of cells according to their pH of growth (Table 1, rightmost column).

3.2 Metabolic pathways of S. meliloti affected by the extracellular pH during growth in batch cultures

Modification of the pH of the culture medium from 7.0 to 6.1 resulted in changes in several compounds of central carbon (C) metabolism (Fig. 2). Acidic conditions produced an increase in the concentration of the first intermediates in the glucose-degradation pathways, including glucose-6-P, gluconolactone, gluconate, and gluconate-6-P. All these metabolites belong to a set of reactions shared in common between the oxidative branch of the PPP and the Entner–Doudoroff pathway (EDP; Fuhrer et al. 2005). Several three-carbon (triose) compounds derived from glucose catabolism also underwent changes in cytosolic concentration at the more acid extracellular pH. Whereas glyceraldehyde-3-P, glyceraldehyde-2-P, phosphoenolpyruvate (PEP), and lactate all had higher concentrations in acid-grown cells, the concentration of pyruvate was lower at pH 6.1. No significant changes were observed in the metabolites of the tricarboxylic-acid cycle during batch-culture growth of the bacteria under both pH conditions.

Several amino acids and/or their intermediates also became significantly varied in concentration upon alteration of the extracellular pH (Fig. 3). At pH 6.1, serine levels increased, but alanine concentrations were lower. In addition, several intermediate compounds in the biosynthesis of methionine became elevated at acid pH. Likewise, the levels of shikimate—a biosynthetic precursor of aromatic amino acids—were higher at pH 6.1: whereas 2-isopropylmalate—an intermediate in the biosynthesis of branched-chain amino acids—along with urea—an end product in arginine metabolism—both became decreased at acid pH.

3.3 Enzyme activities related to some of the observed changes in the metabolome

3.3.1 Lactate dehydrogenase

In view of the variation in the cytosolic concentration of lactate at changes in the extracellular pH of the batch
cultures, we investigated the total l-lactate dehydrogenase (LDH) activities in rhizobia grown at pHs 6.1 and 7.0 as indicated in Materials and Methods (Fig. 4, Panel a). The *S. meliloti* genome contains four genes encoding for putative lactate dehydrogenases: SMc01455 (*dld*), SMc01712 (*lldD2*), and SMc01740 (*lldD1*), all chromosomally located, along with SMb20850 (*lldD3*) carried by the pSymB megaplasmid (Capela et al. 2001). Rhizobial cells grown at pH 6.1 contained a much higher LDH activity than that observed at pH 7.0. This result was consistent with the higher concentration of lactate and lower level of pyruvate recorded in the rhizobia grown at pH 6.1 than the corresponding values for those intermediates at pH 7.0.

### 3.3.3 Urease

As we indicated above in the section on metabolic pathways, the concentration of urea varied according to the extracellular pH in the growth medium. Urea metabolism has been reported to be involved in maintaining

### 3.3.2 Myo-inositol dehydrogenase

ATR(+) rhizobia had a higher concentration of myo-inositol than the ATR(−) rhizobia grown at pH 7.0 (Table 1). The catabolism of myo-inositol in *S. meliloti* is mediated by a myo-inositol dehydrogenase encoded by the gene *idhA* (SMb20899) located in the pSymB megaplasmid. This gene plays a key role in the symbiosis of the rhizobia with *Medicago sativa* plants (Kohler et al. 2010) and also in the symbiosis of other rhizobia, such as *Sinorhizobium fredii* USDA191 (Jiang et al. 2001) and *Rhizobium leguminosarum* bv. *viciae* (Fry et al. 2001) with their corresponding host plants. Figure 4, Panel b depicts the markedly higher activity of myo-inositol dehydrogenase in the ATR(+) rhizobia than in the ATR(−) rhizobia, thus pointing to a role of myo-inositol catabolism in the response to acid stress.

### Table 1

|   | p value       | Fold change | log_{2}(FCa) | VIP scores |
|---|---------------|-------------|--------------|------------|
| 1 | Gluconate-6-P | 4.30 × 10^{-10} | 26.29 | 4.72 | 1.59 |
| 2 | Glycerate-3-P | 1.46 × 10^{-5} | 6.85 | 2.78 | 1.47 |
| 3 | Gluconate    | 9.61 × 10^{-5} | 2.84 | 1.51 | 1.40 |
| 4 | Phosphoenolpyruvate | 1.66 × 10^{-3} | 2.37 | 1.24 | 1.31 |
| 5 | Serine       | 2.31 × 10^{-3} | 3.39 | 1.76 | 1.44 |
| 6 | Homoserine   | 3.08 × 10^{-3} | 14.37 | 3.85 | 1.41 |
| 7 | Fructose     | 4.49 × 10^{-3} | 2.29 | 1.19 | 1.40 |
| 8 | Glycerate    | 0.01 | 1.81 | 0.86 | 1.31 |
| 9 | O-succinyl-homoserine | 0.01 | 1.80 | 0.85 | 1.36 |
| 10 | Fructose-6-P | 0.01 | 1.44 | 0.53 | 1.24 |
| 11 | Glyceral-2-P | 0.01 | 1.90 | 0.93 | 1.28 |
| 12 | 2-Isopropylmalate | 0.01 | 0.61 | −0.72 | 1.17 |
| 13 | Urea        | 0.01 | 5.95 | 2.57 | 1.25 |
| 14 | Pyruvate    | 0.01 | 0.63 | −0.66 | 1.14 |
| 15 | Homocysteine | 0.02 | 1.68 | 0.75 | 1.20 |
| 16 | Glucose-6-P | 0.02 | 1.35 | 0.43 | 1.14 |
| 17 | Lactate     | 0.02 | 1.91 | 0.93 | 1.04 |
| 18 | Alanine     | 0.03 | 0.53 | −0.91 | 1.10 |
| 19 | Sucrose     | 0.03 | 1.89 | 0.92 | 1.08 |
| 20 | Gluconolactone | 0.03 | 1.39 | 0.48 | 1.11 |
| 21 | Shikimate   | 0.05 | 1.53 | 0.61 | 1.05 |
| e22 | α-Glycerophosphate | 0.06 | 1.29 | 0.37 | 1.09 |
| e23 | Methylcitrate | 0.06 | 1.48 | 0.56 | 1.17 |
| e24 | Proline     | 0.06 | 2.15 | 1.10 | 1.12 |
| e25 | Uracil      | 0.06 | 1.42 | 0.51 | 1.05 |
| e26 | Myoinositol | 0.06 | 1.47 | 0.56 | 1.16 |

*The table shows significantly different metabolites according to the univariate (p ≤ 0.05) or the multivariate variable-importance-in-projection (VIP) values. Those metabolites whose VIP values are ≥1 and p values ≥0.05 are marked with asterisks (*)

a FC, fold change in concentration at pH 6.1 relative to the value at pH 7.0
intracellular-pH homeostasis in several microorganisms—e.g., *Helicobacter pylori*, *Streptococcus* spp., and *Brucella* spp. (Mobley et al. 1995; Chen et al. 2000; Bandara et al. 2007). To investigate if a similar role might also be operative in *S. meliloti*, we tested the urease activity in ATR(+) and ATR(−) rhizobia. Figure 4, panel c accordingly indicates that the urease activity was substantially lower in the ATR(+) cells compared to activity measured in the cells not adapted to acidity.

### 3.4 Quantification of PHB and EPSs in ATR(+) and in ATR(−) rhizobia

In addition to synthesizing the structural polysaccharides found mainly in the bacterial membranes and cell walls, rhizobia are able to elaborate several secreted polysaccharides, such as the EPSs and cyclic β-glycans, along with polysaccharides that are accumulated internally, such as PHB or glycogen (Zevenhuizen 1981). Because these polymers fulfil diverse functions in free-living cells as well as participating in signalling processes and establishing symbiosis, the biosynthesis or degradation of the EPSs and PHB is highly regulated by environmental conditions (Dilworth et al. 1999; Mendrygal and Gonzalez 2000; Tavernier et al. 1997; Povolo and Casella 2009). In order to determine the influence of acidity on the carbon-storage status of cells that had been grown in batch culture at the two pHs, we quantified the PHB content. The data in Table 2 demonstrate that the amount of PHB/cell dry weight was significantly lower in the ATR(+) rhizobia at pH 6.1 than in the ATR(−) cells at pH 7.0 (*p* ≤ 0.01). In contrast, the EPS production was significantly higher (Student *t* test, *p* ≤ 0.05) at pH 6.1 (63.3 ± 25.1 mg mg⁻¹ dry weight) than at pH 7.0 (15.8 ± 4.8 mg mg⁻¹ dry weight).

### 3.5 Determination of the internal redox state

The intracellular content of PHB (through a balance of synthesis and degradation) is controlled by the cellular availability of NADH (Uchino et al. 2007). Thus, because of
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1. Discussion

The acid-stress response in *S. meliloti* has proven to be a multigenic trait (Draghi et al. 2016), where several molecular changes operate in the acid-challenged rhizobia. Accordingly, several attempts have been made—mainly through the use of genetic, transcriptomic, or proteomic approaches (Reeve et al. 2004; Tiwari et al. 2004)—to characterize the biochemistry of the rhizobial responses to extracellular acidity. Acidic batch cultures of rhizobia were of particular interest since under this growth condition bacterial cells enter into the so-called ATR(+) state and display both an increased acid tolerance and an improved competitiveness for nodulation (Draghi et al. 2010). In the present experiments, a metabolomic approach was used to characterize ATR(+) rhizobia compared to control ATR(−) cells grown at neutral pH. The results accordingly demonstrated significant changes in several intermediates of the central C metabolism (Table 1). In *S. meliloti* C sources are catabolized through two main pathways, the Entner–Douderoff and the pentose-phosphate. The absence of phosphofructokinase in *S. meliloti* enables the use of only the reverse Embden–Meyerhoff (EM) pathway in gluconeogenesis, in this manner providing precursors for polysaccharide biosynthesis (Geddes and Oresnik 2014). The lower half (C3-compounds) of the EM pathway is formally fully operative. The increases in both fructose-6-P and glucose-6-P in the acidic batch cultures are consistent with a recent report on the metabolome of *S. meliloti* grown in a chemostat under acidic conditions where an increased production of EPS was also observed (Hellweg et al. 2009; Geddes et al. 2014).

As was shown in this work (Table 2), the analysis of the [NADH]/[NAD+] ratio indicated that the relative amount of the reduced form of this dinucleotide decreased severely under acidity in batch cultures, thus suggesting a higher demand for reducing power in the acid-stressed rhizobia. A limited availability of reducing power is furthermore in agreement with the observed decrease in the PHB content per bacterial cell at low pH. Such a limitation in reducing power might be the origin of the observed increases in different metabolites of the PPP that would be consistent with a higher activity of this pathway at low pH. Increases in several transcripts of the PPP had also been observed in *Staphylococcus aureus* in acid batch cultures (Bore et al. 2007), thus indicating that the observation presented here might correspond to a more general response to acidity in bacteria. An increase in the oxidative reactions of the PPP (i.e., glucose-6-P → glucono-1,5-lactone and then gluconate → ribulose-5-P) would be expected to generate a C overflow which must be eliminated via secondary metabolites like EPS and possibly also through lactate production (Paczia et al. 2012), which intermediate proved to be elevated in the metabolome of the acid-grown rhizobia. The synthesis of lactate in rhizobia has been previously reported as a fermentative response under different stresses such as low temperatures or during the oxygen depletion of batch cultures (Sardesai and Babu 2000; Encarnacion...
et al. 1995). That several triose-related compounds were increased in the acid-grown rhizobia in batch cultures was remarkable since glycerate-3-P, glycerate-2-P, and phosphoenolpyruvate are upstream intermediates in the biosynthesis of lactate. The intracellular concentrations of several amino acids and related precursors became significantly altered at low pH. The decreased concentration of alanine or 2-isopropylmalate (a precursor in the synthesis of leucine) in acid-grown cells is probably a consequence of the low concentration of pyruvic acid. In contrast, the intracellular concentration of proline increased at low pH, as has also been reported to happen in other bacteria under osmotic stress (da Costa et al. 1998). The concentration of shikimate, an intermediate in the synthesis of aromatic amino acids, was elevated at low pH. Shikimate consumption requires phosphorylation by the shikimate kinase encoded by the aroK gene (Dunn 2015). In Escherichia coli this reaction is rate-limiting in this pathway and the expression of aroK is under the control of a two-component CpxR/CpxA system that exerts a negative transcriptional control of aroK under stress conditions affecting envelope integrity, such as alkaline stress (Hunke et al. 2012; Vogt and Raivio 2012). Santos et al. (2010) have suggested that the loci SMB20560 and SMB20561 are possible S. meliloti homologues to CpxR/CpxA, respectively. If a regulatory pathway similar to the one observed in E. coli is present and operative in S. meliloti, the changes reported by those authors in membrane lipids under acid stress would likely have impacted membrane structure so as to change the concentration of shikimate via a Cpx-like form of aroK regulation (Chen E. et al. 2009). Nevertheless, the means by which the observed increases in intracellular shikimate could promote acid tolerance in rhizobia is not yet clear. Another two-component system that is responsive to acid stress in S. meliloti is the ExoS/ChvI (Hellweg et al. 2009), which loci at low pH positively regulate several genes of the myo-inositol catabolic pathway including iolB, iolC, iolD, and iolE (Sohlenkamp and Geiger 2016). Under continuous cultivation at pH 6.1 we have also observed an increased transcription of idhA (Draghi et al. 2016), the gene that encodes a myo-inositol dehydrogenase (Jiang et al. 2001), which activity was found here to be increased...
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by more than eightfold (Fig. 4, Panel b). That in the reaction sequence from glucose to the first intermediate of the myo-inositol degradation pathway a net NADH is gained might be relevant in view of the decreased intracellular NADH/NAD+ ratio under acidity.

In order to maintain cytoplasmic-pH homeostasis, bacteria may use different mechanisms addressed at counteracting the effects of increased hydrogen-ion concentrations in the extracellular medium (Lund et al. 2014). In certain microorganisms the hydrolysis of urea to NH₄⁺ and CO₂ by urease has been reported to contribute to a sequestering of hydrogen ions from the cytoplasm (Lund et al. 2014). Since ATR(+) rhizobia exhibited a lower urease activity than those grown at neutral pH, the hydrolysis of urea for the purpose of neutralizing intracellular acidity is unlikely to be operative in S. meliloti. The lower urease activity that we observed here (Fig. 4, Panel c) is consistent with the decreased transcription previously reported for the ureC locus encoding the urease alpha-subunit (Draghi et al. 2016). Nonetheless, other urea-independent deamination reactions could contribute to a neutralization of intracellular hydrogen ions by generating ammonium ions. The methionine biosynthetic pathway, for example, generates those ions during the deamination of cystathionine (Ferla and Patrick 2014). In addition, that pathway generates precursors of the membrane-lipid diacylglycerol- N,N,N-trimethylhomoserine, which intermediate has been involved in changes in the composition of the S. meliloti membranes under other stress conditions (Geiger et al. 1999). Both the accumulation of the several intermediates of methionine biosynthesis demonstrated in this work and the previously reported increases in their transcripts (Hellweg et al. 2009) support a role for this pathway in facilitating rhizobial growth under conditions of acidity. Further analyses will be required to elucidate the degree of contribution of all these reactions to acid tolerance in S. meliloti.

Acid-stressed S. meliloti manifested several changes in different metabolic pathways while growing in acidic batch cultures. Though the precise relationship between the observed changes and acid tolerance could not always be established, alterations in specific metabolic pathways could be identified in several instances that were related to responses to different abiotic stresses reported in other bacteria. Since ATR(+) rhizobia, whose metabolome we characterized in this work, are also more competitive for noduleation than the non-adapted strains (Draghi et al. 2010), improvements in cultural conditions in accordance with the modifications reported here could serve the practical purpose of obtaining symbiotically more competent inoculants for acidic soils without a loss of viability.
Table 2 Measurement of intracellular carbon storage and reducing-power balance

| PHB content (mg mg⁻¹ dry weight) | NADH/NAD+ ratio |
|----------------------------------|-----------------|
| pH 7.0 0.045 ± 0.0023            | 12.25 ± 2.67*   |
| pH 6.1 0.018 ± 0.001**           | 2.68 ± 0.19     |

The table shows polyhydroxybutyrate (PHB) content and the balance of intracellular reduced power (NADH/NAD+) ratio in S. melliloti of the ATR(+)/ phenotype versus the non-acid-adapted phenotype. The results are the means of triplicate experiments ± the SDs.

*Significant differences at p values ≤ 0.05
**Significant differences at p values ≤ 0.01 by the Student t test

Acknowledgements This research was partially supported by the National Science and Technology Research Council (PIP 2014/0420, Consejo Nacional de Investigaciones Científicas y Técnicas—CONICET, Argentina) and National Agency for Science and Technology Promotion (PICT 2012/1719). W.O.D., M.F.D.P., F.A., M.I.L., and A.L. are members of CONICET. The authors are grateful to Dr. Donald F. Haggerty for editing the final version of the manuscript.

Compliance with ethical standards

Conflict of interest None of the authors have any conflict of interest to declare.

Research involving human or animal participants This article does not contain any studies with human or animal subjects.

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Supplementary Figure 1. The permutation plot (n = 999 iterations) for the validation of the OPLS-DA model (R² = green circles, Q² = blue squares). Figure A shows the values plot for Class N (pH 7.0). Figure B shows the values plot for Class A (pH 6.1).
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**Supplementary Table 1.** Complete list of metabolites detected and quantified by gas-chromatography–mass-spectrometry. Table values are the mean and the standard deviation of the quantified metabolites at each pH condition. Values are expressed as µM.

| Amino-acid metabolism | pH 7.0       | pH 6.1       |
|------------------------|--------------|--------------|
| Compound               | KEGG ID      | Mean  | Std. Dev. | Mean  | Std. Dev. |
| Alanine                | C00041       | 1.54391 | 0.69036   | 0.774288 | 0.507864  |
| Arginine               | C00062       | 0.02715 | 0.005166  | 0.018269 | 0.019114  |
| Asparagine             | C00152       | 0.22203 | 0.214452  | 0.199272 | 0.176639  |
| Aspartate              | C00049       | 2.98409 | 1.516756  | 2.94633  | 0.969774  |
| ß-Alanine              | C00099       | 0.04241 | 0.042957  | 0.09243  | 0.164     |
| Glutamate              | C00025       | 25.1339 | 28.78122  | 20.81099 | 26.1459   |
| Glutamine              | C00064       | 0.0082  | 0.00957   | 0.02314  | 0.025316  |
| Glycine                | C00037       | 2.56421 | 0.914013  | 5.465661 | 5.314584  |
| Isoleucine             | C16434       | 0.21796 | 0.095336  | 0.277431 | 0.129578  |
| Leucine                | C00123       | 0.2741  | 0.163127  | 0.266022 | 0.152769  |
| Lysine                 | C00047       | 2.83557 | 3.25799   | 2.051535 | 3.93744   |
| Methionine             | C00073       | 0.07448 | 0.034714  | 0.103581 | 0.063256  |
| Ornithine              | C01602       | 0.08068 | 0.042987  | 0.256858 | 0.472891  |
| Phenylalanine          | C02057       | 0.04781 | 0.048175  | 0.037332 | 0.049408  |
| Proline                | C00148       | 0.13059 | 0.085878  | 0.273119 | 0.163547  |
| Shikimate              | C00493       | 0.05158 | 0.030165  | 0.070095 | 0.014236  |
| Serine                 | C00716       | 0.13411 | 0.099494  | 0.432434 | 0.160313  |
| Compound                              | KEGG ID  | pH 7.0 Mean | pH 7.0 Std. Dev. | pH 6.1 Mean | pH 6.1 Std. Dev. |
|--------------------------------------|---------|-------------|------------------|-------------|------------------|
| alpha-Ketoglutarate                  | C00026  | 1.63041     | 0.757613         | 1.545703    | 0.248305         |
| Citrate                              | C00158  | 6.1772      | 3.493511         | 3.591422    | 1.244466         |
| Dihydroxyacetone phosphate           | C00111  | 0.59156     | 0.145182         | 0.319366    | 0.234114         |
| Fumarate                             | C00122  | 1.92154     | 0.65651          | 1.789196    | 0.566214         |
| Glycerate-2-P                        | C00631  | 2.23376     | 1.136395         | 3.866435    | 1.115064         |
| Glycerate-3-P                        | C00197  | 0.33752     | 0.19751          | 2.084727    | 0.897085         |
| Lactic acid                          | C00186  | 13.1205     | 7.725381         | 22.5249     | 8.030693         |
| Compound                  | KEGG ID | pH 7.0 Mean | pH 7.0 Std. Dev. | pH 6.1 Mean | pH 6.1 Std. Dev. |
|--------------------------|---------|-------------|------------------|-------------|------------------|
| Fatty-acid metabolism    |         |             |                  |             |
| 2-Hydroxyglutarate       | C01087  | 0.28278     | 0.095611         | 0.375639    | 0.180518         |
| Maleic acid              | C01384  | 0.00055     | 0.000855         | 0          | 0                |
Methylcitrate  

| Other metabolic pathways | pH 7.0 | pH 6.1 |
|--------------------------|--------|--------|
| Compound | KEGG ID | Mean | Std. Dev. | Mean | Std. Dev. |
| Myoinositol | C00137 | 0.45246 | 0.24126 | 0.612776 | 0.212814 |
| Myoinositol-P | C01177 | 0.02671 | 0.025913 | 0.039085 | 0.029138 |

| Nucleotide Metabolism | pH 7.0 | pH 6.1 |
|-----------------------|--------|--------|
| Compound | KEGG ID | Mean | Std. Dev. | Mean | Std. Dev. |
| Thymine | C00178 | 0.44776 | 0.301848 | 0.472676 | 0.099166 |
| Uracil | C00106 | 1.92841 | 0.985978 | 2.524029 | 0.918242 |