ubiF is involved in acid stress tolerance and symbiotic competitiveness in *Rhizobium favelukesii* LPU83

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Abstract

The acidity of soils significantly reduces the productivity of legumes mainly because of the detrimental effects of hydrogen ions on the legume plants, leading to the establishment of an inefficient symbiosis and poor biological nitrogen fixation. We recently reported the analysis of the fully sequenced genome of *Rhizobium favelukesii* LPU83, an alfalfa-nodulating rhizobium with a remarkable ability to grow, nodulate and compete in acidic conditions. To gain more insight into the genetic mechanisms leading to acid tolerance in *R. favelukesii* LPU83, we constructed a transposon mutant library and screened for mutants displaying a more acid-sensitive phenotype than the parental strain. We identified mutant *Tn*833 carrying a single-transposon insertion within *LPU83_2531*, an uncharacterized short ORF located immediately upstream from *ubiF* homolog. This gene encodes a protein with an enzymatic activity involved in the biosynthesis of ubiquinone. As the transposon was inserted near the 3′ end of *LPU83_2531* and these genes are cotranscribed as a part of the same operon, we hypothesized that the phenotype in *Tn*833 is most likely due to a polar effect on *ubiF* transcription.

We found that a mutant in *ubiF* was impaired to grow at low pH and other abiotic stresses including 5 mM ascorbate and 0.500 mM Zn^{2+}. Although the *ubiF* mutant retained the ability to nodulate alfalfa and *Phaseolus vulgaris*, it was unable to compete with the *R. favelukesii* LPU83 wild-type strain for nodulation in *Medicago sativa* and *P. vulgaris*, suggesting that *ubiF* is important for competitiveness. Here, we report for the first time an *ubiF* homolog being essential for nodulation competitiveness and tolerance to specific stresses in rhizobia.

Keywords Acid tolerance · *ubiF* · *Rhizobium favelukesii* LPU83 · Stress · Competitiveness

Introduction

Rhizobia are Gram-negative bacteria that live in soils and symbiotically associate with the roots of leguminous plants to fix atmospheric nitrogen and, in turn, receive carbon compounds derived from plant photosynthates. While, in general, the rhizobium-legume symbiosis shows different degrees of specificity according to the associated species, the nodulation of *Medicago sativa* (alfalfa) is known to be particularly restricted to *Ensifer meliloti* (formerly *Sinorhizobium*

meliloti) and other few rhizobia [1–3] including the unusual and genetically closely related strains *Rhizobium favelukesii* Or191 (previously named *Rhizobium* sp. Or191) [4] and the *Rhizobium favelukesii* LPU83-type strain isolated in the USA and Argentina, respectively [5, 6]. However, neither of the latter strains is able to establish a full N_2-fixing symbiosis on *Medicago*, *Melilotus*, *Trigonella* or *Phaseolus vulgaris* [6–8]. Some features of these alfalfa-nodulating rhizobia include acid tolerance [6]; extended host range for nodulation [9]; inefficiency of nitrogen fixation in *M. sativa*, *Medicago truncatula* and *P. vulgaris*; and their extremely low genetic diversity independent of their geographical origin [9, 10]. In addition, analysis of exopolysaccharides—necessary for the establishment of an effective symbiosis—in *R. favelukesii* LPU83 revealed an identical composition to those in *E. meliloti* [11]. All these characteristics, along with their remarkably successful competition with the N_2-fixing symbionts for nodulation, point to this type of rhizobium

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as a detrimental agent in agricultural soils when it coexists with *E. meliloti* [5]. We have previously shown that under acidic conditions, the strain *R. favelukesii* LPU83 increased its competitiveness to nodulate alfalfa against the efficient symbiont *E. meliloti* [12]. Contrasting *E. meliloti*, the attachment of *R. favelukesii* LPU83 to alfalfa roots is almost not influenced by pH or Ca^{2+} concentration [13]. Although we have characterized the behaviour of these rhizobia in soil microcosms, we have, so far, no data available on the persistence and ecology of these bacteria in acidic fields, or on how the symbiosis between alfalfa and the efficient rhizobia is affected.

The low performance of alfalfa in acidic soils results from several conditions that affect the host plant, their rhizobia and the symbiotic interactions between them [14–17]. Acid tolerance of rhizobia has long been considered a phenotypic characteristic that would positively affect persistence and symbiosis under low pH. For this reason, the identification and manipulation of the genetic determinants of acid tolerance is a high-priority task towards the phenotypic improvement of rhizobial inoculants for acid soils. We and others have previously identified and characterized genes associated with tolerance to acidity (*act* genes) in alfalfa-nodulating rhizobia [18–23]. In addition, other factors like the production of exopolysaccharides have been pointed out as critical for tolerance to acid conditions and other stresses in *E. meliloti* [24, 25]. On the other hand, transcriptomic and proteomic analyses of *R. favelukesii* LPU83 under acid conditions revealed the differential expression of 1294 and 336 genes, respectively [11, 26]. The affected genes belonged to different categories, with a prominent number of those involved in cell envelope modification, cell permeability and reverse proton efflux. This response was consistent with the one observed in *Rhizobium tropici* when exposed to low pH [27] and seems to be a general bacterial response when exposed to acidic environments [28].

To gain more insight on the molecular basis of the acid tolerance in *R. favelukesii* LPU83, we performed transposon mutagenesis and selected acid-sensitive mutants. We focused on *Tn*833, which displayed a remarkable lower ability to grow on acid media. We find that the transposon was inserted within a short ORF located upstream an *ubiF* homolog and constructed mutants to characterize their acid tolerance, symbiosis capabilities and competitiveness in the nodulation of the host plant.

Materials and methods

Bacterial strains and media

The bacterial strains and plasmids used in this work are listed in Table 1. The rhizobia were grown at 28 °C on tryptone yeast medium [35] or yeast extract mannitol medium [36]. The screening of acid-sensitive mutants and the growth curves was performed on GS minimal medium [6]. *Escherichia coli* strains were grown at 37 °C on Luria–Bertani medium [37]. For the agarized media, 15 g of agar per litre of the medium was added. When required, the media were supplemented with 120 μg/ml neomycin (Nm), 400 μg/ml streptomycin (Str), 200 μg/ml ampicillin (Ap), 10 μg/ml tetracycline (Tc) for *E. coli* or 2 μg/ml for *Rhizobium* and 25 μg/ml kanamycin (Km). The plasmids were introduced into rhizobia from *Escherichia coli* S17-1 by biparental mating or from *E. coli* DH5α by triparental mating with pRK2013 as a helper plasmid.

Growth curves in acidic conditions

Starter cultures were grown in GS minimal medium. GS medium was supplemented with 20 mM MES buffer (2-[*N*-morpholino]ethanesulfonic acid) to adjust the pH to the range of 5.0 to 6.0. Twenty millimolar PIPES (piperazine-*N*,*N*′-*bis*(2-ethanesulfonic acid)) was added to the GS medium to maintain the pH within the range of 6.5 to 7.0. The bacterial cultures on the liquid medium were grown at 28 °C, with shaking at 250 rpm. The kinetics of bacterial growth were studied in the GS minimal medium supplemented with MES or PIPES depending on the pH of the experiment. The pH was approximated with HCl or KOH before autoclaving and adjusted more precisely after the addition of filter-sterilized vitamins and micronutrients. The pH of the cultures during bacterial growth was monitored each time that the optical density was measured.

DNA manipulations

Plasmid preparations and DNA cloning, bacterial transformations, restriction endonuclease digestions, agarose gel electrophoresis and molecular hybridization were carried out as described by Sambrook et al. [37]. Total genomic DNA was prepared as described by Simon et al. [29].

Screening for acid-sensitive mutants

Transposon mutagenesis on *R. favelukesii* LPU83 strain was performed using transposon *Tn5*-B20 [30], according to the protocol for *Tn5* mutagenesis of *Rhizobium* sp. described by Rossbach et al. [38]. Mutants were screened for acid sensitivity by using toothpicks to streak individual colonies on GS plates at both acid and neutral pH levels. Transconjugants that grew poorly on GS medium at pH 5.0 but grew well at pH 7.0 were selected for further analysis.

Table 1 Strains and plasmids used in this study

Strains/plasmids	Description	Reference
Strains		
R. favelukesii LPU83	Wild-type, spontaneous Sm^r; is the specie's type strain (=CECT 9014^T=LMG 29160^T); previously named *Rhizobium* sp. LPU83	Del Papa et al. (1999) [6]
*Tn*833	*Tn*5 mutant of wild-type LPU83, Sm^r Nm^r	This study
LPU83_2531$^-$	*LPU83_2531* mutant in *R. favelukesii* LPU83, Sm^r Nm^r	This study
ubiF$^-$	*ubiF* mutant in *R. favelukesii* LPU83, Sm^r Nm^r	This study
E. coli DH5	*recA1*, *lacU169 80dlacZ* M15; host strain used in cloning experiments	Bethesda Research Laboratories
E. coli S17-1	MM294, RP4-2-Tc::Mu-Km::*Tn*7 chromosomally integrated	Simon et al. (1991) [29]
E. coli TOP10	F′ {lacIq*Tn*10(TetR)} *ncrA _(mrr-hsdRNS-ncrBC) _80lacZ_M15 _lacX74 recA1*	Invitrogen
Plasmids		
Tn5B20	Promoter probe transposon that carries the promoterless reporter genes *lacZ* and *npt*II as a selective marker	Simon et al. (1989) [30]
pCR-Topo	PCR-cloning vector Ap^r, Km^r, *lacZ*	Invitrogen
pK18mob	Km^r *ori* ColE1, Mob+, *lacZ*+used for directed insertional disruption	Schäfer et al. (1994) [31]
pK18mob-ORF1	pK18mob with a 232-bp internal fragment of *LPU83_2531* cloned as a blunt insert, Km^r	This study
pK18mob-ubiF	pK18mob with a 427-bp internal fragment of ubiF cloned as a blunt insert, Km^r	This study
pGEMT	Multicopy vector, Ap^r	Promega
pBBR1-MCS5	Mobilizable vector, *lacZalpha*, Gm^r	Kovach et al. (1994) [32]
pBBR1M CS5::*ubiF*	pBBR1MCS5 with *ubiF* cloned as a *Hind*III-*Eco*RI insert, Gm^r	This study
pFAJ1708	Cloning vector, Tc^r	Dombrecht et al. (2001) [33]
pLow1	Low-copy number plasmid, Cm^r	Hansen et al. (1997) [34]
pLow1-833	pLow1 plus 22-kbp *Kpn*I fragment carrying *Tn*5 insertion of mutant *Tn*833	This study

PCR amplification

PCR amplifications were carried out in a total reaction volume of 25 µl containing PCR buffer (50 mM KCl, 20 mM Tris HCl, pH 8.0), 200 µM each of dNTPs, 3 mM MgCl$_2$, 2 µl of DNA template and 1 U Taq DNA polymerase (Gibco, BRL). All amplifications were performed with an Idaho capillary air thermocycler under the following temperature conditions: an initial 1 min denaturation at 94 °C followed by 35 cycles of 94 °C for 10 s, 52 °C for 10 s and 72 °C for 30 s, and a final 1-min extension at 72 °C. PCRs were separated in 1.5% w/v agarose gels containing ethidium bromide (1 µg/ml).

DNA sequencing and sequence analysis

The characterization of the insertion site of transposon *Tn*5B20 was carried out by cloning a 22-kbp *Kpn*I restriction fragment into the low-copy plasmid pLOW1 [34] digested with the same enzyme. The digestion mixtures were joined and electroporated in *E. coli* DH5α. The nucleotide sequence was obtained by a primer-walking strategy using specific deoxyoligonucleotides and plasmid DNA as the template. The reaction products were separated and analysed by an automatic laser–induced fluorescent DNA sequencer (Pharmacia). The sequence was compared to the complete genome of *R. favelukesii* strain LPU83 that is available with the following accession numbers: HG916852 (chromosome), HG916853 (pLPU83a), CBYB010000001-58 (pLPU83b), HG916854 (pLPU83c) and HG916855 (pLPU83d). All sequence data were downloaded from public databases. Sequence comparisons and alignments were performed through BLAST (www.ncbi.nlm.nih.gov/blast) and ClustalX [39] software, respectively.

Construction of *R. favelukesii* LPU83*ubiF$^-$* and LPU83_*2531$^-$* mutants

An internal region of *ubiF* or ORF *LPU83_2531* was amplified by PCR with Pfx polymerase and DNA from strain LPU83 using primers UBIF-Fw (GGGCAATATCACGCGTTT) and UBIF-Rv (GAGTTGCGCACCTTCCTC), or orfF (GGCTTTGGCGTGCTGAT) and orfR (CCGCGAAGCTGTCGATAC) to amplify internal fragments of 427 bp and 232 bp, respectively. Each blunt PCR fragment corresponding to the gene internal sequence was cloned into vector pK18mob (Km^R, *lacZ*) previously digested with *Sma*I [31]. The resulting plasmid was transferred by conjugation to strain LPU83 to yield mutants LPU83*ubiF$^-$* and LPU83_*2531$^-$* by site-specific insertional mutagenesis. All PCR amplifications were performed as described above

except that the Pfx polymerase was used at its optimal elongation temperature of 68 °C.

All internal fragments cloned into pK18mob were confirmed by sequencing. The resulting recombinant plasmids were finally transferred by conjugation from *E. coli* S17-1 to strain *R. favelukesii* LPU83. Putative mutants that had been generated after the site-specific integration of the plasmid (single crossover) were selected by their expected Str^r, Km-Nm^r phenotype. After plasmid transfer from *E. coli* S17-1 to *R. favelukesii* LPU83 by conjugation, the correct plasmid integration was confirmed by PCR using M13 primers and specific oligonucleotides annealing to the flanking regions of the rhizobial genome.

Complementation of the LPU83*ubiF*⁻ mutant

The *ubiF* gene from *R. favelukesii* LPU83 was PCR amplified using UBIFHindIII (TTTAAGCTTTGAAGGAGG AACGGTGCAATGAAGAC) and UBIFRevEcoRI (TTT TGAATTCATCCTGCTTGCGATCAAATC) primers and then digested with *Eco*RI and *Hind*III. The DNA fragment was ligated into the replicative vector pBBR1MCS5 (Gm^r) [32] or pFAJ1708 (Tc^r) [33], both having been previously digested with the same two enzymes. The resulting recombinant plasmids (pBBR1MCS5::*ubiF* or pFAJ1708::*ubiF*) were individually electroporated into *E. coli* DH5α. After several unsuccessful attempts to introduce the *ubiF* construction in *E. coli* cells, the DNA ligation mix was transferred by electroporation to LPU83*ubiF*⁻ to not to use *E. coli* as a recipient strain and to search for the complemented strain, but we were not able to have positive clones.

Plant nodulation tests

Medicago sativa seeds (alfalfa, cv. Super Monarca, obtained from Instituto Nacional de Tecnología Agropecuaria, Argentina) were surface sterilized for 10 min with 20% v/v commercial bleach (NaClO concentration equivalent to 11 g active Cl_2 per litre) followed by six washes with sterile distilled water. The sterilized seeds were germinated on 1.5% w/v water agar. Two- to four-day-old seedlings were transferred to gamma irradiation–sterilized plastic growth pouches (Mega Minneapolis International, Minneapolis, MN, USA) containing 10 ml of nitrogen-free Fåhraeus-modified mineral solution, pH 7.0 [40] or pH 5.6. Three days later, the primary roots were inoculated with 10^6 colony-forming units (CFU) of rhizobia by dripping 100 μl of a bacterial suspension onto the root from the tip towards the base. The rhizobia were obtained from log-phase yeast extract mannitol medium cultures. The plants were cultured in a growth chamber at 22 °C with a 16-h photoperiod. The CFU contained in the inoculum were estimated by plate counts. The competition was evaluated at 4 weeks post-inoculation.

Nodulation assays with common beans cv. Negro Jamapa were carried out in 200-ml plastic pots containing vermiculite and Fåhraeus mineral solution, pH 7.0 [41]. Surface-sterilized seeds were germinated on 1.5% w/v water agar, and two small seedlings were transferred to each pot. The seedlings were inoculated with ca. 10^7 rhizobia/pot. Plant roots were analysed for the presence of nodules 30 days after inoculation. The results are given as the average number of nodules per plant. Where present, the error bars indicate the standard deviation ($\sigma/\sqrt{n}$). Results are taken from a representative experiment among a set of three.

Sensitivity assays

For all assays, exponential cultures ($OD_{600} = 1$) grown in GS medium at pH 7.0 supplemented with antibiotics, when required, were centrifuged at 640 g and the cell pellet was resuspended in saline solution (0.89% w/v NaCl). An aliquot of cells was used to inoculate flasks containing GS either with or without the addition of 100 μM $CuSO_4$, 10% w/v sucrose, 0.5 M NaCl, 500 μM $ZnSO_4$ or 5 mM ascorbic acid. The cultures were then incubated at 28 °C and 250 rpm for 6 h or 24 h. After treatment, cultures were plated on TY media for CFU determination. CFU were compared to non-treated samples (control). Photosensitivity was tested as described by Nakahigashi et al. [42].

Results

Isolation of an acid-sensitive mutant and identification of an *act* gene in *R. favelukesii* LPU83

In order to identify novel genetic determinants of tolerance to acidity in alfalfa-nodulating rhizobia, random transposon mutagenesis was performed with transposon *Tn5*B20 on the acid-tolerant strain *R. favelukesii* LPU83. The collection of transposon mutants was selected in TY solid medium supplemented with Sm and Nm. More than 3000 mutants were streaked on GS solid medium at both pH levels 7.0 (control) and 5.0. Acid-sensitive mutants were expected to show either no growth or reduced growth in the minimal medium at pH 5.0, but near wild-type growth at pH 7.0. Mutants that had completely lost their ability to grow under acid conditions (pH 5.0) but had preserved normal growth at neutral pH were not found. We only found one mutant that displayed an impaired growth selectively under acidic conditions, *Tn*833, which was selected for further characterization. When mutant *Tn*833 was examined by Southern hybridization analysis utilizing an IS50 probe, single *Eco*RI- and *Kpn*I-hybridizing signals were found that corresponded to fragments of 15 kb and 22 kb, respectively (data not shown).

In order to identify the disrupted gene in mutant *Tn*833, the 22-kb *Kpn*I restriction fragment that contained the *Tn5*B20 was cloned into the low-copy number plasmid pLOW1 and a 10-kb sequence of the rhizobial DNA bordering the transposon was obtained (Fig. 1). The *Tn5*B20 transposon was inserted close to the 3′ end of *LPU83_2531*, whose translation product corresponds to a hypothetical protein containing a domain of the DUF934 superfamily, identified in several rhizobia (*Rhizobium leguminosarum* bv. viciae, *R. leguminosarum* bv. trifolii, *Rhizobium etli* and *E. meliloti*, among others) and other bacteria. We identified a homolog of *ubiF* downstream *LPU83_2531* gene. *ubiF* encodes a hydroxylase of 2-octaprenyl-6-methoxyphenol, an enzyme that catalyzes the sixth step in the biosynthesis of ubiquinones from the precursor chorismate under aerobic conditions [43]. Ubiquinone is required for ATP production through the electron transport chain, and its function is important to maintain proper cell respiration. *ubiF* is well conserved among rhizobial species, but its role in acid tolerance has not been yet investigated. The LPU83 *ubiF* ortholog presented the highest identity with the homologous genes present in *R. etli* CIAT 652 and in *R. leguminosarum* bv. viciae 3841, both bean-nodulating rhizobia. In *R. favelukesii* LPU83, *LPU83_2531* and *ubi*F are present in a single copy in the genome. *ubiF* is the last gene of an operon comprising *LPU83_2531* and three other genes located upstream that are cotranscribed from a single promoter (analysis performed using RNA-seq data from Nilsson et al. [44]. The genes in the operon are *cysG1* that codifies for uroporphyrin III C-methyltransferase protein, *cysI* that codifies for putative sulfite reductase (NADPH) protein from the cysteine biosynthetic pathway, *LPU83_2531* which is an open reading frame interrupted by *Tn5*B20, *LPU83_2533* which is an open reading frame with unknown function and *ubi*F putative hydroxylase of 2-octaprenyl-6-methoxyphenol from the quinone biosynthetic pathway. Due to the well-known role of UbiF in proton transport and to the proximity of the transposon to the 5′ end of *ubiF* gene, we hypothesize that the phenotype observed in *Tn*833 is likely due to the disruption of this gene rather than *LPU83_2531* or the disruption of both genes.

## Mutants in *LPU83_2531* and *ubiF* genes exhibit altered growth kinetics at low pH

In order to evaluate the role of LPU83_2531 and UbiF in acid tolerance, mutants *LPU83_2531⁻* and *ubiF⁻*, respectively, were obtained by site-directed integration, as described in the "Materials and methods". Both mutants displayed similar colony morphology to the WT strain in rich and minimal media. When grown in GS broth at pH 7.0, no significant differences between the WT and *Tn*833 and *LPU83_2531* mutants were observed during the first 30 h, although *ubiF⁻* was considerably affected in this condition (Fig. 2). Incubation at pH 5.0 showed remarkable differences in growth kinetics for all mutants (Fig. 2). Interestingly, the mutants were not able to grow beyond 10^9 CFU/ml, and *LPU83_2531⁻* and *ubiF⁻* were also affected in log phase, being the last one more impacted (Fig. 2). Although cultures were buffered to maintain the initial pH, an increase in the pH of the extracellular medium of about 0.2 to 0.5 pH units was found under both pH conditions.

To complement the mutants with *ubiF* endogenous gene, we tried to clone the open reading frame from *R. favelukesii* LPU83 in different expression vectors. However, several attempts using different *E. coli* vectors failed, suggesting that the expression of *ubiF* from *R. favelukesii* LPU83 is toxic for *E. coli*. Attempts made at cloning *ubiF* in *R. favelukesii* LPU83 resulted in non-viable colonies.
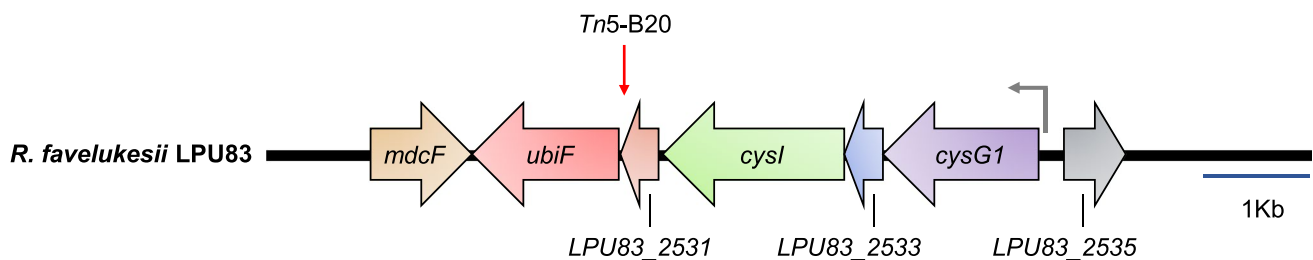
Fig. 1 Genetic organization of the DNA region interrupted by transposon *Tn5*B20 insertion in the *Tn*833 mutant. The red arrow indicates the position where transposon was inserted (genome position 2,469,710), and the grey arrow shows the position of the predicted promoter of the operon containing *LPU883_2531* (ORF1) and *ubiF* (genome position 2,473,610). The genome coordinates are relative to the reference genome of *R. favelukesii* LPU83. The genes in the region nearby are as follows: *mdcF*, malonate transport protein; *ubiF*, putative 2-polyprenyl-6-hydroxyphenol hydroxylase from the quinone biosynthetic pathway (UQ, coenzyme Q); *LPU83_2531*, open reading frame interrupted by *Tn5*B20; *cysI*, putative sulfite reductase (NADPH) protein from the cysteine biosynthetic pathway; *cysG*1, uroporphyrin III C-methyltransferase protein; *LPU83_2535*, unknown function
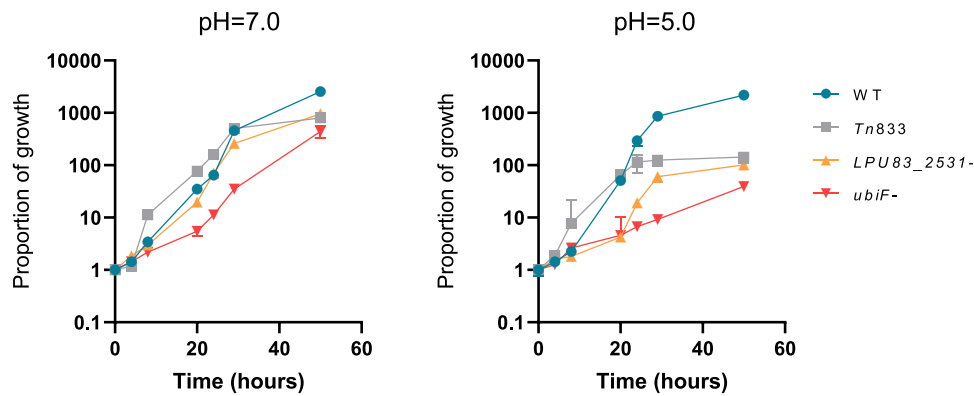
Fig. 2 Disruption of *LPU83_2531/ubiF* impairs growth in acid media. Growth kinetics in neutral (pH = 7.0) or acid (pH = 5.0) GS medium for the WT and mutants are shown. The proportion of growth was calculated as the CFU at each time point relative to time zero. Error bars represent the average of at least two independent experiments. The variations in the pH throughout each experiment were no greater than 0.6 units

LPU83_2531⁻ and *ubiF⁻* mutants are affected in other stress conditions

Rhizobial mutants that express a higher sensitivity to hydrogen ions frequently show a lower tolerance to increased concentrations of heavy metals [45]. Such a common phenotypic pattern strongly suggests the presence of some related, but unknown yet, biochemical alterations in all mutants irrespective of their primary *act* genetic alteration. To investigate the response of the mutants to other stresses, rhizobia were exposed to the presence of heavy metals (100 mM Cu^{2+} and 0.5 mM Zn^{2+}), osmotic stress (0.5 M NaCl or 10% w/v sucrose) and reducing conditions (5 mM ascorbic acid). Though no differences in cell viability between the mutants and the parental strain were observed in the presence of Cu^{2+}, sucrose

or NaCl (data not shown), there were differences in the other conditions. In presence of high concentrations of Zn^{2+}, both mutants and the WT strain were importantly affected (Fig. 3). However, the impact was more severe in the mutants, particularly in *ubiF⁻*, where no CFU were detected after 24 h of incubation. On the other hand, while the WT was able to grow in the presence of ascorbic acid, the mutants were impaired to grow in this condition and CFU decreased dramatically after 24 h, being undetectable in the case of *ubiF⁻* after this time (Fig. 3). These results indicate that *ubiF* or *LPU83_2531/ubiF* genes are involved in tolerance to other stresses beyond acidity. Finally, concerning the effects of physical agents on *ubi* mutants, it is worth mentioning that the photosensitive phenotype described for *ubiF* mutants in *E. coli* [42] was not observed in our LPU83 derivatives.
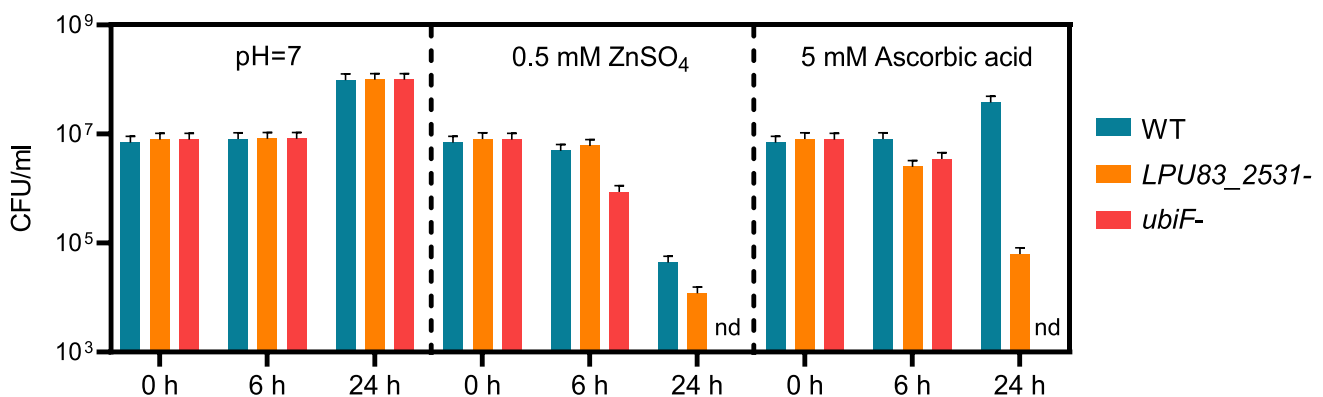
Fig. 3 Lack of *LPU83_2531/ubiF* impacts on the survival of *R. favelukesii* LPU83 in stressing conditions. Survival of *ubiF⁻* and *LPU83_2531.⁻* mutants and the WT strain was evaluated in the presence of high concentrations of $ZnSO_4$ and ascorbic acid. CFU counting in medium at pH = 7.0 was used as a control. Log-phase cultures were diluted to a concentration of 10^7 CFU/ml and incubated at 28 °C and 280 RPM. For each condition, the number of CFU was determined after 6 h or 24 h. nd, not detected

UbiF is important for plant nodulation competitiveness

To determine if the observed decrease in acid tolerance has any effect on the rhizobial ability to associate with alfalfa, the symbiotic properties of the mutants at neutral and acidic pH levels were evaluated. We performed single-inoculation assays using alfalfa plants in a nitrogen-free medium at neutral pH and determined the number of nodules at different time points. We found that both *LPU83_2531⁻* and *ubiF⁻* achieved a similar number of nodules per plant than the WT. However, the nodulation kinetics of *ubiF⁻* were slightly different than the parental strain, showing a delay in the formation of primary roots (Fig. 4). Despite the number of nodules, a more important and desirable feature in soils is the ability to compete with other rhizobia and colonize roots. Thus, to evaluate the competitiveness of the mutants, we performed coinoculation assays infecting alfalfa and common bean plants with a similar CFU number of the WT and each of the mutant strains, as described in the "Materials and methods". Interestingly, we found that *LPU83_2531⁻* and *ubiF⁻* were nearly fully outcompeted by the WT strain in *M. sativa* plants (Fig. 5) and completely outcompeted in *P. vulgaris*, where the entire bacterial recovery from those nodules corresponded to the WT. These results suggest that *ubiF*, and possibly *LPU83_2531*, plays an important role in competition in alfalfa and the common bean.

Discussion

Acid soils are seen as problematic in the matter of plant cultivation as acidity impacts both the plant and the associated rhizobia, having negative consequences in crop yield
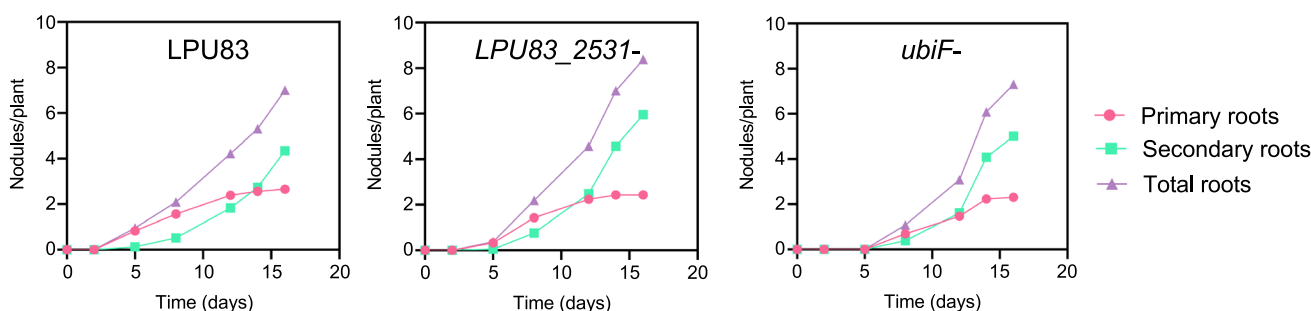
Fig. 4 Alfalfa nodulation kinetics of *R. favelukesii* LPU83 and its derivative strains. The nodules from the principal roots (pink), secondary roots (green) and total roots (purple) were determined at different time points as indicated in the "Materials and methods". The results, expressed as the average number of nodules per plant, are taken from a representative experiment within a set of three. The standard deviations throughout were lower than 20% in all cases

Fig. 5 Loss of *LPU83_2531/ubiF* dramatically reduces the ability to compete with the WT strain. Nodulation competitiveness of *LPU83_2531⁻* (**A**) or *ubiF⁻* (**B**) mutants was evaluated against the *R. favelukesii* LPU83 WT strain in alfalfa plants. The results are presented as the mean value of the percentage of nodules occupied by each strain coinoculated from a 1:1 mixture. The occupancy of each strain in the nodules was determined by differential growth on TY solid plates supplemented with selective antibiotics
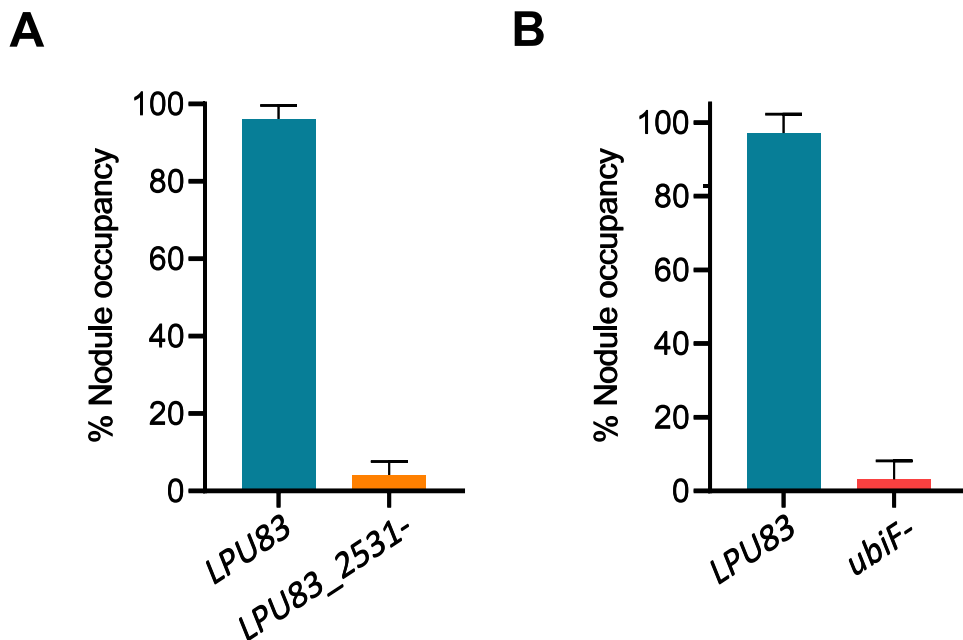
[17]. To address this issue, we and others have directed our research towards the identification of acid-tolerant rhizobia [6, 46–48]. On the other hand, the study of the genes and mechanisms underlying acid tolerance in these bacteria is critical to improve bioinoculants and to have better outcomes. In this regard, here, we performed random transposon mutagenesis on the acid-tolerant strain *R. favelukesii* LPU83 to identify novel determinants of acid tolerance. By screening on solid acid media, we found a mutant, *Tn*833, which was severely affected on its ability to grow at pH 5.0 but subtly disturbed in neutral conditions and further characterized its phenotype.

It is important to note that out of more than three thousand *Tn*5-screened mutants, only one clone appeared to be specifically affected in their growth at low pH without significant changes in the ability to grow in neutral culture media. Mutants that had completely lost their ability to grow under acidic conditions but grew well at neutral pH were not found, reinforcing the concept that tolerance to acidity in rhizobia is a multigenic phenotype that is related to the general bacterial physiology [16, 44, 49]. Although the transposon in *Tn*833 was inserted within the ORF of the unknown function *LPU83_2531*, the acid-tolerant phenotype in the mutant is probably due to the effect on the downstream gene, *ubiF*, cotranscribed with *LPU83_2531* [44]. Since *LPU83_2531⁻* is the upstream gene in a probable operon, the polarity of a plasmid insertion in *LPU83_2531⁻* might decrease the expression of *ubi*F and this proposed inhibition could contribute to the phenotype of the both mutants (Tn833 and *LPU83_2531⁻*). UbiF is part of the aerobic biosynthetic pathway of ubiquinone, a key component of the electron transport chain that plays a key role in energy-generating processes in bacteria [50]. Then, it is reasonable to think that ubiquinone plays a role in acid tolerance, considering its implication in H^+ transfer through the plasma membrane. Disruption of its function could lead to a malfunction of the electron transport chain and an imbalance of protons between the cytosol and the outer membrane space. In agreement with that, it was found that an *ubiF* mutant in *E. coli* was more susceptible to grow under acidic conditions and other stresses [51], supporting our findings.

As an alternative pathway for the biosynthesis of ubiquinone under anaerobic conditions, *E. coli* uses three other hydroxylases along with the oxygen atoms of water [52, 53]. In *Rhizobium*, neither the structure of the ubiquinone biosynthetic route nor the presence or absence of an alternative pathway has been determined. Because of the natural requirement of oxygen in rhizobia, a complete blockage of ubiquinone production should not be possible (only a few of rhizobia may use menaquinone as an electron carrier under symbiosis condition, as this quinone is characteristic of obligate anaerobes). The increased concentrations of hydrogen ions, heavy metals and osmotically active compounds in the extracellular medium are all factors that generate biochemical stress to the bacterial cell via different mechanisms. Proper preservation of the redox balance is crucial in rhizobia to adapt to the extracellular acidity [54]. Considering the phenotype of the mutant described in this work, these alterations are likely a result of primary changes in the biosynthetic route of ubiquinone. Although the quinone pool is often considered a simple group of reduction-equivalent carriers among membrane-protein complexes, these compounds are now clearly known to participate in defence mechanisms against oxidative stress [43] and are critical to preserving the regular activity of multiple cellular processes.

By using a transcriptomic approach, an increased expression of an *ubiF* homolog has been observed in *S. meliloti* in the presence of NO [55]. We show here that the alteration in *ubiF* strongly impacts bacterial behaviour under several stressful conditions and during the interaction with the host plant. Concerning the participation of quinones in the phenomena of tolerance to particular stress situations, a pleiotropic phenotype in *ubiCA* mutants has been observed, in which, for example, the tolerance to temperature is increased: interestingly enough, those mutants are likewise more resistant to phleomycin compounds and linoleic acid [43]. Consistent with our observations, the analysis of *ubiC*A mutants of *E. coli* also showed that these strains were highly sensitive to the action of the reducing agent dithiothreitol, possibly as a consequence of the incapacity of the altered respiratory chain to maintain the redox balance within the periplasm [43]. Interestingly, in our case, *LPU83_2531⁻* and *ubiF⁻* mutants did not show any significant difference in their tolerance to the oxidative stress generated by 400 μM H_2O_2 (data not shown). Such observation weakens the possibility that the low competitiveness for nodulation displayed by the mutants is due to a reduced antioxidant ability necessary to face the peroxides produced by the plant during rhizobial root infection [56–58]. However, it cannot be ruled out that during the development of symbiosis, rhizobia could be exposed to other stresses (i.e. low pH) for which the *ubi* mutants are known to be impaired.

The identification of *ubiF* as a novel *act* gene in the acid-tolerant strain *R. favelukesii* LPU83 presented here is an important contribution towards unravelling the mechanisms underlying acid tolerance in rhizobia. Future experiments should be directed to explore whether the stressing conditions that disturb the nodulation competitiveness of these mutants operate initially in the rhizosphere, subsequently in the rhizoplane, and/or after the bacterium enters the host during infection thread development and also to determine how low pH, Zn^{2+} and ascorbic acid sensitivity could be connected.

Data Availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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