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Proteomic analysis of *Rhizobium freirei* PRF 81^T reveals the key role of central metabolic pathways in acid tolerance

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ABSTRACT

Soil acidity strongly affects microbial diversity and represents a constraint to legume production. *Rhizobium freirei* is a common bean symbiont recognized by its tolerance to environmental stresses, including low pH. The protein expression profiles of *R. freirei* PRF 81^T grown in pH 6.8 and 4.8 were analyzed to clarify the mechanisms responsible for acid tolerance in this species. Bacteria were grown up to exponential phase in tryptone-yeast (TY) medium at pH 6.8 and 4.8. Whole-cell protein extracts were separated by two-dimensional electrophoresis (2-DE), and spots that showed statistical difference in their relative volumes (%vol) between the treatments were selected and excised for identification by MALDI-TOF mass spectrometry. Data showed that protein synthesis was increased at pH 4.8, which consequently raises energy demand. Differential expression of membrane-associated proteins suggested an increased proton extrusion and decreased influx, while central metabolism seemed to be through inducing acid-consuming catabolic pathways and preventing fatty acid biosynthesis. Consequently, the respiratory chain was stimulated along with the production of reactive oxygen species (ROS). The broad range of metabolic pathways modulated by acidified pH endorses the adaptive response to environmental stresses as a multigenic character in *R. freirei* PRF 81. Moreover, our data emphasize the key role of central metabolism in acid stress tolerance.

1. Introduction

Soil acidity represents a current constraint to legume production (Graham and Vance, 2003). The legume-rhizobium interaction is fundamental to ecology and agriculture and can help crop species to cope with stressful environmental conditions (Buckley and Schmidt, 2002). Soil acidity is also described as the main factor affecting microbial diversity and structure (Fierer and Jackson, 2006), besides interfering with the nodulation and symbiosis with legume host, and nitrogen fixation capacity (Hungria and Vargas, 2000). The *Rhizobium freirei* strain PRF 81 shows many interesting features, mainly its high tolerance to environmental stresses and high efficiency in fixing N_2 in association with the common bean (*Phaseolus vulgaris*), that led to its use in commercial inoculants for this legume in Brazil (Hungria et al., 2000).

Despite the economic and environmental importance of rhizobia, a limited number of studies attempted to elucidate the mechanisms of tolerance to abiotic stresses employed by these bacteria (Gomes et al., 2012a,b), and most of the studies are related to clinical and industrial microorganisms such as *Escherichia coli*, *Lactobacillus* spp., and *Streptococcus mutans* (Lund et al., 2014). To better understand the biological processes involved in stress response, differential proteomics has been successfully employed (Gomes et al., 2012b; Batista and Hungria, 2012; Luche et al., 2016). Here differential proteomics was employed to investigate the acid tolerance responses of *R. freirei* PRF 81.

2. Materials and methods

Rhizobium freirei PRF 81^T, isolated from common bean nodules in Brazil, is deposited at "Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja" (WFCC Collection # 1213, WDCM Collection # 1054). Further information about the strain is

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available elsewhere (Hungria et al., 2000; Ormeño-Orrillo et al., 2012; Dall'Agnol et al., 2013; Gomes et al., 2015). The whole protein extract was achieved after growing the bacterium in TY under two conditions: control (pH 6.8) and with acid stress (pH 4.8). The procedures of total protein extraction (I), 2-DE electrophoresis and gel analysis (II), mass spectrometry (III), and *in silico* identification of proteins (IV), were made as described by Gomes et al. (2012b); except for using: the automatic data acquisition mode on the mass spectrometer (III), and the newer Mascot software version 2.4 adopting peptide tolerance of 100 or 150 ppm (IV). Microbes Online (Alm et al., 2005) and the Integrated Microbial Genomes system (Markowitz et al., 2006) were also used for protein characterization.

3. Results and discussion

Although *R. freirei* PRF 81 grow at pH 4.0 (Hungria et al., 2000; Dall'Agnol et al., 2013), the pH 4.8 was chosen because the growth kinetics at this pH was not significantly different from the growth kinetics observed at pH 6.8 (data not shown). This choice is also supported by the similar growth kinetics results of *R. tropici* CIAT 899 Graham et al. (1994), which shares genotypic (Ormeño-Orrillo et al., 2012) and morphological similarities with *R. freirei* (Dall'Agnol et al., 2013). Also, a recent study evaluated the response of *R. freirei* CIAT 899 grown under acid conditions and the pH of choice was 4.5 (Guerrero-Castro et al., 2018).

Thirty-six proteins were successfully identified (Table 1 and Supplementary Material S1) and classified according to functional groups of Clusters of Orthologous Groups (COG) (Table 1) (Galperin et al., 2015). In relation to cell location, thirty-two proteins were classified as cytoplasmic, spots 23 and 35 as outer membrane and periplasmic proteins, respectively; location of spots 8 and 11 were not determined by the software used. Additional data are available in Supplementary Material S2.

3.1. Induction of protein synthesis

Bacterial exposure to acidic conditions can shift cytoplasmic pH to levels too low to be regulated by primary responses such as buffering or ionic flux. In these cases, inducible responses become important for the reestablishment of the normal pH (Lund et al., 2014). Such responses may include intensification of aerobic respiration due to the higher energy demand, as suggested by the differential expression of energy-related genes observed in *Sinorhizobium meliloti* (Draghi et al., 2016). In this study, the profile of protein spots was also modified in *R. freirei* PRF 81 grown at low pH, and transcriptional and translational processes were intensified in this condition (Table 1).

The 50S ribosomal protein L9, RplI, is responsible for maintaining translation fidelity, while the elongation factor P, EF-P, is involved in relieving stalled ribosomes (Keiler, 2015; Naganathan et al., 2015). Increased expression of RplI and EF-P were observed at pH 4.8 in *R. freirei* PRF 81 and in *S. meliloti* exposed to pH 6.1, in addition to other translation-related proteins (Draghi et al., 2016). The importance of these proteins for cell survival is reinforced by the observation that *E. coli* deficient for RplI and EF-P are usually non viable (Naganathan et al., 2015).

3.2. Glutathione involvement in acid pH responses

Glutathione (GSH) is essential for protecting rhizobia against environmental stresses (Riccillo et al., 2000; Sobrevals et al., 2006) and is involved in nodulation and nitrogen fixation (Harrison et al., 2005; Cheng et al., 2017). GSH synthetase (GshB) and serine hydroxymethyltransferase (SHMT) are enzymes involved in GSH biosynthesis (Sobrevals et al., 2006; Schirch et al., 1985). Previous reports showed that *R. tropici* CIAT 899 under acid shock increases *gshB* expression (Muglia et al., 2007) and is particularly sensitive to acidic conditions when *gshB* is mutated (Riccillo et al., 2000). In contrast, GshB and SHMT are down-regulated in *R. freirei* PRF 81 grown at low pH (Table 1).

R. freirei was grown in TY medium which contains yeast extract, rich in GSH (Sobrevals et al., 2006). Noteworthy, GSH itself, and not its anabolic pathway, is required for stress tolerance, and exogenously supplied GSH can modulate metabolic and symbiotic responses (Cheng et al., 2017) and lead to increased stress tolerance (Corticeiro et al., 2006). Therefore, the TY medium may be supplying the bacterial GSH demand, thus leading to decreased GSH synthesis and down-regulation of GshB and SHMT at pH 4.8.

3.3. Reduced cellular susceptibility to proton influx

RopB, an up-regulated outer membrane protein in acid pH (Table 1), have an important role in maintain membrane stability (Vanderlinde and Yost, 2012) and is a main structural component in some rhizobia (de Maagd et al., 1989). *Rhizobium leguminosarum* bv. *viciae ropB* mutants are more sensitive to low pH and other abiotic stresses (Foreman et al., 2010) and *Agrobacterium tumefaciens* growing under acidic conditions presented an increased expression of a homologous *ropB* gene (Li et al., 2002; Yuan et al., 2008).

Although the F_1F_0-ATPase channel has been reported as induced by acidic conditions (Len et al., 2004; Draghi et al., 2016), the subunit AtpD was down-regulated at pH 4.8 in *R. freirei*; in contrast, the NADH dehydrogenase subunit C of the Complex 1 (NuoC) was up-regulated. Up-regulation of *nuoC* was previously reported at the transcriptional level in *S. meliloti* at pH 6.1 (Draghi et al., 2016), and in *E. coli* exposed to acid shift from pH 7.6 to 5.5 (Kannan et al., 2008) or growing at pH 5.0 (Maurer et al., 2005).

In the *R. freirei* genome, both F_1F_0-ATPase and the Complex 1 are composed of several subunits (Duary et al., 2010; Spero et al., 2015) encoded by the same operons (Ormeño-Orrillo et al., 2012). The down-regulation of AtpD and the up-regulation of the NuoC suggest a mechanism that decreases proton intrusion and increases proton extrusion to control the internal pH (Fig. 1). In *Staphylococcus aureus*, decreased expression of the *atp* operon was observed 2 min after exposure to pH 4.5, and increased expression of *nuo* genes after 10 min (Bore et al., 2007). These opposing mechanisms are needed to raise the cytoplasmic proton content in *E. coli* exposed to alkaline pH (Maurer et al., 2005).

Although pH 6.0 limits the growth of *S. meliloti* (Draghi et al., 2016), *R. freirei* (Dall'Agnol et al., 2013) and *S. aureus* (Bore et al., 2007) can grow at pHs lower than 5.0. While *R. freirei* and *S. aureus* show down-regulation of ATPase and up-regulation of Complex 1 components under acidic pH, *S. meliloti* induces these two proteins (Draghi et al., 2016).

The up-regulation of the Complex 1 suggests that respiration was stepped up in *R. freirei* grown at pH 4.8, as previously reported in *E. coli* at pH 5.0 (Maurer et al., 2005). *S. meliloti* O_2 consumption is more than 5-fold higher at pH 6.1 in comparison with neutral pH (Draghi et al., 2016). In addition, the proton extrusion catalyzed by the Complex 1 is coupled to the conversion of $NADH + H^+$ into NAD^+ (Spero et al., 2015), essential for the maintenance of the central metabolism (Geddes and Oresnik, 2014).

Considering that increased protein synthesis raises energy demand (Draghi et al., 2016) and ATPase was down-regulated in *R. freirei*, besides its growth kinetics was not significantly affected at pH 4.8, it is likely that the ATP supply was being provided by other alternative sources such as catabolic reactions. These reactions consume NAD^+, thus explaining the induction of the Complex 1. These results are in accordance with the observations in *E. coli* showing the induction of *nuo* genes after acid shift is delayed (Kannan et al., 2008), suggesting that Complex 1 activation is a consequence of de-energization following acid stress, as a secondary response of acid-associated metabolism.

Table 1
Proteins of *Rhizobium freirei* PRF 81 whole-cell extract differentially expressed in low pH conditions, arranged according to COG functional categories. Spots on Table 1, Supplementary Material S1 and Supplementary Material S2 are marked with the same number.

Spot	Protein (sequence coverage[†]) – Differential expression[‡]	Control	pH 4.8
C – Energy production and conversion			
1	Putative aldehyde dehydrogenase (28%) ↑	0.77539 ± 0.075	1.22461 ± 0.112
2	ATP synthase subunit beta (51%) ↓	1.12627 ± 0.073	0.87374 ± 0.128
3	Methylmalonate-semialdehyde dehydrogenase (19%) ↓	1.27442 ± 0.148	0.72558 ± 0.085
4	Phosphoenolpyruvate carboxykinase (33%) ↑	0.77009 ± 0.047	1.22991 ± 0.207
5	NADH dehydrogenase subunit C (36%) ↑	0.79663 ± 0.154	1.20337 ± 0.164
6	NAD(P)-dependent oxidoreductase (29%)↓	1.25314 ± 0.035	0.74686 ± 0.122
E – Amino acid transport and metabolism			
7	Serine hydroxymethyltransferase (46%) ↓	1.14436 ± 0.074	0.85564 ± 0.080
8	Spermidine/putrescine ABC transporter (27%) ↓	1.13657 ± 0.105	0.86342 ± 0.013
9	Aspartate-semialdehyde dehydrogenase (40%) ↑	0.88641 ± 0.102	1.11360 ± 0.041
10	Histidinol-phosphate aminotransferase (46%) ↓	1.56135 ± 0.221	0.43865 ± 0.016
11	Amino acid ABC transporter substrate-binding (24%) ↑	0.76501 ± 0.175	1.23499 ± 0.077
12	Amino acid aminotransferase (41%) ↑	0.87784 ± 0.050	1.12217 ± 0.095
G – Carbohydrate transport and metabolism			
13	Glucose-6-phosphate 1-dehydrogenase (17%) ↑	0.87594 ± 0.048	1.12406 ± 0.096
14	KHG-KDPG bifunctional aldolase (68%) ↑	0.70449 ± 0.210	1.29551 ± 0.230
15	Fructose-bisphosphate aldolase (28%) ↓	1.13196 ± 0.067	0.86804 ± 0.078
I – Lipid transport and metabolism			
16	Acetyl-CoA carboxylase biotin carboxylase subunit (56%) ↓	1.30584 ± 0.051	0.69416 ± 0.122
H – Coenzyme transport and metabolism			
17	Hypothetical protein RHSP_82341 (22%) ↓	1.13414 ± 0.043	0.86586 ± 0.036
18	Porphobilinogen synthase (26%) ↓	1.09488 ± 0.029	0.90512 ± 0.039
19	Glutathione synthetase (31%)↓	1.14761 ± 0.058	0.85239 ± 0.061
20	Flavin-dependent oxidoreductase (20%) ↓	1.31606 ± 0.273	0.68394 ± 0.101
P – Inorganic ion transport and metabolism			
21	Aromatic ring-hydroxylating dioxygenase subunit alpha (30%) ↓	1.21173 ± 0.118	0.78827 ± 0.087
M – Cell wall/membrane/envelope biogenesis			
22	UTP-glucose-1-phosphate uridylyltransferase (60%) ↓	1.10184 ± 0.077	0.89816 ± 0.086
23	Outer membrane protein RopB (23%) ↑	0.81340 ± 0.038	1.18661 ± 0.049
K – Transcription			
24	YebC/PmpR family DNA-binding transcriptional regulator (37%) ↑	0.80793 ± 0.039	1.19207 ± 0.011
J – Translation, ribosomal structure and biogenesis			
25	Elongation factor P (24%) ↑	0.78581 ± 0.108	1.21419 ± 0.045
26	50S ribosomal protein L9 (36%) ↑	0.83891 ± 0.066	1.16109 ± 0.134
27	Peptide chain release factor 2 (14%) ↓	1.21705 ± 0.125	0.78295 ± 0.076
28	Glutathione S-transferase (25%) ↑	0.75530 ± 0.078	1.24470 ± 0.170
29	ATP-dependent Clp protease proteolytic subunit (25%) ↓	1.06938 ± 0.040	0.93062 ± 0.073
T – Signal transduction			
30	Hypothetical protein RHSP_68990 (58%) ↓	1.14602 ± 0.032	0.85397 ± 0.015
V – Defense mechanisms			
31	Anti-oxidant protein (28%) ↑	0.63131 ± 0.074	1.36869 ± 0.144
R – General function prediction only			
32	Zinc-dependent protease (19%) ↓	1.19710 ± 0.029	0.80290 ± 0.166
33	GTP-binding protein CobW (21%) ↓	1.21741 ± 0.074	0.78259 ± 0.056
S – Function Unknown			
34	Hypothetical protein RHSP_28418 (46%) ↓	1.38591 ± 0.136	0.61409 ± 0.050
35	Putative outer membrane protein (36%) ↓	1.14387 ± 0.149	0.85613 ± 0.058
NO related COG			
36	Cyclase (21%) ↓	1.28959 ± 0.163	0.71041 ± 0.060

Source: the author.
[†] Sequence coverage of identified protein on Mascot platform (Matrix Science).
[‡] ↑: higher expression in acid pH; ↓: lower expression in acid pH.

3.4. The central metabolic pathways and acid stress response

The up-regulation of Glucose-6-phosphate 1-dehydrogenase and KHG-KDPG bifunctional aldolase proteins at pH 4.8 (Table 1) can be associated with a responsive induction of central metabolic pathways. These enzymes catalyze irreversible reactions involved in the conversion of glucose into pyruvate through the Entner-Doudoroff (ED) pathway (Geddes and Oresnik, 2014) (Fig. 1), the preferential pathway for glucose metabolism in rhizobia (Fuhrer et al., 2005). An increased concentration of ED pathway metabolites when *S. meliloti* was cultured at pH 6.1 was previously reported (Draghi et al., 2017).

The phosphoenolpyruvate (PEP) carboxykinase Pck, has a key role in catalyzing the reversible conversion of oxaloacetate into PEP (Fig. 1) and drives the metabolism towards catabolic tricarboxylic acid (TCA) cycle or anabolic gluconeogenesis (Klaffl et al., 2013). Although Pck regulation is not fully known, reducing conditions are known to favor anaplerotic reaction of Pck (Klaffl et al., 2013), as promoted by peroxidases and reductases, including Alkyl hydroperoxide reductase (Ahp) (Machová et al., 2014).

Expression of both PckA and AhpC were induced in *R. freirei* PRF 81

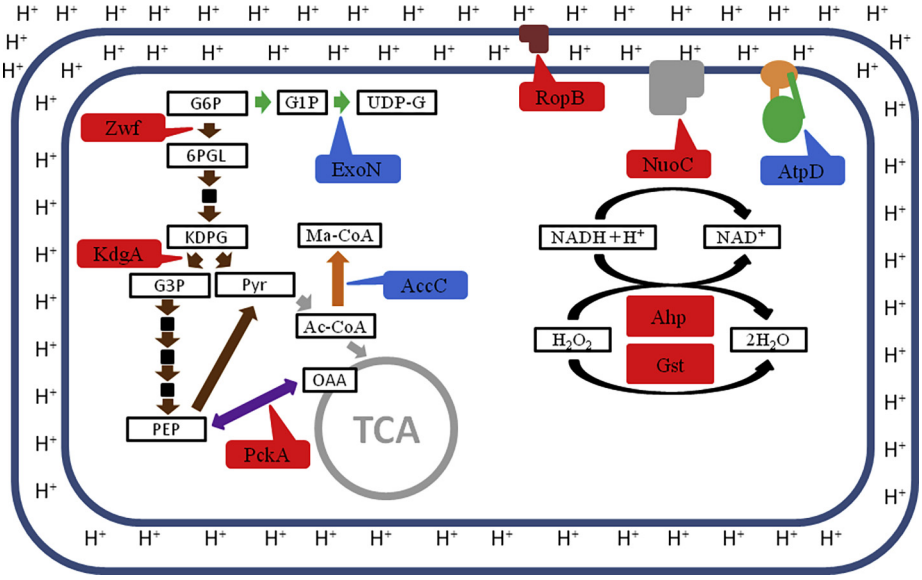

Fig. 1. Schematic representation of the main molecular mechanisms of *R. freirei* PRF 81's adaptive response to acidified pH. Red and blue balloons correspond to up- and down-regulated proteins of the *R. freirei* proteome, respectively. Arrows correspond to enzymatic reactions. The central metabolism is represented by: the Entner-Doudoroff (ED) pathway (brown), fatty acid biosynthesis (orange), tricarboxylic acid (TCA) cycle (gray), and Gluconeogenesis (purple). Green arrows correspond to exopolysaccharide (EPS) biosynthesis pathway and black arrows represent antioxidant reactions. Metabolites are in the black boxes: glucose-6-phosphate (G6P), glucose-1-phosphate (G1P), UDP-glucose (UDP-G), 6-phosphogluconolactone (6PGL), 2-Keto-3-Deoxy 6-Phosphogluconate (KDPG), glyceraldehyde-3-phosphate (G3P), pyruvate (Pyr), phosphoenolpyruvate (PEP), acetyl-CoA (Ac-CoA), Malonyl-CoA (Ma-CoA), and oxaloacetate (OAA). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

grown at acid pH (Table 1). In *A. tumefaciens* cultured under an acidified condition (pH 5.5), the *pckA* gene was also induced (Liu et al., 2005; Yuan et al., 2008). In contrast with the environmental bacteria *A. tumefaciens* and *R. freirei*, *E. coli* did not show differential expression of AhpC in a pH range from 4.9 to 9.1 (Stancik et al., 2002). Therefore, the reducing system may be driving Pck mainly towards the anaplerotic direction of catabolic TCA, thus increasing acid consuming. In addition, the down-regulation of fructose-bisphosphate aldolase Class I protein, which is involved in gluconeogenesis (Scamuffa and Caprioli, 1980), reinforces the hypothesis pro-catabolism rather than anabolism in *R. freirei* under acidic pH, although further research is needed to verify this hypothesis.

Produced by several *Rhizobium* species, succinoglycan is a type of exopolysaccharide (EPS) whose biosynthesis is dependent on the products of *exo* genes (Janczarek, 2011). In *S. meliloti,* the acid pH resulted in increased expression of *exo* genes (Hellweg et al., 2009; Lucena et al., 2010) and EPS production (Draghi et al., 2016). On the other hand, there is no evidence that EPS improves bacterial acid tolerance, as reported in a study with a *S. meliloti* mutant for *exoY* exposed to low pH (Dilworth et al., 1999). Moreover, a study with *Rhizobium ciceri* exposed to pH 5, 6, 7, 8, and 9 indicated that EPS yield is dramatically decreased under acidic pH (Küçük and Kivanç, 2009).

The ExoN protein was down-regulated in *R. freirei* PRF 81 grown at pH 4.8. Corroborating our result, ExoN2 protein expression is decreased in *S. meliloti* at pH 6.1 (Draghi et al., 2016), and *R. tropici* CIAT 899 strongly reduces EPS production under acidic pH 4.7 and 5.7 (Staudt et al., 2012). Therefore, the down-regulation of ExoN is in accordance with the inclination towards catabolism in low pH conditions.

The Acetyl-CoA carboxylase (Acc), a key enzymatic complex that catalyzes the first step in fatty acid biosynthesis (Rathnasingh et al., 2012), was down-regulated in *R. freirei* at pH 4.8 (Fig. 1). In *H. pylori,* a highly acid tolerant species, *accA* tended to be less expressed at pHs lower than 6.0 (Shao et al., 2008). Taken together, these data suggest that these bacteria bypass fatty acid biosynthesis in low pH conditions. To this end, the metabolism is probably driven to catabolism, catalyzing acid consuming rather than fatty acid biosynthesis, thus alleviating internal acidification.

3.5. Reactive oxygen species (ROS) formation and antioxidant defense

Although not fully elucidated, the correlation between acidity and oxidative stresses is often suggested (Bruno-Bárcena et al., 2010; Rangel, 2011; Ormeño-Orrillo et al., 2012). A large number of oxidative stress genes show pH-dependent expression with most being induced in acid or inhibited in alkaline conditions (Shao et al., 2008; Draghi et al., 2016). Moreover, aerobic respiration steps up at pH 5 (Maurer et al., 2005). A proteomic analysis of *Yarrowia lipolytica* pointed out that one of the major cellular responses of this yeast species to low pH environment is the enhanced expression of TCA cycle enzymes and increased levels of ROS (Guo et al., 2016).

The respiratory chain is considered the major source of ROS (Pastor et al., 2009), and the Complex 1 is a known producer of endogenous superoxide (O_2^-) (Frick et al., 2015). Acid environmental conditions may lead to increased aerobic respiration and ROS formation, with consequent induction of oxidative stress.

ROS can be converted into hydrogen peroxide (H_2O_2) and Glutathione S-transferase (GST) belongs to a cluster of enzymes involved in some processes directly connected to the oxidative stress response, by efficiently reducing H_2O_2 (Imlay, 2013; Todorova et al., 2007). The up-regulation of GST at pH 4.8 suggests that this enzyme may be protecting the cell against oxidative damage.

Ahp is also involved in oxidative stress response; however, in contrast with catalases, Ahp degrades H_2O_2 into H_2O consuming NADH (Fig. 1) and does not lead to the formation of oxidizing species. Ahp is, therefore, the most effective scavenger during low-level H_2O_2 stress (Imlay, 2013). Nevertheless, Ahp depends on the NADH supply from metabolism and becomes saturated when intracellular H_2O_2 exceeds 20 μM (Seaver and Imlay, 2001). Since Ahp was the most induced protein, it may be simultaneously detoxifying H_2O_2 and supplying metabolism with NAD^+.

3.6. Overall response to acid pH

The wide range of *R. freirei* PRF 81 response to acid stress is summarized in the scheme depicted in Fig. 1. Several changes were revealed at translational level, including processes involved in protein biosynthesis, proton transport, proton motive force modulation, central carbon metabolism, ROS generation and antioxidative defense against ROS, among others.

Only few rhizobial species are able to grow in pH lower than 5.0 (Hungria and Vargas, 2000; Ribeiro et al., 2012; Dall'Agnol et al., 2013); this may be a key to symbiotic efficiency, since the pH in determinate nodules is slightly acid (approximately 6.4 in younger nodules and 5.5 in older or stressed nodules) (Becana and Klucas, 1992).

4. Conclusions

This study revealed that acidic growth conditions affect several *R. freirei* PRF 81's cellular processes and the response elicited by these bacteria to this stress. Our results emphasize the key role played by central metabolism in the acid tolerance response of *R. freirei* PRF 81 that may contribute with studies aiming to improve *R. freirei* PRF 81 symbiotic abilities under adverse soil conditions and to optimize the development and industrial production of inoculants.

Similar strategies seem to be employed by the related species *R. tropici* when exposed to pH 4.5, including changes on carbohydrate metabolism and energy generation processes, proton extrusion, reduced membrane permeability to protons, among others (Guerrero-Castro et al., 2018). In *S. meliloti* Rm 2011, a strain sensitive to low pH, it was observed the acid-induced expression of *fixN1* and *fixG* only at the transcriptional level (Draghi et al., 2016). Since both nitrogen-fixation genes are located in symbiotic plasmid pSymA, which is recognized to be involved in several stress responses, the authors indicate that the cellular role of *fix* genes may exceed their action in nitrogen fixation.

The molecular mechanisms discussed here suggest that the adaptive response of *R. freirei* PRF 81 to acid pH is a multigenic character, and acid tolerance responses seem to be orchestrated to alleviate internal acidification by inducing acid consuming, proton extrusion, reduction of proton influx, and oxidative stress defense, while decreasing fatty acid and EPS biosynthesis. The prevalence of differential cytoplasmic proteins highlights the crucial role of the central metabolism in the tolerance of *R. freirei* PRF 81 to acid stress.

Competing interests

The authors declare that they have no competing interests.

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Authorship policy

Conceived or designed the study: Batista JSS, Gomes DF, Hungria M.
Performed research: Batista JSS, Gomes DF, Silva LP, Tullio LD.
Analyzed data: Batista JSS, Gomes DF, Silva LP, Tullio LD.
Contributed with new methods or models: Batista JSS, Gomes DF, Tullio LD.
Wrote the paper: Batista JSS, Hungria M, Tullio LD.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2018.11.014.

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