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Genomic analysis of *Sinorhizobium meliloti* LPU63, an acid-tolerant and symbiotically efficient alfalfa-nodulating rhizobia

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The growth and persistence of alfalfa (*Medicago sativa*), a perennial legume capable of producing high yields of high-quality forage, is reduced in moderately acidic soils. The low performance of alfalfa at low pH is due to numerous factors that affect the host plant, their rhizobia, and the symbiotic interaction. *Sinorhizobium meliloti* LPU63 was isolated from acid topsoil (in Argentina) and showed to be a highly competitive and efficient N_2-fixing rhizobium under both neutral and moderately acidic soil conditions. In this study, we obtained a draft of the LPU63 genome sequence using Illumina HiSeq4000. The whole genome phylogenetic analysis confirmed the taxonomic position of LPU63 as a *S. meliloti* strain and the multilocus sequence analysis confirmed that LPU63 is not related to the strains used in Argentina in bioformulations. The genomic analysis showed that beyond the canonical chromosome, pSymA, and pSymB, LPU63 strain has an accessory plasmid that codes for a *rep*ABC origin of replication and a conjugative T4SS, suggesting that this plasmid could be self-transmissible. In addition, the complete denitrification pathway (i.e., the gene clusters *nap, nir, nor*, and *nos*), including *nap*C and *nos*Z, which could be used as an alternative respiration route under hypoxic conditions with moderate N_2O emissions was found. Also, genes associated with plant growth-promoting activities (PGPR) and the degradation of phenylacetic acid (PAA) were identified. LPU63 is a highly melanogenic strain, a property that could enhance its survival under soil conditions, and the genome data showed a particular arrangement of the genes involved in melanin production. The information regarding LPU63 activities compatible with plant-growth promotion phenotypes, together with other characteristics mentioned here (melanin production, potential moderate N_2O emissions), constitute the basis of future experiments toward the rational design of a novel bioinoculant for the environmentally sustainable production of alfalfa.

KEYWORDS

rhizobia, *Sinorhizobium meliloti*, accessory genome, denitrification pathway, PGPR-related genes, symbiosis

1 Introduction

Nitrogen is a crucial element for plant growth and production. Inadequate access to bioavailable nitrogen limits crop productivity and, consequently, food production. Several legume plants can fulfill their demand for nitrogen by establishing a symbiotic association with nitrogen-fixing soil bacteria known as rhizobia. An important goal of nitrogen-fixing symbiosis research is to understand how to increase plant productivity while achieving a parallel drastic reduction of greenhouse gas emissions. However, developing more productive and environmentally sustainable symbiosis is not simply a matter of choosing both best symbionts because symbiotic productivity can be limited by suboptimal soil conditions for their interaction and for nitrogen fixation. For example, soil's acidity significantly reduce the productivity of legumes mainly because of the detrimental effects of hydrogen ions on the plants and their N_2-fixing rhizobial symbionts (Munns, 1970; O'hara et al., 1989). *Sinorhizobium meliloti* and *Sinorhizobium medicae* (the symbionts of *Medicago*, *Melilotus*, and *Trigonella* spp.) are extremely sensitive to low pH (Glenn and Dilworth, 1994), slowing down and even stopping their growth at pH 5.5 or below (Howieson et al., 1992; Reeve et al., 1993). On the basis of empirical observations, the *in vitro* acid tolerance of rhizobia has been considered a positive trait for the bacteria to perform well under acidic conditions in the field (Howieson et al., 1988). Therefore, screening for acid-tolerant isolates and those that colonize or persist in acidic soils gave rise to novel strains with enhanced survival and/or symbiosis in moderately acid conditions (Thornton and Davey, 1984; Richardson and Simpson, 1989; Graham et al., 1994; Del Papa et al., 1999; Segundo et al., 1999; Del Papa et al., 2003).

S. meliloti LPU63 is an autochthonous strain isolated from an acid soil in Argentina (Castelar, Buenos Aires). It has a "mid-acid tolerant" phenotype (able to grow under laboratory conditions at pH 5.6), whereas the majority of strains isolated from this region were shown to be sensitive to acidic pH (not able to grow under laboratory conditions at pH 5.6) (Del Papa et al., 1999). This strain proved to be a competitive and effective nitrogen-fixing rhizobium in neutral and moderately acidic soil conditions (Segundo et al., 1999; Del Papa et al., 2003). Based on these results, strain LPU63 was proposed as an efficient inoculant for use in the cultivation of alfalfa both in acid soils and in optimal edaphic conditions. Although several phenotypic aspects of *S. meliloti* LPU63 have been described in relation to its tolerance of acid stress and symbiotic association with alfalfa (Segundo et al., 1999; Del Papa et al., 2003), the underlying molecular mechanisms that enable acid pH-tolerant inoculants to outperform other strains in acidic soils are still unclear.

Although reducing the use of synthetic nitrogen fertilizers due to biological nitrogen fixation (BNF) is considered a favorable alternative for the environment, the generation of greenhouse gases during alfalfa cultivation has been recognized as a possible negative effect. These gases are generated both by the plant and by the incomplete denitrification process carried out by the rhizobia (Horchani et al., 2011; Ruiz et al., 2022). In oxygen-limited conditions, many bacterial species can switch from O_2 respiration to nitrate respiration (NO_3^-) to produce energy through a process called denitrification (Torres et al., 2018). The complete denitrification pathway comprises the reduction of nitrate (NO_3^-) or nitrite (NO_2^-) to molecular nitrogen (N_2) with formation of the gaseous intermediates nitric oxide (NO) and nitrous oxide (N_2O). NO contributes to the depletion of the ozone layer and N_2O is a potent greenhouse gas (Solomon, 2007). In particular, *S. meliloti* B399, used for more than 50 years as a commercial inoculant in Argentina, produces high levels of N_2O, due to the absence of N_2O reductase genes (*nos*), involved in the conversion of N_2O to N_2 (Brambilla et al., 2018; Brambilla et al., 2019). For these reasons, priority should be given to the selection of inoculant strains efficient in BNF and *nos*$^+$ to mitigate the emission of N_2O in the cultivation of alfalfa (Brambilla et al., 2019). It is unknown whether strain LPU63 codes for the complete denitrification pathway. Recently, it has been shown that the overexpression of the *nap* operon (coding for the enzymes involved in the first step of the denitrification pathway), promotes nodulation when the host plant develops in a hypoxic environment (Pacheco et al., 2023).

In addition to the ability to establish a nitrogen-fixing symbiosis, other metabolic activities of rhizobia generate a beneficial effect on the host plant, called PGPR activities. These activities include the solubilization of phosphates, production of auxins, synthesis of siderophores and enzymatic activities such as chitinase, cellulase, protease, amylase, and pectinase (Paterson et al., 2017; López et al., 2018). The identification of PGPR activities in LPU63 would increase its value as a potential inoculant of alfalfa.

S. meliloti LPU63 was also described as a proficient melanin producing strain (Del Papa et al., 1999). The identification and analysis of the melanin biosynthesis gene cluster were carried out in the *Sinorhizobium meliloti* CA15-1 strain (Chizhevskaya et al., 2018). It was shown that the melanin production did not affect efficiency of symbiosis with *Medicago sativa*, most probably melanin is important at the stages of adaptation of the free-living cells in the environment. These pigments, mostly derived from aromatic amino acids such as tyrosine, are produced by several types of bacteria and contribute to numerous relevant biological roles: protection from UV radiation, oxidants, and free-radicals (Meredith and Sarna, 2006); and chelation of metals (Hong and Simon, 2007); among other roles which ultimately improve the survival of bacteria in stressful conditions (Pavan et al., 2020). In *S. meliloti*, copper-induced melanin production was linked to bacterial resistance to predation by the soil bacterium *Myxococcus xanthus* (Contreras-Moreno et al., 2020). Therefore, production of melanin is other trait that could enhance the survival of rhizobial strains in soil conditions.

To answers these outstanding questions, in this study, we obtained the *S. meliloti* LPU63 draft genome and presented an analysis of relevant genome properties and features. We also analyzed the roles of various genetic determinants in the development of symbiotic relationships with the host plant and in its survival in soil conditions.

2 Materials and methods

2.1 Bacterial strains and growth conditions

The strains used in this study are listed in Table 1. Rhizobial strains were grown at 28°C in tryptone yeast (TY) medium (Beringer, 1974). *Gluconacetobacter diazotrophicus* Pal5 was grown in LGI medium (Cavalcante and Dobereiner, 1988) at 28°C.

2.2 Genomic DNA preparation, sequencing, and assembly

Genomic DNA was extracted using the AccuPrep Genomic DNA Extraction Kit (Bioneer) following the manufacturer's protocol. Insert libraries from *S. meliloti* LPU63 genomic DNA were performed using standard Illumina protocols and further sequenced at SNPsaurus (Eugene, USA) using an Illumina HiSeq 4000 instrument. All raw reads obtained from SNPsaurus were analyzed with FastQC (Andrews, 2010), and a high proportion of Nextera adapters was found. Adapters were removed and reads were quality filtered using Trimmomatic (Bolger et al., 2014) (at Galaxy Australia server). Next, filtered reads were assembled with SPAdes, Velvet Optimizer (Zerbino, 2010), and Unicycler. Medusa (Bosi et al., 2015), using Unicycler assembly as the target, Multi-CSAR (Chen et al., 2018) and CISA (at Orione galaxy portal, http://orione.crs4.it) were used to improve the assembly. A comparison of assembly quality was done with Quast and the Medusa (http://combo.dbe.unifi.it/medusa) refined assembly was selected. Mummer (Delcher et al., 2003) was used to look for artifacts produced during the scaffolding step. Misassembled Medusa scaffolds were manually broken.

2.3 Genome annotation and analysis of the codon usage profiles

The genome was annotated using the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2013). TXSScan was used to search for secretion systems or flagellar genes in the genome of LPU63 at the Galaxy Pasteur server (Abby et al., 2014).

For analysis of the codon usage profiles of *S. meliloti* LPU63 contigs, modal codon usages for different gene sets were calculated as previously described by (Davis and Olsen, 2009) with the details reported by López et al. (2019). Factorial correspondence analysis of Relative Synonymous Codon Usage frequencies (RSCU) of the individual genes and modal codon usage frequencies from *S. meliloti* genomes, *S. meliloti* accessory plasmids, and *S. meliloti* LUP63 contigs was calculated by the use of CodonW software (http://codonw.sourceforge.net/).

TABLE 1 Strains used in this study.

Strain	Source/Reference
Sinorhizobium meliloti LPU63	(Del Papa et al., 1999)
Sinorhizobium meliloti 1021	(Meade et al., 1982)
Sinorhizobium meliloti GR4	(Casadesús and Olivares, 1979)
Sinorhizobium meliloti AK83	(Biondi et al., 2009)
Sinorhizobium meliloti SM11	(Stiens et al., 2006)
Sinorhizobium meliloti BL225C	(Galardini et al., 2011)
Sinorhizobium medicae WSM419	(Howieson and Ewing, 1986)
Rhizobium etli CE3	(Noel et al., 1984)
Gluconacetobacter diazotrophicus Pal5	(Bertalan et al., 2009)

2.4 Taxonomic classification

LPU63 assembly was analyzed with MiGA to identify the most closely related genomes available in the NCBI Genome database (Rodriguez-R et al., 2018). A genome-based taxonomy was performed with TYGS (Meier-Kolthoff and Göker, 2019) comparing LPU63 genome with available genomes of type strains. Next, a whole-genome phylogenetic analysis was performed with EGDAR (Efficient Database framework for comparative Genome Analyses using BLAST score Ratios) (Blom et al., 2009) in order to include non-type strains of rhizobia. Genomes used for the analysis are listed in Table S1. A multilocus sequence analysis (MLSA) was conducted in MEGA X (Kumar et al., 2018) using the housekeeping genes *aap*J, *aap*M, *dna*K, *fcl*, *gyr*B, *hyd*R, *ndv*B and *rho* as described in Brambilla et al. (2019). The concatenated DNA sequences of the housekeeping genes were aligned with ClustalW (Gap Opening Penalty: 15.00; Gap Extension Penalty: 6.66). Next, the evolutionary history was inferred by using a Neighbor-joining phylogenetic tree constructed using the Tamura-Nei model with 1,000 bootstrap replications.

2.5 Pan-genome

To determine the accessory genome of *S. meliloti* LPU63 a pan-genome analysis was done with Roary (Page et al., 2015), using default parameters and the genomes of *S. meliloti* strains selected following the MiGA ANI results (KH46, RMO17, BL225c, AK83, SM11, 2011, HM006, KH35c, WSM1022, CCMM_B554, GR4, and B401; Table S1).

2.6 Identification of phenylacetic acid degradation and denitrification pathway gene clusters

Genes involved in PAA degradation and the denitrification pathway were identified using BLASTp. Gene cluster comparison figures were created with Clinker (Gilchrist and Chooi, 2021). The final figures were edited with Inkscape.

2.7 PGPR activities

Homologous genes involved in plant growth promotion traits were identified by BLASTp as described by Bruto et al. (Bruto et al.,

2014). The solubilization of mineral phosphate was tested on plates containing the National Botanical Research Institute's phosphate growth medium (NBRIP), according to Nautiyal (Nautiyal, 1999). *Gluconacetobacter diazotrophicus* Pal5 was used as a positive control.

2.8 Melanin production

Genes involved in melanin production were identified using BLAST. Melanin production was determined by the method of Cubo et al. (1988), as modified by Zhang et al., (1991). Briefly, bacteria were cultured in TY media supplemented with 40 μg mL^{-1} $CuSO_4$ and 100 μg mL^{-1} of L-tyrosine for 3 days at 28°C, then 10% (w/v) SDS was added, and plates were further incubated for 1 day at room temperature.

2.9 Plasmid profiles

In order to compare bacterial plasmid profiles *in situ* lysis gel assay described by Eckhardt (1978) was used with the modifications described by Martini et al. (2015).

2.10 PCR

The *mco*A gene was amplified using PCR with *Pfu* DNA polymerase (Embiotec, Quilmes, Argentina) and primers *mco*A-Fw (GCACGTTTGGGAGGTTTG) and *mco*A-Rv (CTAGTGATG GCCGGTAAC) designed using *S. meliloti* LPU63 sequence information, *S. meliloti* LPU63 total DNA as template and following standard procedures. The PCR product was purified and sequenced at Macrogen (South Korea).

3 Results and discussion

3.1 General characteristics and genome annotation

In this study, we performed a high-throughput sequencing of the whole genome of *S. meliloti* LPU63. The draft genome sequence and annotation of strain LPU63 is available at GenBank (BioProject accession number: JAFFTL000000000.1; BioSample: SAMN17951857; SRA accession: SRX10118759; Assembly: ASM2410269v1). The sequencing run (2 × 150 bp) resulted in 724,343 paired end reads yielding approximately 93.0 Mb of sequence information. A total of 710,276 paired end reads that passed the quality check were used for the assembly. The G+C content of the assembled genome of strain LPU63 was 62.00% and the size was estimated to be 6,931,924 bp (Table S2). The assembled genome (coverage of 15×) was composed of 160 large contigs (> 1,000 bp). The size of the contig N50 was 107,219 bp. The genome was annotated applying the NCBI Prokaryotic Genome Annotation Pipeline, which predicted 6,374 protein-coding sequences, 50 tRNA genes and 3 rRNA genes (one 5S, one 16S and one 23S) (Table S2).

3.2 Phylogenetic analysis

LPU63 was previously identified as a *S. meliloti* strain based only on 16S rRNA sequencing. To accurately identify its species, a genome-based taxonomy was performed with TYGS, comparing the LPU63 genome with available genomes of type strains. This study showed that LPU63 belongs to the same species cluster as *S. meliloti* NBRC 14782 type strain (deposited as USDA 1002), in agreement with previous results. The dDDH value between the two strains was 87.1% (TYGS formula d_4, equivalent to GGDC formula 2), which is above the cut-off required to assign both strains as the same species (≥70% dDDH). The closest available genome deposited in NCBI was *S. meliloti* KH35c (NZ_CP021825), as identified by MiGA.

Next, we expanded this phylogenetic analysis in order to include non-type strains of rhizobia, to analyze the relatedness of strain LPU63 with previously characterized rhizobia. A whole-genome phylogenetic tree was constructed using EDGAR (Figure 1). Strain LPU63 and *S. meliloti* KH35c were found in a cluster that diverged from the one containing laboratory strains, such as 1021, and commercial strains like B399 and B401, the latter two extensively used in Argentina as alfalfa inoculants. This result suggests that LPU63 is not related to strains previously isolated from Argentina, but it is more closely related to strain KH35c, originally isolated from France.

The clustering of stain LPU63 and KH35c, and their divergence from other *S. meliloti* strains in the whole-genome phylogenetic tree is noteworthy (Figure 1). In a recent screening of 13 *S. meliloti* strains, KH35c was described as one of the most competitive for nodule occupancy against strain BL225C (Bellabarba et al., 2021). Interestingly, *S. meliloti* LPU63 was previously reported as a good competitor for nodule occupancy against the native rhizobia population and even the acid tolerant and highly competitive *Rhizobium favelukesii* LPU83^T strain (Segundo et al., 1999), which is an acid tolerant alfalfa-nodulating rhizobia. Bellabarba et al. (2021) performed a k-mer-based GWAS analysis that linked a 26 kb region present in the KH35c genome putatively associated with its competition capability against BL225C. This region includes genes involved in the synthesis of the redox cofactor F_{420} (*fbi* operon) and several Bra/Liv type ABC transporters involved in uptake of branched-chain amino acids. The presence of F_{420} cofactor is linked to several important processes such as persistence, antibiotic biosynthesis (tetracyclines, lincosamides, and thiopeptides), and prodrug activation, possibly increasing the fitness of these strains (Greening et al., 2016; Ney et al., 2017). The *S. meliloti* genome encodes many ABC uptake and export systems (Greening et al., 2016; Ney et al., 2017), it has been reported an attenuated competitive phenotype in the *liv*M mutant. Considering that *liv*M gene encodes the permease subunit of the Bra/Liv complex, it was then proposed that this complex may provide a significant advantage in the competition, guaranteeing a higher supply of amino acids in rhizospheres' environments and increasing

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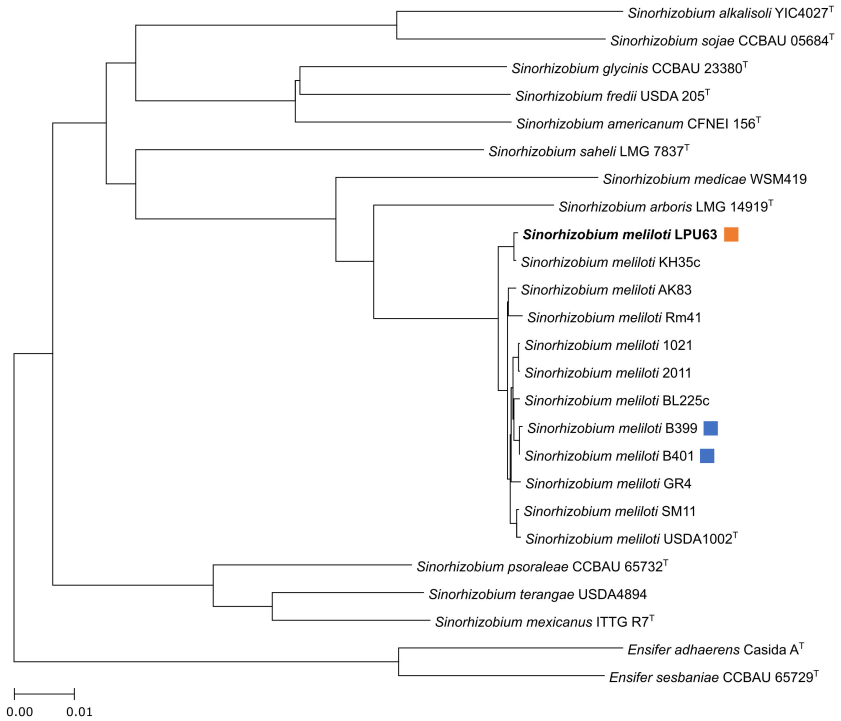

FIGURE 1
Whole genome phylogenetic analysis. Phylogenetic study based on whole genomic sequences of *S. meliloti* LPU63 and related rhizobia performed with EDGAR. Superscript T indicates that strains are type strains. The orange squares indicate the strain analyzed in this work; the light blue squares denote rhizobia that is used for commercial inoculant production in Argentina. *S. meliloti* LPU63 clusters together with *S. meliloti* KH35c.

strain competitiveness (van Dillewijn et al., 2001; Pobigaylo et al., 2008). We also found this 26 kb region present in LPU63, which is highly conserved in content and synteny (Figure S1), pointing to these genes as attractive candidates to study in the competition of rhizobia for the host plant.

Lastly, a MLSA based phylogenetic tree with chromosomal housekeeping genes was constructed in order to compare strain LPU63 with the recently described INTA strains isolated from different eco-regions of Argentina (Brambilla et al., 2019). The genomes of these strains have not been sequenced yet, however, Bambrilla et al., (2019) reported the sequence of several housekeeping genes, that we here use for the MLSA analysis. We included the orthologs from strain LPU63 of these housekeeping genes. This phylogenetic study showed that LPU63 diverges from the INTA-1 to INTA-6 strains (Figure 2). Altogether, these findings clearly indicate that strain LPU63 is not a re-isolation of a previously described *S. meliloti* strain.

3.3 Genome overview: identification of replicons, syntenic regions, core and pan-genome

The *S. meliloti* 1021 reference genome has a chromosome and two megaplasmids, pSymA and the pSymB chromid, but no other small accessory plasmids (Galibert et al., 2001). However, several *S. meliloti* strains carry smaller accessory plasmids (also called cryptic plasmids), that code for genes implicated in adaptation to the specific

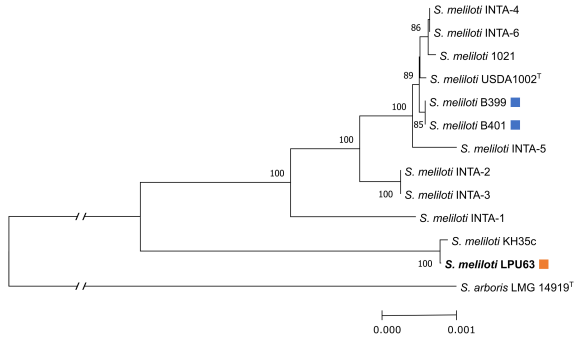

FIGURE 2
MLSA phylogenetic analysis. Phylogenetic study based on the DNA sequence of 8 chromosomal housekeeping genes from several *S. meliloti* strains. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei, 1993) and are in the units of the number of base substitutions per site. This analysis involved 13 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 18017 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018). Superscript T indicates that strains are type strains. The orange squares indicate the strain analyzed in this work; the light blue squares denote rhizobia that is used for commercial inoculant production in Argentina. *S. meliloti* LPU63 diverges from other strains isolated from Argentina (INTA1 to INTA6, B399, and B401).

niche they occupy, some of which have been shown to affect nodulation and metabolic potential (Crook et al., 2012; Mazur and Koper, 2012). This is the case of the accessory plasmid pSmeSM11a, that among other genes codes for *acd*S, suggested to modulate the levels of the phytohormone ethylene and potentially enhancing the nodulation capacity of the rhizobia (Stiens et al., 2006). Cryptic plasmids can also attenuate nitrogen fixation in alfalfa, as it is the case for *S. meliloti* SAF22 carrying the plasmid pRmSAF22c (Velazquez et al., 1995). Accessory plasmids are generally thought to be transient components (easily gained or lost) from the species pan-genome.

LPU63 contigs were mapped to the corresponding replicon of *S. meliloti* KH35c, the closest available strain with a complete sequenced genome (Table S3). Most of the contigs were related to the chromosome or the pSymA and pSymB megaplasmids. Also, several contigs mapped to the accessory plasmid A of strain KH35c (Table S3). This result is consistent with the plasmid profile shown in Figure 3, which indicates that LPU63 carries an accessory plasmid of approximately 200 kb. This accessory plasmid was named pLPU63a.

Considering that LPU63 was isolated from acidic soil, it is tempting to speculate that genes coded in this accessory plasmid could provide functions to overcome this specific abiotic stress. Therefore, we tried to reconstruct the sequence of this accessory plasmid with cBar and PlasFlow. However, the results were unreliable, due to the presence of sequencing reads from the pSymA and pSymB megaplasmids. This shows that the assembly of the accessory plasmid with these methods is not possible as they are not able to distinguish the reads belonging to the megaplasmids or the cryptic plasmid. Therefore, we designed a strategy to look for which contigs potentially belong to the LPU63 accessory plasmid. All contigs were first used in a BLASTn search against sequences of accessory plasmids isolated from several *S. meliloti* strains (López et al., 2019). Contigs that showed a query coverage larger than 5 kb were selected. Next, considering that samples of purified cryptic plasmid are usually contaminated with pSym or chromosomal DNA, we discarded the contigs that mapped to these replicons of *S. meliloti* KH35c. Contigs that passed this test and putatively belong to plasmid pLPU63a are shown in Table S4. Genes that code for a conjugative T4SS, plasmid replication and a module of the *rep*ABC partitioning system were identified in the pLPU63a putative contigs suggesting that this plasmid could be self-transmissible (Table S5). These results should be interpreted with caution, as our analysis are unable to distinguish whether these contigs belong to only one or more than one accessory plasmid of the strain LPU63, since it is challenging to reconstruct plasmids from short read sequencing.

In addition, the BLAST analysis of gene content of the contigs putatively assigned to pLPU63a indicated that more genes found on the accessory genome were found only on other *Sinorhizobium* criptic plasmids or pSymA, rather than pSymB or the chromosome. Nelson et al. (2018) suggests that the accessory plasmids and pSymA have greater ability to exchange genetic elements than the other replicons. Whereas the pSymA is enriched in genes associated with signal transduction (COG-T) and energy production (COG-C) compared to pLPU63a, the latter is enriched in genes promoting trafficking and transport (COG-U).

Previous studies have shown that cryptic plasmids of *S. meliloti* have different codon usage compared to the chromosome or the pSyms (Lopez et al., 2019). To confirm the designation of LPU63 contigs to the accessory plasmid, we performed a correspondence analysis of RSCU. We included all the KH35c replicons (chromosome, pSyms, and accessory plasmid), all accessory plasmids of *S. meliloti* available on the NCBI database, and LPU63's contigs. As shown in Figure 4, LPU63

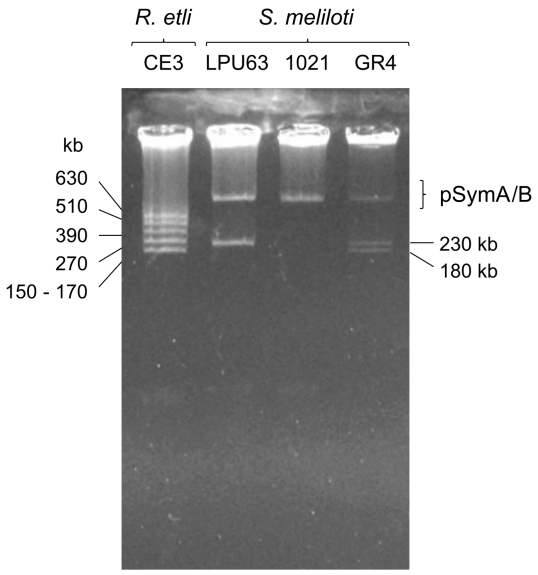

FIGURE 3
Plasmid profiles obtained in gels of *in situ* lysis. *S. meliloti* LPU63 carries an accessory plasmid of approximately 200 kb. Plasmid sizes were estimated from *R. etli* CE3 which carries plasmids of known sizes. *S. meliloti* GR4 harbors two accessory plasmid of sizes 176 kb and 226 kb. *S. meliloti* 1021 does not have any accessory plasmids.

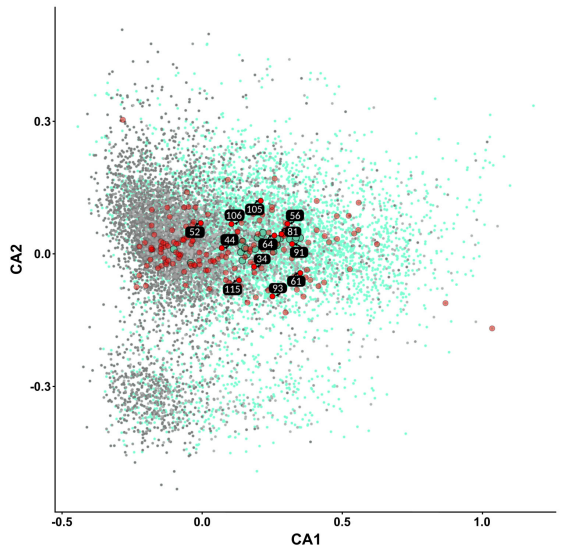

FIGURE 4
Analysis of the codon usage profiles of *S. meliloti* LPU63 contigs. Correspondence analysis of the Relative Synonymous Codon Usage (RSCU) frequencies of genes from *S. meliloti* KH35c genome, *S. meliloti* accessory plasmids and *S. meliloti* LPU63 contigs. KH35c chromosomal and pSyms genes (gray), *S. meliloti* accessory plasmid genes (turquoise), and LPU63 contigs (red). Small dots represent the RSCU of individual genes. Big dots represent modal codon usage frequencies.

contigs (big red dots) assigned as accessory plasmid had a modal codon usage that clustered together with other cryptic plasmids of *S. meliloti* (big turquoise dots).

Finally, to determine the accessory genome of *S. meliloti* LPU63 a pan-genome analysis was done using the genomes of *S. meliloti* strains listed in Table S1. The pangenome is a collection of all DNA sequences of a species that contains sequences shared by all individuals (core genome) and is also able to display sequence information unique to each individual (variable genome). LPU63 singletons (unique, exclusive genes) found are listed on Table S6.

3.4 Analysis of symbiosis-related and nitrogen fixing proteins of LPU63

LPU63 genes involved in nodulation and nitrogen fixation were identified by BLAST search using the already characterized *S. meliloti* 1021 genes. Nod factor biosynthesis genes (*nod, noe*, and *nol*) are involved in the synthesis of hosts specific lipochitooligosaccharides which are indispensable for symbiosis initiation. All the fundamental genes involved in the synthesis of the Nod factor and genes that codified enzymes involved in nitrogen fixation and their corresponding regulators were identified (Table S7 and Table S8). Most of the genes were mapped to the putative pSymA replicon in strain LPU63. This result is consistent with the location of the symbiotic-related genes directly involved in symbiotic nitrogen fixation (such as the *nod*, *nif* and *fix* genes) to the pSymA megaplasmid in other *S. meliloti* strains. By examining the genomic data of *S. meliloti* strains 1021 and LPU63, we can locate these Nod factor biosynthesis genes (*nod*M, *nol*FG, *nod*N, *nod*D1ABCIJ, *nod*QPGEFH, *syr*M, *nod*D3, *noe*BA, *nod*L) into their correct order and they present a syntenic organization. The conservation of blocks of order within these two chromosomes is one of the most reliable criteria for establishing the orthology of genomic regions and can reflect important functional relationships between genes. As expected, also LPU63 contain orthologs of the key nitrogen fixation genes (*nif*H, *nif*D, *nif*K, *nif*A, *nif*BEN, *fix*ABCX, *fix*NOPQ, *fix*LJ, *fix*K, *fix*GHIS, and *fdx*N), mainly co-located on megaplasmid pSymA, with the Nod factor biosynthesis gene clusters. As shown in Table S7 and Table S8, Nod and nitrogen fixation proteins of LPU63 present more than 96% and 92% sequence identity respectively with their homologous. The exception with lower protein identity are NodQ2 and FixT2 (57.4% and 76.5% protein identity, respectively).

Other genes putatively involved in competition or interaction with their host plant, such as genes involved in secretion systems and flagella, were identified with TXSScan (Table S9). Several chromosomally encoded flagellum genes were found in LPU63. In *S. meliloti* flagellum motility has been associated with competitiveness for nodule occupation (Bernabéu-Roda et al., 2015). Several T1SS coding genes were identified in the chromosome and in pSymB. We also found putative conjugative bacterial type IV secretion systems (T4SSs), one located in pSymA and another in pLPU63a.The T4SSs are the specific nano machine for bacterial horizontal gene transfer. These devices mediate the dissemination of genes *via* horizontal gene transfer and contribute to the bacteria's evolution. In addition, Nelson et al. (2017) showed that *Sinorhizobium* uses a T4SS during the initiation of symbiosis with *Medicago* spp. and alters *Medicago* cells in planta during symbiosis. Previous studies have shown that the *S. meliloti* 1021 megaplasmid pSymA is self-transmissible (Pérez-Mendoza et al., 2005). Therefore, the pSymA and pLPU63a of LPU63 could be also conjugated *via* a T4SS.

Two putative Tight adherence (Tad) gene clusters were identified in the genome of LPU63, one encoded in the chromosome and the other on pSymA. *S. meliloti* 1021 also contains two gene clusters coding for Tad: *flp*-1, which is chromosomally-encoded, and *flp*-2, located on the pSymA megaplasmid (Zatakia et al., 2014). These gene clusters are homologous to the Tad loci present in *Caulobacter crescentus* (Skerker and Shapiro, 2000) and *Aggregatibacter actinomycetemcomitans* (Kachlany et al., 2000). Flp-1 was found to be important for the plant nodulation of *S. meliloti* 1021, indicating that this pilus might enhance the attachment of the bacteria to plant roots (Zatakia et al., 2014). The reason for *S. meliloti* to possess two distinct Tad loci is still unclear, but the maintenance of the *flp*-2 locus on pSymA suggests a specific role during the free-living or symbiotic state. Further studies are required to understand the role of *flp*-2 in the early stages of the symbiotic process.

3.5 Genes involved in acid tolerance

The study of rhizobial strains under acidic conditions showed that acid-tolerant related traits are multigenic or strain-dependant (Glenn et al., 1999; Draghi et al., 2016). As a first step to try to unravel the molecular determinants involved in the acidic tolerance of strain LPU63, we searched for orthologs of genes already characterized in other rhizobia that showed to play a role in the resistance to low pH or that were upregulated in this condition (Table S10). LPU63 codes for several genes putatively involved in acid tolerance, such as the two-component systems (TCS) ActSR (Tiwari et al., 1996) and ActJK (Albicoro et al., 2021; Albicoro et al., 2023). Most of the genes found to be important for acid tolerance in *Rhizobium* strains had orthologs in *S. meliloti* LPU63.

3.6 Denitrification pathway

Rhizobia can use nitrate as the final electron acceptor, in a process known as denitrification. Although this metabolic pathway is crucial for the survival of rhizobia in anoxic environments, it is considered one of the main drawbacks in the use of these bacteria as inoculants, because this process involves the generation of greenhouse gases. Particularly, the use of *S. meliloti* strains that have the *nap-nir-nor* gene cluster, which can start the denitrification process, but lack the *nos* gene cluster (involved in the last step of this pathway, converting the greenhouse N_2O into the inert N_2) is not desirable. This is the case of the *S. meliloti* B399 strain, extensively used for decades in Argentina as alfalfa inoculant, which has recently been shown to produce high levels of N_2O during

symbiosis (Brambilla et al., 2020). We were therefore interested in determining the integrity of the denitrification pathway in strain LPU63. Our results show that LPU63 codes the complete denitrification pathway, in an almost identical gene arrangement as the reference strain 1021 (Figure 5, Table S11). The genome sequence revealed the presence of the *nap*EFDABC, *nir*K, *nor*ECBQD and *nos*RZDFYLX denitrification genes (Torres et al., 2014). As such, it is possible to speculate that the N_2O emission of this strain could be comparable to the levels of strain 1021, which are significantly lower than strain B399 (Brambilla et al., 2020).

Alfalfa root nodules produce N_2O and Nap has an important role on its release from nodules in response to nitrate and flooding (Pacheco et al., 2023). Recently, a novel strategy to avoid these undesirable metabolic pathways was the screening of *S. meliloti* strains with spontaneous mutations in *nap*C, which encodes the nitrate reductase structural protein NapC involved in the first step of N_2O production *via* the denitrification process. These *nap*C minus strains are consequently unable to start the denitrification pathway (Brambilla et al., 2020). While these strains are regarded as eco-friendly, due to the extremely low N_2O production in the symbiosis with alfalfa, it remains to be proven whether they are able to survive in field conditions, especially when O_2 is limited.

Altogether, strain LPU63 could be regarded as a compromise solution, emitting low N_2O levels but capable, under low O_2 environments, of utilizing nitrate or nitrate as final electron acceptors, a useful trait for the survival of this bacterium in adverse soil conditions.

3.7 Phenylacetic acid degradation pathway

For the aerobic degradation route of phenylacetic acid (PAA), in a first stage, PAA is activated to PAA-CoA in the presence of ATP and Mg^{2+} by a CoA-ligase. Subsequently, it was postulated that the enzymatic complex composed of the proteins encoded in the *paa*ABCDE operon would catalyze the hydroxylation of the aromatic ring of PAA-CoA. This complex would give rise to a non-aromatic product that is subsequently metabolized in a complex sequence of reactions.

Comparative genomic studies of numerous bacterial species involved in the nodulation of legume plants have positioned the *paa*ABCDE cluster as a possible molecular determinant of the interaction between plants and rhizobia (Seshadri et al., 2015). This cluster belongs to a group of genes involved in the degradation of PAA. Therefore, the presence of the catabolic cluster could promote some adaptive advantages for the rhizobium. PAA is the auxin produced in greatest quantity by many plant species (Cook, 2019), and has been detected in root exudates in different plants, including legumes (Rovira, 1969). Thus, the available PAA levels imply the possible use of this aromatic compound as an alternative carbon source for rhizobia, consequently comprising an adaptive advantage in the colonization of the rhizosphere. Alternatively, the metabolism of PAA by rhizobia could intervene in the modulation of responses from the host plant. In addition, PAA is one of the degradation products of plant-derived flavonoids by *S. meliloti* (Rao and Cooper, 1994) and the expression of the *paa*ABCDE gene cluster was found to be increased in nodule bacteria (Barnett et al., 2004). *S. meliloti* PAA degradation genes were also found to be induced by oxidative stress (Lehman et al., 2018) and by the entry into stationary phase (Sauviac et al., 2007). PAA catabolism ultimately serves as a carbon source that provides intermediates for the TCA cycle.

The presence of *paa* genes in the strain LPU63, including the genes that code for catalytic enzymes involved in PAA degradation, transport, and transcriptional regulation, is currently unknown. Due to the emerging role of this pathway in rhizobia-legume interactions, we performed a BLAStp search in the LPU63 genome and related rhizobia for gene homologues involved in PAA metabolism previously characterized in *Escherichia coli* (Table S12). As shown in Figure 6, the analysed rhizobia have a conserved gene cluster for the degradation of PAA. Our analysis also indicate similarity at protein level and the presence of syntheny between the *paa*-cluster found in LPU63 and WSM419, despite them belonging to different *Sinorhizobium* species. Particularly, *S. meliloti* and *S. medicae* have genes that code for the PaaABCDE, PaaG, and PaaZ proteins, involved in the enzymatic steps required for the hydroxylation and rupture of the aromatic ring present in PAA, a crucial step for the degradation of this molecule. These

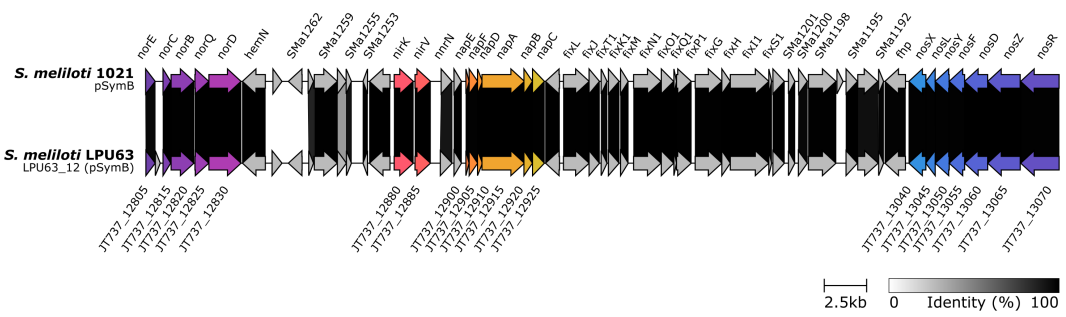

FIGURE 5
S. meliloti LPU63 genome codes for the complete denitrification pathway. Synteny plot of the genomic region coding for the denitrification pathway (*nor, nir, nap* and *nos* gene clusters) performed with Clinker.

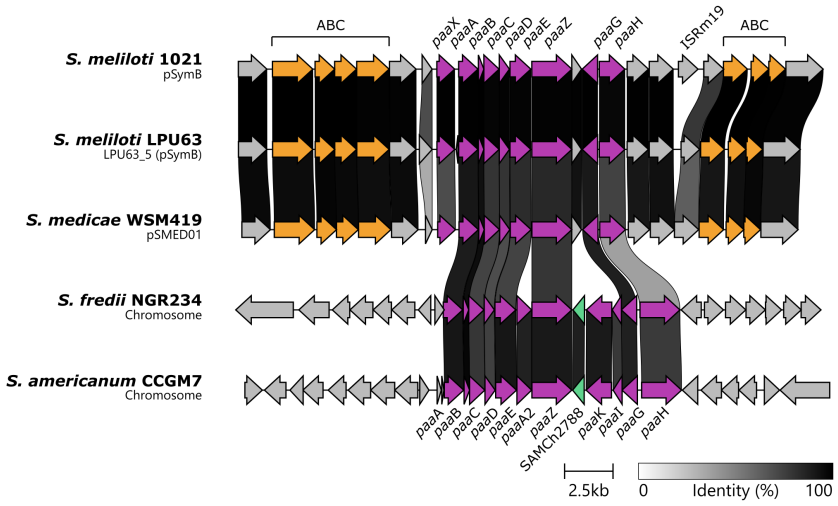

FIGURE 6
Conservation of the PAA catabolic gene cluster in rhizobia. Synteny plot of the PAA degradation gene cluster (violet arrows) of several rhizobia performed with Clinker. Two ABC transporter gene clusters putatively involved in PAA transport are depicted with orange arrows.

strains also code for a homologue of the *paa*X gene, a transcriptional repressor that responds to intracellular PAA-CoA levels, and two ABC transporters that could be involved in the uptake of PAA. *S. fredi* and *S. americanum* have a more complete PAA degradation gene cluster, including PaaK, which catabolizes the first step in this pathway by generating the PAA-CoA intermediate, and PaaI. Interestingly, in these strains *paa*X seems to have been replaced by a different transcriptional regulator (SAMCCGM7_Ch2788 in *S. americanum* CCGM7).

The identification of a clear *paa*K homolog remains elusive in the *S. meliloti* strains analyzed. In this species, either there is another protein that catabolizes the CoA activation of PAA, or this step is not required. However, the conservation of the PAA catabolic gene cluster and its respective expression in *S. meliloti* implies that it confers some adaptive advantage on the rhizobium, even in the absence of *paa*K.

The ability to degrade PAA is emerging as a relevant feature in host-bacteria interactions. For example, the PAA degradation gene cluster is the largest region acquired by horizontal gene transfer in the *Mycobacterium abscessus* genome (Ripoll et al., 2009). PAA degradation was found involved in the pathogenicity of *Burkholderia cenocepacia* (Law et al., 2008). Both *M. abscessus* and *B. cenocepacia* are opportunistic pathogens in cystic fibrosis patients. To our knowledge the functional role of PAA degradation in the rhizobia-legume symbiosis has not yet been investigated.

3.8 Melanin production

S. meliloti LPU63 was described as a melanin producing strain (Del Papa et al., 1999). The production of melanin is involved in overcoming abiotic and biotic stress, a trait that could enhance the survival of this strain in soil. In rhizobia, the synthesis of melanin is linked to two genes, the *mco*A (encode a multicopper oxidase) and *mep*A (encode a melanin production protein with tyrosinase activity)(Mercado-Blanco et al., 1993; Chizhevskaya et al., 2018). In our genomic sequencing of LPU63, the 5' end of *mco*A was found at the end of contig LPU63_25 and the 3' end at the beginning of contig LPU63_173. We designed primers to PCR-amplify the complete *mco*A gene and the amplicon sequencing confirmed the proposed junction of contigs LPU63_25 with LPU63_173. Interestingly, in strain LPU63 there is a frameshift in *mco*A producing two separate ORFs, designed as *mco*A1 and *mco*A2 (Figure 7A) which may be functional because melanin production is observed in strain LPU63 (Figure 7B). The same genetic arrangement is found in *S. meliloti* HM006.

The production of melanin by LPU63 was compared with other previously reported melanin producing strains with an intact *mco*A gene (such as *S. meliloti* GR4) (Mercado-Blanco et al., 1993; Castro-Sowinski et al., 2002) following the protocols previously described. The medium was supplemented with $CuSO_4$ and L-tyrosine to attain the maximum melanin yield because previous reports had shown that absence of the tyrosinase cofactor Cu in culture medium, results in the synthesis of inactive tyrosinase (Cabrera-Valladares et al., 2006). LPU63 produced high amounts of melanin in the presence of $CuSO_4$ and L-tyrosine, with levels similar to those of strain BL225C and SM11 (Figure 7B). Thus, the split of *mco*A into two different genes does not seem to have a detrimental effect on melanin production. The proficient melanin production by strains LPU63, BL225C and SM11 could be due to a high level of *mco*A and *mep*A transcripts or to the presence of additional genes involved in melanin synthesis. The genomic comparisons revealed that, in rhizobia, two hypothetical proteins are conserved in the *mco*A-*mep*A region, suggesting a putative role in melanin production.

Other than their biological role and, due to their particular chemical structure, microbial melanins are used in diverse industrial applications such as: X-ray, γ-ray and UV absorption; semiconduction; cation exchange; bioremediation, among other applications (Singh et al., 2021). Interestingly, LPU63 produced

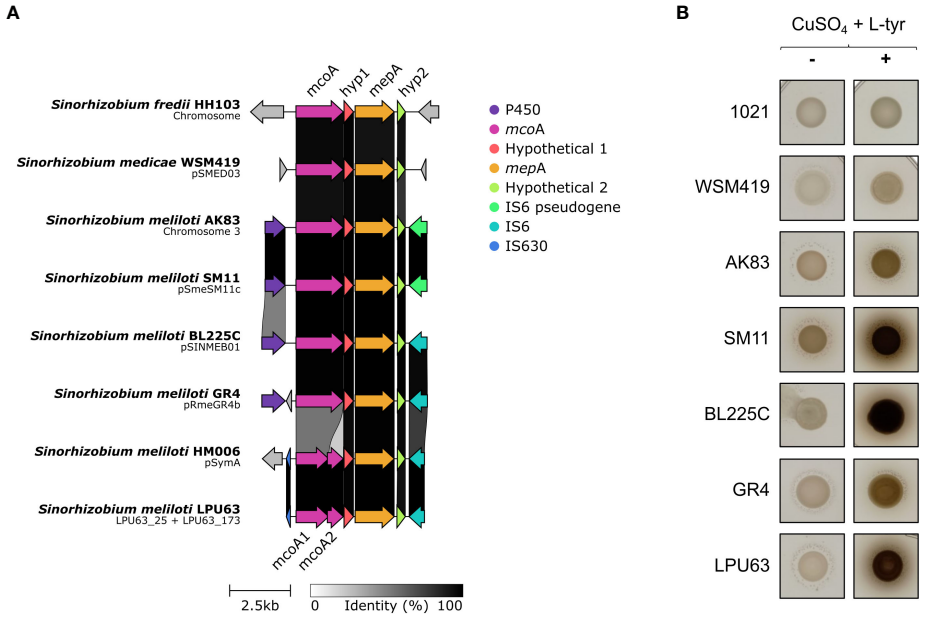

FIGURE 7
Melanin production by *S. meliloti* LPU63 and related rhizobia. **(A)** Analysis of the gene cluster involved in melanin production. In strains LPU63 and HM006 the *mco*A gene is split in two genes by a frameshift mutation. Two genes coding for hypothetic proteins (Hyp1 and Hyp2) are highly conserved in rhizobia. **(B)** Melanin production was evidenced by culturing strains in TY media supplemented with 40 μg mL^{-1} $CuSO_4$ and 100 μg mL^{-1} L-tyrosine for 3 days at 28°C and then 10% (w/v) SDS was added. Positive strains show a dark brown pigment.

melanin even in a medium with $CuSO_4$ as low as 4 μg mL^{-1} in the absence of L-tyrosine, suggesting that this strain could be used in the cost-effective industrial production of melanin.

3.9 Plant growth promoting rhizobacteria activities

In addition to nitrogen fixation, the presence of additional bacterial activities that promote plant growth present in LPU63 could enhance its value as a potential bioinoculant of alfalfa. Therefore, we searched for PGPR coding genes following the method of Bruto et al. (Bruto et al., 2014).

Solubilizing bacteria convert unavailable forms of P (both inorganic and organic) into available nutrients that can be absorbed. The phosphate solubilisation process helps meet the P requirements of plants. Soil-dwelling bacteria solubilize mineral phosphates by secreting gluconic acid. This compound is produced from glucose by a periplasmic glucose dehydrogenase (GDH) that uses pyrroloquinoline quinone (PQQ) as a redox coenzyme. We found that LPU63 codes for the *pqq*BCDE gene cluster necessary for the PQQ cofactor biosynthesis, involved in phosphate solubilization. Then, we confirmed the *in vitro* phosphate solubilization capacity of strain LPU63 (Figure 8), which shows a clear halo around the colony in NBRIP media, as the *G. diazotrophicus* Pal5 positive control and *S. meliloti* AK83 used as the reference strain for the *pqq* gene cluster identification.

Some soil bacteria produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which promotes plant growth by sequestering and cleaving plant-generated ACC, thereby reducing the level of ethylene in the plant. This allows the plant to be more resistant to a wide variety of environmental stresses. In addition, *acd*S was detected in LPU63 strain (Table 2). These PGPR activities are consistent with those found in other *S. meliloti* strains (Bruto et al., 2014). Interestingly, it was recently reported that the transfer of an ACC deaminase gene from *S. medicae* WSM419 to *S. meliloti* 1021 increases nodulation and improves plant growth in the symbiosis with *Medicago truncatula* (Ghosh et al., 2021). In this study, it was also shown the expression of *acd*S in symbiotic and in free-living bacteria confer improvements to nodule formation and symbiotic productivity. The above results indicate the relevance and potential benefits of the *acd*S in LPU63 strain.

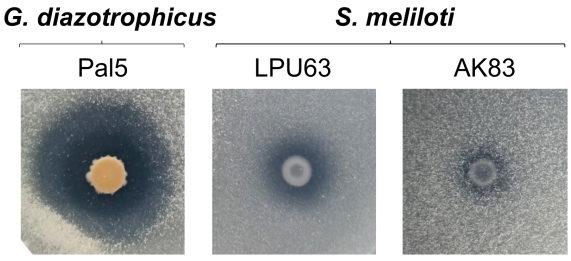

FIGURE 8
Mineral phosphate solubilization. Solubilization of mineral phosphate was tested on plates containing NBRIP growth medium. *Gluconacetobacter diazotrophicus* Pal5 was used as a positive control. The presence of a transparent halo is considered as a positive test. *S. meliloti* LPU63 and AK83 (*pqq* gene cluster reference strain) solubilize mineral phosphate.

TABLE 2 PGPR-related genes identified in *S. meliloti* LPU63.

Gene	Function	*S. meliloti* reference strain	Accession number	LPU63 locus tag	Coverage	E-Value	% ID
*pqq*B	Phosphate solubilization	AK83	Sinme_3967	JT737_01840	100%	0	99.34%
*pqq*C		AK83	Sinme_3966	JT737_01835	99%	0	100%
*pqq*D		AK83	Sinme_3965	JT737_01830	100%	1.00E-65	100%
*pqq*E		AK83	Sinme_3964	JT737_01825	100%	0	99.73%
*acd*S	ACC desaminase	AK83	Sinme_5642	JT737_29415	100%	0	98.23%
*nif*D	Nitrogen fixation	1021	SMa0827	JT737_29980	100%	0	100%
*nif*H		1021	SMa0825	JT737_29975	100%	0	100%
*nif*K		1021	SMa0829	JT737_29985	100%	0	99.61%

We also searched for genes homologs to *phl*ABCD (required for 2,4-diacetylphloroglucinol synthesis), *idp*C (required for auxin synthesis) and *bud*ABC (required for acetoine/2,3-butanediol synthesis). None of these PGPR-related genes were found. A study based on ancestral character reconstruction has proposed that PGPR-plant cooperation may have been established separately in various taxa, yielding PGPR strains that use different gene assortments (Bruto et al., 2014). According to our study, LPU63 strain (like many PGPR strains) is multifunctional, i.e., it harbors more than one phyto-beneficial trait, then all PGPR activities detected make strain LPU63 an even more attractive candidate for use as an alfalfa inoculant.

4 Conclusion

Inoculant selection should be viewed as a continuous process, that complements the choice and implementation of new plant cultivars, as well as to changes in the cropping areas, in the soil and in environmental conditions. The genetic stability of the bacterial strains should also be considered since long-term storage could involve genotypic and phenotypic changes that could alter the rhizobium´s capacity to fix nitrogen, among other relevant characteristics.

This work presents an in-depth analysis of the genome of an economically and eco-friendly promising strain LPU63. The genome of LPU63 is represented by a chromosome, two megaplasmids, and also has an accessory plasmid, pLPU63a. This potentially conjugative replicon contained *tra*A, the T4SS gene cluster, and type IV coupling protein. Our analyses based on ANIb and core phylogenetic trees supported the taxonomy of the strain LPU63 as *S. meliloti*.

In addition to the previously reported outstanding competitiveness, persistence and efficiency of strain LPU63 in acidic and neutral conditions, the information regarding LPU63 activities compatible with plant-growth promotion phenotypes, together with other characteristics examined here (presence of *paa* cluster, high-level production of melanin and the presence of the complete denitrification pathway that could be related with moderate N_2O emissions), constitute the basis of future experiments tending toward the rational design of a novel bioinoculant for environmentally sustainable alfalfa production. This could impact by increasing the stability of forage production and could lead to an increase in the extension of the cultivation area.

The detailed genome annotation of strain LPU63 provides a rewarding resource, allowing the use of comparative genomics as a powerful tool to figure out the genetic mechanisms that could contribute to the phenotype of this strain and sustain the rhizobium–*Medicago* symbiotic interaction.

Further analysis of the genomic features in beneficial rhizobia could clarify how some bacterial strains establish a highly efficient symbiosis or present superior performance in certain edaphic environments.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

The authors confirm their contribution to the paper as follows: Study conception and design: MD. Methodology: JC, MS, CV, SL. Software and formal analysis: JC, ML and AL. Investigation: JC, MS, CV, WD. Data curation: ML. Writing and original draft preparation: JC and MD. All authors contributed to the discussion, provided comments on the manuscript, and approved the submitted manuscript version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2023.1175524/full#supplementary-material

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