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LETTER TO THE EDITOR

Microevolution Rather than Large Genome Divergence Determines the Effectiveness of Legume–Rhizobia Symbiotic Interaction Under Field Conditions

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Abstract Despite the vast screening for natural nitrogen-fixing isolates by public and private consortia, no significant progresses in the production of improved nitrogen-fixing inoculants for alfalfa production have been made in the last years. Here, we present a comprehensive characterization of the nitrogen-fixing strain *Ensifer meliloti* B399 (originally named *Rhizobium meliloti* 102F34), probably the inoculant most widely used in alfalfa production since the 1960s. Complete nucleotide sequence and genome analysis of strain B399 showed that the three replicons present in this commercial strain and the model bacterium *Ensifer meliloti* 1021 are extremely similar to each other in terms of nucleotide identity and synteny conservation. In contrast to that observed in B399-treated plants, inoculation of plants with strain 1021 did not improve nitrogen content in different alfalfa cultivars under field conditions, suggesting that a small genomic divergence can drastically impact on the symbiotic phenotype. Therefore, in addition to the traditional screening of natural nitrogen-fixing isolates, the genome engineering of model strains could be an attractive strategy to improve nitrogen fixation in legume crops.

Keywords Microevolution · Legumes · Rhizobia · Commercial inoculants · Field conditions

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Introduction

Alfalfa is the most important forage crop in the temperate areas of the world (Garcia et al. 2014; Jozefkowicz et al. 2016). This main crop has a mutually beneficial symbiotic relationship with nitrogen-fixing *Ensifer* strains (Oldroyd et al. 2011). The strain *Ensifer meliloti* B399, initially named *Rhizobium meliloti* 102F34 (Burton 1972; Ditta et al. 1980) and included in the microbial collection of the National Institute of Agricultural Technology (www.inta.gob.ar) of Argentina since the nineties (Segundo et al. 1999; Soto et al. 2013), has been extensively used as an efficient nitrogen-fixing commercial inoculant for alfalfa production (e.g., Nitragin 102-F-34). The extremely high capacity of strain B399 to fix nitrogen in a wide range of alfalfa varieties under different environments (Burton 1972; Sanz-Sáez et al. 2012a, b) has positioned this strain among the main inoculants for alfalfa production worldwide during the last fifty years. In fact, despite the large efforts to produce better nitrogen-fixing inoculants for alfalfa production through public and private consortia, the efficiency of strain B399 has not been significantly improved. Consequently, strain B399 remains the inoculant recommended by public institutions of agronomy in alfalfa-producing countries. In this research letter, we report the complete genome sequence of the commercial strain B399, the extremely high genomic similarity between commercial strain B399 and model bacterium *Ensifer meliloti* 1021 (Galibert et al. 2001), and their contrasting abilities to fix nitrogen in symbiosis using a variety of alfalfa elite germplasm under field conditions. Our results indicate that small genomic divergence can radically impact on the symbiotic process under field conditions.

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Methods

The genomic DNA of *Ensifer meliloti* B399 was isolated from overnight cultures using Wizard Genomic DNA Purification Kit (#A1120, Promega, USA). Genome sequencing was performed at the Instituto de Agrobiotecnología de Rosario, Argentina (INDEAR). As a result, a total of 16.04 mega reads (1.62 Gp) were obtained from Illumina Hiseq 1500 technology (www.indear.com). De novo assembly and annotations were carried out using Geneious 10.0.1 (www.geneious.com) and NCBI prokaryotic genome annotation pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok), respectively. The nucleotide sequences of the three replicons of *E. meliloti* B399 obtained here were deposited at the EMBL nucleotide sequence database, accession numbers: CP019488 for the chromosome, CP019486 for megaplasmid A, and CP019487 for megaplasmid B. Genomic alignments of B399 against other strains were performed using LASTZ plugin in Geneious software (www.geneious.com). The accession numbers used in these alignments were NC_003047, NC_003037, and NC_003078 for 1021; NC_015590, NC_015591, and NC_015596 for AK83; NC_017322, NC_017324, and NC_017323 for BL225; NC_017325, NC_017327, NC_017326 for Sm11; NC_019845, NC_019848, and NC_019849 for GR4; NZ_CP009144, NZ_CP009145, and NZ_CP009146 for RMO17; NC_018700, NC_018683, and NC_018701 for Rm41. Monoculture field trials were conducted at five localizations within the Pampean region of Argentina. Alfalfa plants were non-inoculated or inoculated by strains B399 and 1021 using 10^5 bacterial cells per seed. Total nitrogen content in plant matter was quantified 6 months after the sowing of alfalfa seeds as previously described (Fox et al. 2016).

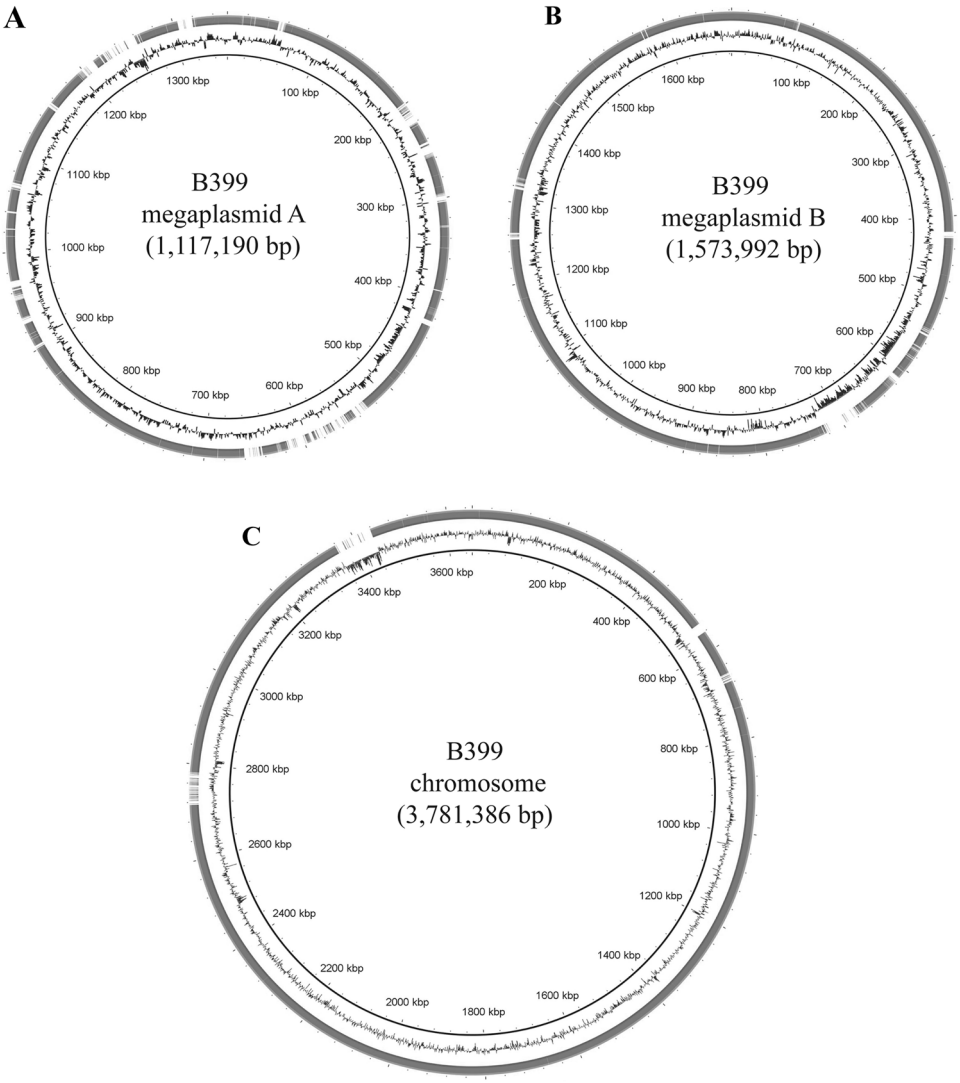

Fig. 1 Tripartite 6.4-megabase (Mb) genome of *Ensifer meliloti* B399 comprises a 1.1-Mb megaplasmid A (**a**), a 1.5-Mb megaplasmid B (**b**), and a 3.7-Mb chromosome (**c**). The genomic analyses of GC% and the percent of nucleotide identity (id.%) of commercial strain B399 against model strain 1021 were performed using the BLAST ring image generator (brig.sourceforge.net), where id.% = 100, 75, 50, and >50% are represented by *black*, *dark gray*, *light gray*, and *white colors*, respectively

Results

The genome of strain B399 (6,472,568 bp) includes the three typical replicons of *E. meliloti*, consisting of a circular chromosome encoding housekeeping functions (3,781,386 bp), the nitrogen fixation-related megaplasmid A (1,117,190 bp), and the rhizosphere colonization-related megaplasmid B (1,573,992 bp) (Fig. 1). The average percent GC content for the genome of strain B399 (62.3%) is close to the average for *E. meliloti* (61.0–62.3%) (Table 1) (Sugawara et al. 2013), and the genomic alignments show that strain B399 has high nucleotide coverage and identity to other *E. meliloti* strains, including model strain 1021 (Table 2). Specifically, strains B399 and 1021 have nucleotide identities of 99.8% for the chromosome, 98.0% for megaplasmid A, and 98.7% for megaplasmid B (Table 2), suggesting the close relationship between these strains. In addition, genome pairwise comparisons of strains B399 and 1021 showed an extremely conserved synteny without any large-scale DNA rearrangement within the three replicons (Fig. 2), including variable and conserved megaplasmid A and B, respectively (diCenzo et al. 2016; Galardini et al. 2013). In agreement with previous reports about the plant growth promotion ability of commercial strain B399 in alfalfa (Sanz-Sáez et al. 2012a, b), high levels of nitrogen content were observed in alfalfa plants inoculated with strain B399 (Fig. 3), suggesting that these alfalfa plants are incorporating large amounts of nitrogen provided by biological nitrogen fixation under field conditions (Fig. 3). In contrast to that

Table 1 Genome features of the commercial inoculant *Ensifer meliloti* B399

Features	Values
Genome size	6,472,568 bp
G+C content	62.3%
Total genes	6482
Coding genes	6034
rRNA	9
tRNA	61

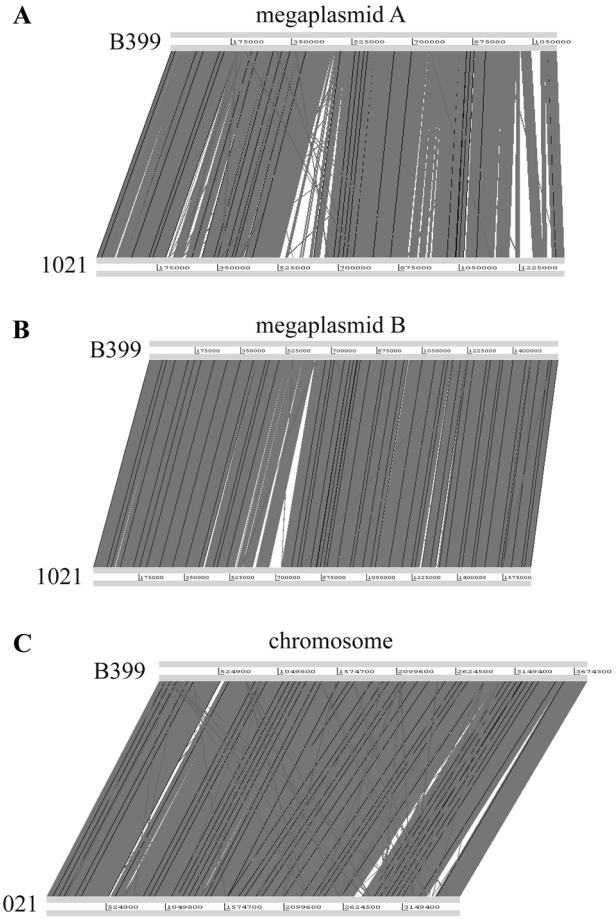

Fig. 2 Genome comparison of commercial strain B399 against model strain 1021. Syntenic regions are shown with *gray* connections. *Black lines* indicate inverted gene regions. **a** Megaplasmids A. **b** Megaplasmids B. **c** Chromosomes

Table 2 Similarity of the commercial bacterium B399 to other *Ensifer* strains

B399 vs	1021	AK83	BL225	Sm11	GR4	RMO17	Rm41
Chr							
Cover %	91.5	79.3	80.9	80.7	90.9	91.1	79.8
id. %	99.8	99.6	99.8	99.7	99.7	99.8	99.3
pA							
Cover %	96.5	47.3	70.4	84.8	80.0	91.0	87.2
id. %	98.0	98.3	98.0	98.2	97.6	98.8	98.5
pB							
Cover %	99.7	61.5	76.0	92.0	93.4	93.0	92.1
id. %	98.7	98.4	98.6	98.6	98.3	98.6	98.5

Similarity of commercial strain B399 against other *Ensifer* strains for chromosome (chr), megaplasmid A (pA), and megaplasmid B (pB) replicons. Ref-seq coverage% (cover%)

The pairwise% identity (id.%)

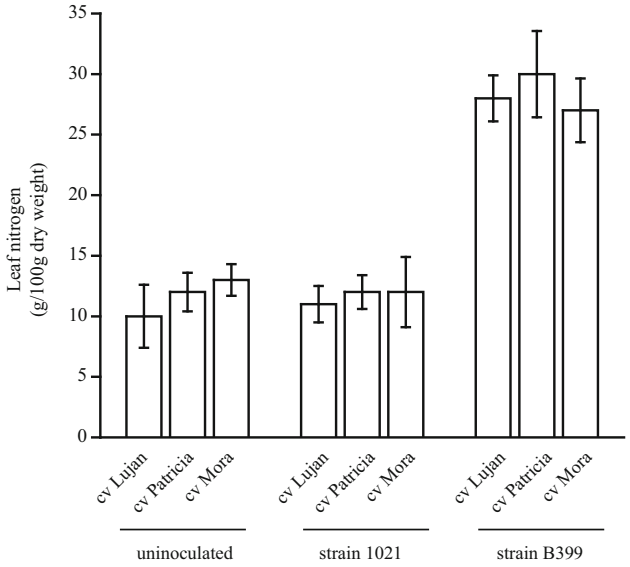

Fig. 3 Quantification of nitrogen content in strain B399-inoculated alfalfa plants under field conditions. Total nitrogen content in leaves from 6-month-old alfalfa plants without inoculation or inoculated with model strain 1021, commercial strain B399. Three alfalfa cultivars were analyzed: cv Lujan, cv Patricia, and cv Mora. All values are mean ± SEM ($n = 24$)

observed in B399-treated plants, inoculation of alfalfa with model strain 1021 did not improve nitrogen content under field conditions (Fig. 3).

Discussion

Since the report of the complete genome sequence of the model strain *E. meliloti* 1021 (Galibert et al. 2001), genetic and genomic data of non-commercial strains belonging to this species have increased dramatically (diCenzo et al. 2016; diCenzo and Finan 2015; Galardini et al. 2013; Schneiker-Bekel et al. 2011; Sugawara et al. 2013). However, little is known about the genomic and phenotypic relationships between non-commercial and commercial strains. Genomic and phenotypic data presented here suggest that the contrasting efficiency of strains B399 and 1021 as nitrogen-fixing alfalfa inoculants could be due to small genomic differences. Considering these results and the little progress in the production of improved commercial inoculants for alfalfa culture by traditional screening of natural nitrogen-fixing isolates in the last years, we propose the use of the genome engineering of model strains by directed evolution as an alternative strategy to improve nitrogen fixation in alfalfa and in other important legume crops for which the classic screening of natural nitrogen-fixing strains for phenotypic variants has failed.

Alfalfa, commonly known as the "Queen of Forages," is particularly rich in proteins and vitamins, and thus, the basis of meat and milk productions worldwide. This legume species is considered one of the first crops and has been used in Asia for more than 3500 years. During the domestication of alfalfa, the deep green color associated with high nitrogen content has been enhanced by human selection. The results presented in this article provide evidences, for the first time, that the ability of symbiotic nitrogen fixation was enhanced during the domestication of alfalfa, probably by the selection of small changes into the genome of its nitrogen-fixing endosymbiont *E. meliloti*.

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