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Ensifer meliloti elite strain U143 used as alfalfa inoculant in Uruguay: Characterization and draft genome sequence

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ABSTRACT

Ensifer (syn. *Sinorhizobium) meliloti* U143 is an effective nitrogen-fixing strain isolated from Uruguayan soils. For decades, this strain has been used as an inoculant for different alfalfa cultivars. Here we report for the first time a characterization of the U143 elite strain that includes the preliminary genomic sequence, its annotation, and physiological parameters related to its symbiotic efficiency and nitrate respiration capacity. Through Illumina sequencing, the genome of the U143 strain was sequenced. The genome length was 6,801,966 bp, and it contained two megaplasmids, an average GC content of 62.15 %, and 6522 protein-coding sequences. In the symbiotic plasmid, we identified *nap*, *nir*, *nor,* and *nos* sequences that explain the ability of the U143 strain to respire NO_3^- in free-living and microaerobic conditions. Field assays performed in two locations for two years showed that alfalfa inoculated with the U143 strain produced 41 % more total shoot dry matter than the non-inoculated control, and between 61.3 % and 66.5 % of shoot N in alfalfa inoculated with strain U143 derived from nitrogen fixation.

1. Introduction

The rhizobia associated with legumes perform biological nitrogen fixation (BNF) which helps to maintain pasture productivity and reduce the need for nitrogenous fertilizers, thereby avoiding the negative effects of fertilizer use (Iannetta et al., 2016). For these reasons, the interest has been renewed in using inoculants for rhizobia-legume symbiosis in food and forage crops (Laranjo et al., 2014). Among forage legumes, *Medicago sativa* (alfalfa), in symbiosis with rhizobia of the *Ensifer* genus, can fix 210–350 kg of N ha^{-1}. $year^{-1}$ depending on the rhizobial strains and cultivars (Carlsson and Huss-Danell, 2003; Wang et al., 2018).

The genus *Ensifer* (syn. *Sinorhizobium*), alpha-proteobacteria belonging to the Rhizobiaceae family, includes different species of gram-negative motile bacilli (Diagne et al., 2017; Le Quéré et al., 2017). The *Ensifer* group includes free-living species as well as legume roots associated with nitrogen-fixing bacteria (Fagorzi et al., 2020), mainly from *Medicago, Melilotus,* and *Trigonella* genera (Galardini et al., 2013).

In Uruguay, a total of 125,000 ha are dedicated to cultivating alfalfa, with an annual planting rate of 25,000 ha (DIEA-MGAP, 2022) primarily on slightly acid soils with a pH of 5.5–5.7 (Tabares-da Rosa et al., 2019). The registered information on the inoculants use for this legume in Uruguay allows us to identify three periods. The inoculation history of alfalfa includes the use of the U45 strain (1964–1990), a mixture of U137 and U143 strains (1991–2003), and the current use of the U143 strain (2004-present) as the only commercial inoculant (Altier et al., 2013). Nevertheless, there is no record of the precise location where the U143 strain was isolated (Fabiano et al., 2023).

In acidic soils, alfalfa inoculated with the U143 strain showed improvements of 50 % in biomass production (Rebuffo et al., 2000). This symbiotic pair produced 240 % more biomass in the first harvest, and 80 % more than the total biomass produced in the first year, with 70 % of plant N coming from biological nitrogen fixation (Racca et al., 2013). In this scenario, alfalfa inoculation allows crop stability and productivity improvement in acidic soils, in which Oregon-type parasitic and competitive strains in alfalfa have recently been identified (Berais-Rubio et al., 2022).

This study describes physiological characteristics and genome sequence annotation of *E. meliloti* U143 elite strain, the only commercial

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alfalfa inoculant used in Uruguay.

2. Material and methods

2.1. Bacterial strain and cultural conditions

Ensifer meliloti U143 strain (Tabares-da Rosa et al., 2019) was cultivated at 28 °C in YMA or YM broth. The culture in YM was incubated with orbital shaking (180 rpm) until an $OD_{620\ nm} \sim 0.8$. The ability of the U143 strain to respire NO_3 was determined in YM with 10 mM KNO_3 incubated with an initial O_2 concentration of 2 % according to (Torres et al., 2014). As a nitrate respiration positive control, *Bradyrhizobium japonicum* USDA110 was used.

2.2. Genome sequencing and annotation

Genomic DNA was extracted using QIAamp DNA Micro Kit (QIAGEN, Germany). DNA purity and concentration were determined by absorbance measurements at 260/280 nm with a NanoDrop spectrophotometer (Thermo Scientific). Whole-genome sequencing (Novaseq-Illumina, paired-end, PE, 2 × 151 bp) was performed at Macrogen (Korea).

Library construction and Illumina PE (2 × 151) sequencing were performed at a sequencing facility (Mr. DNA, USA). Reads quality was visually inspected using fastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), while Trimmomatic v0.39 (Bolger et al., 2014) was used for quality control and adapter trimming. Blast (Altschul et al., 1990) was used to discard potential contaminants. Read *de novo* assembly was performed using Unicycler v0.4.7 (Wick et al., 2017), which functions as a SPAdes-optimiser (Bankevich et al., 2012). The generated contigs were subjected to scaffolding using sspace v2.0 (Boetzer et al., 2010). After that, assembly completeness was evaluated using BUSCO v5.1.2, with the dataset 'bacteria_odb10'. Assembly statistics were estimated using quast v5.0.2 (Gurevich et al., 2013). Genome annotation was carried out using two software: Prokka bacterial genome annotation tool (Seemann, 2014) and RAST (Rapid Annotation Using Subsystem Technology (Aziz et al., 2008); Overbeek et al., 2014). The assembled contigs were subjected to the plaSquid tool (Giménez et al., 2022), for plasmid detection and classification. U143 genome sequence is available in the NCBI BioProject accession number PRJNA967351.

2.3. Genomic and phylogenetic analysis

The genome sequence data were uploaded to the Type Genome Server (TYGS) for a whole genome-based taxonomic analysis (Meier--Kolthoff and Göker, 2019). To determine the closest type strain genomes, the U143 genome was compared against all type strain genomes available in the TYGS database via the MASH algorithm, a fast approximation of intergenomic relatedness (Ondov et al., 2016). The genomes of *Ensifer* strains B399, B401, 1021, WSM1022, WSM 419, and CCMM B554 (FSM-MA) were also included in the analysis. The strains with the smallest MASH distances were chosen. All pairwise comparisons among the genomes were conducted using Genome Blast Distance Phylogeny (GBDP) for the phylogenomic inference, and accurate intergenomic distances were inferred under the algorithm 'trimming' and distance formula d5 (Meier-Kolthoff et al., 2013). The resulting intergenomic distances were used to infer a balanced minimum evolution tree rooted at the midpoint. Trees were inferred with FastME 2.1.6.1 from GBDP distances (Lefort et al., 2015). Branch support was inferred from 100 pseudobootstrap replicates each.

The genomic relatedness analysis was performed using the average nucleotide identity (ANI, (Goris et al., 2007)) determined with BLASTn (ANIb) and MUMmer (ANIm) algorithms, the correlation indexes of tetra-nucleotide signatures, and tetra correlation search (TCS), using the JSpeciesWS server (Richter et al., 2016).

Additionally, a set of closely related strains was determined via the 16S rDNA gene sequences. The 16S rDNA gene sequence was extracted from the U143 genome using RNAmmer (Lagesen et al., 2007), and it was BLASTed (Camacho et al., 2009) against the 16S rDNA gene sequence of each of the strains available in Type Genome Server (TYGS) database. The best 50 matching strains (according to the bitscore) for the U143 genome were found, and the precise distances were calculated using the Genome BLAST Distance Phylogeny approach (GBDP) under the algorithm 'coverage' and distance formula d5 (Meier-Kolthoff et al., 2013). These distances were finally used to determine the closest strain genomes for the U143 genome.

The *nifH* and *nodA* gene sequences were also extracted from the U143 genome and were analyzed by comparison with *nifH* and *nodA* gene sequences of reference strains available in GenBank (http://www.ncbi.nlm.nih.gov). Sequence alignment with the ClustalW algorithm and phylogenetic tree constructions (Maximum likelihood) was done using MEGA 11 software (Tamura et al., 2021) using a multilocus sequence analysis (MLSA). Statistical support for tree nodes was evaluated by bootstrap analyses using 1000 replicates.

2.4. Symbiotic efficiency assay in field conditions

To evaluate the field performance of the U143 strain, trials were set up at INIA Experimental Unit "Glencoe" (32°01′S, 57°09′W) in the Basalto agroecological region in a Typic Hapludert soil with pH 5.7 and at "La Estanzuela" Research Station (34°34′S, 57°69′W). Trials were sown in the fall of 2020 in 5 × 1,25 m plots, following a complete randomized block design with four replicates per location. Plots were placed 1 m apart from each other to prevent contamination. Seed inoculation was made at the recommended commercial doses (200 g of inoculum per 25 kg of alfalfa seed). The sowing rate was 20 $kg.ha^{-1}$, and the experiments were fertilized with 80 kg P_2O_5 ha^{-1} annually.

All biomass above 5 cm was harvested mechanically in a 2.5 m^2 area whenever alfalfa height reached 15–20 cm. Herbage production was determined over two consecutive years in the case of the Glencoe trial, while the La Estanzuela trial was evaluated over three years. On average, plants were harvested 5–6 times per year. Harvest fresh matter was weighed and dried at 60 °C in a forced air oven for 72 h to estimate its dry matter content (DM). Data were analysed using a generalized linear model with location, treatments, and location by treatments interaction as fixed effects. Normality assumptions were analysed by plotting residuals.

Subsequently, the $\delta\ ^{15}N$ determination was carried out following the procedure used by (Irisarri et al., 2019), sampling shoots (above 5 cm from the crown) of 6-month-old plants. Biological nitrogen fixation was assessed using the ^{15}N natural abundance method, following the approach proposed by Unkovich et al. (2010). This method relies on the relative abundances of ^{15}N in the sample and the standard (atmosphere) and is expressed as values $\delta^{15}N = [(^{15}N/^{14}N)\ sample)/(^{15}N/^{14}N)\ standard)] - 1) \times 1000$ (Högberg, 1997). The percentage of total atmospheric nitrogen derived from fixation (%Ndfa) by legumes was calculated using the following equation: $\%Ndfa = 100\ x\ (\delta^{15}N reference - \delta^{15}N legume) / (\delta^{15}N reference - B)$. In this equation, B represents the $\delta^{15}N$ value of the legume growing with atmospheric N_2 as the sole nitrogen source, while $\delta^{15}N$ reference and $\delta^{15}N$ legume are the $\delta^{15}N$ values obtained for the reference plant (non-fixing plant) and the legume, respectively.

3. Results

3.1. Draft genome U143 strain reported

Illumina sequencing yielded a total of 13,347,362 paired-end reads. After quality inspection and read trimming, a total of 11,039,276 paired-end reads showed an overall Phred quality > 30 and read length > 80 bp, and were kept for posterior analysis. De novo assembly and posterior scaffolding yielded a total of 127 contigs longer than 1000 bp, covering a

total length of 6801,966 bp, and a mean GC content of 62.15 % (Fig. 1). From the 127 contigs, 55 (4,111,637 bp) correspond to the bacterial chromosome, while 46 and 26 (1,142,264 and 1,548,065 bp, respectively) correspond to the plasmids, according to the plaSquid analysis. From the assessed 124 Single-Copy Orthologs, 123 (99.2 %) were completed and in single-copy in our assembly, while one (0.8 %) was duplicated. No fragmented or missing BUSCOs were retrieved. Assembly statistics were L50: 18, N50: 105,034 bp, and N's per 100 kbp: 40.51. Genome annotation using Prokka yielded 6522 coding sequences.

Genome annotation carried out with RAST showed that the most abundant subsystem was amino acids and derivatives, followed by carbohydrates (Fig. 2). Among the CDS related to N metabolism, the U143 strain has *nap*, *nir*, *nor,* and *nos* denitrifying reductase clusters. This set of four clusters includes some clusters involved in the denitrification at the periplasmic space (*nap* cluster); the reduction of nitrite to nitric oxide (*nir* cluster); the reduction of nitric oxide to nitrous oxide (*nor* cluster); and the transformation of nitrous oxide into nitrogen (*nos* cluster).

3.2. Phylogenetic analysis

The 16S rDNA phylogenetic tree indicates that the U143 strain belongs to the *E. meliloti* species, as it groups together with the reference strain 2011 and the B399 strain used commercially in Argentina (Fig. 3).

The phylogenomic analysis of *Ensifer* sp. strains showed that 2011, 1021, B399, B401, and U143 strains group together in the *E. meliloti* supercluster (Fig. 4A). The genomic relatedness of U143 and *E. medicae* USDA1037 strains was 86.6 %, and 88.3 % for ANIb and ANIm, respectively; while the genomic connexion between U143 and *E. meliloti* 2011 strains was 98.3 % for ANIb, 98.8 % for ANIm, and 89.8 % dDDH (formula d4 estimated with TYGS). Moreover, U143 shared z-scores $>$ 0.99977 with *E. meliloti* 2011 in TCS and tetra-nucleotide signature, indicating that the U143 strain belongs to *E. meliloti* species.

The *E. meliloti* 2011 and U143 strains, and *E. medicae* USDA1037 strain complete genomes overlapping showed that most of the coding genes are common to all strains (85 %). In turn, the 2011 and U143 strains presented a greater overlap of homologous genes (12 %), while in *E. medicae* around 2 % of these genes were exclusive (Fig. 4B).

3.3. Symbiotic genes

The maximum likelihood tree based on symbiotic *nodA* and *nifH* genes partial sequences showed *E. meliloti* and *E. medicae* strains in different clusters (Fig. 5). The parasitic alfalfa *R. favelukesii* ORY1 strain is separated from *Ensifer* sp., but closer to *E. meliloti* and *E. medicae* (Fig. 5).

Although U143, CCMM B554, and WSM1022 strains form a subcluster separated from other *E. meliloti* strains such as B399 and 1021 (Fig. 5), they all differ in 2.1 % of aminoacidic concatenated sequences.

3.4. Nitrate respiration

In free-living and microaerobic conditions, the U143 strain was able to grow from nitrate respiration. On the fourth day of culture under the indicated conditions, the $OD_{620\ nm}$ increased from 0.049 ($\pm$ 0.003) to 0.33 ($\pm$ 0.024), meanwhile, the positive control *B. japonicum* USDA110 strain increased from 0.04 ($\pm$ 0.002) to 0.31 ($\pm$ 0.03).

3.5. Symbiotic efficiency of U143 strain on alfalfa

The symbiotic efficiency of the U143 strain was assessed through aerial dry matter production in two locations for two years. In addition, the N fixed proportion due to BNF was also determined. Location, treatment, and location by treatment interaction showed significant effects for all the variables measured at the field trials (Table 1). Location by treatment interaction was due to the magnitude difference between the treatments, where no crossover was observed. When alfalfa was inoculated with the U143 strain, higher herbage production was recorded for all the variables (Table 2). Responses resulted in 60 %, 27

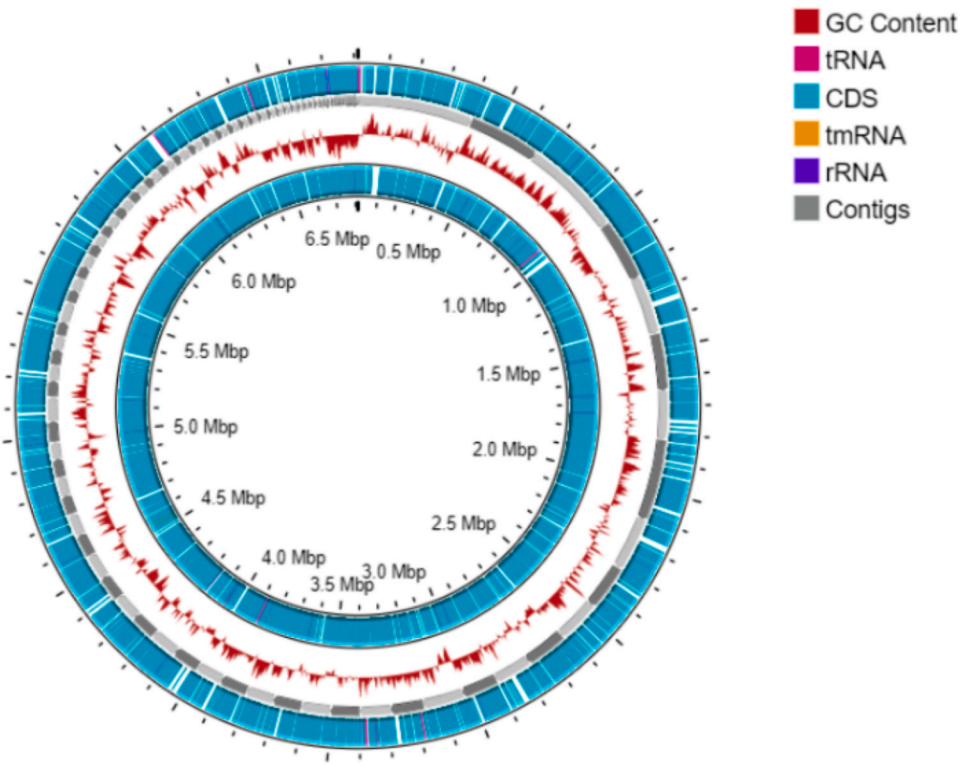

Fig. 1. Circular bacterial genome containing coding sequences (CDS), tRNA, rRNA, tmRNA, and GC content skew. The map was generated using Proksee online software.

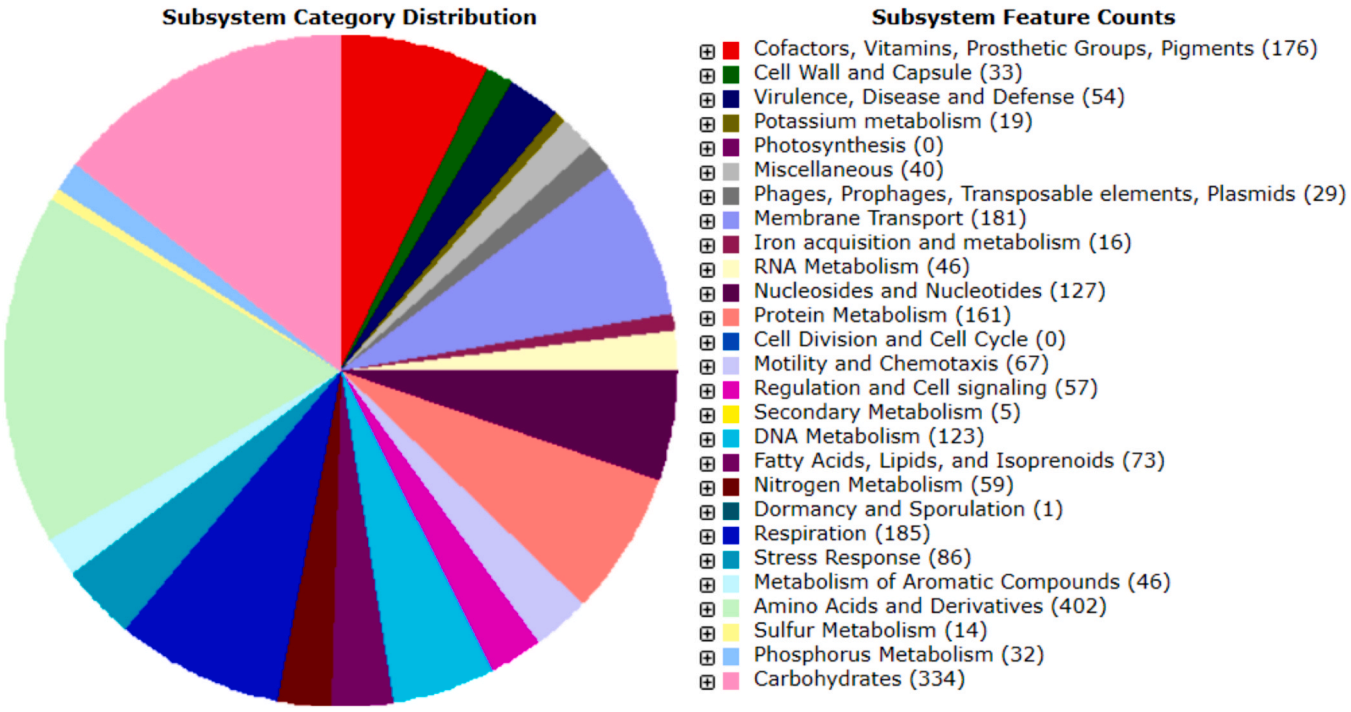

Fig. 2. Bacterial genome representation subsystem category distribution of coding sequences (CDS) from U143 strain, generated through RASTtk pipeline. The number of CDS in the subsystem is shown in the brackets.

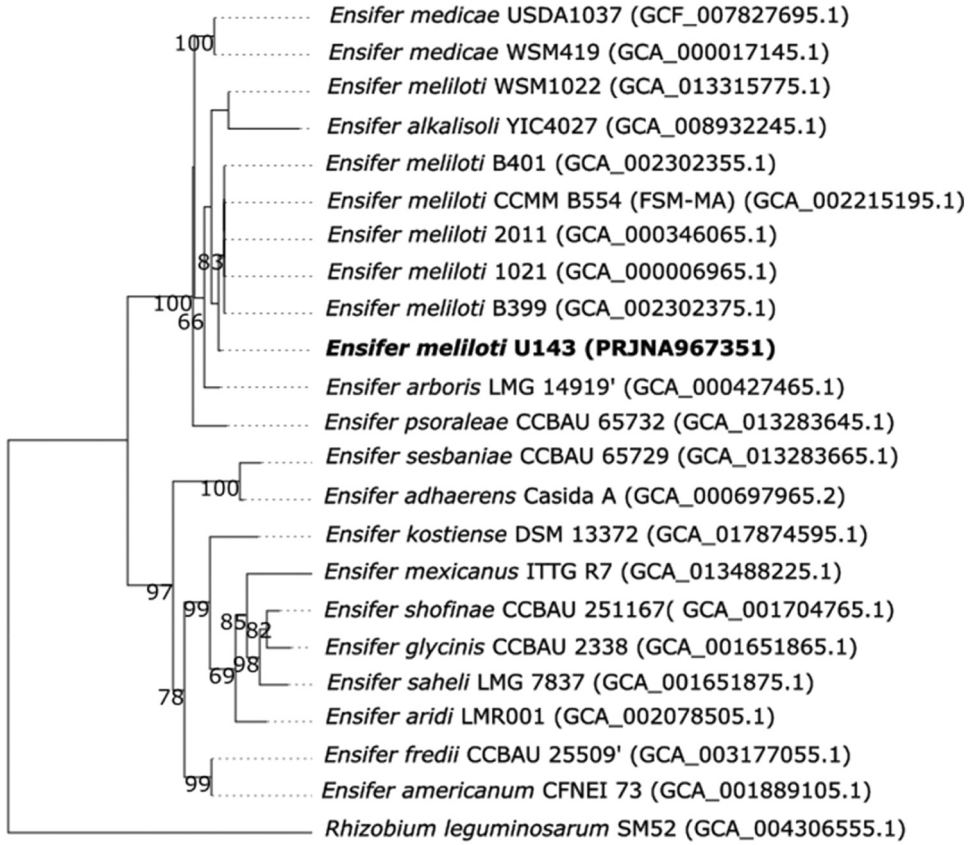

Fig. 3. Tree based on 16S rDNA. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 69.2 %. The NCBI Accession Numbers are shown in brackets. With bold letters is indicated the U143 strain.

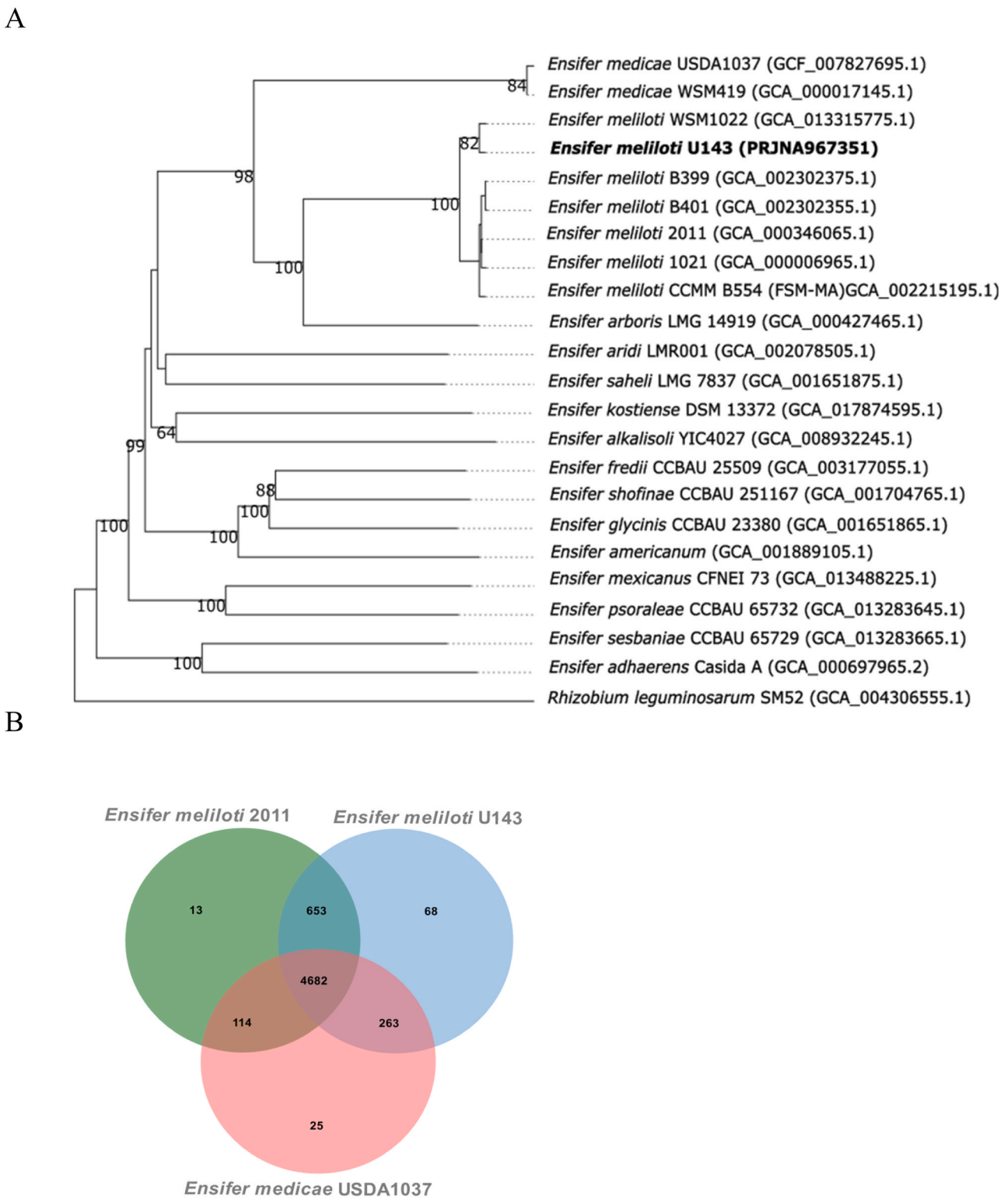

Fig. 4. Phylogenetic analysis and Venn diagram of homologous genes in three *Ensifer* sp. strains A. Tree based on genome sequences. The numbers above branches are GBDP pseudo-bootstrap support values > 60 % from 100 replications, with an average branch support of 76.3 %. The NCBI Accession Numbers are shown in brackets. With bold letters it is indicated the U143 strain. B. Venn diagram of homologous genes in *E. meliloti* 2011 and U143 strains, and *E. medicae* USDA1037. Overlapping regions represent homologous genes shared by 2 or 3 strains.

%, and 41 % increments for shoot DM production to year 1, year 2, and for total DM production respectively (Table 2).

The determination of δ ^{15}N in shoot showed that the proportion of N derived from BNF in 6-month-old plants inoculated with U143 strain was 66.5 % and 61.3 % at Glencoe and La Estanzuela trials respectively.

4. Discussion

The genome size of the U143 strain falls within the expected range of previously sequenced *E. meliloti* strains (6.65–8.94 Mbp) (Nagy Mihály et al., 2017; Terpolilli et al., 2013). The complete genome of the U143

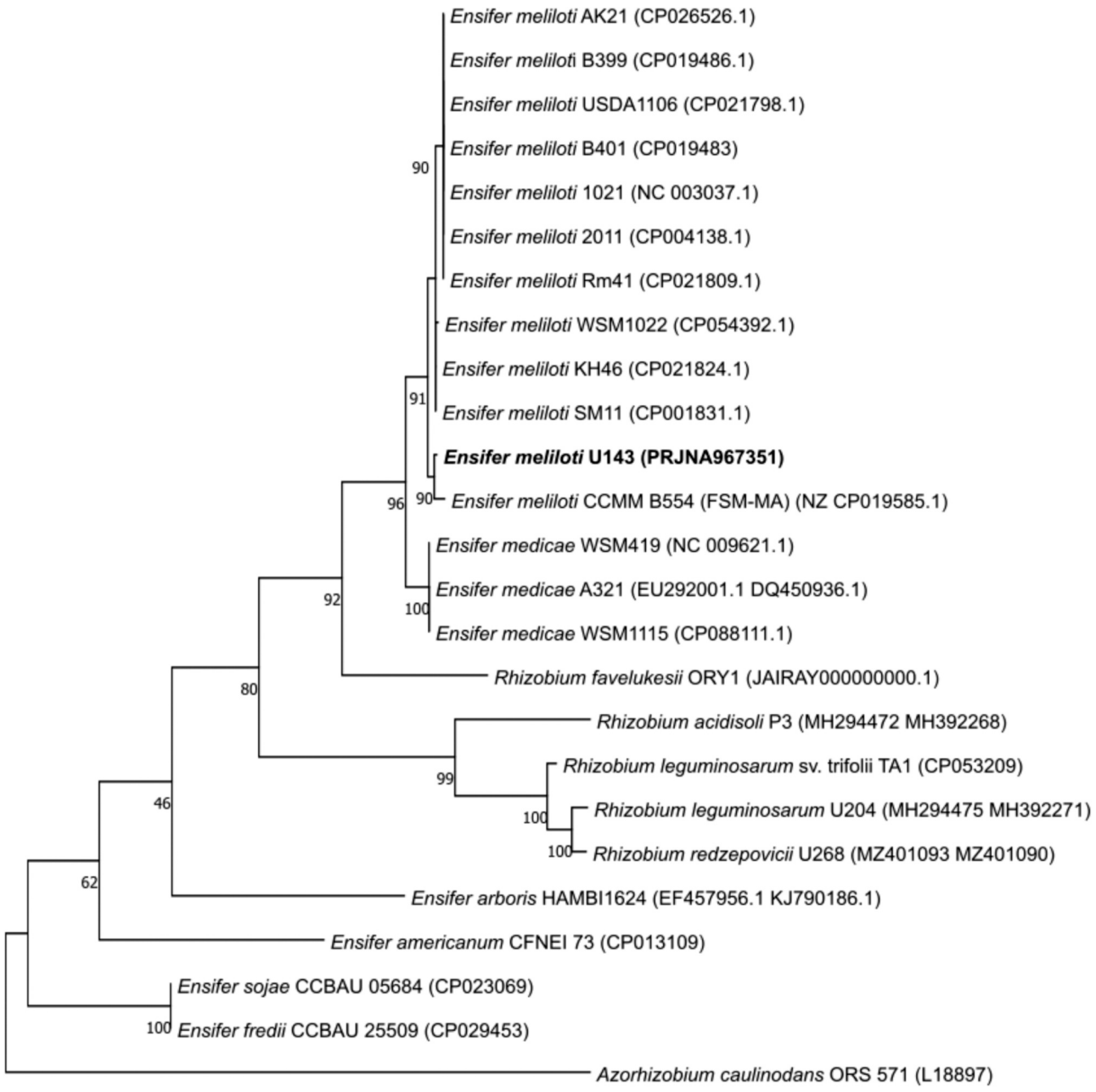

Fig. 5. MLSA of symbiotic genes *nodA* and *nifH*. Maximum likelihood tree inferred from partial sequences alignments of *nodA* (387 nt) and *nifH* (317 nt) concatenated genes. Bold letters indicate U143 strain isolated from Uruguayan soil used as commercial inoculant. Bootstrap values of 50 or more (based on 1000 replicates) are indicated.

Table 1
Analysis of variance presenting *p* values and the significance level for each variation source for field-measured variables. Shoot dry matter (Shoot DM).

Source of variation	Shoot DM year 1	Shoot DM year 2	Total Shoot DM
Location	< 0,0001***	0,0067**	0,0017**
Treatment	0,0001***	0,0029**	0,0007***
Location*Treatment	0,0004***	0,0069**	0,3076^{ns}

ns = not significant; * = significant at 5 %; **=significant at 1 %; *** = significant at 0.1 %.

Table 2
Annual and total shoot dry matter production (kg. ha^{-1}) for U143 and control (without inoculation) treatments. Shoot dry matter (Shoot DM).

Treatments	Shoot DM Year 1	Shoot DM Year 2	Total DM
No inoculation	2398 b	6020 b	8262 b
U143	3844 a	7638 a	11,639 a

Different letters means significant difference at *p* value < 0,05.

strain includes a chromosome and two megaplasmids, namely the symbiotic and accessory plasmids, as other *Ensifer* sp. (Terpolilli et al., 2013). Our analysis shows that the genome architecture of the U143 strain matches reference *E. meliloti* 1021 and 2011 strains. However, it differs from the *E. medicae* WSM419 strain as it contains an additional third plasmid (Baxter et al., 2021). We cannot completely rule out the existence of small plasmids in the U143 strain with the current methodology.

The strains 1021 and 2011 were isolated from *M. sativa* in Australia and derived from the unstable symbiotically SU47 strain (Sallet et al.,

2013; Meade et al., 1982). Similarly, the strains with stable symbiotic efficiency, identified as U143 and U137, were reisolated from the symbiotically unstable U45 strain, isolated in Uruguay around 1956 (Chatel, 1982). This strain was used as a commercial alfalfa inoculant in Uruguay between 1964 and 1990 (Fabiano et al., 2023), in Australia (Howieson and Ewing, 1986), and in South Africa (Bloem et al., 2002). The U143 strain was found to group together with the CCMM B554 strain, which was isolated from *M. arborea* in Morocco (Nagy Mihály et al., 2017), and the WSM 1022 strain isolated from *M. orbicularis* in Grecia (Terpolilli et al., 2013), based on the comparison of *nodA* and *nifH* symbiotic gene sequences.

In the symbiotic plasmid of the U143 and the 1021 strains, four sets of genes involved in nitrate respiration were located: *napC* cluster (nitrate to nitrite reduction in periplasmic space), *nir* cluster (reduction of nitrite to nitric oxid), *nor* cluster (reduction of nitric oxide to nitrous oxide), and *nos* cluster (reduction of nitrous oxide into nitrogen). The 1021 strain and the U143 strain both use nitrate as a respiratory substrate when incubated with 2 % O_2 at the beginning of the culture (Torres et al., 2014). The impact of denitrification caused by N_2O emissions from endosymbiotic bacteria in agricultural production has been understudied (Torres et al., 2014). Therefore, new inoculants should be developed to include strains with "climatically intelligent" characteristics to reduce these emissions.

In this study, field trials were conducted to evaluate the alfalfa cv. Estanzuela Chaná yield inoculated with the U143 elite strain. The results showed that the inoculated plants produced 60 % more yield than the non-inoculated ones in the first year, and 41 % more in the 2-year dry matter accumulation. Between 61 % and 66 % of plant nitrogen was acquired through biological nitrogen fixation, estimated by ^{15}N natural abundance. Our results are in accordance with previous research, where alfalfa inoculated with the U143 strain showed improvements of 50 % in biomass production (Rebuffo et al., 2000). This symbiotic pair produced 240 % more biomass in the first harvest, and 80 % more than the total biomass produced in the first year, with 70 % of plant N coming from BNF (Racca et al., 2013). A higher response to inoculation is expected when rhizobial concentrations are lower than 300 rhizobia per gram of soil, while no responses are expected when the concentrations are greater than 1000 rhizobia per gram of soil (Herridge et al., 2008). Thus, the high response to inoculation obtained in Uruguayan soils suggests a low prevalence of efficient strains, or large populations of inefficient-parasite strains, making alfalfa inoculation necessary to overcome implantation and production problems (Racca et al., 2013; Castro-Sowinsky et al., 2002).

5. Conclusions

The genome sequence of *Ensifer meliloti* species elite strain U143, used for decades as a commercial alfalfa inoculant, can expand comparative genomics possibilities and help identify genetic determinants of symbiotic capacities.

Despite being selected under different conditions and cultivars, the U143 strain remains highly efficient in alfalfa. Therefore, inoculating alfalfa with this strain is necessary to overcome implantation and production problems.

Author contributions

Conceptualization, J.M.; writing original draft preparation and visualization, A.B-R; M.M-R, C.V.F, and R.R. writing, review, and editing were developed by all authors; supervision, J.M., R.R project administration, J.M.; funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jorge Monza reports financial support was provided by Instituto nacional de investigaciones agropecuarias (INIA). Jorge Monza, Maria Morel-Revetria, Rafael Reyno reports financial support was provided by Sistema Nacional de Investigadores (SNI). Jorge Monza, Maria Morel-Revetria, Andres Berais-Rubio reports financial support was provided by PEDECIBA. Andres Berais-Rubio reports financial support was provided by Comision Academica de Posgrado (CAP) Universidad de la Republica.

Data Availability

Data will be made available on request.

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