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Phenogenetic profile and agronomic contribution of *Azospirillum argentinense* Az39^T, a reference strain for the South American inoculant industry

Maroniche GA[1*], Puente ML[2*], García JE[2], Mongiardini E[3], Coniglio A[4], Nievas S[4], Labarthe MM[1], Wisniewski-Dyé F[5], Rodriguez Cáceres E[6], Díaz-Zorita M[7] and Cassán F[4]

[1]Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata (UNMdP), CONICET, Balcarce, Buenos Aires, Argentina.

[2]Instituto de Microbiología y Zoología Agrícola, Instituto Nacional de Tecnología Agropecuaria (INTA), Hurlingham, Buenos Aires, Argentina.

[3]Instituto de Biotecnología y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata (UNLP), CONICET, La Plata, Buenos Aires, Argentina.

[4]Laboratorio de Fisiología Vegetal y de la Interacción Planta-Microorganismo, Instituto de Investigaciones Agrobiotecnológicas (INIAB-CONICET), Universidad Nacional de Río Cuarto (UNRC), Río Cuarto, Córdoba, Argentina.

[5]Universite Claude Bernard Lyon 1, Laboratoire d'Ecologie Microbienne, UMR CNRS 5557, UMR INRAE 1418, VetAgro Sup, 69622 Villeurbanne, France

[6]Private advisor, Buenos Aires, Argentine.

[7]Facultad de Agronomía, Universidad Nacional de La Pampa (UNLPam), CONICET, Santa Rosa, La Pampa, Argentina.

* Contributed equally to the work

Abstract

Azospirillum sp. is a plant growth-promoting rhizobacteria largely recognized for its potential to increase the yield of different important crops. In this work, we present a thorough genomic and phenotypic analysis of *A. argentinense* Az39^T to provide new insights into the beneficial mechanisms of this microorganism. Phenotypic analyses revealed the following *in vitro* abilities: growth at 20–38 °C (optimum, 28 °C), pH 6.0–8.0 (optimum, pH 6.8), and in the presence of 1% (w/v) NaCl; production of variable amounts of PHB as intracellular granules; nitrogen fixation under microaerophilic conditions; IAA synthesis in the presence of L-tryptophan. Through biochemical (API 20NE) and carbon utilization profiling (Biolog) assays, we proved that *A. argentinense* Az39^T is able to use 15 substrates and metabolize 19 different carbon substrates. Lipid composition indicated a predominance of medium and long-chain saturated fatty acids. A total of 6 replicons classified as one main chromosome, three chromids, and two plasmids, according to their tRNA and core essential genes contents, were identified. Az39^T genome includes genes associated with multiple plant growth-promoting (PGP) traits such as nitrogen fixation and production of auxins, cytokinin, abscisic acid, ethylene, and polyamines. In addition, Az39^T genome harbor genetic elements associated with physiological features that facilitate its survival in the soil and competence for rhizospheric colonization; this includes motility, secretion system, and quorum sensing genetic determinants. A metadata analysis of Az39^T agronomic performance in the pampas region, Argentina, demonstrated significant grain yield increases in wheat and maize, proving its potential to provide better growth conditions for dryland cereals. In conclusion, our data provide a detailed insight into the metabolic profile of *A. argentinense* Az39^T, the strain most widely used to formulate non-legume inoculants in Argentina, and allow a better understanding of the mechanisms behind its field performance.

Keywords

Plant growth-promoting mechanisms; Inoculants; *Azospirillum argentinense* Az39^T genome; Rhizosphere colonization; Crop yield improvement

1. Introduction

Azospirillum is one of the best characterized plant growth-promoting rhizobacteria nowadays (PGPR) (Hungria et al., 2013; Cassán and Diaz-Zorita, 2016). This genus belongs to the class *Alphaproteobacteria*, order *Rhodospirillales* (Pfennig and Truper, 1971), and it was recently included in the new family *Azospirillaceae* as the type genus (Tarrand et al., 1978; Baldani et al., 2015). *Azospirillum* comprises non-fermentative, free-living, Gram-negative diazotrophic bacteria that do not form spores but produce intracellular granules of poly-β-hydroxybutyrate. It was described for the first time under the name of *Spirillum lipoferum* by Beijerinck (1925). Afterwards, Tarrand et al. (1978) reclassified the genus to *Azospirillum* describing two species, *A. lipoferum* and *A. brasilense*. Nowadays, the genus includes 24 species: *A. agrícola*, *A. argentinense*, *A. baldaniorum*, *A. brasilense*, *A. canadense*, *A. cavernae*, *A. doebereinerae*, *A. fermentarium*, *A. formosense*, *A. griseum*, *A. halopraeferens*, *A. humicireducens*, *A. largimobile*, *A. lipoferum*, *A. melinis*, *A. oryzae*, *A. palustre*, *A. picis*, *A. ramasamyi*, *A. rugosum*, *A. soli*, *A. thermophilum*, *A. thiophilum* and *A. zeae* (Nievas et al., 2023). In recent years, new species have been described and proposed as candidates for this genus, including *A. aestuarii*, *A. endophyticum*, *A. massiliensis*, *A. oleiclasticum*, *A. palatum*, and *A. tabaci*. This results in a total of 32 species on the list published at the List of Prokaryotic names with Standing in Nomenclature (https://lpsn.dsmz.de).

In the last two decades, inoculants based on several species, but mainly on *A. brasilense*, and currently on *A. baldaniorum* and *A. argentinense*, have been commercialized in different South American countries, including Argentina, Brazil, Uruguay, and Paraguay (Okon and Labandera-Gonzalez, 1994; Cassán and Díaz-Zorita, 2016). In Argentina, the first inoculant was registered in 1996 and it was formulated using *A. argentinense* Az39^{T} . This strain was isolated from surface-sterilized roots of wheat (*Triticum aestivum* L.) grown in Marcos Juárez (Córdoba, Argentina) by Enrique Rodríguez Cáceres, researcher at the Instituto Nacional de Tecnología Agropecuaria (INTA), and deposited at the BPCV-IMYZA-INTA Culture Collection (WDCM31), Argentina (Rodríguez Cáceres et al., 2008). Although it was initially considered as a member of *A. brasilense* species, it was later

taxonomically reclassified into a separate species, termed *A. argentinense*, mainly due to multi-locus sequence analyses and whole-genome relatedness indexes obtained by newly available genomic data (dos Santos Ferreira et al., 2022).

Due to its remarkable ability to increase maize and wheat yields under field conditions, Az39^T is the most used strain as the active ingredient in registered inoculants, being present in about 75% of these products in South America. It is considered a reference strain and a model strain for studying PGPR due to the accumulated extensive data on its behavior in the laboratory and under field conditions (Cassán et al., 2020).

The plant growth promotion by *Azospirillum* is achieved by multiple mechanisms; this includes nitrogen biological fixation, the production of phytohormones such as auxins, gibberellins, and cytokinins (Cassán et al., 2014, 2020; Peng et al., 2020; Pankievicz et al., 2021; Zaheer et al., 2022), and the synthesis of nitric oxide (NO), a well-known signal molecule shown to participate in several physiological and developmental plant processes (Creus et al., 2005). *Azospirillum* also synthesizes other plant-growth regulators including polyamines (Hartmann and Baldini, 2006; Perrig et al., 2007), which were reported to modulate plant growth under saline stress (Cassán et al., 2009). This bacterium can also promote plant growth by mitigating abiotic stresses (García et al., 2017; Fukami et al., 2018). The main benefit of inoculated plants is evidenced by root morpho-physiological changes, entailing significant increases in root surface area (Dobbelaere et al., 1999; Speepen et al., 2007; Molina-Favero et al., 2008; Mora et al., 2023). Consequently, an increase in water and nutrient absorption rates, early growth, higher emergence rates and plant vigor, and early physiological maturity may be observed under field conditions.

The presence of *Azospirillum* has been largely associated with an altered plant growth, but mainly with an improved root growth during early developmental stages, with varying impacts on cereals grain yields depending on differences in water and nutrient availability throughout the crop cycle (Okon and Labandera, 1994; Díaz-Zorita et al., 2015).

Over the last 40 years, *A. argentinense* Az39^T has been one of the most important strains for the biofertilizers' industry both in Argentina and South America. In this study, a deep analysis of the *A. argentinense* Az39^T genome content and phenotypic profile was carried out and compared with the genomes and phenotypic profiles of some close intra-genus and intra-species relatives. The focus was put on properties relevant for rhizosphere colonization and plant growth-promotion. Concurrently, the impact of introducing this strain in agriculture over the past two decades was assessed via a metadata analysis of its agronomic performance. Our global objective was to comprehend the phenogenomic basis of its well-known plant growth-stimulating capability and to furnish a valuable tool for its utilization in academy or industry.

2. Materials and Methods

2.1. Bacterial strains and sequences

The strains used in this work were Az39^T, Az19, REC3, BR11975, MTCC4035 and MTCC4036 of the *A. argentinense* species (dos Santos Ferreira et al., 2022), and the type strains of the phylogenetically closest three species, *A. baldaniorum* Sp245^T (dos Santos Ferreira et al., 2020), *A. brasilense* Sp7^T, and *A. formosense* CC-Nfb-7^T (Lin et al., 2012), all of which are deposited in the Bacterial Culture Collection (WDCM31) at the INTA-IMYZA, Castelar, Argentina. These strains were used in comparative analyses as required, to discern which of the observed traits are exclusive to *A. argentinense* species and which ones are particular to Az39^T strain. They were routinely cultured in RC medium (Rodrıguez Cáceres, 1982). The Genbank accessions used for the genomic analyses herein performed were GCA_000632475.2/GCF_000632475.1 (Az39^T), GCF_002895265.1 (REC3), GCF_008369925.1 (Az19), GCF_016622125.1 (BR11975), GCF_005222165.1 (MTCC4035), GCF_005222185.1 (MTCC4036), GCF_003119195.2 (Sp245^T), GCF_008274945.1 (Sp7^T) and GCF_019780885.1 (CC-Nfb-7^T). Further details about the origin of *A. argentinense* strains other than Az39^T are provided in Santos Ferreira et al. (2022).

2.2. Morphological and microbiological characterization

Gram staining was performed as described by Murray et al. (1994). Colonies on RC medium after 5 days of growth were photographed with 10X magnification (Olympus, Japan)**.** To determine Az39^T growth profiles, tubes with 20 mL of chemically defined NYA (Nfb supplemented with 1 g L^{-1} NH_4Cl as a defined nitrogen source, and 0.05 g L^{-1} yeast extract to supplement for vitamins and other minor nutrients; García et al., 2020) or rich LB medium (Sambrook et al., 2001) were inoculated with 0.2 mL of starter cultures (grown in the same media until the stationary phase) and incubated at 30 °C for 72 h with 180 rpm shaking. Temperature, pH, and NaCl concentration of the culture media were modified to establish the optimal conditions. Final growth was estimated by optical density at 600 nm and by enumerating viable cells using the drop plate method, as described in Di Salvo et al. (2022), for further comparative analyses with type strains of closely related species regarding temperature and culture media effects.

PHB quantification was conducted in liquid NYA and LB culture medium, according to Alves et al. (2017). Cell morphology was analyzed by scanning electron microscopy (ESEM, FEI ESEM Quanta 200) after growth for 48 h in the NYA medium at 28 °C.

Cellular fatty acid methyl esters were extracted and identified according to the protocol of the Sherlock® Microbial Identification system (MIDI, version 4.5), using the RTSBA6 aerobe library (Sasser, 2001). To this purpose, samples were prepared according to the manufacturer's recommendation from a single colony developed on trypticase soybean agar (TSA) for 24 h at 30 °C. The enzymatic activities and carbon sources assimilation ability were assayed using the API 20NE kit (bioMérieux®) and Biolog-GENIII MicroPlate system (Biolog®) kits, according to the manufacturers' instructions.

Auxins production was analyzed in *A. argentinense* strains Az39^T, Az19 and REC3, as well as the type strains of closely related species, by reverse-phase HPLC according to Rivera et al. (2014). Briefly, stationary cultures of each strain obtained in LB medium were centrifuged at 13,500x*g* for

10 min to collect the supernatant. Identification and quantification of indole-3-acetic acid (IAA) in thar fraction was conducted by reverse-phase HPLC (Jensen et al., 1995).

The nitrogen fixation capacity was evaluated for Az39^T and type strains of closely related species by the acetylene-reduction assay (ARA) as described by Hardy et al. (1973). Briefly, vials (30 mL) containing 10 mL of semi-solid NFB medium were inoculated with each strain, sealed with rubber septa, and incubated at 30 °C in a dark incubator. After 72 h, 10% (v/v) of the air phase was replaced with acetylene (Koch and Evans, 1966) and the vials were re-incubated. The amount of ethylene released was monitored for a total of 24 h in a gas chromatograph (FID Gas Chromatograph), according to Reis and Döbereiner (1998).

Biofilm formation was assessed by the crystal violet method (O'Toole and Kolter, 1998). Static cultures in 96-microwell plates with 0.2 mL of NYA medium were incubated for 48 h at 28°C or 36°C. Nitrogen source was unchanged (1 g L^{-1} NH_4Cl) or modified to 0.27 g L^{-1} of NH_4Cl ("low NH_4") or to 2 g L^{-1} of KNO_3 ("NO_3"). Experimental data were analyzed by ANOVA, followed by Tukey's post hoc test at $p<0.05$.

2.3. Genome analysis

Pulse-field gel electrophoresis (PFGE) was performed on DNA obtained from cells grown in LB medium until mid-log phase; cell treatment to obtain undigested DNA and electrophoretic runs were performed as described previously (Vial et al., 2006).

All the genomic analyses included in this manuscript are novel and based on the previously sequenced and annotated genome of *A. brasilense* Az39 (currently *A. argentinense* Az39^T) (Rivera et al., 2014). The KEGG (https://www.genome.jp/kegg/) and RAST (https://rast.nmpdr.org/) servers were used for general functional analysis of the genes. These analyses were focused on genes associated with plant growth-promotion and rhizosphere colonization traits such as auxin biosynthesis, denitrification, mobility and secretion systems. When required, protein sequences were re-analyzed with CDSearch (Lu et al., 2020).

Mobile elements integrated into the Az39 genome were identified using ISSAGA (Varani et al., 2011). Prophages were detected using Phaster (Arndt et al., 2016), Prophet (Reis-Cunha et al., 2019), and Phigaro (Starikova et al., 2020). Secretion systems components were detected using the KEGG server and TXSS script (Abby et al., 2016). Genome visualization maps were constructed with Proksee (Grant et al., 2023). Operons and gene cluster synteny were analyzed with MultiGeneBlast (Medema et al., 2013).

2.4. Metadata analysis of *A. argentinense* Az39^T agronomic performance

To assess the contribution of inoculated *A. argentinense* Az39^T in grain crops, we analyzed the results from 638 wheat and 318 maize's field trials performed in the pampas region of Argentina during 18 and 12 growing seasons, respectively. These trials were conducted in experimental sites under regular agricultural practices including crop rotations, mostly under no tillage and with adequate NP fertilization to avoid nutrient deficiency.

In all locations with replicated strip trials, the grain production of crops developed from Az39^T-treated seeds was compared to that of control crops without this treatment. In all trials, the inoculant was prepared in a liquid carrier and stored at room temperature for 3 to 6 months before the on-seed application, and had a mean title of 1 x 10^9 CFU mL^{-1} at the time of application. The seeds were inoculated at a rate of 10 mL kg^{-1} (wheat) and 12 mL kg^{-1} (maize).

At physiological maturity, duplicated 1 to 3 m^2-sampling units were randomly chosen within a uniform 100 m^2 area to measure grain production. The weight of the grains was corrected to 0.14 g g^{-1} of moisture content. The data analysis was based on descriptive statistics (mean and dispersion parameters), analysis of variance (ANOVA), and mean comparison by the least significance difference test (LSD), based on a factorial design with seasons and inoculation treatments as main factors and using each location as a replication. Among all the growing seasons and locations, the environmental effects on grain yields were evaluated through comparative regression analyses between treatments (non-inoculated vs. inoculated), according to Jennrich (1995) using Statistix 9.1

(Analytical Software, 2015). The agronomic success of the inoculation was estimated based on both the percentage of sites with positive responses (positive response: Az39^T-inoculated treatments yielded at least 50 kg ha^{-1} more than the control) and the net yield increment obtained (kg ha^{-1}).

3. Results and Discussion

3.1. Morphological and microbiological characterization

The morphological characterization of Az39^T cells carried out by microscopy revealed Gram-negative rods, slightly curved, with an average size of 2.3 x 0.7 µm (**Figure S1**). Remnants of exopolysaccharides (EPS) attaching Az39^T cells to the surface were detected in the ESEM images (**Figure S1A and B**). Mature colonies of strain Az39^T on RC medium displayed the typical phenotype of azospirilla cells: dry, scarlet, rough, eventually showing radial ridges and irregular borders (**Figure S1C**). Growth profile of Az39^T according to different culture conditions was characterized in both a chemically defined medium (NYA) and a rich medium (LB), which are those usually used to culture this strain (dos Santos Ferreira et al., 2022; García et al., 2017; Molina et al., 2020); the results are summarized in **Figure 1**.

Insertion Figure 1

Though bacterial growth was possible in both culture media over a wide range of pH (5 to 9) , the highest growth was achieved in LB at a pH of about 7-8 (**Figure 1A**). The presence of increasing NaCl concentrations in the medium (1% to 5%) had little impact on Az39^T growth, causing only a slight growth decrease at 5% in the NYA medium (**Figure 1B**). The growth rate at different temperatures was followed by measuring the final OD_{600nm} and by counting viable cells, and similar results were obtained: while Az39^T reached increasing cell densities from 15 °C up to about 28 °C, where it reached a plateau level that was kept even at more than 33-35 °C, in LB growth was stimulated over 20 °C and temperatures over 35 °C had a negative impact on cell multiplication

(**Figure 1C and 1D**). Overall, Az39^T growth in LB medium was more restricted and variable (**Figure 1A to D**) than in NYA, possibly due to 1% NaCl content. Growth limitations in LB at higher temperatures were detected also in other related *Azospirillum* species such as *A. baldaniorum* Sp245^T and *A. formosense* CC-NFb-7^T, being *A. brasilense* Sp7^T an exception in this regard (**Figure 1E**). Biofilm formation by Az39^T was tested in the NYA medium. We found that the presence of 200 mM of NaCl or an incubation temperature of 36 °C diminished biofilm formation (**Figure 1F**). Likewise, biofilm production was strongly affected by N amount or source, with significantly less production under a higher C:N ratio or nitrate as the N source (**Figure 1G**).

Table S1 summarizes our findings about *A. argentinense* Az39^T general metabolic profile compared with those of related species including *A. baldaniorum* Sp245^T, *A. brasilense* Sp7^T, and *A. formosense* CC-Nfb-7^T. We used the commercial API 20NE and Biolog GENIII systems. Strains analyzed by API 20NE were able to use 13 substrates including sugars (D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, and D-maltose), potassium nitrate, urease, esculin ferric citrate, 4-nitrophenyl-βD-galactopyranoside, potassium gluconate, malic acid, and trisodium citrate. Specifically, Az39^T also demonstrated the ability to use adipic acid. On the other hand, Biolog GENIII system showed that the strains evaluated were able to metabolize 38 different carbon substrates and commonly assimilated 12 substrates. In the sugar test, the strains grew in the presence of sucrose and D-mannose (Sp7^T, Sp245^T, and CC-Nfb-7^T), α-D-Glucose and L-Rhamnose (Sp245^T and CC-Nfb-7^T), and D-Fructose (Az39^T, Sp245^T, and CC-Nfb-7^T), which is common among nitrogen-fixing bacteria (Dhevendaran et al., 2013). This metabolic versatility has facilitated the isolation of *Azospirillum* members from diverse environments, thereby showing their colonization capabilities and interactions with various plant species (Nievas et al., 2023).

In the amino acid test, the strains were able to metabolize L-Alanine, L-Aspartic acid, L-Glutamic acid, and L-Pyroglutamic acid (Az39^T, Sp7^T, Sp245^T and CC-Nfb-7^T), and L-Histidine and L-Serine. (Sp7^T, Sp245^T, and CC-Nfb-7^T). This is in agreement with Gonzalez-Lopez et al. (1995), who reported that free-living nitrogen-fixing bacteria produce and metabolize significant amounts of

amino acids, particularly the genera *Azotobacter* and *Azospirillum.* Carboxylic acid test showed that all strains were able to metabolize γ-amino-butyric acid and acetic acid substrates. This is expected in rhizobacteria since the use of these compounds as substrates allows an increased microbial biomass and activity around the root (Doornbos et al., 2012).

We found that *A. argentinense* Az39 produced higher amounts of PHB in the NYA medium supplemented with NH_4 than in the rich medium LB (1250.9 ± 4.1 AU vs. 131.7 ± 4.5 AU). Martínez et al. (2023) reported recently that *A. brasilense* produces the greatest amount of PHB when malic acid and ammonia chloride are used as the carbon and nitrogen sources, respectively.

Lipid composition analysis following the Sherlock Microbial Identification System Protocol showed that Az39^T and Sp7^T strains have a predominance of medium and long chain saturated fatty acids such as lauric, myristic, palmitic, and stearic acids (41.2% and 36.5%, respectively), and a lower proportion of unsaturated fatty acids (13.4% Az39T, 28.9% Sp7^T), while Sp245^T and CC-Nfb-7^T strains showed an inverse pattern (**Table S2**).

The nitrogen fixation capacity of Az39^T was confirmed and showed higher values than that of *A. baldaniorum* Sp245^T and *A. formosense* CC-Nfb-7^T, while *A. brasilense* Sp7^T was the strongest nitrogen fixer (**Table S1 and Figure 3b**).

The first mechanism proposed for explaining the capacity of *Azospirillum* to promote plant growth was biological nitrogen fixation (Okon et al., 1983). Bashan and Holguin (1997) and Bashan and Levanony (1990) showed that the contribution of nitrogen fixation by *Azospirillum* reached less than 20% of the total N increase in the plant. Reynders and Vlassak (1979) and Tien et al. (1979) were the first to suggest that *Azospirillum* produces indole-3-acetic acid (IAA), which enhances plant growth. In our experiments, Az39^T was a stronger IAA producer than Sp7^T when these strains were grown in LB. However, its capacity was lower with respect to Sp245^T and CC-Nfb-7^T (**Table S1, Figure 4**).

3.2. Genome analysis

A deep characterization of the *A. argentinense* Az39^T genome was carried out in this study. A PFGE analysis confirmed the genome structure previously deduced from the genome sequence (Rivera et al., 2014), clearly revealing 6 replicons (**Figure 2**). According to rRNA and tRNA content, maintenance and replication systems, as well as core essential genes content (Harrison et al., 2010), the different replicons could be classified as a chromosome (biggest replicon), 3 chromids (p1, p2, p4), and 2 plasmids (p3 and p5). Members of the genus *Azospirillum* may have up to 10 megareplicons, including the chromosome, plasmids, and chromids (Maroniche et al., 2016). Moreover, the genome of *A. baldaniorum* Sp245, composed of 7 replicons, was also shown to harbor 3 chromids (p1, p2, p4) of similar sizes to those of *A. argentinense* Az39^T (Winsiewski-Dyé et al., 2011).

Insertion Figure 2

Details on Az39^T genome components are provided in **Table 1**. The mean G+C content is 68.6%, varying from 65.6 to 69.5% depending on the replicon. Eight rDNA and 87 tRNA loci were detected almost exclusively in the chromosome and chromids, except for one tRNA locus, located in plasmid p5.

Insertion Table 1

The search for mobile genetic elements (MGE) in the genome revealed the presence of several simple and composite transposons scattered across the genome (**Table 1**), including the one reported to carry out denitrification functions (Jang et al., 2019) in the replicon p2, and one associated to arsenic tolerance genes (Vezza et al., 2020) in p3 (**Figure S2**). Prophage signatures were only detected in certain replicons (**Table 1**), and some of them overlapped with transposons (**Figure S2**). For example, the two prophages detected in the 163 kb plasmid p5, which is an equivalent to the polysaccharide

synthesis gene-rich plasmid pRhico of *A. brasilense* Sp7 (Vanbleu et al., 2004), were close to each other and overlapped with a composite transposon (**Figure S2**). This is in line with previous findings reporting the presence of bacteriophage genes in pRhico (Boyer et al., 2008), which can induce genomic rearrangements with other replicons (Katsy and Petrova, 2015).

Regarding CRISPR-Cas components, *cas5* (ABAZ39_18645) and *cas8* (ABAZ39_18650) genes of the class 1 subtype I-C (Nidhi et al., 2021) were found encoded immediately downstream of a transposon in replicon p1. However, sequence comparison with other *Azospirillum* Cas proteins indicate that Az39 Cas8c C-terminal domain is disrupted by the transposon insertion (**Figure S2**), raising the question whether Az39 CRIPSR-Cas system is fully functional.

Most *Azospirillum* genomes characterized to date comprise from 6.33 to 8.1 megabases, with G-C contents varying between 68.2% and 70.7%. They have multiple copies of the 16S rRNA gene, which are heterogeneous within a single genome (Maroniche et al., 2016). More than 7,000 genes, 7,000 proteins, and 300 pseudogenes have been identified in this genus. *Azospirillum* contains a core genome that codifies 2,328 proteins, representing 30-38% of total proteins. These core proteins have mostly (74%) an ancestral origin (Wisniewski-Dyé et al., 2011). The non-ancestral part of core proteins is codified by genes involved in signal transduction, carbohydrate and amino acid metabolism, and transport and adaptability to changing environments (Borland et al., 2015), like the soil and the rhizosphere.

Finally, we compared the number of shared clusters between Az39^T and the type strains of closely *Azospirillum* related species (**Figure S3A**) and between Az39^T and other strains within *A. argentinense* species (**Figure S3B**). *A. argentinense* Az39 shares 4,980 orthologous gene clusters with three closely related species (*A. brasilense* Sp7^T, *A. baldaniorum* Sp245^T, and *A. formosense* CC-Nfb-7T), whereas 19 clusters and 509 singletons are strain-exclusives (**Figure S3A**). The comparison of Az39 ^{T} with five other strains within the *A. argentinense* species (Az19, Rec3, BR11975, MTCC4035, and MTCC4036) revealed that they share 4892 orthologous gene clusters, wuth 2 clusters and 166 singletons strain-exclusive (**Figure S3B**).

3.3. Phenogenomics of plant growth-promotion and rhizosphere lifestyle

3.3.1. Genomic determinants involved in nitrogen metabolism

Nitrogen metabolism, and in particular nitrogen fixation, is an important PGP trait of *Azospirillum* that has been extensively studied (Steenhoudt and Vanderleyden, 2000). Genomic analysis indicated that, as expected, Az39^T carries a nitrogen fixation island in its chromosome, with 55 annotated genes (**Figure 3A**).

Insertion Figure 3

Az39^T showed higher nitrogen fixation values than the type strains of its closely related species *A. baldaniorum* Sp245^T and *A. formosense* CC-Nfb-7^T, while *A. brasilense* Sp7^T was the strongest *in vitro* nitrogen fixer (**Figure 3B**). Since the overall architecture of the *nif* cluster is similar across the genus, the differences in nitrogen fixation observed experimentally are probably due to differences in regulation mechanisms at gene expression or nitrogenase activity levels.

All the genes known to be required for ammonia and nitrate assimilation (de Souza and de Oliveira Pedrosa, 2015) were identified in the Az39^T genome (**Table S3**). Regarding denitrification, two different gene clusters coding for periplasmic nitrate reductase complexes were detected, while the rest of the enzymatic functions (*i.e.* NIR, NOR, and NOS) were found to be coded in single loci. However, the copper-containing nitrite reductase coding gene *nirK* was predicted as a disrupted pseudogene (**Table S3**) or as coded by two separate ORFs (**Figure 3C**), depending on the annotation pipeline. As it may be observed on **Figure 3C**, this is not a particularity of strain Az39^T; it was also detected in the genome of *A. argentinense* Az19 (Garcia et al., 2020) and other *A. argentinense* strains. The detection of nitrite in the supernatant of Az39^T biofilms grown in the presence of nitrate (unpublished data) suggests that this *nirK* genetic setup is functional.

3.3.2. Phytohormones biosynthetic genes

3.3.2.1. Auxins

The production of auxins is a well-characterized plant growth-promoting mechanism in the *A. brasilense* species complex (Cassan et al., 2014). The main auxin produced by azospirilla is indole-3-acetic acid (IAA), which is synthesized by the indole 3-pyruvic acid pathway (IPyA) (Steenhoudt and Vanderleyden, 2000). Az39^T genome contains genes coding for indole-3-pyruvate decarboxylase (*ipdC*), histidinol-phosphate aminotransferase (*hisC*), and indole-3-acetaldehyde dehydrogenase (*aldA*) (**Figure 4** and **Table S3**), all of which have been experimentally proven to be involved in IAA synthesis (Castro-Guerrero et al., 2012; Costacurta et al., 1994; Xie et al., 2005). Although IAA synthesis by the indole-3-acetonitrile (IAN) pathway has not been experimentally proven in *Azospirillum*, a nitrilase-coding gene *nitA* is present in Az39^T genome (**Table S3**). According to the comparative genomic analysis of the *ipdC* loci carried out (**Figure 4A**), there is no major divergence in the *ipdC* coding sequence between *A. argentinese* Az39^T and related species (unpublished data). However, the *iaaC* gene, located downstream of *ipdC* in *A. baldaniorum* Sp245^T genome and known to code for a repressor of IAA biosynthesis (Malhotra and Srivastava, 2008; Vande Broek et al., 2005), was absent from Az39^T and the other strains (**Figure 4A**). To further understand the role of this gene, we tested the impact of *iaaC* absence on IAA production using two different culture media: the minimal medium MMAB supplemented with L-tryptophan (L-Trp) and LB. All strains produced similar levels of IAA in the former (unpublished results), but IAA synthesis was higher in *A. baldaniorum* Sp245^T and CC-Nfb-7^T cultures than in other strains in LB medium (**Figure 4B**). This higher IAA level might be attributed to the presence of unknown *ipdC*-inducing compounds in this complex medium.

Nevertheless, the results demonstrated that, under our tested conditions, *iaaC* presence has no consequence on IAA levels. We next performed a more detailed analysis of the *ipdC* promoter that unveiled striking differences between strains. Both Az39^T and Sp7^T promoters contained 150 bp and

23 bp deletions, respectively (**Figure 4C**), that disturbed the inverted repeat motif present between -58 and -38 positions, required for the normal expression of *ipdC* and essential for IAA-mediated inducibility (Vande Broek et al., 2005). Moreover, the 150 bp deletion in $Sp7^T$ promoter has already been shown to negatively impact *ipdC* expression (Rothballer et al., 2005). Thus, taken together, the results indicate that *A. argentinense* $Az39^T$ has a lower capacity for IAA production in a rich medium than other related species (*i.e.*, *A. baldaniorum* and *A. formosense*), possibly due to a 23 bp deletion in the *ipdC* promoter that interferes with normal IAA synthesis induction.

In a previous paper, Rivera et al. (2018) confirmed that $Az39^T$ produces IAA only in the presence of L-Trp, being unable to degrade auxins, to conjugate IAA with sugars or L-amino acids, and to hydrolyze such conjugates to release free IAA. They found that bacterial growth and/or IAA biosynthesis were inhibited by the presence of several L-amino acids, probably by both diversion of the cellular metabolism and *ipd*C gene expression regulation. At the plant growth promotion level, recent results suggest that root architecture in *A. thaliana* inoculated with $Az39^T$ do not depend exclusively on root IAA, as this bacterium induces root changes through both IAA-dependent and IAA-independent mechanisms (Mora et al., 2023). In relation to the IAA-dependent mechanisms, the bacterial production of this molecule generates significant changes in the root morphology such as the reduction of the main root length, increase in the number and length of lateral roots, and increase in the length of root hairs (Mora et al., 2023).

3.3.2.2. Other phytohormones

It has been previously reported that *A. argentinense* $Az39^T$ is able to secrete cytokinins (CKs) when cultured *in vitro* (Perrig et al., 2007). The analysis of $Az39^T$ genome showed the presence of *miaA* and *miaB* genes (**Table S3**), which participate in the biosynthesis of methylthiolated CKs (2MeS-CKs) through the tRNA degradation pathway (Gibb et al., 2020). Experimental confirmation is still needed to establish if *A. argentinense* $Az39^T$ produces CKs in this way or by other yet-uncharacterized

pathway. Notably, *miaA* has been pointed out as responsible for the cytokinin-dependent response of plants to *Pseudomonas fluorescens* G20-18 inoculation (Grosskinsky et al., 2016).

Other phytohormones that have been identified in *A. argentinense* Az39^T culture supernatant include abscisic acid (ABA) and ethylene (ET) (Perrig et al., 2007). The genetic basis of ABA biosynthesis in bacteria is still unknown, and thus was not analyzed. On the other hand, there are two ET biosynthesis pathways described in microbes. The first one, first described in *Pseudomonas* but later found to be present in other bacterial groups, involves the activity of an ethylene-forming enzyme (EFE) (Eckert et al., 2014). This enzyme could not be found encoded in the Az39^T genome. The second source for ET biosynthesis in microbes is 2-keto-4-methylthiobutyric acid (KMBA), which is produced from L-methionine by a NADH:Fe(III)EDTA oxidoreductase (Shipston and Bunch, 1989). Although this enzyme was not predicted in the Az39^T genome, we detected an acireductone dioxygenase gene (**Table S3**) that could theoretically produce KMBA from 1,2-dihydroxy-3-keto-5-methylthiopentene (DHK-MTPene) through the methionine salvage pathway, which, in turn, would be transformed into ET by light (Shipston and Bunch, 1989). Since no genes for DHK-MTPene synthesis were detected, experimental evidence will be required to confirm this hypothesis.

Polyamines (PA) are polycationic compounds involved in plant growth regulation, development, and stress mitigation. The most common PA produced by bacteria are putrescine and spermidine, whereas cadaverine is less abundant (Dunn and Becerra-Rivera, 2023). Indeed, the presence of PA (*i.e.*, putrescine, spermidine, spermine, and cadaverine) was experimentally detected in culture supernatants of *A. argentinense* Az39^T (Perrig et al., 2007). The finding of cadaverine production during Az39^T-rice interaction suggests that this PA may be involved in plant growth promotion and abiotic stress mitigation (Cassán et al., 2009). In accordance with this experimental evidence, genes for putrescine, spermidine, homospermidine, and cadaverine biosynthesis were identified in the *A. argentinense* Az39^T genome (**Table S3**).

3.3.3. Rhizospheric lifestyle genes

Azospirillum rhizospheric and endophytic lifestyles are governed by several mechanisms, leading to efficient niche colonization (Nievas et al., 2023). These mechanisms include biofilm formation, motility, chemotaxis, phytohormone and other signaling molecules production, and cell-to-cell communication, likely involved in regulating *Azospirillum* interactions with the surrounding microbial community.

3.3.3.1. Motility

Thanks to a functional flagellar system, *A. argentinense* Az39^T can swim and swarm in liquid and semisolid media, respectively (Cassán et al., 2021). However, the importance of its flagellar system is not limited to motility: it was recently demonstrated that flagellin of Az39^T is a key factor mediating root growth promotion in *Arabidopsis thaliana* (Mora et al., 2023). We identified in the Az39^T genome five gene clusters predicted to encode flagellar components, and an orphan chromosomal gene coding for flagellin.

Extensive gene duplications in key flagellum components, including stators, basal body, hook, and filament proteins, were detected in this strain (**Table S3 and Figure 5**). This agrees with the ability of closely related *Azospirillum* strains to synthesize one polar and several lateral flagella (Filip'echeva et al., 2018; Wang et al., 2001). Likewise, several genes coding for putative regulators of flagellar synthesis were found in these cluster (**Figure 5**), including the three component system CtrA-CckA-ChpT, which could function as the master regulator of a flagellar system (Smith and Hoover, 2009), and the class II and III regulators FlbD-FliX and FlbT-FlaF (**Table S3**), which may control the synthesis of flagellar systems in a similar way as they do in *Caulobacter crescentus* and *Bradyrhizobium diazoefficiens*, respectively (Dardis et al., 2021; Mangan et al., 1999; Mongiardini et al., 2017; Muir et al., 2001).

The FlbT-FlaF regulator pair (ABAZ39_30550/5) of the cluster IV (**Figure 5**) is a strong candidate to participate in controlling the lateral system synthesis, considering its link to the flagellin gene ABAZ39_30545. This gene is homologous to *laf1* of *A. brasilense* Sp7, already shown to be involved

in lateral flagellar filament formation (Moens et al., 1995). However, cross-regulation between the polar and lateral systems cannot be ruled out, as observed in *A. brasilense* Sp7 and *A. baldaniorum* Sp245 (Filip'echeva et al., 2018; Wang et al., 2001). Further studies are necessary to decipher how these regulators work in *A. argentinense* Az39^T.

As already mentioned, an IAA-independent mechanism related with *A. thaliana* root growth promotion was recently detected in Az39^T and attributed to Az39^T flagellin, which induced an increased number of root hairs and root surface (Mora et al., 2023). In line with these concepts and considering that polar-flagellum flagellin from *A. argentinense* also behaves as a microbe-associated molecular pattern (MAMP) (Elías et al., 2021), it is very likely that flagellin from Az39^T could be involved in the signaling processes resulting in root architecture modification by the increase in the production of root hairs, in addition to phytohormone-mediated mechanisms.

3.3.3.2. Secretion systems

Rhizosphere colonization by PGPR often relies on the secretion of effectors to the extracellular space or into neighboring competitors, or host cells (Lucke et al., 2020). The type I secretion system (T1SS), one of several secretion systems described in Gram-negative bacteria, has been shown to be important for host colonization. This secretion system consists of ABC transporters that translocate effectors, such as virulence or biofilm modulating-factors, out of the cell (Lucke et al., 2020).

Genome annotation predicted that Az39^T harbor several T1SS loci, including hemolysin, adhesin, and hemophore-type complexes (**Table S3**), and only one of them (hemolysin type) was associated to a transposable element (**Figure S2**). Besides, two tight-adherence (Tad) gene clusters were predicted within the Az39^T genome (**Table S3**). The Tad pilus apparatus encoded by these proteins, which are a special form of type II secretion system (T2SS), was considered an important feature for adhesion and biofilm formation (Wisniewski-Dyé et al., 2011). In addition, putative autotransporter proteins of the class a and b type V secretion systems (T5aSS and T5bSS, respectively) were detected (**Table**

S3). These proteins might also be relevant during surface colonization since they contain domains with homology to pertactin, FHA, and ShdA adhesins (Meuskens et al., 2019).

Type VI secretion systems (T6SS) are molecular machineries used to inject effectors into neighboring prey cells. Two gene clusters are present in chromid p1 (T6SS1) and plasmid p3 (T6SS2) of *A. argentinense* Az39^T (**Table S3**). The cluster T6SS1 was recently characterized and shown to be important for attachment to the microalga *Chlorella sorokiniana*. (Cassán et al., 2021). A mutant of Az39^T, defective in T6SS1, showed impaired attachment to eukaryotic cells and this reduction was correlated to the reduced production of metabolites by the microalga (Cassán et al., 2021).

3.3.3.3. Quorum mechanisms

The quorum sensing (QS) mechanism is used by a vast group of rhizobacteria as a communication strategy to orchestrate their successful establishment in the rhizosphere (Zhuang et al., 2023). *A. argentinense* Az39^T capacity for QS has been already assessed (Gualpa et al., 2019). Despite the genome of this strain does not carry genes for QS signals synthesis, a *luxR* orphan gene in chromid p4 was identified (**Table S3**), opening the hypothesis that this strain could detect via an acyl-homoserine-lactone (AHL)-binding domain (PFAM03472) exogenous AHL signals produced by other bacteria, and trigger a response to their presence. In addition, it was demonstrated that Az39^T exerted a strong quorum quenching (QQ) activity. Two candidate genes responsible for this activity were identified in Az39^T genome (**Table S3)**, which are predicted to code for a N-acyl-homoserine lactone acylase and an aliphatic amidase (Gualpa et al., 2019). Nevertheless, the relevance of QS signals for *A. argentinense* Az39^T rhizosphere competence has not been established yet. But considering the evidence available for other *Azospirillum* species (Boyer et al., 2008; Fukami et al., 2018), it could likely modulate Az39^T traits important for root colonization.

3.4. Metadata analysis of *A. argentinense* Az39^T agronomic performance

3.4.1. Cereal inoculation

A wide range of conditions accounting for the most frequent seasonal and site-specific variability sources in the pampas region (Argentina) were covered in our metadata analysis. Wheat grain production varied from as low as 850 kg ha^{-1} to as high as 8990 kg ha^{-1} maize yield ranged from 2020 kg ha^{-1} to 18,654 kg ha^{-1}. In both crops, seed application of $Az39^T$ increased the harvested grain production (**Figure S4**).

In average, inoculated wheat and maize yielded 231 kg ha^{-1} and 432 kg ha^{-1} over their controls without inoculation ($p<0.01$). These values are equivalent to 5.4% and 4.5% of the wheat and maize attainable yields, respectively.

As **Table 2** shows, the grain yield increment in wheat due to on-seed application of $Az39^T$ was significant across the complete range of the observed attainable yield distribution. The number of sites with positive response to inoculation, defined as those with a grain yield increments of >50 kg ha^{-1}, was uniform in all the range of studied conditions and averaged 77 % of positive sites. Yield increases due to $Az39^T$ inoculation were significant in all attainable yield categories (**Table 2**).

A different response pattern was observed in maize. The probability of finding differences due to $Az39^T$ introduction was not significant at both extremes of yield data distribution (<5% and >95% of the attainable yield), while in the range from 5107 to 13189 kg ha^{-1}, which corresponds to the central 90%, grain yields increases were significant (**Table 2**).

The lowest proportion of sites with a positive response (56%) was observed for observed yields under 5107 kg ha^{-1}, with a mean response rate of 2.9%. In sites with greater grain yields up to 13,189, the mean response rate was 4.9%.

Both wheat and maize crops showed higher net yield increases due to $Az39^T$ inoculation in the more productive sites (**Table 2**). In light of these findings and based on the diverse mechanisms that $Az39^T$ can deploy to favor the maintenance of optimal plant growth, we interpret that the introduction of this microbe allowed an improved use efficiency of key resources like water (Alvarez and Díaz-Zorita, 2020) and nutrients (Saubidet et al., 2002; Díaz-Zorita and Fernandez Canigia, 2009) by these crops.

However, at the lowest productive sites, the occurrence of severe limitations for plant growth tend to reduce the beneficial effects of Az39^T introduction, resulting in less consistent responses.

It has been documented thatAz39^T inoculation improved early growth of wheat plants, shoot growth, and tillering (Dobbelaere et al., 2001) favoring the total shoot biomass production and the formation of fertile tillers. Also, it was reported that these yield components were directly and strongly related with wheat grain yields (Frederick and Bauer, 1999). In this regard, the less consistent responses to Az39^T inoculation in maize crops could be explained by the usual occurrence of climate restrictions, mainly water scarcity coinciding with high temperature periods during summer in the pampas region, which is expected to impact on the availability of key resources for grain formation. Low maize yields in the pampas were found to be closely related to severe water and temperature stresses at flowering, and differences in plant early growth had limited positive, and sometimes even negative effects on grain production (Maddonni, 2012).

Most of *Azospirillum* sp. contributions to plant growth refer to increased shoot and root growth, with minimal mitigating effects on heat or intensive water stresses. Moreover, at those sites and studies where the abundance of growth resources and minimal restrictions allowed optimal maize production, close to the maximum attainable yields (>13189 kg ha^{-1}), the contribution of introducing this microbe was limited. Summing up, the results of this meta-analysis suggest that within the range of the more frequent production levels of representative cereals cultivated in the pampas region in Argentina such as wheat and maize, the contribution of Az39^T incorporation to grain yield cannot be predicted with certainty since yield is a multifactorial-dependent outcome highly affected by eventual abiotic stresses.

3.4.2. Soybean co-inoculation

It was reported that the practice of legume co-inoculation with rhizobia and *Azospirillum* could improve plant performance and the establishment of the rhizobia-legume symbiosis under both controlled and field conditions (Remans et al., 2008; Puente et al., 2018, 2019). Hungria et al. (2013)

and Nogueira et al. (2018) reported increases in soybean grain yield of over 15% and 200 $kg.ha^{-1}$, respectively, through a combined *Bradyrhizobium* and *Azospirillum* inoculation. Studies on soybean co-inoculation in Argentina and Brazil also found increases in the nodulation rate of over 5% (Hungria et al., 2015) and 10% (Benintende et al., 2010; Morla et al., 2019). Torres et al. (2022) evaluated the interactions between *B. japonicum* E109 and *A. argentinense* $Az39^T$ and elucidated their impact on the *Bradyrhizobium*-soybean symbiosis, plant growth, and crop yield. They found an improved ability of E109 to survive on soybean seeds, with a cell recovery of 25% and 10% after 4 hours and 6 days post-inoculation, respectively. As a result of the greater bacterial survival, several symbiosis parameters including nodule number, size, and biomass as well as nodulation percentage also significantly increased. In agreement with these observations, soybean grain yield under field conditions showed 17.3% greater than single E109 inoculation. The physiological changes observed in *B. japonicum*-E109 behavior and their impact on the soybean symbiosis depended, at least partly, on the $Az39^T$ capacity of both to release IAA into the culture medium, thus increasing the catabolism and production of exopolysaccharides by E109 (Torres et al., 2018, 2021), and to increase the number of root hairs, as previously proposed (Molla et al., 2000).

3.5. Impact on plant microbiome

A. argentinense $Az39^T$ is the recommended strain in Argentina for the formulation of bioproducts, which are widely employed at the field level to inoculate non-legume crops and to co-inoculate legumes. Despite its extensive use, the capacity of this bacterium to colonize the rhizosphere and its impact on the plant microbiome is scarcely documented.

To assess whether $Az39^T$ inoculation modifies the bacterial microbiota associated with the maize rhizosphere, Coniglio et al. (2022) conducted a high-throughput analysis of the 16S rRNA gene sequences. Their findings confirmed that the three most abundant genera associated with $Az39^T$ inoculation were *Burkholderia*, *Massilia*, and *Sphingobium*. An increase in the relative abundance of some members of the *Rhizobiales* order (*Mesorhizobium* and *Rhizobium*) was observed in inoculated

plants. The co-occurrence networks indicated a positive interaction between *Azospirillum* and *Pseudomonas*, along with a negative correlation with *Hyphomicrobium*. The aforementioned study constitutes the first exploration of *A. argentinense* Az39^T capacity to colonize the rhizosphere and its effects on the plant microbiome, suggesting a positive relationship between *Azospirillum* inoculation and certain PGPR bacterial genera.

Conclusions

It is known that members of the bacterial genus *Azospirillum* can promote the growth of a great variety of plants, an ability harnessed by the industry to create bioproducts aimed at enhancing the yield of economically relevant crops. *A. argentinense* Az39^T (formerly *A. brasilense* Az39) was isolated in 1982 from surface-sterilized roots of wheat seedlings in Marcos Juarez, Argentina, and gradually became the PGPR most usually utilized to formulate non-leguminous inoculants in South America due to its ability to increase maize and wheat yield of under agronomic conditions. In this work, a deep phenotypic and genotypic profiling of this strain was undertaken.

Genome analysis of Az39^T confirmed the presence of six replicons that could be clearly qualified as 1 chromosome, 3 chromids, and 2 plasmids; these replicons altogether conferred the capacity for coding 6,311 proteins. Genome dynamics was also deduced from the presence of numerous transposons (simple and composite), several prophage signatures, and an incomplete cas5-8 CRISPR-Cas cluster. The presence of genes relevant for an efficient rhizosphere colonization was also evidenced, this included five flagellum-coding clusters, two Tad pilus, several T1SS, and two T6SS. As for nitrogen metabolism, genes for ammonia and nitrate assimilation, a full denitrification pathway, and a nitrogen fixation island were identified. The nitrogen fixation capacity of Az39^T proved to be higher than that of Sp7^T and CC-Nfb-7^T but lower than that of Sp245^T.

Az39^T can produce several compounds relevant for plant growth promotion such as cytokinins, polyamines, and, particularly, auxins, that together with bacterial flagellin seem to behave as key effectors for root growth promotion and rhizobia-legume symbiosis establishment. Metabolic tests

showed that Az39^T can grow with numerous compounds as sole carbon sources, can reduce nitrate and nitrite, and possess several enzymatic and assimilatory capacities. The high metabolic versatility, coupled to the ability to produce high amounts of PHB (when malic acid and ammonia chloride are provided as carbon and nitrogen sources, respectively) and to form biofilm, may explain, at least in part, its proficiency for rhizosphere colonization.

The metadata analysis of the agronomic performance of this bacterium revealed that seed inoculation with Az39^T provide better growth conditions for dryland summer and winter cereals as well as legumes co-inoculated with bradyrhizobia, increasing their efficiency for utilizing the applied nutrients and sequestering soil carbon. The information here presented will hopefully serve as a guide for further exploration of the plant growth-promoting potential of *A. argentinense* Az39^T; this will be facilitated by the recent development of molecular tools for the mutagenesis of this strain (Cassán et al., 2021; Mora et al., 2023) and specific PCR primers, which allow the accurate identification, traceability, and monitoring of Az39^T establishment in the rhizosphere (Coniglio et al., 2020; Nievas et al., 2023).

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Availability of data and material

All DNA sequencing data are available at the NCBI database (genomes). All data can be found within the manuscript and its supporting materials.

Conflicts of interest

The authors report no conflicts of interest.

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Figure Legends

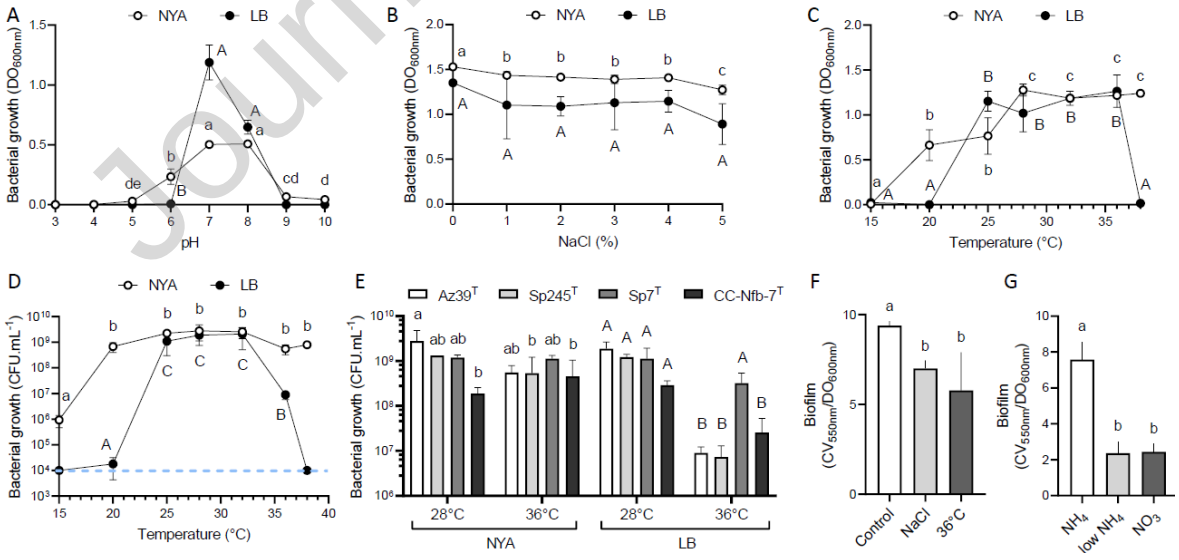

Figure 1. *A. argentinense* Az39^T growth in different physicochemical conditions. Az39^T was cultured in NYA and LB at different initial pH values (A), sodium chloride concentrations (B), and

temperatures (C) until the stationary phase (~72 h), and its growth was estimated by optical density at 600 nm. Temperature and culture medium effects on bacterial multiplication was further determined by the drop plate method, expressing viable cell numbers as $CFU \cdot mL^{-1}$ (D). Using this same method, Az39^T growth at 28 °C and 36 °C was compared with that shown by the related strains *A. baldaniorum* Sp245^T, *A. brasilense* Sp7^T, and *A. formosense* CC-Nfb-7^T grown at the same conditions (E). Biofilm formation was studied in standard NYA medium (control), in the presence of 200 mM NaCl, at 36°C (F), and under different N conditions including a higher C:N ratio and nitrate (instead of NH_4^+) as the N source (G). Different letters indicate significant differences according to ANOVA plus Tukey's multiple comparison test ($p<0.05$). The blue dashed line in (D) denotes the lower threshold for UFC detection.

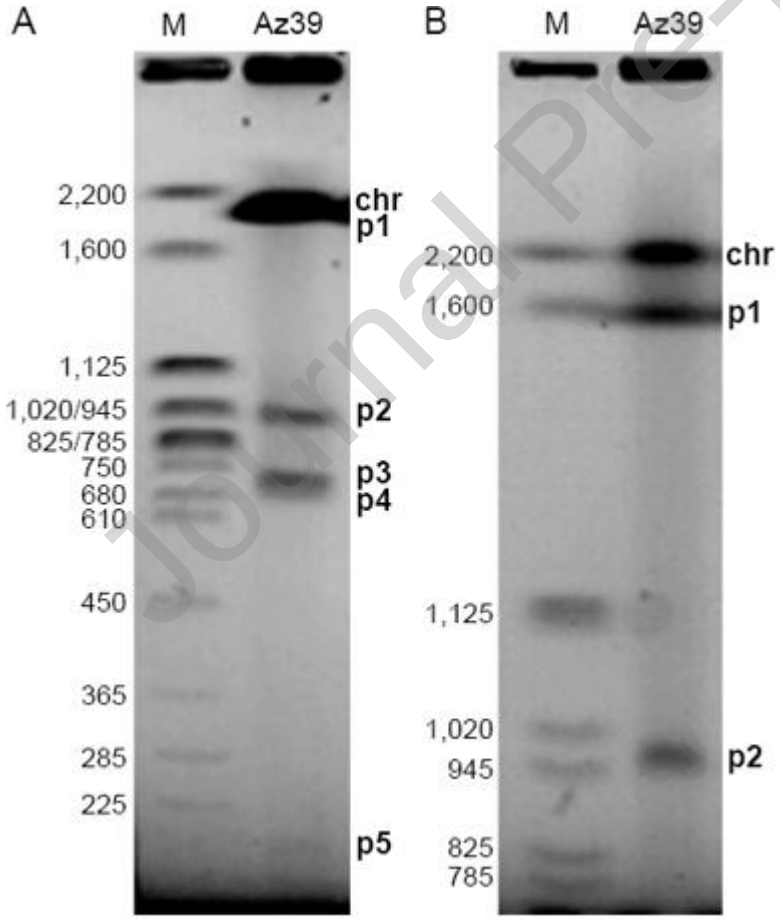

Figure 2. Visualization of Az39^T replicons by PFGE. Undigested DNA of *A. argentinense* Az39^T was analyzed by pulsed-field gel electrophoresis (PFGE) as detailed by Vial et al. (2006). To resolve

all the bands, electrophoresis was performed with classic (A) and long (B) runs. (M), *Saccharomyces cerevisiae* marker.

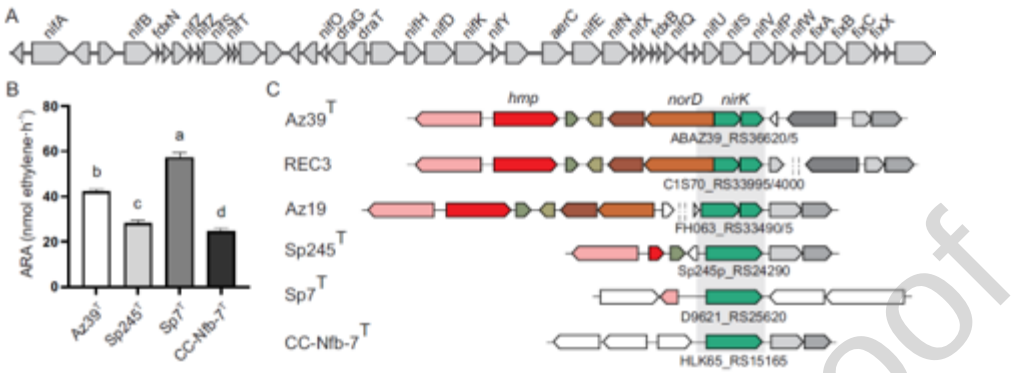

Figure 3. Nitrogen fixation and denitrification in Az39T and related strains. (A) Schematic representation of Az39^T nitrogen fixation genomic island. (B) Nitrogen fixation activity of Az39^T and related species, as measured by the acetylene reduction assay (ARA). One-way ANOVA plus Tukey's test (p<0.05) was used to detect significant differences. (C) Synteny analysis of the nitrite reductase *nirK* gene in *A. argentinense* Az39^T and related species. White symbols indicate unrelated genes; symbols with the same color denote homolog genes. The *nirK* locus identificator is indicated for each strain below the corresponding symbol (green).

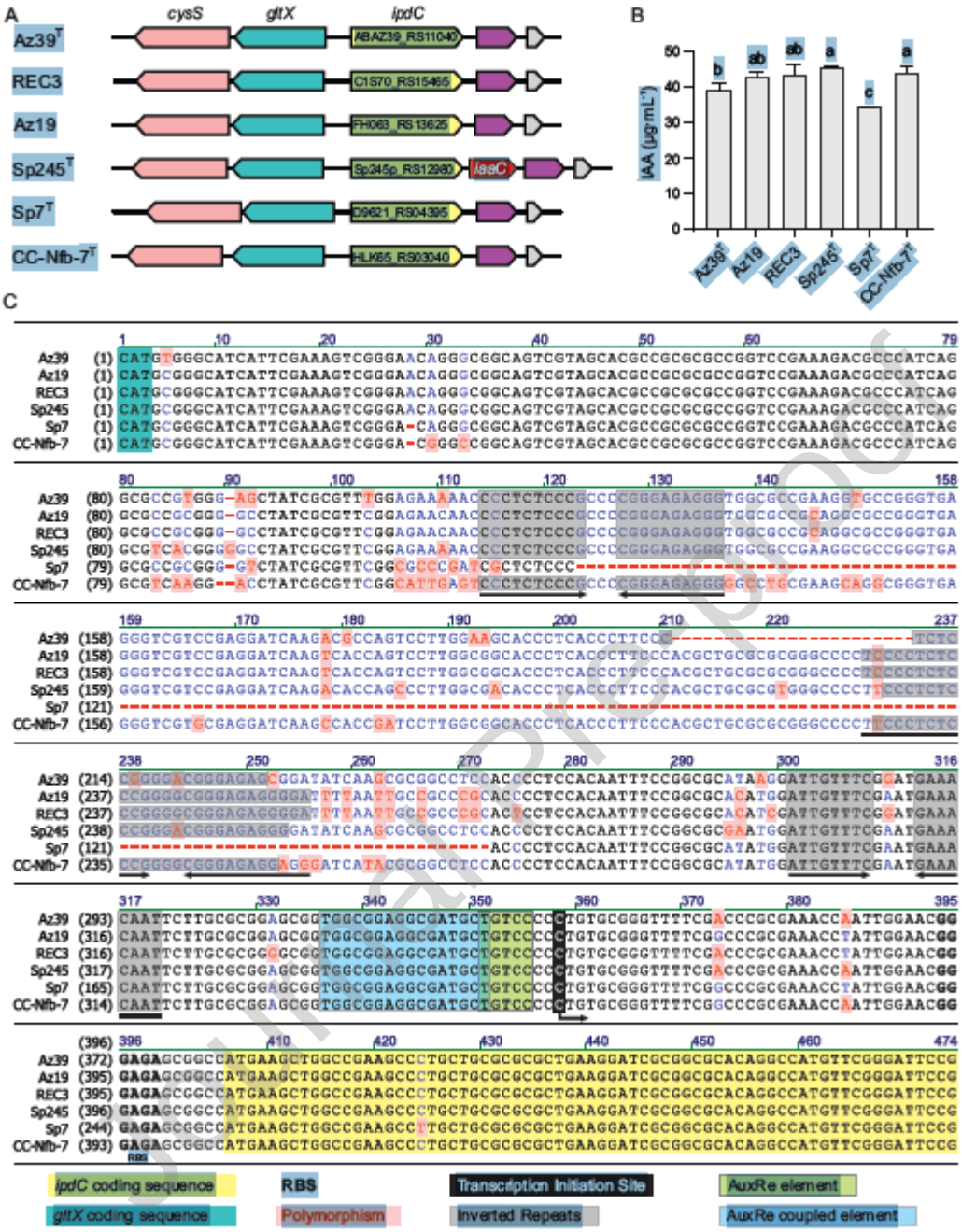

Figure 4. Comparative analysis of the *ipdC* gene and IAA synthesis in Az39^T and related strains.

(A) Synteny of the *ipdC* genomic context. Locus identifiers are detailed inside each *ipdC* symbol. (B) IAA production in LB medium measured by HPLC in the culture supernatants. Two-way ANOVA plus Tukey's test (p<0.05) was used to detect significant differences. C). DNA multiple alignment of the *ipdC* promoter and upstream regulatory region. Relevant features in the sequence are highlighted or bolded and indicated below.

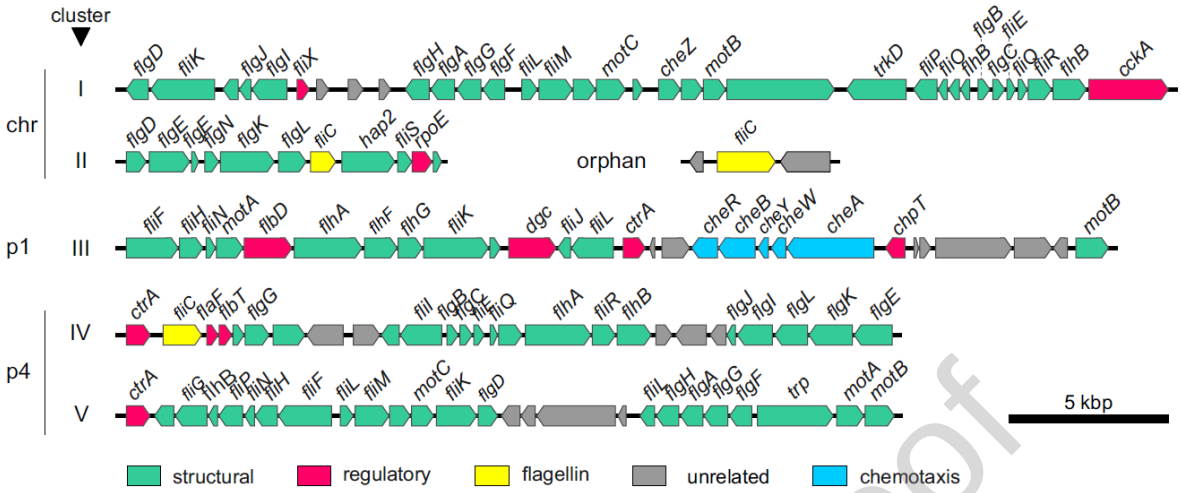

Figure 5. Flagellar gene clusters predicted in the Az39^T genome. Schematic representation of the five clusters and the orphan gene predicted to encode flagellar components. Color pattern: structural genes (green), regulator proteins (red), flagellins (yellow), chemotaxis (light blue), unrelated genes (grey). The genomic distribution of these clusters is indicated on the left.

Tables headers

Table 1. *A. argentinense* Az39^T genome characteristics.

Table 2. Metadata analysis of *A. argentinense* Az39^T agronomic performance: main results. The analysis covered 638 wheat and 318 maize field trials conducted in the pampas region, Argentina, along 18 and 12 growing seasons, respectively. To refine our analysis, we established five observed yield categories (mean values of percentile ranges); most sites evaluated corresponded to the three central categories. Positive sites: sites with a grain yield increment of inoculated plots over control plots >50 kg^{-1} ha^{-1}. Increment: net increment over the control. Response (%). LSD: least significant difference test.

Table 1. *A. argentinense* Az39^T genome characteristics

Replicon	Chromosome	Chromid p1	Chromid p2	Plasmid p3	Chromid p4	Plasmid p5	Total
Size (bp)	3,064,393	1,901,707	933,960	686,487	641,573	163,159	7,391,279
G+C content (%)	68.4	68.4	68.6	69.5	69.2	65.6	68.6
CDS	2,763	1,605	744	534	557	108	6,311
Protein-coding	2,613,012	1,618,308	813,561	612,081	552,601	133,137	6,342,700
Coding %	85.3	85.1	87.1	89.2	86.1	81.6	85.8
rRNAs	2	4	1*	-	2	-	8
tRNAs	44	26	4	-	12	1	87
Pseudogenes	26	20	14	7	6	3	76
Transposons	5	7	1	3	1	2	19
Prophages	4	2	0	2	0	2	10
NCBI accession	CP007793	CP007794	CP007795	CP007796	CP007797	CP007798	

*Incomplete operon

Table 2: Mean values of percentile ranges in the observed wheat and maize grain net yields from control and *A. argentinense* Az39^{T} inoculated from 638 and 318 field sites performed in the pampas region (Argentina) respectively. Positive sites: sites with grain yield increment > 50 kg ha^{-1}, LSD: Least significant difference test.

wheat grain yield	**Range of the observed distribution (%)**				
	0- 5	**5-25**	**25-75**	**75-95**	**95-100**
Attainable yield (kg ha^{-1})	< 1904	1904-3451	3451-5141	5141-6336	6336-8990
Studied sites	31	128	319	128	32
Positive sites	21	91	223	97	24
Increment (kg ha^{-1})	149	175	202	328	446
Response (%)	9.1	4.6	6.2	5.6	5.9
LSD, p-value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
maize grain yield	**Range of the observed distribution (%)**				
	0- 5	**5-25**	**25-75**	**75-95**	**95-100**
Attainable yield (kg ha^{-1})	< 5107	5107-7644	7644-11091	11091-13189	13189-18654
Studied sites	16	63	159	63	17
Positive sites	9	47	128	48	12
Increment (kg ha^{-1})	106	383	460	536	281
Response (%)	2.9	4.8	5.5	4.4	-0.8
LSD, p-value	0.370	0.01	< 0.001	< 0.001	0.320

Author contributions

All authors contributed substantially to the discussion of the content, writing the article and editing the manuscript before submission.

Guillermo Maroniche; Mariana Puente; Julia García; Elias Mongiardini, Anahí Coniglio; Sofía Nievas, María Labarthe; Florence Wisniewski-Dyé F and Martín Díaz-Zorita contributed with conceptualization, methodology, formal analysis and investigation

Enrique Rodriguez Cáceres and Fabricio Cassán contributed with writing- review and editing, as well as visualization.

Guillermo Maroniche, Mariana Puente and Fabricio Cassan contributed with project administration, resources, supervision and funding acquisition.