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Research Article

RESEARCH ARTICLE

Global transcriptome analysis of *Rhizobium favelukesii* LPU83 in response to acid stress

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One sentence summary: The information garnered here provides a cornerstone for new studies and design of modified rhizobia with improved persistence and symbiotic activity at low pHs.

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ABSTRACT

Acidic environments naturally occur worldwide and inappropriate agricultural management may also cause acidification of soils. Low soil pH values are an important barrier in the plant–rhizobia interaction. Acidic conditions disturb the establishment of the efficient rhizobia usually used as biofertilizer. This negative effect on the rhizobia–legume symbiosis is mainly due to the low acid tolerance of the bacteria. Here, we describe the identification of relevant factors in the acid tolerance of *Rhizobium favelukesii* using transcriptome sequencing. A total of 1924 genes were differentially expressed under acidic conditions, with ∼60% underexpressed. *Rhizobium favelukesii* acid response mainly includes changes in the energy metabolism and protein turnover, as well as a combination of mechanisms that may contribute to this phenotype, including GABA and histidine metabolism, cell envelope modifications and reverse proton efflux. We confirmed the acid-sensitive phenotype of a mutant in the *braD* gene, which showed higher expression under acid stress. Remarkably, 60% of the coding sequences encoded in the symbiotic plasmid were underexpressed and we evidenced that a strain cured for this plasmid featured an improved performance under acidic conditions. Hence, this work provides relevant information in the characterization of genes associated with tolerance or adaptation to acidic stress of *R. favelukesii*.

Keywords: acid stress; rhizobia; transcriptomics; RNA-Seq

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INTRODUCTION

Nitrogen is one of the most relevant nutrients for plant growth, and its availability limits crop yields. Some microorganisms called rhizobia are capable of using molecular nitrogen (N_2) and converting it into ammonium, making it an available source of this nutrient (Raymond *et al.* 2004). Every year, ∼40 million tons of nitrogen is introduced into agricultural systems by symbiotic nitrogen fixation (SNF) (Herridge, Peoples and Boddey 2008). This association provides benefits to both participants: plants obtaining a source for assimilable nitrogen, and microorganisms acquiring photosynthates to metabolize for an energy source. The rhizobia–leguminous symbiosis is highly specific and involves a complex process (Helal and Sauerbeck 1989; Perret, Staehelin and Broughton 2000) ending with the production of a new organ in the roots of the plant, the nodule, which provides the environment to carry out the SNF. Meanwhile, nitrogen fertilization entails harmful effects on the environment; SNF is a relevant factor for the functioning of the ecosystems as well as for maintaining the sustainability of agricultural practices (Savci 2012).

Alfalfa globally covers >32 million hectares (Michaud, Lehman and Rumbaugh 1988; Li *et al.* 2011) and provides the main forage source for cattle. This crop can obtain most of its nitrogen needs from SNF (Burity *et al.* 1989). This supply is, however, impaired by several stresses that restrict the development and functioning of the symbiotic system (Zahran 1999). Particularly, acidity is a known stress factor limiting the SNF in alfalfa (Brockwell, Pilka and Holliday 1991; Glenn and Dilworth 1994). A rising concern is that arable land pH decreases as a consequence of inappropriate agricultural management (Graham 1992; Martikainen and De Boer 1993; Von Uexküll and Mutert 1995). This soil acidification is responsible for ∼50% of the yield losses in wheat and barley crops, as well as in legume crops (Mahler and McDole 1987; Graham 1992; Bordeleau and Prévost 1994; Vassileva *et al.* 1997). Thus, low soil pH (acting mainly on the low acid tolerance of rhizobia; Bordeleau and Prévost 1994) impairs the development of the rhizobia–legume symbiosis, diminishing the nitrogen supply to the crop and concomitantly, producing yield loss.

The advance in the development of alfalfa–rhizobia associations able to tolerate acidic soil conditions could be the key to overcome this stress factor. Several groups have tried to identify acid-tolerant strains to unravel the genetic mechanisms involved in the resistance to low pHs (Graham *et al.* 1982; Goss *et al.* 1990; del Papa *et al.* 1999; Priefer *et al.* 2001; Orr 2009). Initially, several genes related to acidity tolerance were identified using Tn5 mutant libraries in *Ensifer* (*Sinorhizobium*) *medicae* (Goss *et al.* 1990; Tiwari *et al.* 1996; Reeve *et al.* 2002). Examples of these are *act* genes, so-named by acid tolerance, and included genes encoding the two-component system (TCS) *actS–actR* (Tiwari *et al.* 1996). Among the possible targets of the response regulator ActR are the *cbbS* gene, involved in CO_2 fixation and the *narB* gene, involved in nitrate assimilation, as well as *fixK* and *nifA* nitrogen fixation regulatory genes (Fenner *et al.* 2004). Expression of *actA* and *actP* genes, which encode an acyl apolipoprotein transferase and a copper-transporting ATPase, respectively, were also identified at low pH (Tiwari *et al.* 1996; Reeve *et al.* 2002). Another relevant identified gene in acid conditions is *lpiA*, associated with membrane components and which acid activation was essential for enhanced cell viability in *E. medicae* under lethal acid conditions (Tiwari *et al.* 2004). This gene was also induced at low pH in *Rhizobium tropici*, and it was described as necessary for increasing the competitiveness in nodulation (Vinuesa *et al.* 2003). These first results indicated that acid tolerance is not a phenotypic response in which only a few genes are involved.

Nowadays, high-throughput analyses have allowed to progress in the knowledge of gene expression and regulation in response to stress. Several approaches revealed the mechanisms involved in acid defense of *Ensifer meliloti* (Hellweg, Puhler and Weidner 2009; Draghi *et al.* 2016). The complex physiological response of *E. meliloti* includes differential expression of genes associated with exopolysaccharide (EPS) biosynthesis, chemotaxis and motility, as well as central carbon metabolism and respiration. An analogous response to acidic conditions is evidenced in *Agrobacterium tumefaciens*, a species phylogenetically closely related to *E. meliloti* (Yuan *et al.* 2008). Another study in *E. meliloti* evidenced that *rpoH* gene (heat stress-related sigma factor) plays an important role in acid response and regulates several genes associated with this response (Mitsui *et al.* 2004; de Lucena, Puhler and Weidner 2010). Thus, a multigenic stress response was observed when a systemic approach was used to unraveling cellular responses to acid pH.

Other rhizobial species display a differential acid-stress response. In *Mesorhizobium loti*, the acidic response includes the expression of several genes associated with ABC transporters and proteins of the cell envelope, but does not include the overexpression of those genes typically associated with acid tolerance in rhizobia such as *act* and *exo* genes (Laranjo, Alexandre and Oliveira 2014). In *R. leguminosarum* bv. *trifolii*, sensitive strains to low pH showed to have an altered proton permeable membrane, and, in some cases, also a lower proton ejection activity than tolerant strains (Chen, Gartner and Rolfe 1993). Besides, it has been observed that rhizobia grown under acidic conditions have higher cytoplasmic levels of glutamate and K^+, accompanied by alterations in the levels of polyamines, which is similar to what occurs in situations of osmotic stress (Graham 1992; Fujihara and Yoneyama 1993). Thus, the current acid tolerance model in rhizobia comprises several systems to sense pH changes in different ways and respond to them, reflecting the complexity of the acid response.

The improvement of SNF in alfalfa requires a better understanding of the development of symbiotic system at low pH. The root infection by *Ensifer* spp. strains would be limited by its own boundaries, imposed by the absence of effective defense mechanisms against pH values lower than 5.6–5.4. The search for acid defense mechanisms in rhizobia adapted to acidity could be essential to recognized defense systems that protect cells from high proton concentrations. With this knowledge, it would be possible to obtain commercial effective strains to improve the development of alfalfa in acid soils. *Rhizobium favelukesii* is an alfalfa-nodulating rhizobia that can withstand exposure to low pH (pH 4.6), even though inefficient for nitrogen fixation (del Papa *et al.* 1999). This rhizobium is genetically related at chromosome level to the bean-infecting rhizobia, like *Rhizobium etli*, and at pSym level to the alfalfa-infecting rhizobia, like *E. meliloti* (Torres Tejerizo *et al.* 2011). The remarkable acid tolerance and the interesting evolutionary history of *R. favelukesii* (Torres Tejerizo *et al.* 2011) make this rhizobium an appropriate research subject to unraveling acid stress mechanisms, by comparing them with efficient alfalfa-nodulating bacteria like *E. meliloti* and *E. medicae* strains. In this work, we performed a comprehensive transcriptional study to obtain a global dimension of the acid response

of *R. favelukesii* LPU83. The obtained results provide new targets that will allow knowing in-depth new mechanisms involved in the adaptation of bacteria to changes in environmental pH.

MATERIALS AND METHODS

Bacterial strains and plasmids: culture and growth conditions

Table S1 (Supporting Information) lists strains, plasmids and primers used in this work. *Escherichia coli* was grown on LB medium (Luria, Adams and Ting 1960) at 37°C. *Rhizobium favelukesii* LPU83 and its mutants were cultivated in sucrose–glutamate (SG) minimal medium (del Papa *et al.* 1999) at 28°C and 160 rpm, for the transcriptomic analysis. The medium pH was buffered with a combination of HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] and MES [2-(*N*-morpholino) ethanesulfonic acid] both at 10 mM (Graham, Draeger and Ferrey 1994). Growth at acidic (4.6) and neutral (7.0) pHs was evaluated through measurements of the optical density at 600 nm (OD_{600nm}). For general purpose, *R. favelukesii* LPU83 were grown in tryptone–yeast extract (TY) medium (Beringer 1974) and the growth kinetics (28°C, 200 rpm) analyzed by monitoring OD_{600} in a microplate reader (BMG LABTECH, Germany). For the solid media, 15 g of agar was added per liter of TY or LB medium. The final antibiotic concentrations per ml of either medium were 50 μg kanamycin (Km) for *E. coli* or 400 μg streptomycin (Sm) and 120 μg neomycin (Nm) for *R. favelukesii* LPU83.

RNA extraction

Rhizobium favelukesii LPU83 was cultivated (SG medium) at pH 4.6 and pH 7.0 (Nilsson *et al.* 2019). Then, RNAprotect (Qiagen, Hilden, Germany) reagent was added and cells harvested, and the pellet was frozen in liquid nitrogen. The commercial RNeasy® Protect Bacteria Mini Kit (Qiagen, Hilden, Germany) was used for the RNA extraction and DNA was removed with DNase I (Qiagen, Hilden, Germany). The depletion of the ribosomal RNA was performed with a commercial kit (Ribo-Zero rRNA removal kit, Illumina Inc., San Diego, USA).

RNA sequencing and data analysis

The cDNA library was made with the commercial kit TruSeq® mRNA Sample Preparation (stranded) (Illumina Inc., San Diego, USA). The samples were sequenced by means of Illumina HiSeq platforms available at the Center for Biotechnology (CeBiTec, Bielefeld University, Germany). After that, the quality of the sequences was evaluated with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and the reads were mapped unambiguously to the reference genome of *R. favelukesii* LPU83 (Wibberg *et al.* 2014) with Bowtie2 (Langmead and Salzberg 2012). Finally, the results were visualized through ReadXplorer 2.0 (Hilker *et al.* 2014). This software also provided the statistical tests for differential gene expressions, i.e. Deseq2 (Love, Huber and Anders 2014). The statistical significance of relative quantitative changes between the conditions of neutral and acid pH in replicate data was determined by Deseq2 at an adjusted *P*-value < 0.05. The resulting raw count files for the six transcriptomes have been deposited in GEO and are available via accession GSE141742. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to assign a position to each coding sequence (CDS) in the respective pathways and the STRING version 9.1 (Szklarczyk *et al.* 2017) to predict protein–protein interactions.

Construction of the cured symbiotic plasmid strain

To cure the symbiotic plasmid (pSym, pLPU83b) of *R. favelukesii* LPU83, a plasmid incompatibility technique was used. For this purpose, a copy of the replication and partition region (*repABC*) of the pSym was cloned in the plasmid pCR2.1-Topo. In the resultant plasmid, pT-*repABC*, the *mob* cassette was cloned as a *KpnI*-fragment from plasmid pUC19*mob*, obtaining the pT-*repABC-mob* plasmid. By conjugation, pT-*repABC-mob* was transferred to *R. favelukesii* LPU83-H, a derivative of LPU83 that carries the gentamicin resistance gene (Gm) inside the *nodH* gene of the symbiotic plasmid. The loss of the symbiotic plasmid in LPU83-H was monitored for loss of resistance to Gm. To verify that genomic rearrangements resulting in insertions of pSym fragments into another replicon had not occurred during the deletion process, the cured strain designated *R. favelukesii* LPU83-pSym$^-$ was sequenced by Illumina MiSeq platform of the Department of Environmental Sciences of the University of Aarhus, Denmark. The resulting reads were filtered and trimmed according to their quality using Trimmomatic with default settings (Bolger, Lohse and Usadel 2014) and were assembled using gsAssembler (version 2.8).

Symbiotic plasmid loss assay

Rhizobium favelukesii LPU83-H (Gm resistance integrated in symbiotic plasmid) was grown at acidic (4.6) and neutral (7.0) conditions in SG medium to saturation. Each culture was serially diluted and 100 μL plated on TY and TY-Gm agar plates. After incubation at 28°C, the ratio of the number of colonies grown on TY-Gm and TY was calculated and represents the fraction of plasmid-bearing cells.

Construction of single-insertion mutant in the *braD* gene

First, a central region (~100 bp) of the *braD* gene was amplified by PCR with the Pfu DNA polymerase (PBL, Quilmes, Argentina). The fragment obtained was subsequently cloned into the vector pK18mob (a suicide vector in rhizobia), previously digested with *SmaI*. The resulting plasmids were conjugated into *R. favelukesii* LPU83 via *E. coli* S17-1 (Simon, Priefer and Pühler 1983). Transconjugants were selected for resistance to Sm and Nm. Mutants were verified by PCR with the primers LPU83_3205-right and M13-Rv. Table S1 (Supporting Information) lists the primers used.

EPS extraction and quantification

Rhizobium favelukesii LPU83 was cultivated at pH 4.6 and pH 7.0 in SG medium. Cultures were centrifuged for 45 min at 10 000 × *g*. The EPS in the supernatant was precipitated with three volumes of ethanol overnight at −20°C and stored until processed. Then, the samples were centrifuged for 45 min at 10 000 × *g* and the pellets were resuspended in water. The quantification was performed by the anthrone method (Loewus 1952). The statistical analysis was done by a *t*-test using three independent biological replicates.

RESULTS AND DISCUSSION

Transcriptome response of *R. favelukesii* LPU83 under acidic conditions

Under natural conditions, microorganisms are exposed to a constantly changing environment. Accordingly, adaptive processes are guided by multiple factors (Aertsen and Michiels 2004). Hence, we conducted a comprehensive analysis of global changes, first in the proteome (Nilsson *et al.* 2019) and then, in the transcriptome levels under acidic conditions. The previous proteomic analysis demonstrated the multigenic nature of the *R. favelukesii* acid-response. The findings indicated the participation of a diverse set of cellular proteins, as had been observed for other types of stress (Guerrero-Castro, Lozano and Sohlenkamp 2018; Teixeira-Gomes, Cloeckaert and Zygmunt 2000). Despite a significant number of proteins (336) that were identified as differentially expressed under acidic conditions, these results were not sufficient to fully unravel the acid response of *R. favelukesii*.

In this work, we conducted a comparative RNA-seq analysis under acidic and neutral conditions. Total RNA was extracted at an OD_{600nm} of 0.5 from cell cultures grown in SG medium at a pH value of 7.0 (control condition) and 4.6 (acid-stress condition). These conditions were chosen to mimic the previously determined acid stress condition to evaluate the proteomic acid response of *R. favelukesii* LPU83 (Nilsson *et al.* 2019). The transcriptomes were sequenced and quantified as indicated in Materials and Methods. A total of 7700 CDSs are annotated in the *R. favelukesii* LPU83 genome, of which 7693 genes were represented in this RNA-seq study (Table S2, Supporting Information). Among the 7693 identified genes, 1924 (25%) were found to be differentially expressed genes (DEG: fold change $\geq$ 2, $\log_2$*fold change* equal to or less than –1 or equal to or greater than 1, and a *P*-adjusted value of $\leq$ 0.05) (Figure S1 and Table S3, Supporting Information). At pH 4.6, 810 genes were detected as differentially overexpressed and 1114 underexpressed (Table S3, Supporting Information). The DEGs were distributed through all replicons (chromosome and four plasmids, pLPU83a, pLPU83b, pLPU83c, pLPU83d) but most of them were found to be located in the chromosome (~50% of DEGs, Fig. 1A). When the number of DEGs was normalized according to the number of CDSs harbored in each replicon, the replicon with the higher representation was strikingly the symbiotic plasmid (pLPU83b) (Fig. 1B), while the remaining replicons showed similar DEG rates. In order to elucidate the possible functional relationships between DEGs and to explain the altered expression of the genes under acidic conditions, a functional analysis was performed. Cluster of orthologous groups (COGs) is a widely used classification scheme to depict orthologous proteins (Tatusov *et al.* 2000, 2003). DEGs were categorized into cellular-functional groups according to assigned COGs. It was possible to assign COGs to 1322 out of 1924 DEGs (68.7%). These genes were distributed over 21 of the 22 functional categories (Fig. 2; Table S3, Supporting Information). Figure 2 summarizes the proportion of DEGs over- and underexpressed in each COG category. The functional categories with the highest number of overexpressed genes under acidic conditions were amino-acid transport and metabolism (E) and energy production and conversion (C) (Fig. 2), which is in agreement with proteomic results. Both COG categories were also described as relevant in other rhizobia under acidic conditions (Draghi *et al.* 2016; Guerrero-Castro, Lozano and Sohlenkamp 2018; Nilsson *et al.* 2019). These categories were followed by COG categories of cell envelope (M) and cell motility (N), among others. The wide distribution of DEGs in all functional categories denotes the complexity of the acid response. Moreover,

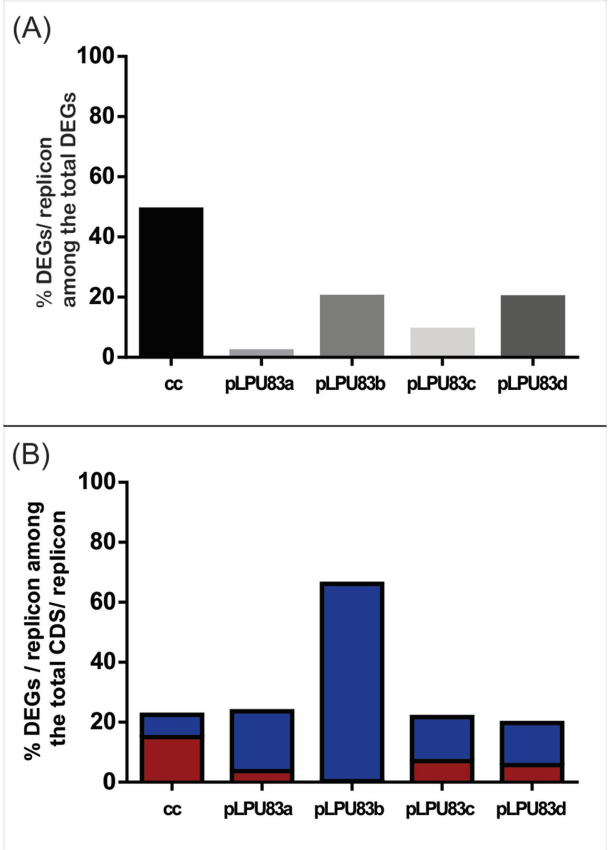

Figure 1. Genomic distribution of genes differentially expressed under acid stress in *R. favelukesii* LPU83. *Rhizobium favelukesii* LPU83 contains a chromosome and four plasmids. **(A)** The percentage of DEGs of each replicon among the total number of DEGs is plotted for each of the replicons. **(B)** The percentage of DEGs of each replicon with respect to the total number of CDSs in each replicon is plotted for each of the replicons. The proportion of underexpressed/overexpressed genes is also plotted in panel B. Blue, underexpressed DEGs; red, overexpressed DEGs. cc, chromosome; pLPU83a, pLPU83b, (symbiotic, pSym), pLPU83c and pLPU83d.

the number of DEGs without COG assignments (615 DEGs—~32%) and with unknown function (655 DEGs—~34%) indicates the contribution of unknown mechanisms in acid tolerance of *R. favelukesii* LPU83.

To get a deeper insight into the knowledge and to obtain statistically supported data of the possible pathways altered under acid conditions, a functional-enrichment analysis was performed by means of the STRING tool (https://string-db.org) (Szklarczyk *et al.* 2017). All the genes that were differentially expressed were included in the analysis. Figure 3 summarizes the networks of those predicted associations for the overexpressed proteins (networks for underexpressed proteins are shown in Figure S2, Supporting Information). This analysis indicated that several pathways listed in the KEGG were significantly enriched such as oxidative phosphorylation, flagellar system, bacterial chemotaxis, among others (Fig. 3). An exhaustive analysis of the principal pathways is detailed in the following section.

Metabolic stress response

Our results support the previously described evidence indicating that the acid-response of rhizobia is a multigenic response. Several bacteria share some metabolic response to cope with environmental conditions such as acid stress,

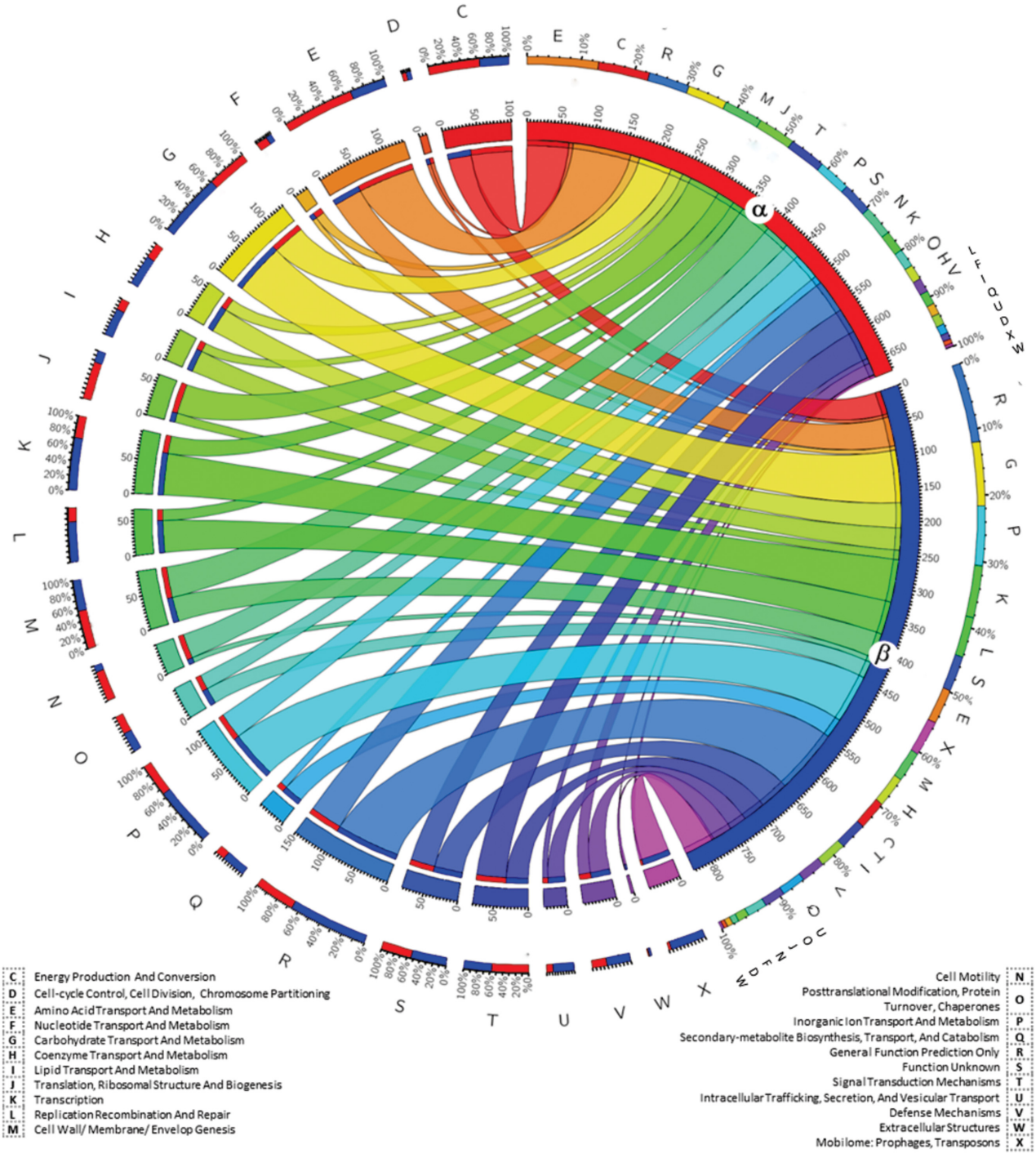

Figure 2. Representation of the different COGs containing the total DEGs under acidic conditions in *R. favelukesii* LPU83. Underexpressed (beta, blue) and overexpressed (alfa, red) DEGs are represented by circularly arranged segments, whose length is proportional to the total DEG, respectively. The outer rings segments are stacked bar plots that represent relative contribution of each COG to over- or underexpressed DEGs, respectively. Ribbons connect the DEG segments with each COG category (letters from C to X); the width of the ribbons is proportional to the DEGs number in each COG. The outer rings segments are stacked bar plots that represent relative contribution of each over- or underexpressed DEG to each COG, respectively. The figure was performed with the visualization tool Circos (Krzywinski *et al.* 2009).

which usually involves changes in metabolisms as cellular respiration, amino acid and protein synthesis, motility and chemotaxis and repair mechanisms (Boor 2006; Yuan *et al.* 2008; Hellweg, Puhler and Weidner 2009; Draghi *et al.* 2016).

Oxidative phosphorylation

Increases in oxidative phosphorylation under acidic conditions have been evidenced in other rhizobia (Tiwari *et al.* 2004; Yuan *et al.* 2008; Draghi *et al.* 2016). Our transcriptomic analysis identified numerous DEGs associated with oxidative phosphoryla-

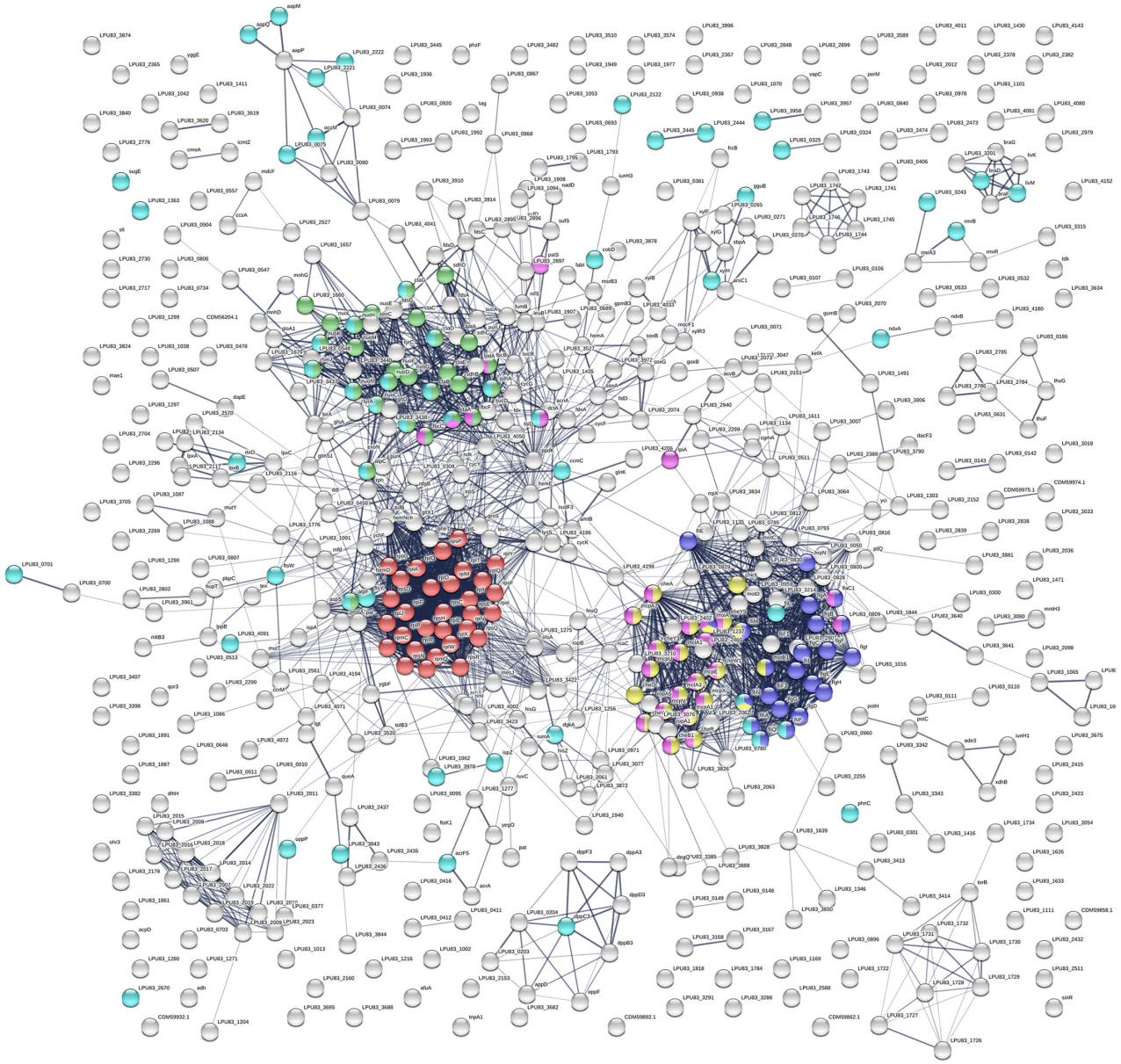

Figure 3. Networks of overexpressed genes under acidic conditions in *R. favelukesii* LPU83. The figure delineates the specific interactions (connecting lines) between the proteins of the overexpressed DEGs (circles). The aggregated circles are colored depicting the significantly enriched KEGG categories determined by STRING analysis. Red circles, ribosomal proteins (false-discovery rate [FDR], 4.99×10^{-6}); dark blue circles, flagellar assembly (FDR, 4.990×10^{-6}); green circles, oxidative-phosphorylation proteins (FDR, 4.99×10^{-6}); yellow circles, bacterial chemotaxis (FDR, 1.44×10^{-5}); purple circles, two-component system (FDR, 0.0249); light blue circles, cell membrane (0.00017).

tion, in agreement with our proteomic results (Nilsson *et al.* 2019). Most of the genes involved in the electron transport chain (complexes I, II, III and IV) showed a significant transcriptional increase under acid stress (Fig. 4; Table S3, Supporting Information). This result is in agreement with previous results in *E. meliloti* and *A. tumefaciens* (Yuan *et al.* 2008; Draghi *et al.* 2016). Increases in the oxidative phosphorylation in both rhizobia suggest a high energy demand in the face of acidic challenge. Finally, it is especially noteworthy that two genes encoding the 'b' and 'epsilon (ε)' subunits of F0F1 ATP synthase (*atpF* and *atpC*) were upregulated under acidic conditions (Fig. 4). In agreement, it was recently reported an increased expression of two subunits of F0F1 ATP synthase, among them the epsilon subunit, under acidic conditions in *Rhizobium tropici* CIAT 899 (Guerrero-Castro, Lozano and Sohlenkamp 2018). The ATP synthase (F0F1-ATPase) is capable of synthesizing ATP using a proton flow according to the electrochemical gradient from outside to inside the cell. Previously, it has been described for several species that reverse flux of protons through the F0 subunit against the electrochemical gradient promoted by ATP hydrolysis can be used as a mechanism to maintain the intracellular pH under acidic conditions (Diez *et al.* 2004; Barriuso-Iglesias *et al.* 2013; Papadimitriou *et al.* 2016).

Carbohydrate metabolism

The category of carbohydrate transport and metabolism grouped 24 DEGs. The previous proteomic analysis revealed the increase of the ribose-5-phosphate isomerase A (encoded by *rpiA*) of

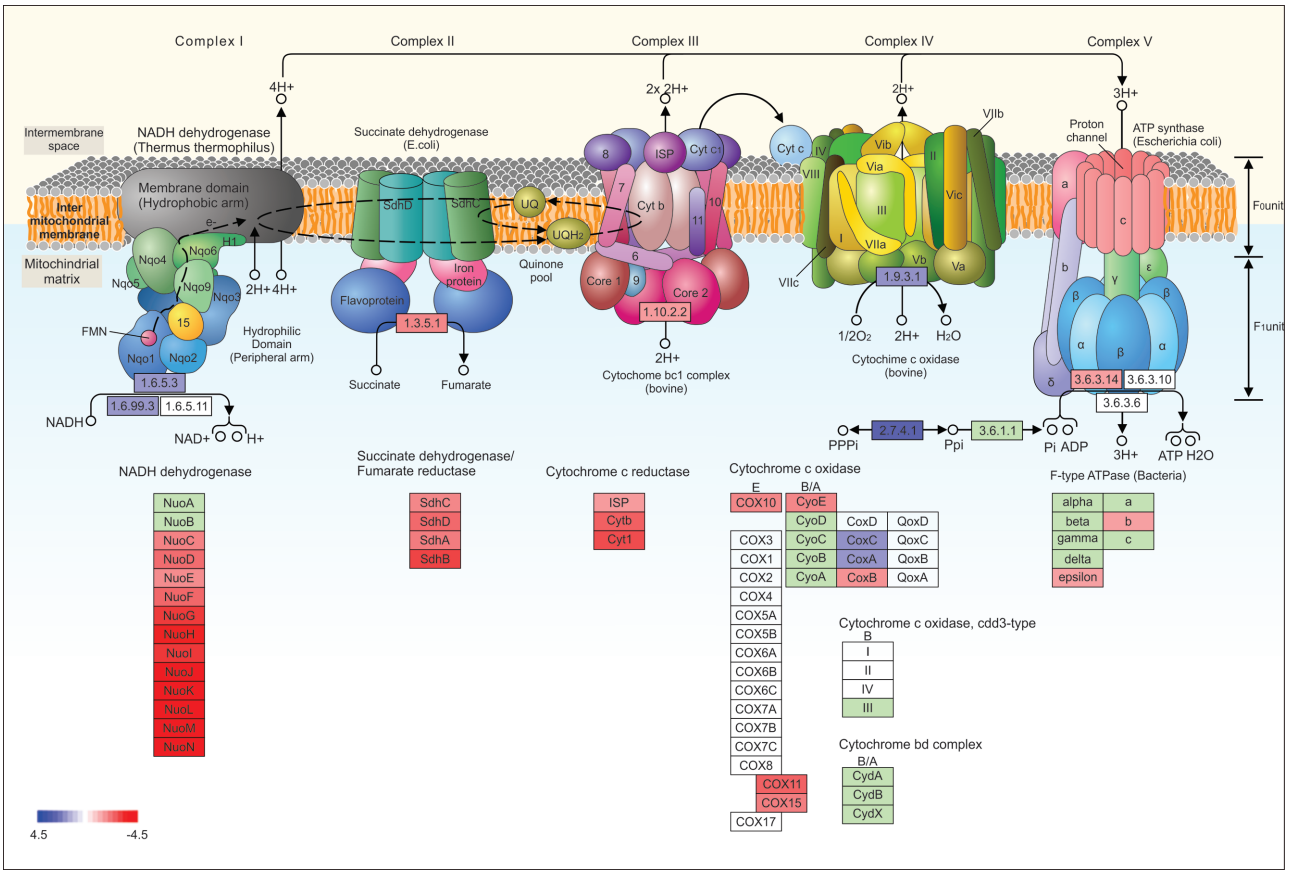

Figure 4. Expression of DEGs involved in the oxidative phosphorylation in response to acid stress in *R. favelukesii* LPU83. The scheme was made through the KEGG Mapper tool and edited manually (Kanehisa and Sato 2020). The expression level of DEGs is represented in the gene box with a range of colors between blue and red. The higher level of overexpression corresponds to the more intense red. The lower expression corresponds to the more intense blue. Enzymes without significantly fold change have the box gene colored in green. Gene boxes that are not colored (white) correspond to proteins that are absent in genome of *R. favelukesii*. Column headers B/A stands for bacteria/archaea and E for eukaryotic. The different colors of each protein and of the membrane do not stand for expression values.

pentose-phosphate pathway (PPP) (Nilsson *et al.* 2019). In agreement, the *tktB* gene (transketolase) showed higher transcript abundance under acid stress in *R. favelukesii*. Regarding PPP, metabolome analyses in *E. meliloti* showed that the reduction in the relative amount of NADH vs NAD^+ under acidic conditions may be responsible for the observed enhancement of different metabolites of the PPP (Draghi *et al.* 2017). Thus, in *R. favelukesii* LPU83 cells exposed to acid stress, the use of carbon through the PPP may denote an increase in the generation of intermediates. The L-lactate dehydrogenase, which transcript (*lldD*) was stimulated in acidic condition, catalyzes the reversible conversion of pyruvate to lactate accompanied by H^+ consumption. Hence, this enzyme may contribute to maintaining the intracellular pH consuming H^+ by lactate synthesis. In agreement with this, the lactate dehydrogenase activity and lactate concentration showed an increase in *E. meliloti* under acidic conditions (Draghi *et al.* 2017; Nilsson *et al.* 2019).

Furthermore, ten genes associated with the tricarboxylic acid (TCA) cycle were upregulated under acidic conditions (Table S3, Supporting Information). Therefore, the *R. favelukesii* LPU83 acid-response includes both the TCA cycle and oxidative phosphorylation increased under acid stress, indicating an increase of cell respiration, hypothesized previously with proteomic results (Nilsson *et al.* 2019). The TCA cycle probably is a key pathway for the supply of precursors to satisfy the demand for biosynthetic reactions under acidic conditions, in addition to its role in generation of reduction equivalents for cellular respiration to yield energy under acid stress conditions.

Ribosomal proteins and translation

The COG category corresponding to translation, ribosomal structure and biogenesis contained 61 DEGs with mainly a higher abundance under acidic conditions (Figs 2 and 3). Mainly, this gene group includes several genes encoding ribosomal proteins, aminoacyl-tRNA synthetase, transcripts representing different tRNAs and the gene *tufB*, encoding the translation elongation factor Tu (EF-Tu) (Table S3, Supporting Information). Thus, this increase in the translational system denotes the high turnover of proteins under acid stress in *R. favelukesii*.

Amino acid metabolism

RNA-seq also revealed several DEGs associated with amino acid metabolism, such as alanine, aspartate and glutamate, cysteine and methionine, leucine and isoleucine, valine and histidine metabolism (Table S3, Supporting Information; Fig. 2). The histidine biosynthesis could be involved in the acid response (Broadbent *et al.* 2010; Nilsson *et al.* 2019). Both, the first step of the histidine biosynthesis pathway (increased *hisZ* and *hisG*), and the amount of phosphoribosyl pyrophosphate (PRPP) generated by

the overexpressed PPP enzymes may, together, increase the histidine biosynthesis. In this context, a transcriptomic and physiological study of *Lactobacillus casei* ATCC 334 under acid stress suggested that the accumulation of intracellular histidine may contribute to the intracellular buffer capacity (Wu, He and Zhang 2014). Thus, an increase of the histidine concentration in the cytoplasm could be part of the *R. favelukesii* LPU83 response to acid stress.

Moreover, transcripts of three DEGs, *gltD, glnT* and *glsA* associated with alanine, aspartate and glutamate metabolism, respectively, were found with a remarkably higher abundance under acidic conditions. The gene *gltD* encodes the beta subunit of glutamate synthase that catalyzes the conversion of two glutamates to glutamine and 2-oxoglutarate yielding NAD(P)H^+ and H^+. The gene *glnT* encodes glutamine synthetase. This enzyme incorporates ammonia into glutamate to produce glutamine. Finally, the glutaminase encoded by *glsA* hydrolyzes glutamine to form glutamate and ammonia. These results indicated that these genes may play a role in a cycle of production and consumption of ammonia as a mechanism to deal with acid stress.

Chaperones

Chaperones are usually involved in the stress response and have been described to play an important role in acid tolerance (Brigido and Oliveira 2013). In our transcriptomic analysis, the transcripts of *groES*, *degQ,* LPU83_1275 and LPU83_0110 (annotated as a chaperone protein DnaK and a putative cold shock protein, respectively) were overexpressed under acid stress. Brigido and Oliveira (2013) reported evidence that the *dnaK* and *groESL* induction could be involved in the tolerance to acid pH of chickpea mesorhizobia. The *degQ* gene encodes a periplasmic serine endoprotease DegP-like and remarkably, *degP* has been described as the most strongly induced gene upon acidic conditions in *E. meliloti* (Hellweg, Puhler and Weidner 2009). Furthermore, in *M. loti,* it was proposed that *degP* could be degrading incorrectly folded proteins under acidic conditions. The transcripts for DnaJ (LPU83_pLPU83d_1507) and three heat shock proteins (*hsp,* LPU83_2461 and *hspAT1*), however, showed a decreased expression in acidic conditions in our transcriptomic analysis. Heat shock proteins participate in the protein inactivation prevention and in the degradation of denatured proteins (de Lucena, Puhler and Weidner 2010). Moreover, the transcript expression of *grpE* (HSP-70 cofactor, LPU83_0454) and a peptidase (*clpP1,* LPU83_pLPU83d_0474) that is involved in the SOS response was lower under acid stress. Both expression levels are in opposite with the previously described in *E. meliloti* by de Lucena, Puhler and Weidner (2010).

Two-component systems and transcriptional regulators

The transcriptional analysis revealed that several DEGs associated with TCSs were identified under acidic conditions (five histidine kinases and four response regulators; Table S3, Supporting Information). Among these, the transcription of the TCS *feuP–feuQ* was increased in acidity. In *E. meliloti*, the histidine kinase FeuQ and the response regulator FeuP regulate infection steps during symbiosis and this TCS is a positive regulator of NdvA expression (Griffitts *et al.* 2008). The *ndvAB* operon was overexpressed at low pH in *R. favelukesii*. This operon has been described in *E. meliloti* to be involved in the production and export of extracellular cyclic β-(1,2) glucan. Periplasmic cyclic-glucan production is involved in the symbiotic infection in *E. meliloti* (Griffitts *et al.* 2008). NdvA expression was also described that is stimulated by low osmolarity (62). Besides, the *rrpX* gene, which encodes a peroxide stress-activated histidine kinase, was overexpressed under acidic conditions in *R. favelukesii*. This result could be associated with cross-protection behavior (Shen, Soni and Nannapaneni 2015; He *et al.* 2016) or with the increase in oxidative phosphorylation that leads to increased reactive oxygen species under acidic conditions (Yuan *et al.* 2008). The two-component system *actS–actR,* described as relevant under acidic conditions in *E. meliloti* (Fenner *et al.* 2004), did not show a differential expression in *R. favelukesii*. Nevertheless, the genes positively regulated by *actR*, as *fixK* and *fixOQP* (Fenner *et al.* 2004), were downregulated at low pH. Thus, it is probably that *actS–actR* mechanism does not respond similarly in *E. meliloti* than in *R. favelukesii*.

Another remarkable group refers to transcriptional regulators. Two of them encoding two putative transmembrane anti-sigma factors (LPU83_pLPU83d_0193 and LPU83_2665) were downregulated under acidity. These regulators have an RsiW domain. In *Bacillus subtilis,* the anti-sigma RsiW inhibits the activity of SigW, which is necessary for transcriptional initiation of several stress-activator genes (Devkota *et al.* 2017). The gene encoding RNA polymerase sigma factor SigW (LPU83_2664), however, was also downregulated in *R. favelukesii* LPU83 under acidic conditions. This phenomenon could be a response to regulation of SigW activity. In addition, our transcriptomic data revealed the overexpression under acid stress of *hmrR*, a heavy metal-responsive transcriptional regulator. HmrR regulates the *actP* transcription in *E. meliloti* and *R. leguminosarum* bv. *viciae* (Reeve *et al.* 2002). ActP is a P-type ATPase that belongs to the CPx heavy metal-transporting subfamily and a mutant in this gene showed an acid-sensitive phenotype (Reeve *et al.* 2002). ActP was described to participate in copper homeostasis, being copper toxicity a relevant factor to acid tolerance (Reeve *et al.* 2002). In agreement, *actP* gene was also overexpressed under acidic conditions in *R. favelukesii*.

Motility and chemotaxis

Motility responds differently depending on the species analyzed under acid stress conditions. For instance, a decrease in the chemotaxis and motility was evidenced under acid stress in *E. meliloti, R. leguminosarum* bv. *viciae* and *A. tumefaciens* (Bowra and Dilworth 1981; Yuan *et al.* 2008; Hellweg, Puhler and Weidner 2009). In our transcriptomic analysis, 23 out of 25 flagellar biosynthesis genes (Fig. 5), several genes encoding both chemotaxis TCS (*cheA, cheB, cheW, cheR* and *cheY*) and methyl-accepting chemotaxis proteins (*mcpV*, *mcpX*, *mcpA7* and LPU83_pLPU83d_0676) were all overexpressed under acidic conditions (Table S3, Supporting Information). *Ensifer meliloti* motility, however, decreases under acidity and this phenotype appears to respond to the general stress and not specifically to pH stress (Hellweg, Puhler and Weidner 2009). Since cell motility consumes energy, repression of motility genes could be associated with energy-saving to face the low pH challenge. The flagella genes, in the more distantly related enterobacterium *E. coli*, however, are induced in presence of low pH value (5.0) (Maurer *et al.* 2005). This fact is in agreement with our results (Nilsson *et al.* 2019) but remarkably *R. favelukesii* LPU83 shows a non-motile phenotype (Torres Tejerizo *et al.* 2016). The genes needed for mobility and its regulation, however, are present in *R. favelukesii* LPU83, including master regulators as *visN*, *visR* and *rem* (LPU83_0783, LPU83_0784 and LPU83_0817, respectively; Table S2, Supporting Information). These three regulators did not show significant changes under acidic stress. Thus, questions concerning the functionality of motility related genes remain open.

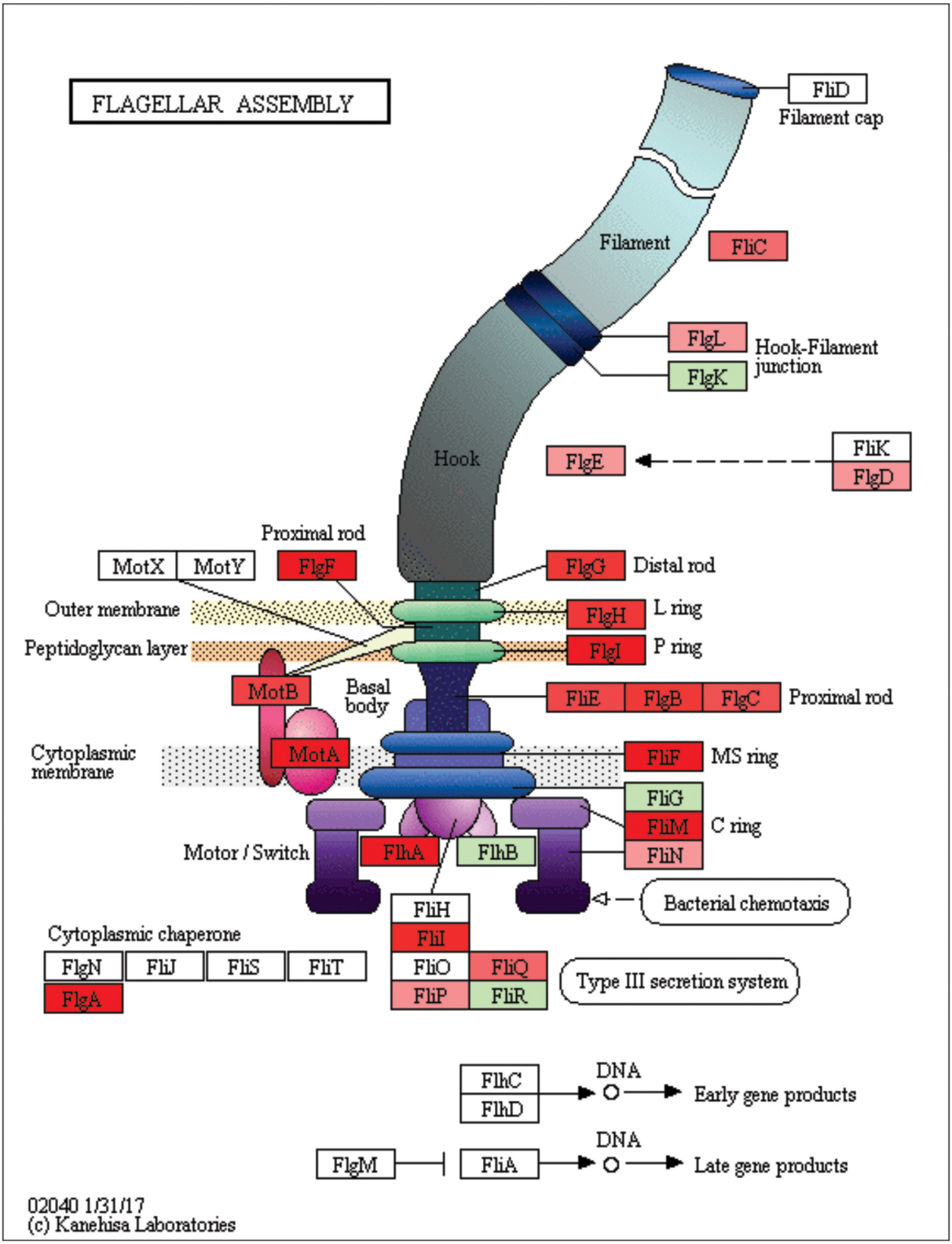

Figure 5. Transcript expression of DEGs involved in the flagellar assembly in response to acid stress in *R. favelukesii* LPU83. Flagellar structure scheme with the differential expression of DEGs. The scheme was performed with KEGG Mapper tool (Kanehisa and Sato 2020). The level of expression of the DEGs is represented with a color range from blue to red. The more intense red represents the higher overexpression level. The more intense blue represents the higher underexpression level. Enzymes without significant fold change have the box gene colored in green. Gene boxes that are not colored (white) correspond to proteins that are absent in genome of *R. favelukesii*. The different colors of each protein and of the membrane do not stand for expression values.

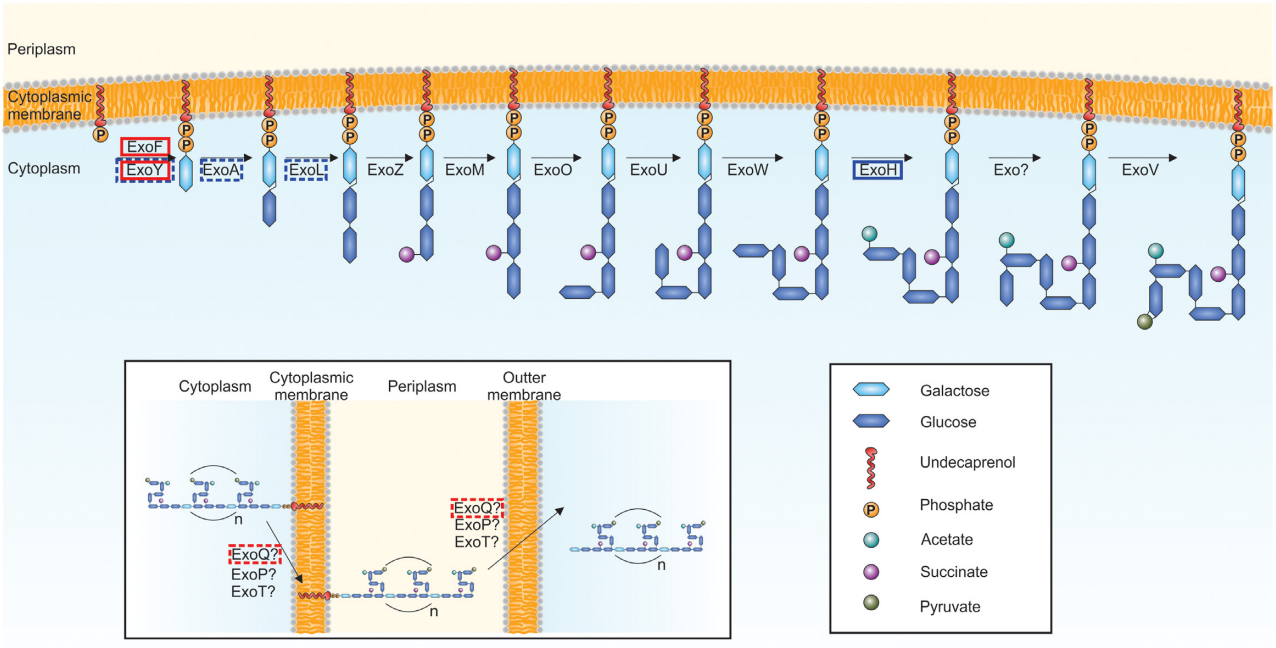

Figure 6. Expression of genes involved in exopolysaccharide biosynthesis in response to acid stress in *R. favelukesii* LPU83. The scheme shows the expression of transcripts (RNA-seq data, boxes with dashed lines) and proteins detected by proteomics (Nilsson *et al.* 2019; boxes with continuous line) involved in exopolysaccharide biosynthesis (*E. meliloti* biosynthetic pathways were used as model; Reuber, Long and Walker 1991). The red color represents a higher expression in acidity. The blue color represents a lower expression under acidic conditions (higher expression in neutrality).

Cell envelope

The function of the cell envelope is crucial in protecting the cell against harmful environmental conditions and represents the main barrier to stress. In particular, many bacteria change their envelope in response to an acidic environment, such as *Shewanella oneidensis*, *E. coli* and *A. tumefaciens* (Jordan, Oxford and O'Byrne 1999; Leaphart *et al.* 2006; Yuan *et al.* 2008). Indeed, the maintenance of the impermeability of the membrane toward the influx of H^+ ions, is critical to carefully control ion channels and/or activity of transport systems (Booth, Cash and O'Byrne 2002). A transcriptomic analysis of *A. tumefaciens* reported that 24% of the genes induced under acid stress are functionally related to the cell envelope. Accordingly, in the transcriptomic analysis of *R. favelukesii* LPU83, a higher number of cell envelope genes were differentially expressed under acidic conditions (Table S3, Supporting Information). Regarding to cell wall biosynthesis, the transcripts for a D-alanine-D-alanine ligase, an undecaprenyl-diphosphatase, a penicillin-binding protein 1C, three possible glycosyltransferases (GTases) and two enzymes responsible for glycosidic-bond cleavage between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan were overexpressed under acidic conditions (*ddl, uppP, pbpC* LPU83_0377, LPU83_1936, LPU83_pLPU83d_0134, *mltB* and LPU83_2746, respectively). Transcripts of the *lppB* and *pal* genes also showed a higher abundance under acid stress conditions. The *lppB* gene encodes the lipoprotein NlpD that regulates the amidase activity. The peptidoglycan-associated lipoprotein PAL, encoded by *pal*, is involved in the maintenance of the extern membrane integrity and the import of organic molecules.

Presumably, other components of the cell envelope may also be altered such as EPS and lipopolysaccharide (LPS). The correlation between the EPS production and the acid response is known in *E. meliloti* and *A. tumefaciens*, among others (Yuan *et al.* 2008; Hellweg, Puhler and Weidner 2009). Our previous proteomic analysis revealed an unclear expression in EPS-biosynthesis proteins. While ExoF and ExoY were overexpressed; proteins encoded by both copies of *exoH* were underexpressed in acidic conditions. Unfortunately, the transcriptomic analysis also showed unclear results regarding EPS biosynthesis. The genes *exoA*, *exoK*, *exoL* and *exoY* were downregulated but exoN was upregulated under acid stress (Fig. 6). Hence, with the aim to elucidate the correlation of EPS biosynthesis and the acid response in *R. favelukesii* LPU83, the amount of EPS secreted was determined under the same conditions than those applied in the transcriptome and proteome analyses by anthrone method (Loewus 1952). The results did not show a significant difference in the polysaccharide amount under the conditions tested (Figure S3, Supporting Information), indicating that total EPS amount under acidic treatment could be similar to that obtained under neutral conditions. In *E. meliloti* has been shown the upregulation of EPS biosynthesis genes and the increase of EPS secreted under acidic conditions (Hellweg *et al.* 2009; Draghi *et al.* 2016). Thus, *R. favelukesii* LPU83 exhibits a different response than that described for *E. meliloti* regarding transcription of EPS genes and possibly EPS production under acid stress.

LPS is the major component of the outer membrane of Gram-negative bacteria (Alexander and Rietschel 2001; Raetz and Whitfield 2002). In rhizobia, it is essential to suppress the plant defense, enabling successful infection and survival within the host cells (Rincon-Zachary 2010). The transcriptomic analysis revealed a group of genes associated with LPS biosynthesis pathway with a high abundance under acidic conditions (*lpxA, lpxB, lpxC, lpxL, kdsB1,* LPU83_2061, LPU83_3077 and LPU83_3872). Moreover, two more genes of the LPS biosynthesis, *lpxD* and *lpxK*, were slightly overexpressed (log_2*fold change*:

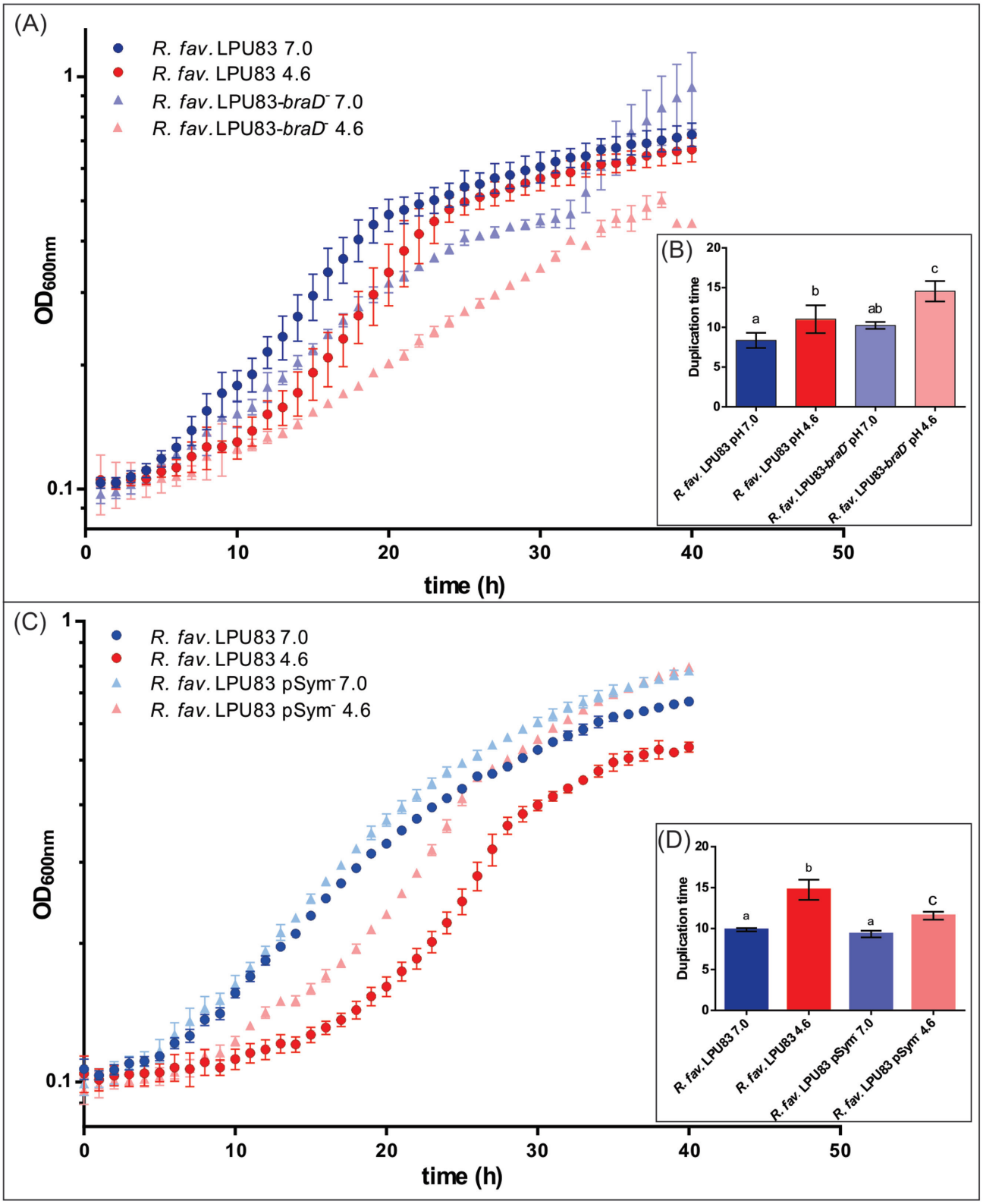

Figure 7. Growth curves and determination of doubling times of *R. favelukesii* LPU83⁻pSym⁻, *R. favelukesii* LPU83-*braD*⁻ and the wild-type strain at acid and neutral pH. **(A)** Growth curves for the mutant and wild-type strain. The optical density at 600 nm (OD_{600}) is plotted on an exponential scale with respect to the time. **(B)** The doubling time in hours is plotted for each of the strains indicated. **(C)** Growth curves for the pSym-cured strain and wild-type strain. The optical density at 600 nm (OD_{600}) is plotted on an exponential scale with respect to the time. **(D)** The doubling time in hours is plotted for each of the indicated strains. The superscript letters indicate significant differences at $P \leq 0.05$ (Tukey test). The statistical data represent three independent biological replicates.

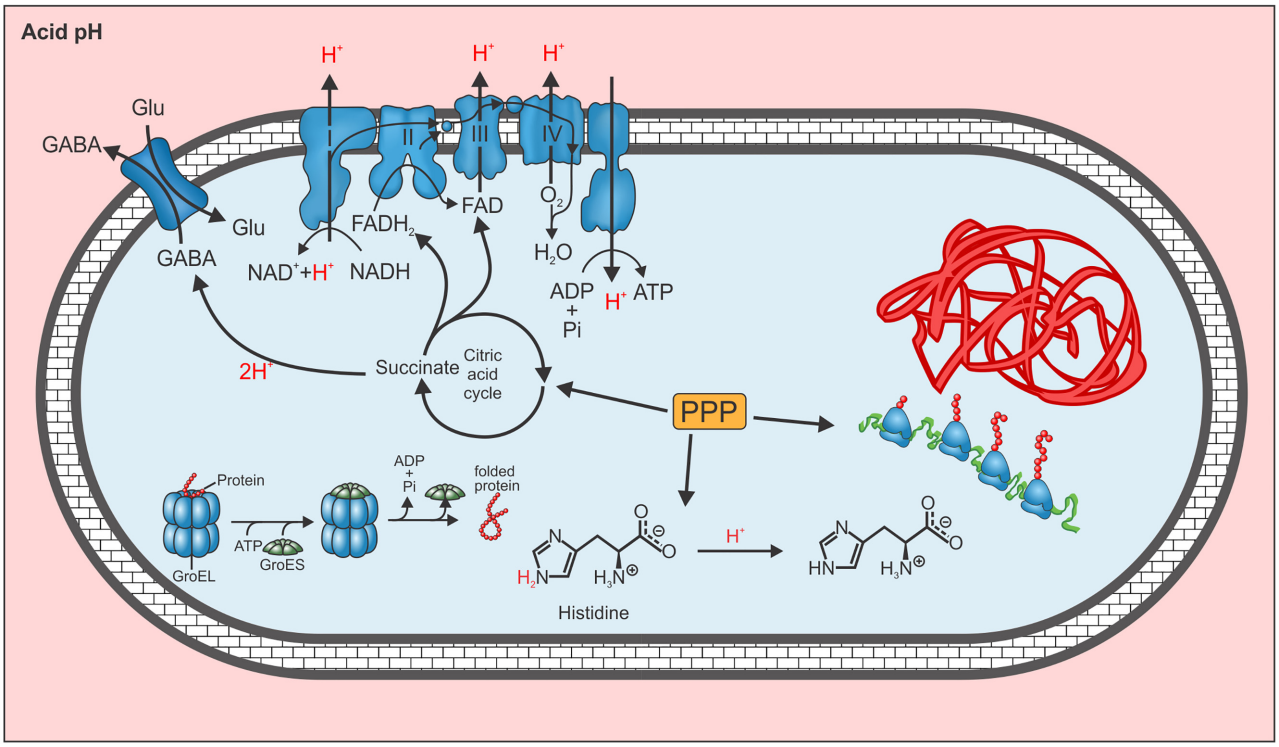

Figure 8. Acid response of *R. favelukesii* LPU83. The figure shows the increased metabolisms under acidic conditions of *R. favelukesii* LPU83. This rhizobium presents a global response as consequence of the cellular deterioration caused by acid stress. This response involves an increase in oxidative phosphorylation, as well as TCA cycle in response to the high energy demand. The PPP, possibly also increased, may contribute to generate the necessary reducing power required for cellular biosynthesis under this condition. In addition, *R. favelukesii* LPU83 could trigger various mechanisms to cope specifically with acid stress, as increase in the histidine concentration, GABA metabolism and changes regarding the cell envelope. Cell contour containing blocks represents modifications in the cell envelope that would contribute to the *R. favelukesii* acid tolerance.

−0.5 for both; *adj*. *P*-value: 0.02 and 0.04, respectively). Likewise, the gene encoding the lipid A export ATP-binding component (LPU83_3342) was also overexpressed under acidic conditions. Moreover, the known gene *lpiA* (low pH-inducible gene A), like in other rhizobia, was upregulated under acidic conditions in *R. favelukesii*. This gene encodes a lysylphosphatidylglycerol synthase, which generates membrane modifications relevant to acid tolerance in *R. tropici* (Vinuesa *et al*. 2003). Furthermore, *lpiA* has been described to function in the adaptation to low pH values (Reeve *et al*. 2004; Reeve *et al*. 2006) in *E. medicae* and to show a continually increased expression under acidic conditions in *E. meliloti* (Hellweg, Puhler and Weidner 2009). Other relevant lipids are the ornithine lipids (OL), which biosynthesis is increased under acidic conditions in *R. tropici* and their presence in the outer membrane is higher in acid stress cells (Vences-Guzman *et al*. 2011). The lipid modification catalyzed by OlsC has been described as relevant in the acid response in *R. tropici* (Rojas-Jimenez *et al*. 2005; Vences-Guzman *et al*. 2011), and a mutant in this gene showed an acid-sensitive phenotype (Vences-Guzman *et al*. 2011). Our transcriptomic analysis revealed that *olsA*, which encodes a phospholipid/glycerol acyltransferase involved in OL biosynthesis, was overexpressed under acidic conditions. In agreement, we have shown that the membrane permeability under acid stress is diminished (Nilsson *et al*. 2019). Hence, *R. favelukesii* LPU83 may specifically modify the structure of the cell envelope and such modifications may contribute to a tighter barrier against the entry of H^+.

Efflux pumps

The expression of several efflux pump genes was altered under acidic stress in *R. favelukesii* LPU83. The genes encoding the efflux pump membrane transporters, AcrAB and RmrAB, were overexpressed in acidity (LPU83_3368, LPU83_3369, LPU83_3677 and LPU83_3678). The gene *bepG* (LPU83_pLPU83d_0800) encodes another efflux pump that was downregulated at low pH. AcrAB has been described as one of five resistance–nodulation–division (RND) systems in *Salmonella* (Blair *et al*. 2015). The efflux transporters of RND systems are located in the inner membrane of Gram-negative bacteria and form a tripartite efflux pump system spanning both the inner and outer membrane (Blair *et al*. 2015). Efflux via AcrB is driven by the proton-motive force and is part of a tripartite complex with the periplasmic adaptor protein AcrA and the outer membrane channel TolC (Blair *et al*. 2015), which in our analysis, was slightly upregulated ($\log_2$*foldchange*: −0.83, *P*-adjusted: 0.00018) under acidity. Moreover, in *Rhizobium etli* CFN42, the operon *rmrAB* was proposed to encode an inducible export system that prevents the accumulation of toxic plant compounds within the bacterial cell, accounting for an advantage to *R. etli* for bean nodulation (Gonzalez-Pasayo and Martinez-Romero 2000). The *rmrAB* mutants showed sensitivity to flavonoids (Gonzalez-Pasayo and Martinez-Romero 2000). In *E. meliloti* was demonstrated that this efflux system is relevant in nodulation competitiveness (Eda, Mitsui and Minamisawa 2011). Also, a *tolC* mutant of a strain of *E. meliloti* showed an increased sensitivity to several abiotic stresses such as osmotic and oxida-

tive stress, as well as a lower succinoglycan production (Cosme *et al.* 2008). Hence, the efflux pumps AcrAB and RmrAB may also contribute to the specific response to acid stress of *R. favelukesii* LPU83 by expulsion of intracellular protons.

Phenotypic analysis

With the aim to validate specific aspects of the *R. favelukesii* LPU83 response to acid stress, experiments to obtain insights into the molecular basis of acid tolerance were performed. Previously, it has been hypothesized that *R. favelukesii* LPU83 is able to produce GABA in order to increase the intracellular pH (Nilsson *et al.* 2019). GABA production was previously described in *E. meliloti* bacteroids (Miller, McRae and Joy 1991). In *R. leguminosarum* bv. *viciae*, a mutant in *gabT* gene, which encode one enzyme of GABA shunt pathways showed an acid-sensitive phenotype and *gabT* expression was increased in the bacteroids (Prell *et al.* 2002). As a first approach to evaluate GABA involvement, an operon encoding a putative GABA transport system (upregulated under acid conditions) was interrupted. The first gene, *braD,* was targeted and the growth behavior of the constructed *R. favelukesii* LPU83 *braD* mutant was evaluated. The mutant showed an acid-sensitive phenotype featuring growth rates that clearly differ from the wild-type strain (Fig. 7A and B). Hence, increased expression of the genes encoding the putative GABA transporter and the sensitive phenotype of *braD* mutant under conditions of acid stress (Fig. 7) contributes to the hypothesis of GABA production under acidic conditions in *R. favelukesii* LPU83.

Independently, the transcriptomic analysis revealed that 60% of *R. favelukesii* LPU83 genes (310) that are harbored on the symbiotic plasmid pLPU83b (pSym) were downregulated under acidic conditions (Fig. 1). This result could be due either a real repression of the pSym genes or that a proportion of the bacteria lose the plasmid. To evaluate this behavior, the pSym loss was tested under both acidic and neutral conditions. The pSym stability does not show significant differences under the evaluated conditions (Figure S4, Supporting Information), this confirms the downregulation of pSym genes. In contrast, several genes of the plasmid pSymA of *E. meliloti* have been described to be overexpressed under acidic conditions in contrast with our results (Draghi *et al.* 2016). Thus, we decided to evaluate the phenotypic behavior of a strain cured for pSym under acid stress conditions with the aim to determine the relevance of this plasmid at low pH. A *R. favelukesii* LPU83 strain cured of the symbiotic plasmid was obtained (*R. favelukesii* LPU83-pSym$^-$) through a plasmid incompatibility approach (Novick 1987). Subsequently, a growth kinetic study of *R. favelukesii* LPU83-pSym$^-$ was performed. The pSym-cured strain showed a significantly lower duplication time under acid stress (Fig. 7C and D). This result is consistent with the downregulation of a high number of the pSym genes. A hypothesis for these results could arise from the energy fitness (Vogwill and MacLean 2015; San Millan and MacLean 2017), suggesting that the maintenance of the symbiotic plasmid represents significant energy cost regarding the growth of the strain under acid stress. This hypothesis is strengthened when considering that *R. favelukesii* LPU83 has a higher energy demand during acid stress. Generally, the maintenance of symbiotic plasmids is not highly energy-consuming since they have evolved together over time without selection pressures for them. In contrast, the symbiotic plasmid of *R. favelukesii* LPU83 has been probably acquired recently during the evolution of the strain (Wibberg *et al.* 2014). Accordingly, the systematic repression of the high number of genes in the symbiotic plasmid under acid stress could be supported by the hypothesis of energetic fitness in *R. favelukesii*.

CONCLUDING REMARKS

The acid response of *R. favelukesii* LPU83 involves a broad metabolic response, as a consequence of the high-energy demand under acidic growth conditions. Indeed, determinants involved in the cellular respiration metabolism, such as the tricarboxylic acid cycle and oxidative phosphorylation, are overexpressed under acidic stress (Fig. 8). Besides, the expression of genes representing the PPP appeared to be modified. These observations indicate that a high proportion of energy, as well as the utilized carbon, is directed toward biosynthesis under acidic conditions. Such an increase in biosynthesis may be supported by the higher expression of enzymes and corresponding transcripts involved in protein synthesis and amino acid biosynthesis. In agreement with this, the *R. favelukesii* LPU83 acid-response also involves a large number of chaperones, proteases and several enzymes associated with cell repair, indicating cell deterioration (Fig. 8). Beyond the metabolic response to acid stress described here, *R. favelukesii* LPU83 triggers several mechanisms to cope with and counteract acid stress, such as mechanisms associated with GABA metabolism (Nilsson *et al.* 2019), increased histidine concentration, changes in cell envelope as peptidoglycan, lipid membrane, among others (Fig. 8). Finally, in response to acid stress, a large number of proteins featuring transcriptional regulator domains (64) as well as a large number of hypothetical proteins (650) were detected through our analyses. Among these unknown functions, key responses regarding acid tolerance may be hidden. Consequently, it is of vital importance to advance on the biochemical study of these proteins and their relationship with acidity tolerance. Thus, the present work provides relevant information on acid tolerance in rhizobia and targets for new biochemical studies that allow in-depth exploration of the mechanisms involved in the acid adaptation of bacteria. The comparison of mechanisms and regulatory networks of the acid-sensitive phenotype of *E. meliloti* with those of the acid-tolerant phenotype of *R. favelukesii* could be a cornerstone to unravel the new determinants necessary for a fruitful symbiosis in acidic conditions. These comparisons could allow us the possibility to obtain strains of *E. meliloti* with a higher acid tolerance but with a genomic background able to perform an efficient SNF in symbiosis with alfalfa.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflict of Interest. None declared.

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