Antonie van Leeuwenhoek
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The *ipdC*, *hisC1* and *hisC2* genes involved in indole-3-acetic production used as alternative phylogenetic markers in *Azospirillum brasilense*

Saúl Jijón-Moreno · Cynthia Marcos-Jiménez · Raúl O. Pedraza · Alberto Ramírez-Mata · I. García de Salamone · Ana Fernández-Scavino · Claudia A. Vásquez-Hernández · Lucia Soto-Urzúa · Beatriz E. Baca

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Abstract Plant growth-promoting bacteria of the genus *Azospirillum* are present in the rhizosphere and as endophytes of many crops. In this research we studied 40 *Azospirillum* strains isolated from different plants and geographic regions. They were first characterized by 16S rDNA restriction analysis, and their phylogenetic position was established by sequencing the genes 16S rDNA, *ipdC*, *hisC1*, and *hisC2*. The latter three genes are involved in the indole-3-pyruvic acid (IPyA) biosynthesis pathway of indole-3-acetic acid (IAA). Furthermore, the suitability of the 16S-23S rDNA intergenic spacer sequence (IGS) for the differentiation of closely related *Azospirillum* taxa and development of PCR protocols allows for specific detection of strains. The IGS-RFLP analysis enabled intraspecies differentiation, particularly of *Azospirillum brasilense* and *Azospirillum lipoferum* strains. Results demonstrated that the *ipdC*, *hisC1*, and *hisC2* genes are highly conserved in all the assessed *A. brasilense* isolates, suggesting that these genes can be used as an alternative phylogenetic marker. In addition, IAA production determined by HPLC ranged from 0.17 to 98.2 μg mg^{-1} protein. Southern hybridization with the *A. brasilense ipdC* gene probe did not show, a hybridization signal with *A. lipoferum*, *Azospirillum amazonense*, *Azospirillum halopreferans* and *Azospirillum irakense* genomic DNA. This suggests that these species produce IAA by other pathways. Because IAA is mainly synthesized via the IPyA pathway in *A. brasilense* strains, a species that is used worldwide in agriculture, the identification of *ipdC*, *hisC1*, and *hisC2* genes by PCR may be suitable for selecting exploitable strains.

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S. Jijón-Moreno · C. Marcos-Jiménez · A. Ramírez-Mata · C. A. Vásquez-Hernández · L. Soto-Urzúa · B. E. Baca (✉)
Centro de Investigaciones en Ciencias Microbiológicas, Benemérita Universidad Autónoma de Puebla (BUAP), 72000 Puebla, Puebla, Mexico
e-mail: beatriz.baca@correo.buap.mx
URL: http://orcid.org/0000-0002-4699-7630

R. O. Pedraza
Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán, San Miguel de Tucumán, Argentina

I. G. de Salamone
Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

A. Fernández-Scavino
Facultad de Química Universidad de la República, 11100 Montevideo, Uruguay

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Introduction

The term rhizosphere is used to describe the portion of soil in which growth of microorganisms is influenced by the presence of the root system (Hartmann et al. 2008). The interactions between plants and microbes are essential for plant health and growth and should be considered when combining high yields with environmentally friendly farm practices. Interactions in the rhizosphere, which is the part of the soil that is highly influenced by roots, are of central importance (Lugtenberg et al. 2013). The composition, abundance and dynamics of the microbial community in the rhizosphere play an important role and may have a positive or negative influence on plant growth. Microbes are essential for the mobilization of plant nutrients and may produce plant growth hormones that are important for plant development (Lugtenberg et al. 2013). Some microorganisms can act as biocontrol agents and protect plants from phytopathogenic bacteria and fungi (Bashan and de Bashan 2010). One of the most studied bacterial genera that is able to promote the growth of several plants of agronomic importance is *Azospirillum* (Baldani et al. 2014). It is included within the plant growth-promoting bacteria (PGPB) group (Bashan and de Bashan 2010). Added to their value as crop inoculants, the potential benefits of PGPB have been used in environmental applications. *Azospirillum* species enhance bioremediation of wastewater treated with microalgae by increasing proliferation and metabolism of the microalgae; hence increasing the effectiveness of the microalgae to clean wastewater better than when used without PGPB (Pérez-García et al. 2011).

There are different mechanisms by which *Azospirillum* can affect plant growth directly, namely fixing atmospheric nitrogen, synthesizing several phytohormones and enzymes, producing nitric oxide and siderophores, and solubilizing mineral nutrients; or indirectly, such as exerting antimicrobial activity, enhancing membrane activity, and by inducting systemic resistance. *Azospirillum* may exhibit more than one of these mechanisms in the same strain. In fact, it has been suggested that the simultaneous and cumulative action of several of these mechanisms explain the beneficial effect observed after inoculation with *Azospirillum*-strains, which has resulted in the "additive hypothesis" (Bashan and de Bashan 2010).

Species of this genus are widely distributed in nature, living in soils of tropical, subtropical and temperate regions. This well-studied PGPB is able to produce indole-3-acetic acid (IAA), gibberellins, cytokinins and abscisic acid and can increase mineral uptake, water status, and growth of plants (Baca and Elmerich 2007; Creus et al. 2004; Bashan et al. 2014). Presently, 18 species have been described for the genus *Azospirillum* (Baldani et al. 2014); however, few of them are reported to exert plant growth-promotion, *Azospirillum brasilense* is the species most frequently used as an inoculant to improve plant yield under field conditions.

A. brasilense synthesizes IAA from tryptophan (Trp) via three pathways: the indole pyruvic acid (IPyA), the tryptamine and the indole acetonitrile pathways (Carreño-López et al. 2000; Spaepen et al. 2007a, b). The best characterized pathway for the conversion of Trp to IAA is the IPyA pathway, in which Trp is transaminated to IPyA via the aromatic amino acid aminotransferases (AATs), subsequently decarboxylated to indole acetaldehyde, and then oxidized to IAA. Two AATs, namely AAT1 and AAT2 have been characterized (Pedraza et al. 2004). The *hisC1* and *hisC2* genes encoding these enzymes have been identified (Castro-Guerrero et al. 2012). The key enzyme in this pathway is phenyl pyruvate decarboxylase (PPDC), which is encoded by the *ipdC* gene (Spaepen et al. 2007a, b; Carreño-López et al. 2000).

Although the 16S rDNA gene is the most widely used, the 16S-23S rDNA intergenic spacer sequence (IGS) region has received increased attention as a target in molecular detection and identification schemes (García-Martínez et al. 1999). In contrast to rDNA genes, which are remarkably well conserved throughout most bacterial species, the IGS region exhibits a large degree of sequence diversity and length variation (García-Martínez et al. 1999). Even within species, the IGS sequence variation may be very high, thus allowing intraspecies strain differentiation, as shown for *Azospirillum* strains (Baudoin et al. 2010).

Even though significant beneficial effects has been demonstrated by this bacterium in laboratory and greenhouse studies (Bashan et al. 2014), it is necessary to perform additional fundamentally research studies to obtain consistent results in field trails. In this study, we identified the *hisC1*, *hisC2*, and *ipdC* genes and determined IAA production in several *Azospirillum* strains, which were isolated from different geographic locations and plants. We proposed that these procedures are useful as genetic markers for identifying *A. brasilense*, a species worldwide used as an inoculant in agriculture.

Materials and methods

Bacterial strains and growth conditions

A. brasilense Sp7 (ATCC 29145^{T}) and Sp245, *Azospirillum lipoferum* Sp59b (ATCC 29707^{T}), *Azospirillum amazonense* Y2 (ATCC 35119^{T}), *A. irakense*, and *A. halopraeferens* were used as references strains; *Pseudomonas stutzeri* A1501 and *Sphingomonas* spp. were used as negative control strains. All other strains tested were isolated as *Azospirillum* strains by phenotypic and biochemical studies (Mascarua-Esparza et al. 1988; Díaz-Zorita and Fernández-Canigia 2009; Di Salvo et al. 2014; Garcia de Salamone et al. 1996; Pedraza et al. 2007; Rariz et al. 2013). The strains were grown in D medium (Nutrient broth 8 g L^{-1}; BD Difco Franklin Lakes NJ), containing 1.15 mM SO_4Mg_2, 0.05 mM $MgCl_2$, 13.4 mM KCl, at pH 6.8. Either Red Congo medium or K-malate medium (Castro-Guerrero et al. 2012) was used to identify and maintain the strains, respectively.

Extraction of DNA and techniques for DNA manipulation

Isolated colonies of *Azospirillum* strains growing in LB* (Luria Broth supplemented with 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$), were lysed with a thermal cellular technique (Pedraza et al. 2007). The DNA obtained for use in PCR reactions was obtained after cell lysis at 95 °C for 10 min (Ausubel et al. 1995; Pedraza et al. 2007). For a more accurate determination, the genomic DNA was also prepared using standard methods (Ausubel et al. 1995). PCR for identifying bacteria from 16S rDNA sequences was performed with the universal 16S rDNA primers 27F and 1492R (Doty et al. 2005). The specific primers for the *Azospirillum* genus designed in this research were rRNA-AzoF and rRNA-AzoR. The reaction mixture consisted of 5 µL of template DNA (5–25 ng) and a 24 µL aliquot of a PCR mix, which contained 1 × buffer (Invitrogen, Carlsbad, CA), 1.25 mM dNTPs, 3.0 mM $MgCl_2$, 5 % DMSO (wt/vol), 250 nM of each primer, and 1 U/25 µL of high-fidelity *Taq* Polymerase (Invitrogen, Carlsbad, CA). The PCR conditions are shown in Table 1. The PCR products were purified using a purification kit (QIAquick, Qiagen, Germany) according to the manufacturer's protocol. The products were checked by electrophoresis in 1 % (wt/vol) agarose gel followed by staining with ethidium bromide.

For Southern hybridization experiments, genomic DNA of *A. lipoferum* Sp59b, *A. lipoferum* USA5b, *A. amazonense* Y1, *A. irakense*, and *A. halopraeferens* was digested with *Eco*RI and *Bgl*II. The digestion products and an internal *ipdC* 1170 bp fragment, which was used as a probe, were blotted onto Hybond N^{+} nylon membranes (Amersham Biosciences, Piscataway, NJ) with a vacuum blotter (Fisher Scientific, Waltham, MA). The DNA was fixed by exposure to a 312 nm transilluminator for 4 min. The pre-hybridization, labeling, purification, and hybridization of the probe were performed as described by Carreño-López et al. (2000).

Amplified ribosomal DNA restriction analysis (ARDRA)

To avoid sequencing identical 16S rDNA genes, 5 µL of the PCR products were digested with the restriction endonuclease *Alu*I (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendation. The restriction reaction was stopped by thermal inactivation at 65 °C for 30 min. Then, 15 µL of the restriction fragment patterns were then analyzed by 3 % agarose gel electrophoresis at 70 V. Strains with an identical ARDRA pattern were considered members of the same Operational Taxonomic Unit (OTU). Representative strains of each OTU were subjected to 16S rDNA gene sequencing. Both DNA strands were sequenced and analyzed to determine their phylogenetic affiliation with described species.

Table 1 Primers used in this study

Primer name	Sequence 5′-3′	Target DNA (product length)	PCR conditions	Reference
Eubacterial 16S rDNA				
27F	GAGAGTTTGATCCTGGCTCAG	16S rRNA (1.5 kb)	95 °C 5 min; 95 °C 1 min; 60 °C 1 min; 72 °C 2 min (35 cycles); 72 °C 5 min	Lane, 1991
1492R	CTACGGCTACCTTGTTACGA			Lane, 1991
rRNA-AzoF	TGAGAAGGGATGCGCAGGCGG	16S rRNA (1.75 kb)	95 °C 5 min; 95 °C 1 min; 72 °C 1 min; 72 °C 2 min (12 cycles); 95 °C 0.5 min; 64 °C 1 min; 72 °C 2 min (22 cycles); 72 °C 5 min	This study
rRNA-AzoR	CCGTGAGAAGGGATGCGCCG			This study
ipdC A. brasilense				
FipdC1	GAGAAGTCGCCGGTCGTCGTCAT	*ipdC* (1170 bp)	95 °C 5 min; 95 °C 1 min; 67 °C 1 min; 72 °C 1.5 min (35 cycles); 72 °C 5 min	This study
RipdC1	CCGCCAGTCGTCCAGGTCATTG			This study
FipdC2	GAAGCTGGCCGAAGCCTTGCTGC	*ipdC* (1597 bp)	95 °C 5 min; 95 °C 1 min; 68 °C 1 min; 72 °C 2 min (35 cycles); 72 °C 5 min	This study
RipdC2	GACGAAGCGGGCCAGCGTGTC			This study
FRegR-2	GTCCGAAAGACGCCCATC	*ipdC* (2139 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 2.5 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 2.5 min (22 cycles); 72 °C 5 min	This study
iaaCR1	TCATAGACGCCGCAGCCCGA			This study
FipdC-int	ACGCAGTTCCAGGTGTTCAA	*ipdC* (2049 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 2 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 2 min (22 cycles); 72 °C 5 min	This study
RiaaC-Stop	TTAGCGGGCCAGCCCGATC			This study
hisC1 A. brasilense and *A. lipoferum*				
FhisC1	GCTGGGACCCGGCAAGAAGG	*hisC1* (961 bp)	95 °C 5 min; 95 °C 1 min; 66 °C 1 min; 72 °C 1 min (35 cycles); 72 °C 5 min	This study
RhisC1	TCC CAG AGC CGC GAC CAG AG			This study
PhisC1-F	GCGCACAAGTACCCTGGCCAG	*hisC1* (582 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 1 min (12 cycles); 95 °C 1 min; 62 °C 1 min; 72 °C 1 min (22 cycles); 72 °C 5 min	This study
PhisC1-R	CAGCGACAGGTCGACCCATGC			This study

Table 1 continued

Primer name	Sequence 5′-3′	Target DNA (product length)	PCR conditions	Reference
hisC1-AzoF	GGTCAAGGAGGTTCTGGACG	*hisC1* (786 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 1 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 1 min (22 cycles); 72 °C 5 min	This study
hisC1-intR	TCGATCATCGCCTCGACATC			This study
hisC1-1-23F	ATGGACCTGCTCAGCCCCCGTCC	*hisC1* (615 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 1 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 1 min (22 cycles); 72 °C 5 min	This study
hisC1-615R	GACGATCTCCGTCCCGTCGCTGTAGT			This study
hisC2 A. brasilense and *A. lipoferum*				
FhisC2	CAACCCGACCGGCACCTACAT	*hisC2* (573 bp)	95 °C 5 min; 95 °C 1 min; 65 °C 1 min; 72 °C 1 min (35 cycles); 72 °C 5 min	This study
RhisC2	GCCTCCGTGCCGATGGTG			This study
FhisC2-Ab	CGCGCCGTAGGTCTGGTAGG	*hisC2* (3134 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 3 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 3 min (22 cycles); 72 °C 5 min	This study
R720-2	CACATCGTCACCACCTTGTC			This study
FhisC2-Ab	CGCGCCGTAGGTCTGGTAGG	*hisC2* (2337 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 2.5 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 2.5 min (22 cycles); 72 °C 5 min	This study
R1hisC2-Stop	TTACGCCGCCAGGAAGTCCTTC			This study
F2.2metX	CCGACAGGTAGGTGATGTGCG	*hisC2* (3708 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 3.5 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 3.5 min (22 cycles); 72 °C 5 min	This study
R720-2	CACATCGTCACCACCTTGTC			This study
Fcm-Azl1	TCCATCTGGTCGAGGTCGCCG	*hisC2* (2066 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 2 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 2 min (22 cycles); 72 °C 5 min	This study
RcodA-Azl1	GCAACGCCGATCTGGTGGTTC			This study

Table 1 continued

Primer name	Sequence 5′-3′	Target DNA (product length)	PCR conditions	Reference
Fcm-Azl2	ATGACCGCCGTCAACACCCC	*hisC2* (2571 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 2.5 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 2.5 min (22 cycles); 72 °C 5 min	This study
RcodA-Azl2	TGCATGTCGGCCAGATGACC			This study
Genus *Azospirillum* rDNA 16S–rDNA 23S internal spacer region				
fAZO	GGCGCATCCCTTCTCACGG	IGS (~500 bp)	95 °C 5 min; 95 °C 1 min; 70 °C 1 min; 72 °C 1 min (12 cycles); 95 °C 0.5 min; 62 °C 1 min; 72 °C 1 min (22 cycles); 72 °C 5 min	Baudoin et al. (2010)
rAZO	GCTTGCGCCACGCGCAGG			Baudoin et al. (2010)

PCR of *ipdC*, *hisC1*, and *hisC2* genes

The primer pairs used for the amplification of bacterial strains are listed in Table 1. These, correspond to the *hisC1* and *hisC2* genes and were designed for analysis of the genomes of *A. brasilense* Sp 245 and *A. lipoferum* 5B (Wisniewski-Dye et al. 2011) and *A. lipoferum* spB510 (Kaneko et al. 2010), as shown in supplementary Fig. S1. The conditions used for corresponding PCRs are also described in Table 1. Representative strains were chosen for further DNA sequencing studies of the *ipdC*, *hisC1*, and *hisC2* genes. That was performed by the Biotechnological Institute of the Universidad Nacional Autónoma de México (IBT-UNAM) using universal and custom oligonucleotides primers.

PCR amplification of IGS (16S-23S rDNA internal spacer region) and RFLP (restriction fragments length polymorphism) analysis

The genomic DNA was prepared as previously described, and the PCR was carried out with 25 ng of DNA. The primers pair fAZO/rAZO for group-specific PCR was previously described by Baudoin et al. (2010) and used with the conditions indicated in Table 1. The reactions were run on a 1 % agarose gel to ensure the amplification was successful. Unsuccessful reactions were attempted a second time. Aliquots (10 µL) of the PCR products were digested with 2 U of restriction endonuclease in 20 µL reaction volumes using the manufacturer's recommended buffer and incubation conditions. The following restriction enzymes were used: *Alu*I, *Hae*III and *Taq*I (Invitrogen, Carlsbad, CA). The restricted DNA was analyzed by vertical electrophoresis in a 7 % polyacrylamide gel (Sigma Aldrich), in TBE buffer at pH 8.0 (89 mM Tris, 89 mM boric acid and 20 mM EDTA) at 4 °C at 70 V. The experiments were performed twice.

Phylogenetic analysis

The sequences were assembled using the DNA Baser sequence assembler software (Heracle Biosoft, Bremen, Germany). Comparisons of sequences in public databases were performed using NCBI (http://www.ncbi.nlm.nih.gov/), using the BLASTN algorithm (Altschul et al. 1997). The sequences were submitted to GenBank and accession numbers were obtained. For

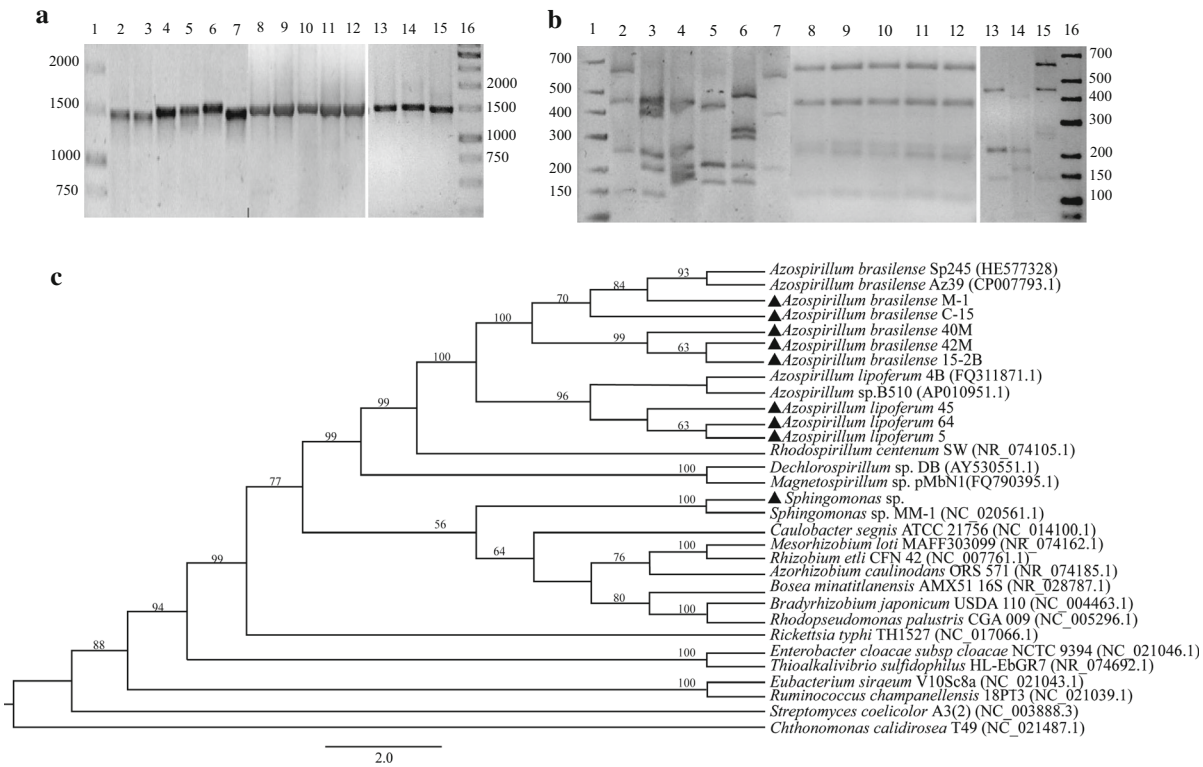


Fig. 1 Amplification of 16SrDNA genes and RFLP patterns, after *Alu*I restriction analysis of *Azospirillum* strains. **a** Amplicons obtained using the 27F/1492R universal primers. *Lane 1* 10 kb molecular marker (Invitrogen); *Lane 2 A. brasilense* Sp7; *Lane 3 A. lipoferum*59; *Lane 4 A. lipoferum*USA5b; *Lane 5 A. halopraeferens*; *Lane 6 A. irakense*; *Lane 7 A. amazonense*; *Lane 8 A. brasilense* UAP154; *Lane 9 A. brasilense* UAP14; *Lane 10 A. brasilense* 8–1; *Lane 11 A. brasilense* M-3; *Lane 12 A. brasilense* 15-2B; *Lane 13 Sphingomonas* spp; *Lane14 Pseudomonas stutzeri* A1501. (The latter two are included as negative controls); *Lane 15 A. brasilense* Sp7 (Included as a positive control); *Lane 16* 10 kb molecular marker (Invitrogen, Carlsbad, CA). **b** RFLP analysis of 16S rDNA from the corresponding genomic amplicons. *Lanes* 1 and 16: 1000 bp DNA ladder molecular marker (Thermo Scientific). The experiments were carried out twice. **c** The phylogenetical tree, based on the neighbor-joining method, was constructed from 16SrDNA sequences. The analysis includes 31 nucleotide sequences. Those obtained from this study are indicated by black triangles. The node numbers are shown as occurrence percentages from 1000 bootstrapped trees

the phylogenetic analysis, the nucleotides sequences and translated *ipdC*, *hisC1*, and *hisC2* genes sequences were used with sequences retrieved from the NCBI. The selected sequences were aligned using ClustalW and used for phylogenetic analysis using MEGA5.2 based on the Maximum likelihood and estimated with a Jones, Taylor, and Thornton (JTT) model (Tamura et al. 2011).

Determination of IAA production by *Azospirillum* strains

To quantify IAA production by *Azospirillum*, cell-free supernatants of tryptophan-supplemented cultures were obtained as described by Szkop and Bielaswki (2013). For inoculum preparation, the bacteria were grown aerobically in 5 mL D medium (initial OD 580 nm = 0.1) on a rotary shaker (145 rpm) at 30 °C for 18 h to obtain bacteria in the exponential phase of growth. One hundred µL of cultures of strain of *Azospirillum* were inoculated in 10 mL of K-malate broth medium and grown aerobically with shaking (145 rpm) at 30 °C for 48 h. To determine IAA production, the cultures were centrifuged at 14,000 × *g* at 4 °C. The cell-free supernatants extracts were filtered through a 0.22 µm filter (EMD Millipore, Billerica, MA) and directly injected into a 5 µm; 250 × 4.6 mm HPLC reverse-phase column, Gold

Table 2 Isolates and reference strains used in this study

Strain	Host plant	Geographic origin	Reference
A. brasilense RLC1[a]	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense RLC2	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense RLC3	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense RLC4	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense RLC5	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense RLC7	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. 2007
A. brasilense RLC8	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. 2007
A. brasilense REC2	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense REC3	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. 2007
A. brasilense REC4	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense REC8	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense PEC3	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense PEC5	*Fragaria ananassa*	Tucumán, Argentina	Pedraza et al. (2007)
A. brasilense M-1[b]	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense UAP14[b]	*Stenocereus stellatus*	Puebla, Pue. México	Mascarua-Esparza et al. (1988)
A. brasilense UAP151	*Zea mays*	Puebla, Pue. México	Mascarua-Esparza et al. (1988)
A. brasilense UAP154	*Zea mays*	Puebla, Pue. México	Mascarua-Esparza et al. (1988)
A. brasilense 40M	*Zea mays*L	Trenque Lauquen, Argentina	Garcia de Salamone et al. (1996)
A. brasilense 42M	*Zea mays*L	Trenque Lauquen, Argentina	Garcia de Salamone et al. (1996)
A. brasilense Az 39	*Triticum turgidum*	Entre Ríos, Argentina	Díaz-Zorita and Fernández-Canigia (2009)
A. brasilense 7-2[a]	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense 12-2B	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense 13-2C	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense 15-2B	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense 23-5B	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense 11-1	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense 8-1	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense H-1	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense M-3	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense A-1	*Saccharum officinarum*	Tucumán, Argentina	This study
A. brasilense UAP46[c]	*Zea mays*	Puebla, Pue. México	Mascarua-Esparza et al. (1988)
A. brasilense C-15	*Panicum maximum* Jacq	Bogotá, Colombia	This study
A. brasilense C-16	*Panicum maximum* Jacq	Bogotá, Colombia	This study
A. brasilense M-1	*Saccharum officinarum*	Tucumán, Argentina	This study
A. lipoferum UAP6[c]	*Opuntia ficus-indica*	Puebla, Pue. México	Mascarua-Esparza et al. (1988)
A. lipoferum USA5b	*Triticum turgidum*	California, USA	Tarrand et al. (1978)
A. lipoferum Sp59b	*Triticum turgidum*	Río de Janeiro, Brazil	Tarrand et al. (1978)
A. lipoferum 5[d]	*Oryza sativa*	INIA[e] Tacuarembó, Uruguay	Rariz et al. (2013)
A. lipoferum 45	*Oryza sativa*	INIA[e] Tacuarembó, Uruguay	Rariz et al. (2013)
A. lipoferum 64	*Oryza sativa*	INIA[e] Tacuarembó, Uruguay	Rariz et al. (2013)
A. halopraeferens	*Leptochloa fusca*	Pujab, Pakistan	Reinhold et al. (1987)
A. irakense	*Oryza sativa*	Diwaniyah, Iraq	Khammas et al. (1989)

Table 2 continued

Strain	Host plant	Geographic origin	Reference
A. brasilense Sp7	*Digitaria decumbens*	ATCC 29145[T]	Tarrand et al. (1978)
A. brasilense Sp245	*Triticum turgidum*	Río de Janeiro, Brazil	Tarrand et al. (1978)
A. amazonense Y2	forage grasses	Mato Grosso du Sul, Brazil	Magalhães et al. (1983)

[a] *A. brasilense* isolates from washed roots (RCL1–8), surface sterilized roots (REC1–7), and stolons (PEC3 and PEC5) of strawberry plants

[b] Surface-sterilized root from Maize, Wheat, and Sugar cane

[c] Surface-sterilized root from *Opuntia*

[d] *A. lipoferum* isolated from sterilized rice roots

[e] INIA (Instituto, Nacional de Investigaciones Agrícolas, Zona Este)

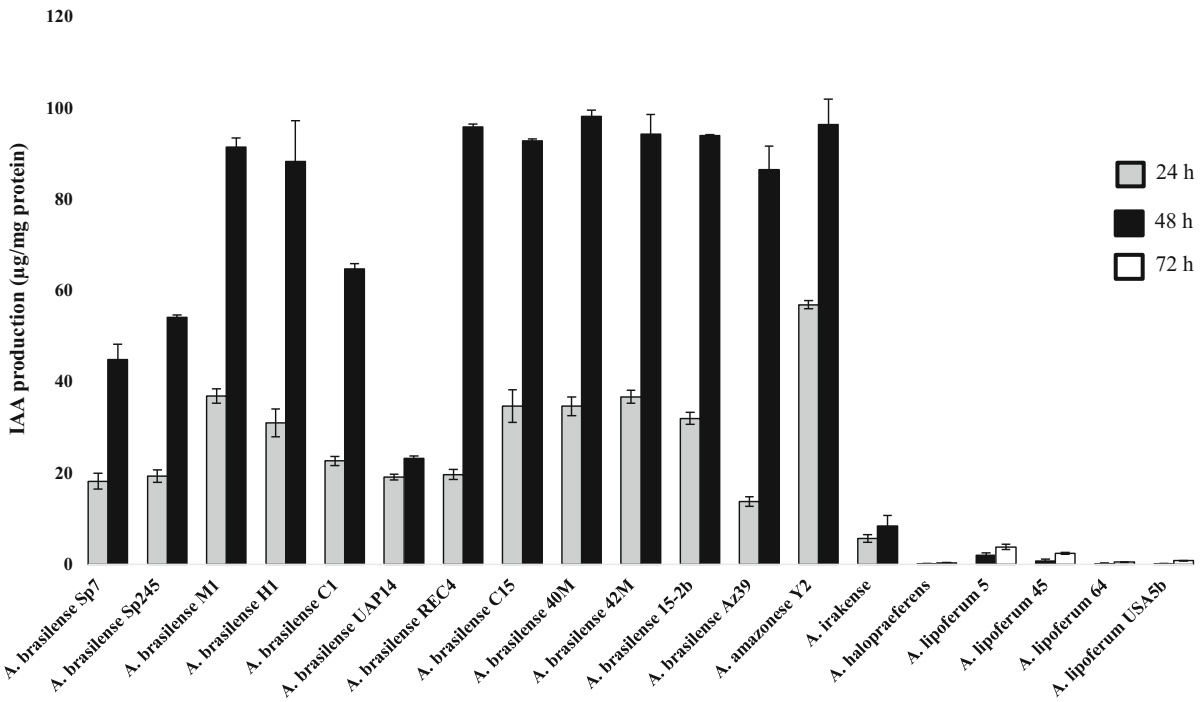


Fig. 2 IAA produced by selected isolates of *Azospirillum* strains that was measured by HPLC. The strains were grown in minimal medium K-lactate, supplemented with 100 µg mL^{-1} L-tryptophan for 24, 48 and 72 h. Three independent replicate experiments were performed per strain, and the values shown are ±SD

Liquid C18 (Beckman Coulter, Brea CA) housed in an Agilent 1200 chromatograph (Agilent Technologies, Santa Clara, CA). A gradient (10–90 %) of an acetonitrile–water system containing 1 % acetic acid was programmed over 12 min at a flow rate of 1 mL min^{-1} and the effluents were detected at 280 nm. The IAA in the cultures was quantified using a standard IAA (Sigma-Aldrich). Extracts from three independent growth studies were analyzed for each treatment. The total protein of the cultures was quantified by the Bradford reagent (BioRad Laboratories, Hercules, CA).

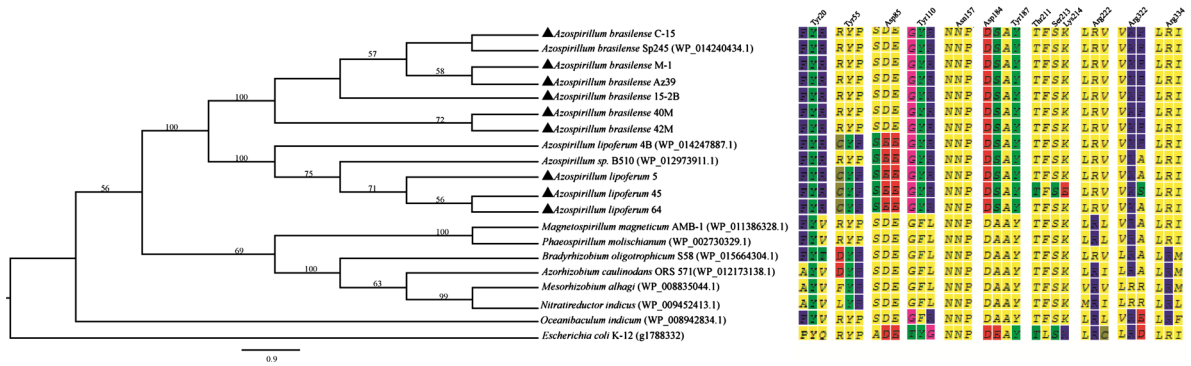


Fig. 3 Maximum Likelihood phylogenetical tree is based on the translated amino acid sequence of the *hisC1* gene (1121 bp). Amplification of *hisC1* genes was obtained using the FhisC1/RhisC1 primers, and amplicons were sequenced as described in the methods section. The sequences obtained in this study are indicated with *black triangles*, and the most closely related sequences obtained from the GenBank. The accession numbers are listed in *parenthesis*. The labeled amino acid residues are distinguished by their similarity to sequence of *Escherichia coli* with known function (Sivaraman et al. 2001). The node numbers are shown as occurrence percentages from 1000 bootstrapped trees; only the values greater than 50 % are shown. The phylogenetic analysis was performed using MEGA 5.2 (Tamura et al. 2011)

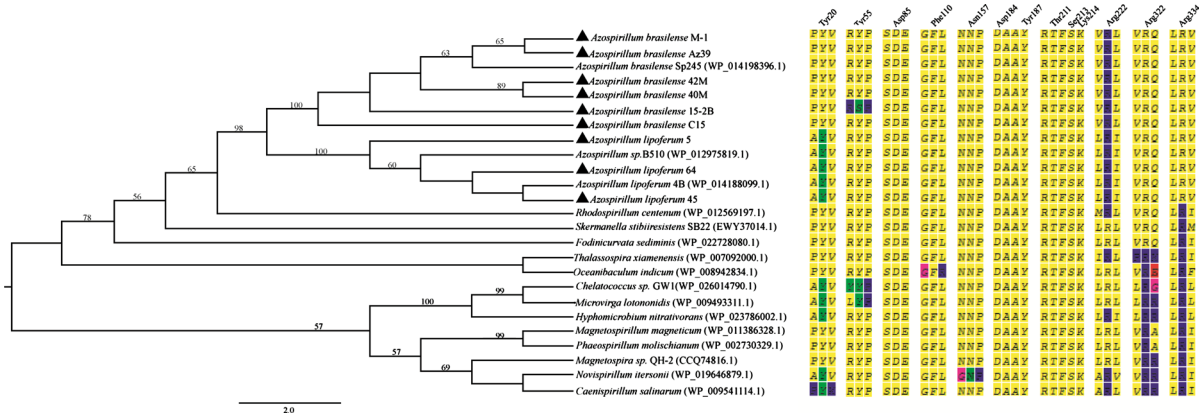


Fig. 4 Maximum Likelihood phylogenetical tree is based on the translated amino acid sequence of the *hisC2* gene (1000 bp). Amplification of *hisC2* genes from *A. brasilense* genomic DNA was obtained using the Fmext/R720.2 primers, and from *A. lipoferum* DNA was obtained using FcmAzl1/or FcmAl2/RcodAzl2. Amplicons were sequenced as described in the methods section. The sequences obtained in this study are indicated with *black triangles*, and the most closely related sequences obtained from the GenBank. The accession numbers are listed in *parenthesis*. The labeled amino acid residues are distinguished by their similarity to sequence of *Escherichia coli* with known function (Sivaraman et al. 2001). The node numbers are shown as occurrence percentages from 1000 bootstrapped trees; only the values greater than 50 % are shown. The phylogenetic analysis was performed using MEGA 5.2 (Tamura et al. 2011)

Results

ARDRA 16S

Forty *Azospirillum* strains that were isolated from different plants and geographic regions were previously identified, together with six type strains, which were used as controls were grouped by ARDRA 16S (Fig. 1a). The sizes of the amplified 16S rDNA fragments ranged from 1400 to 1500 bp. According to their fingerprint-pattern after enzyme restriction, the strains were grouped in different OTUs, which also coincided with the different species of *Azospirillum* used as references. At least two strains from each group were sequenced, shown to be *A. brasilense* and *A. lipoferum*, and considered for further analyses (Fig. 1; Table 2). The nucleotide sequences of the genes 16S rDNA, *ipdc*, *hisC1*, and *hisC2* were

deposited in the GenBank database under the following accession numbers: from KM972378 to KM972392, KP676391 to KP76407 and KP406602.

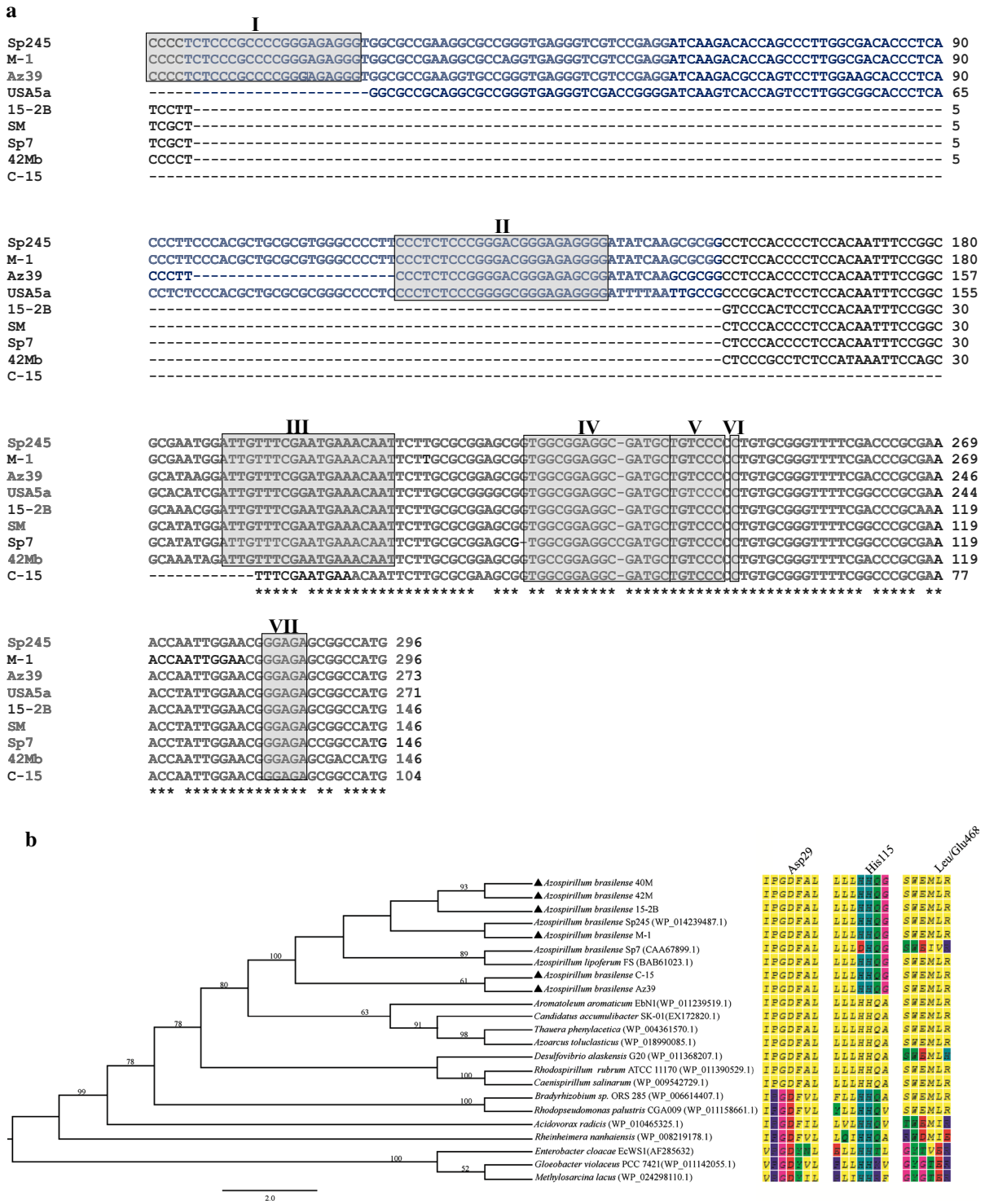
IAA production by *Azospirillum* strains

IAA production is widespread among *Azospirillum* strains and its positive effects on plant-growth have been well documented (Bashan and de Bashan 2010). Quantification of IAA produced by some selected strains was determined, and the results are shown in Fig. 2. The *A. lipoferum*, *A. irakense*, and *A. halopraeferens* strains produced low levels of IAA ranging from 0.25 to 2.4 μg mg^{-1} protein at 48 or 72 h of growth. IAA production by *A. brasilense* and *A. amazonense* Y2 strains ranged from 23 to 98 μg mg^{-1} protein at 24 or 48 h of growth, which was considerably higher than the levels produced by strains of *A. lipoferum*, *A. irakense*, and *A. halopraeferens*. Several isolates of *A. brasilense* produced higher IAA levels than the Sp7 and Sp245 control strains. Only *A. brasilense* UAP14 isolated from the Baja organ pipe cactus *Stenocereus stellatus* in Mexico produced less IAA than the levels produced by the control strains, and wild isolates (Fig. 2 and Table 2).

In silico analysis, amplification, and sequence of *ipdC*, *hisC1*, and *hisC2* genes

Several reports described the principal pathway for IAA production in *A. brasilense* is the IPyA pathway and that the AAT1 and AAT2 enzymes encoded by *hisC1*, and *hisC2* genes, respectively, and the PPDC enzyme encoded by the *ipdC* gene catalyzes the first and second steps of this pathway (Spaepen et al. 2007a, b; Malhotra and Srivatava 2008a; Carreño-López et al. 2000; Pedraza et al. 2004; Castro-Guerrero et al. 2012). Therefore, the *ipdC*, *hisC1*, and *hisC2* genes were identified in *Azospirillum* isolates using the primers designed in this work (Supplementary Fig. S1; Table 1). Amplicons of the expected size, as indicated in Table 1, were obtained from all examined isolates of *A. brasilense* species, and the determination of the nucleotide sequences and their corresponding translate proteins confirmed that the amplicons encodes AATs and PPDC proteins, respectively, with high similarity (95–99 %) at nucleotide levels (98–99 % identity at the amino acid level), although the three genes showed 100 % identity at the nucleotide level to the genomic DNA purified from *A. brasilense* 40 M and 42 M strains. The genomic DNA obtained from *A. lipoferum* USA5b, *A. lipoferum* 59b and the isolates *A. lipoferum* UAP6, *A. lipoferum* 5, 45, and 64 strains yielded amplicons corresponding to the *hisC1,* and *hisC2* genes. The comparison of the AAT1 and AAT2 sequences with sequences recovered from the GenBank data-base facilitated assembly of the phylogenetic trees shown in Figs. 3 and 4. As expected, the phylogenetic trees revealed that all the isolates examined clustered with *Azospirillum* species. As shown in Figs. 3 and 4, Tyr55, Asp85, Asn157, Asp184, Tyr187, Thr211 Ser213, Lys214, and Arg222 are the amino acid residues that bond to PLP. The amino acid residues Tyr20, Arg322, and Arg335 are involved in binding to substrates, as was described previously (Sivaraman et al. 2001). Alignments made with sequences of the AAT1 and ATT2 proteins form *A. brasilense* isolates showed 62 % similarity. The AAT1 and AAT2 protein sequences were grouped in different clades belonging to *A. brasilense* or *A. lipoferum* strains (Figs. 3 and 4). The proteins share approximately 45 % identity (scores $6e{-}10^8$ to $2e{-}10^2$) over their entire lengths and may have resulted from ancient gene duplications, indicating that they belong to a superfamily of aromatic aminotransferases enzymes, namely subfamily Iβ (Jensen and Gu 1996).

The primer pair FipdC- int/RiaaC-Stop was used to amplify the whole *ipdC* promoter region and *ipdC* gene (~2139 bp). The *ipdC* was amplified in 34 isolates belonging to *A. brasilense* species. The amplicons were then subjected to restriction analysis. Very light differences in the restriction patterns were obtained with the enzymes *Bgl*I, *Alu*I, *Nco*I, and *Bst*XI, suggesting that *ipdC* is a conserved gene (Supplementary Fig. S2 a, b, c, and d). We were unable to obtain PCR product from the genomic DNAs obtained from the other *A.lipoferum*, *A. amazonense* Y1, *A. irakense*, and *A. halopraeferens* strains, and *Sphingomonas* spp and *Pseudomonas stutzeri* A1501 that were used as negative control strains, using the primers designed to amplify the *ipdC* gene. To avoid the potential presence of the *ipdC* gene in these strains, hybridization of the genomic DNA, using *ipdC* as the probe, was performed under strict or relaxed conditions, and a hybridization signal was not obtained (data not shown), indicating that the *ipdC* gene is not


a
I
II
III
IV
V
VI
VII
Sp245
M-1
Az39
USA5a
15-2B
SM
Sp7
42Mb
C-15
b
Asp29
His115
Leu/Glu468
Azospirillum brasilense 40M
Azospirillum brasilense 42M
Azospirillum brasilense 15-2B
Azospirillum brasilense Sp245 (WP_014239487.1)
Azospirillum brasilense M-1
Azospirillum brasilense Sp7 (CAA67899.1)
Azospirillum lipoferum FS (BAB61023.1)
Azospirillum brasilense C-15
Azospirillum brasilense Az39
Aromatoleum aromaticum EbN1(WP_011239519.1)
Candidatus accumulibacter SK-01(EX172820.1)
Thauera phenylacetica (WP_004361570.1)
Azoarcus toluclasticus (WP_018990085.1)
Desulfovibrio alaskensis G20 (WP_011368207.1)
Rhodospirillum rubrum ATCC 11170 (WP_011390529.1)
Caenispirillum salinarum (WP_009542729.1)
Bradyrhizobium sp. ORS 285 (WP_006614407.1)
Rhodopseudomonas palustris CGA009 (WP_011158661.1)
Acidovorax radicis (WP_010465325.1)
Rheinheimera nanhaiensis (WP_008219178.1)
Enterobacter cloacae EcWS1(AF285632)
Gloeobacter violaceus PCC 7421(WP_011142055.1)
Methylosarcina lacus (WP_024298110.1)
2.0

◀**Fig. 5** **a** Multiple sequence alignment of the nucleotide sequences of the region upstream of the *ipdC* gene in *A. brasilense* strains. Amplification of *ipdC* genes was using the FRegR-2/iaaCR1 primers, and amplicons were sequenced as described in the methods section. The sequences obtained in this study are indicated with *black triangles*, and the most closely related sequences obtained from the GenBank. The accession numbers are listed in *parenthesis*. The *boxes* indicate the palindromic sequences (*I*, *II*), inverted repeat (*III*), RpoN binding site (*IV*), AuxRE site (*V*), transcription initiation site TIS (*VI*), and the ribosome-binding site RBS (*VII*). The 150 bp insertion found in strains Sp245, M-1, Az39 and USA5a is shown in *blue*. **b** Molecular phylogenetic analysis of PPDC. Maximum Likelihood phylogenetical tree is based on the translated amino acid sequence of the *ipdC* gene (1521 bp). The labeled amino acid residues are distinguished by their similarity to sequences with known function. The node numbers are shown as occurrence percentages from 1000 bootstrapped trees; only the values greater than 50 % are shown. The phylogenetic analysis was performed using MEGA 5.2 (Tamura et al. 2011)

present in these strains. This agrees with the genomic sequence of *A. lipoferum* 4B, *Azospirillum* spB510, and *A. amazonense* Y1, which do not contain the *ipdC* gene (Kaneko et al. 2010; Sant'Anna et al. 2011; Wisniewski-Dye et al. 2011). The nucleotide sequences obtained from all isolates belong to *A. brasilense* and the multiple alignments of the translated proteins include the region comprising the well-conserved thiamine pyrophosphate-binding motif (TPP). *A. brasilense* proteins show a leucine residue in place of a conserved glutamate in the catalytically important amino acids found in other α-keto acid decarboxylases (Asp-His-Glu catalytic triad; Fig. 5b). Hence, the *ipdC* genes were grouped in the cluster corresponding to PPDC protein, as described by Spaepen et al. (2007a, b).

The sequence of the promoter regions were also analyzed, and the multiple nucleotide sequence alignment revealed the occurrence of the auxin responsive *cis*-element AuxRE TGTCNC, and the palindromic sequences required for positive feedback regulation (Fig. 5a; Vande Broek et al. 2005; Malhotra and Srivatava 2008b; Rothballer et al. 2005). The *ipdC* promoter region could be classified into two groups: those whose promoter region is similar to that found in the *A. brasilense* Sp245, and those similar to *A. brasilense* Sp7, in which an insertion of 150 bp is missing (Fig. 5a). Our results do not reveal a correlation between the IAA produced by the strains and the existence of the 150 bp sequence or not in their promoter region. For instance, *A. brasilense* M-1, Az39, 15-2B, 42 M and C-15, isolated in different plants and countries, do not contain the 150 bp region, that was found in the Sp7 and SM (Malhotra and Srivatava 2008b; Rothballer et al. 2005). However, the amount of IAA produced by the former strains was considerably higher than that produced by Sp7 and SM. In contrast, *A. brasilense* Sp245 and M-1 and Az39, contains the *ipdC* regulatory region with the insertion of the 150 bp IAA produced by these last two strains was higher than IAA levels produced by Sp245.

Amplification and RFLP of the 16S-23S rDNA spacer region

The selected isolates were then differentiated at the strain level. An in silico study of the available sequence information of the 16S-23S rDNA spacer region in *A. brasilense* Sp 245 and *A. lipoferum* 4B genomes has shown that the bacteria have multiple copies of the rDNA operons. This indicates that spacer variations between strains and species may be used for differentiation purposes. An in silico analysis using the GenBank 16S-23S spacer region was performed. Using the primers designed by Baudoin et al. (2010) (Table 1), a PCR–RFLP analysis yielded the results shown in Fig. 6. Estimating the sizes of the IGS PCR products of all strains revealed production of several PCR products ranging from >700 to 450 bp, depending on the strain (Fig. 6a). Similar results were published by Baudoin et al. (2010) and Vezyri et al. (2013). The differences in the size of the PCR products may be partly explained by two tRNA genes, and in the variability sequence found the IGS regions by summing the sizes of the restriction fragments (Fig. 6a–d). Each isolate exhibited distinct banding patterns of IGS-PCR–RFLP, suggesting that these isolates may be different strains, with the exception of 40 M and 42 M.

Discussion

The central goal of this study was to determine if the *ipdC*, *hisC1*, and *hisC2* genes are suitable for studying the phylogeny of *Azospirillum* strains that produce high IAA by using physiological and molecular approaches. To date, mainly 16S rDNA and a few other housekeeping genes have been considered for studying species phylogeny because these are conserved, ubiquitous, and universal (Vinuesa et al. 1998;

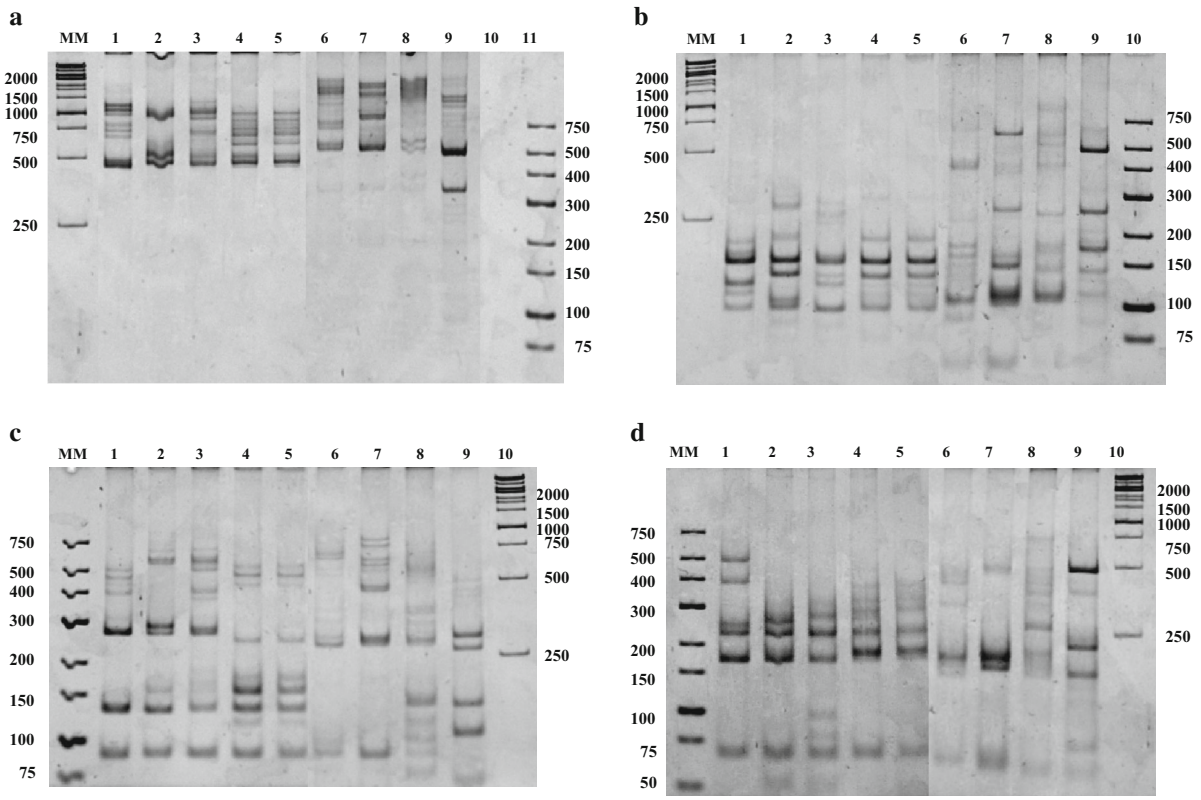


Fig. 6 Amplification of IGS 16SrDNA-23rDNA and PCR-RFLP patterns. **a** PCR-IGS from genomic DNAs. *Lane 1* 10 kb molecular marker (Invitrogen); *Lane 2 A. brasilense* Sp245; *Lane 3 A. brasilense* Sp7 (Included as positive control); *Lane 4 A. lipoferum* 64; *Lane 5 A. lipoferum* 45; *Lane 6 A. lipoferum* 5; *Lane 7 A. brasilense* Az39; *Lane 8 A. brasilense* M42; *Lane 9 A. brasilense* M40; *Lane 10* genomic DNA from *Sphingomonas* (Included as negative control). **b** PCR-IGS pattern from corresponding genomic amplicons digested with the *Hae*III restriction enzyme. **c** PCR-IGS pattern from corresponding genomic amplicons digested with the *Taq*I restriction enzyme. **d** PCR-IGS pattern from corresponding genomic amplicons digested with the *Alu*I restriction enzyme. 1000-bp DNA ladder molecular marker (Thermo Scientific). All experiments were carried out twice

Lin et al. 2011; Venieraki et al. 2011). Different isolates and several reference strains were characterized genotypically at different levels of taxonomic resolution through computer-assisted analysis of the 16S rDNA, PCR–RFLPs, and 16S-23S rDNA intergenic spacer sequence RFLPs. A comparison of the 16SrDNA and *ipdC*, *hisC1*, and *hisC2* genes and their phylogenetic trees revealed that the phylogeny of the three latter genes showed a resemblance to ARDRA characterization based on the 16SrDNA gene. We demonstrated the usefulness of the *ipdC*, *hisC1*, and *hisC2* genes, which are involved in beneficial plant activities, can be applied for phylogeny studies of species of this genus (Pedraza et al. 2004; Castro-Guerrero et al. 2012). In combination with the IGS, this technique has been used by many researchers for demonstrating genetic diversity of bacterial strains and communities associated with plants, particularly with isolates belonging to the *Azospirillum* genus (Baudoin et al. 2010; García-Martínez et al. 1999; Vezyri et al. 2013). Therefore, we combining molecular methods with biochemical and phenotypic studies is also a particularly useful strategy for investigating diversity among *Azospirillum* populations. The data we presented are consistent with previously published data of a partial 16SrDNA sequence. The IGS-PCR–RFLP analysis of strains of different species showed that these were clearly differentiated from each other. Thus, the IGS results exhibits large variability, and it is useful for differentiating genomic groups at the intraspecific level. In fact, this approach is a useful fingerprinting method for

characterizing strains at a higher level than that obtained with ARDRA or 16S rDNA sequencing (Baudoin et al. 2010; García-Martínez et al. 1999; Vezyri et al. 2013).

However, an exception was found with *A. brasilense* 40 M and 42 M. Both strains contained *ipdC*, *hisC1*, and *hisC2* genes with 100 % identity and an identical RFLP pattern after IGS-PCR digestion with the three restriction enzymes that were used (Fig. 6). A recent study of the biochemical and phenotypic properties of these strains showed that 40 M and 42 M, which were isolated from maize in Argentine (Garcia de Salamone et al. 1996), are similar but not physiologically identical and have also shown different. ACC deaminase activity and fatty acid methyl ester profiles of their cells (Di Salvo et al. 2014). Thus, we cannot rule out the possibility that these two isolates are very closely related strains of different biotypes. However, grain-yield performance and aerial biomass production exerted by both strains when inoculated independently of each other, to several cereal crops were not always similar (Di Salvo et al. 2012).

In the promoter region and *ipdC* gene, this gene has been determined only in *A. brasilense* and *A. zeae* (Carreño-López et al. 2000; Spaepen et al. 2007a, b; Venieraki et al. 2011); however, it will be important to assess whether its existence in *Azospirillum* isolates belong to others species. We did not find a correlation between the occurrence of the 150 bp insertion in the promoter region of *ipdC* gene and level of IAA production; (Malhotra and Srivatava 2008a) found that the knock-out *ipdC* gene from *A. brasilense* SM isolated from India exhibited 50 % less IAA production compared with the SM wild-type strain. This suggests that the variability in the copy number of the *ipdC* gene within the same bacterial species may possibly contribute to the variability in IAA production between members of the same species. Our analysis of all of the strains by PCR-RFLP (Fig. S2) and Southern blot indicated that only one copy was present. This could not be the reason for explaining the high level of IAA production obtained with our isolates. However, previous studies have demonstrated that phenotypic variants of *A. brasilense* Sp245 and *A. brasilense* Sp7 collected after prolonged starvation or stocking periods showed quite different PCR pattern and plasmid profiles, as well as phenotypic variations (Lerner et al. 2010; Vial et al. 2006). Therefore, it is conceivable that a long period of preservation and continuous culture may be responsible for modifying IAA production. Determination of IAA production was performed using the method described by Szkop and Bielaswki (2013), which involves the use of non-IAA solvent extraction, and the data obtained herein showed that this is a better method for IAA determination, compared with previous results obtained in our laboratory (Pedraza et al. 2004; Soto-Urzúa et al. 1996).

In conclusion, although the exact mode of action through which *Azospirillum* enhances plant growth is not yet fully understood, the plant growth-promoting activity is mainly attributed to IAA phytohormonal activity (Dobbelaere et al. 2001; Bashan and de Bashan 2010). Our data indicate that isolates from different regions constituted a homogenous population in terms of their *ipdC*, *hisC1*, and *hisC2* genes. Because the *ipdC* gene encodes PPDC, which is a key enzyme in producing the growth hormone IAA, its highly conserved nature makes it an ideal molecular tool for determining *A. brasilense* strains that can be used as inoculants. PCR is a useful and simple method for rapidly discrimination of *A. brasilense* strains and for developing new strain-specific DNA markers for identifying agriculturally important strains. Therefore, the newly designed primers used in this study might be used as alternative molecular marker to identify soil populations of *A. brasilense* strains and monitor their presence after inoculation.

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