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TAXONOMIC DESCRIPTION

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Genome-based reclassification of *Azospirillum brasilense* Sp245 as the type strain of *Azospirillum baldaniorum* sp. nov

Natalia dos Santos Ferreira[1], Fernando Hayashi Sant' Anna[2], Veronica Massena Reis[3], Adriana Ambrosini[2], Camila Gazolla Volpiano[2], Michael Rothballer[4], Stefan Schwab[3], Valter Antonio Baura[5], Eduardo Balsanelli[5], Fabio de Oliveira Pedrosa[5], Luciane Maria Pereira Passaglia[2], Emanuel Maltempi de Souza[5], Anton Hartmann[6], Fabricio Cassan[7] and Jerri Edson Zilli[3,*]

Abstract

Azospirillum sp. strain Sp245^T, originally identified as belonging to *Azospirillum brasilense*, is recognized as a plant-growth-promoting rhizobacterium due to its ability to fix atmospheric nitrogen and to produce plant-beneficial compounds. *Azospirillum* sp. Sp245^T and other related strains were isolated from the root surfaces of different plants in Brazil. Cells are Gram-negative, curved or slightly curved rods, and motile with polar and lateral flagella. Their growth temperature varies between 20 to 38 °C and their carbon source utilization is similar to other *Azospirillum* species. A preliminary 16S rRNA sequence analysis showed that the new species is closely related to *A. brasilense* Sp7^T and *A. formosense* CC-Nfb-7^T. Housekeeping genes revealed that *Azospirillum* sp. Sp245^T, BR 12001 and Vi22 form a separate cluster from strain *A. formosense* CC-Nfb-7^T, and a group of strains closely related to *A. brasilense* Sp7^T. Overall genome relatedness index (OGRI) analyses estimated based on average nucleotide identity (ANI) and digital DNA–DNA hybridization (dDDH) between *Azospirillum* sp. Sp245^T and its close relatives to other *Azospirillum* species type strains, such as *A. brasilense* Sp7^T and *A. formosense* CC-Nfb-7^T, revealed values lower than the limit of species circumscription. Moreover, core-proteome phylogeny including 1079 common shared proteins showed the independent clusterization of *A. brasilense* Sp7^T, *A. formosense* CC-Nfb-7^T and *Azospirillum* sp. Sp245^T, a finding that was corroborated by the genome clustering of OGRI values and housekeeping phylogenies. The DNA G+C content of the cluster of Sp245^T was 68.4–68.6%. Based on the phylogenetic, genomic, phenotypical and physiological analysis, we propose that strain Sp245^T together with the strains Vi22 and BR12001 represent a novel species of the genus *Azospirillum*, for which the name *Azospirillum baldaniorum* sp. nov. is proposed. The type strain is Sp245^T (=BR 11005^T=IBPPM 219^T) (GCF_007827915.1, GCF_000237365.1, and GCF_003119195.2).

The *Azospirillum* genus comprise free-living, Gram-negative bacteria that do not form spores. Bacteria from this genus are able to grow under microaerophilic conditions and their cells are rod or spiral-shaped. The genus was proposed from a reclassification of a group of strains of '*Spirillum lipoferum*', and first described as the species *A. brasilense* and *A. lipoferum* [1]. In 2015, the genus *Azospirillum* was divided and two new genera were created, *Nitrospirillum*, to which *A. amazonense*

Author affiliations: [1]Programa de Pós-Graduação em Agronomia-Ciência do Solo, Universidade Federal Rural do Rio de Janeiro, BR 465 Km 7, 23890-000, Seropédica, Rio de Janeiro, Brazil; [2]Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil; [3]Embrapa Agrobiologia, BR 465 Km 7, 23891-000, Seropédica, Rio de Janeiro, Brazil; [4]Institute of Network Biology, Helmholtz Zentrum Muenchen, Ingolstaedter Landstr. 185764 Neuherberg, Germany; [5]Departamento de Bioquímica e Biologia Molecular, Núcleo de Fixação de Nitrogênio, UFPR, Curitiba, Brazil; [6]Faculty of Biology, Microbe-Host-Interactions, Ludwig-Maximilian-University Muenchen, 82152 Planegg/Martinsried, Germany; [7]Laboratorio de Fisiología Vegetal y de la Interacción Planta-microorganismo. Instituto de Investigaciones Agrobiotecnológicas (INIAB-CONICET), FCEFQyN. Universidad Nacional de Río Cuarto., Ruta 36, Km 601, Río Cuarto, Córdoba, Argentina.
***Correspondence:** Jerri Edson Zilli, jerri.zilli@embrapa.br
Keywords: Genomic metrics; Azospirillum; *Azospirillum brasilense* Sp7; Proteoma- core phylogeny; housekeeping phylogenies.
Abbreviations: ANI, average nucleotide identity; dDDH, DNA–DNA hybridization; OGRI, overall genome relatedness index.
Genome Access numbers of new strains from this study: Sp245^T (BR11005, NZ_VITG01000000, GCF_007827915.1), BR 12001 (NZ_VITE01000000, GCF_007827765.1), Vi22 (VSRJ00000000, GCF_013341015.1), Sp7^T (BR11001, NZ_CP012914, GCF_001315015.1), BR 11017 (WFKA00000000, GCF_013340975.1), BR 11019 (WFKB00000000, GCF_013340985.1), BR 11649 (WFKD00000000, GCF_013340915.1), BR 11002 (NZ_VITX01000000, GCF_007828115.1), CC-Nfb-7^T (WHOR00000000, GCF_013340925.1), TMCY0552^T (WHOS00000000, GCF_013340935.1). 16S rRNA of new strains from this study: BR 11005 (Sp245^T) - MT644282, BR 12001 - MT644283, Vi22 - MT644284, BR 11017 - MT644285, BR 11019 - MT644286, BR 11649 - MT644287, BR 11002 - MT644288.
One supplementary table and one supplementary figure are available with the online version of this article.

Table 1. Information of the strains used in the study

Strain designation		Host	Isolation source	Year of collection (estimated)	Country	Reference
Original	**Other**					
Sp245^T	BR 11005^T, IBPPM 219^T	wheat (*Triticum aestivum*)	surface disinfected roots	1980	Brazil	[10]
AzB 60	BR 12001	signal grass (*Brachiaria decumbens*)	surface disinfected roots	1999	Brazil	unpublished
Vi22	Vi22	sunflower (*Helianthus annuus*)	rhizosphere	2012	Brazil	[21]
Sp7^T	BR 11001^T, ATCC 29145^T, DSM 1690^T, LMG 13127^T	digit grass (*Digitaria decumbens*)	rhizosphere	1974	Brazil	[1]
R3	BR 11017	sorghum (*Sorghum vulgaris*)	roots	1987	Russia	unpublished
R6	BR 11019	cat grass (*Dactylis glomerata*)	roots	1987	Russia	unpublished
Sp42	BR 11649	maize (*Zea mays*)	roots	1974	Brazil	unpublished
Sp Cd	BR 11002, ATCC 29710	bermuda grass (*Cynodon dactylon*)	rhizosphere	1976	USA	[1]
MTCC4038	–	–	agricultural field soil	2006	India	unpublished
SR80	–	wheat (*Triticum aestivum*)	seedlings	1988	Russia	unpublished

was transferred, and *Niveispirillum*, which included *A. irakense* [2]. Nowadays, a total of 21 species belonging to *Azospirillum* are recognized and validated (http://www.bacterio.net/azospirillum.html [3]).

Since the description of the genus *Azospirillum*, different strains aroused the interest for their potential as plant-growth-promoters, notably because their association with cereals of economic importance [1, 4, 5]. As a result of the research conducted firstly by Johanna Döbereiner in Brazil in the 1970s, two main abilities are highlighted: to fix atmospheric nitrogen (N) [6] and produce plant-growth-regulators like auxins, cytokinins, and gibberellins [7, 8]. Therefore, this genus has been one of the most studied plant growth-promoting-bacteria in the last decades and it has been used as inoculant for crops in several countries, reaching millions of hectares in South America alone [3–5, 9].

Azospirillum brasilense is the most studied species of the genus and *Azospirillum* sp. Sp245^T can be considered a model strain due to the knowledge accumulated over it in more than 30 years [3]. This strain was isolated from surface-sterilized roots of wheat (*Triticum aestivum* L.), originary from Rio Grande do Sul State, Southern Brazil [10, 11], and has been considered a promising wheat inoculant in Brazil since the 1980s [12]. In contrast to the rhizosphere soil isolate strain Sp7^T, strain Sp245^T is able to colonize wheat roots endophytically [13]. Despite the phenotypic and physiologic similarities with the *A. brasilense* type strain Sp7^T, strain Sp245^T has been indicated as a distinct species, based on previous DNA analyses [14]. These authors pointed out that the Average Nucleotide Identity (ANI) between the strains Sp245^T and Sp7^T is less than 95–96%, the threshold for the species delimitation. In the same way, spectrophotometric DNA–DNA-analyses demonstrated only 54% DNA–DNA-hybridization between the strains Sp7^T and Sp245^T, which again indicated the necessity for a separate species [15]. In addition, based on spectrometry protein profiling obtained through MALDI-TOF MS analysis it was shown that *A. brasilense* Sp7^T and Sp245^T formed distinct branches [16].

Here we present a phylogenomic study based on *Azospirillum* spp. strains collected in different places around the world. Besides Sp245^T and Sp7^T, other type strains were included with the aim to clarify the taxonomy position of strain Sp245^T. The results led us to propose the new species *A. baldaniorum*, which is described here.

STRAIN COLLECTION

The study included ten *Azospirillum* spp. isolated from roots, rhizosphere soil or bulk soil in different countries and hosts (Table 1) and *A. formosense* CC-Nfb-7^T from agricultural soil in China [17]. All isolates, excepting Vi22, MTCC4038, and SR80 are deposited in the collection of Biological Resource Centre Johanna Döbereiner (WDC364), Embrapa Agrobiologia, Seropedica, Rio de Janeiro-Brazil (www.embrapa.br/agrobiologia/crb-jd). Sp245^T is also deposited in the Bacterial Culture Collection at the Microbiology and Agricultural Zoology (WDCM31), Institute INTA-IMYZA, Castelar, Buenos Aires, Argentina, and in the Collection of Rhizosphere Microorganisms of the Institute of Biochemistry and Physiology of Plants and Microorganisms (WDCM1021), Russian Academy of Sciences (IBPPM RAS), Prospekt Entuziastov, Saratov, Russia (http://collection.ibppm.ru/).

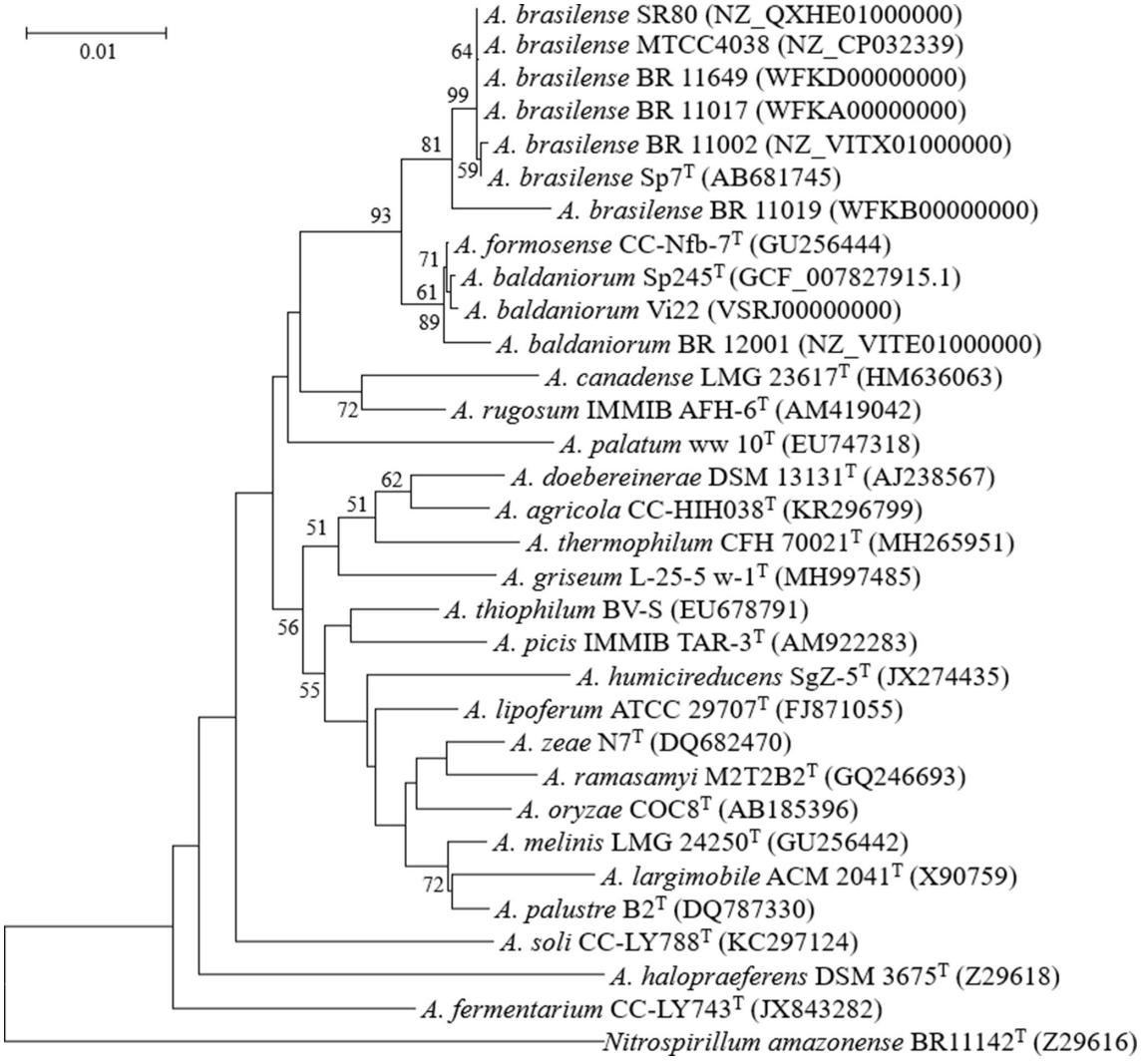

Fig. 1. Maximum-Likelihood (using Jukes-Cantor substitution model) phylogenetic tree based on 16S rRNA gene sequences showing relationships between the new strains and the type species (t) of the genus *Azospirillum*. Bootstrap values were inferred from 500 replicates and are indicated at the tree nodes when ≥50%. GenBank accession numbers are provided in parenthesis. *Nitrospirillum amazonense* BR 11142^T was included as the outgroup. The bar represents one substitution per 100 nucleotide positions.

16S rRNA AND HOUSEKEEPING GENES PHYLOGENETIC RECONSTRUCTION

The genomic DNA was extracted using the Bacterial Genomic DNA Isolation Kit (Wizard; Promega) with a few modifications [18]. The 16S rRNA gene was PCR-amplified using common universal primers (27F/1492R) and sequenced by Sanger's dideoxy DNA sequencing method. The type strain 16S rRNA sequences were retrieved from NCBI nucleotide database (www.ncbi.nlm.nih.gov). The sequences were aligned with Muscle in MEGA X [19]. Concatenation of housekeeping genes was performed by Seaview [20]. The evolutionary distance was calculated using Jukes-Cantor model that was the best fit model. The maximum-likelihood (ML) trees were reconstructed using Mega 10.0.

A BLAST search in NCBI (Standard nucleotide database) showed that the strains Sp245^T, BR 12001, Vi22, BR 11017, BR 11019, BR 11649, BR 11002, beside MTCC4038 and SR80, present high 16S rRNA similarity with some *Azospirillum* type strain, particularly Sp7^T and CC-Nfb-7^T. Thereafter the ML reconstruction with approximately 1200 nucleotides comparing these strains with all *Azospirillum* type strains revealed that they clustered together with *A. brasilense* and *A. formosense* CC-Nfb-7^T (Fig. 1). The strains Sp245^T, BR 12001, and Vi22 grouped together with *A. formosense* CC-Nfb-7^T, while BR 11017, BR 11019, BR 11649, BR 11002, MTCC4038, and SR80 were close to *A. brasilense* Sp7^T.

Considering the low resolution of the 16S rRNA gene for identifying *Azospirillum* spp., we also performed a housekeeping gene analyses using the sequences of the genes *atpD*, *glnII*,

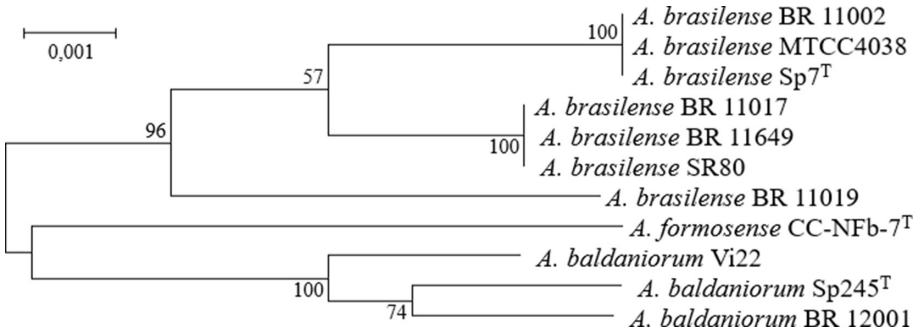

Fig. 2. Unrooted Maximum-Likelihood (using Jukes-Cantor methods) phylogenetic tree based on four concatenated sequences (*atpD*, *glnII*, *recA* and *rpoD*) showing relationships between Sp245^T-cluster, Sp7^T-cluster and CC-NFb-7^T. The sequences of the genes were extracted from the genome listed in Table 2. Bootstrap values were inferred from 500 replicates and are indicated at the tree nodes when ≥50%. The bar represents one estimated substitution per 100 nucleotide positions.

recA and *rpoD*, focusing on the strains *A. formosense* CC-Nfb-7^T and *A. brasilense* Sp7^T, the closest strains to *Azospirillum* sp. Sp245^T. Individual or concatenated reconstructions using these genes (Figs 2 and S1, available in the online version of this article) returned three main clusters, joining the strains Sp245^T, BR 12001, and Vi22 together in one cluster with over 99% similarity; CC-Nfb-7^T was separated from the others with less than 98.7% of DNA sequence similarity; and one group with the strains BR 11017, BR 11019, BR 11649, BR 11002, MTCC4038, and SR80 together with Sp7^T, varying in similarity among them from 98.9–100%. Therefore, taking in account the 16S rRNA and housekeeping gene analyses we have an indication that Sp245^T, together with BR 12001 and Vi22, represents a species different from *A. brasilense* Sp7^T and *A. formosense* CC-Nfb-7^T, as it was firstly observed elsewhere [14].

GENOME ANALYSES

The genome of *A. brasilense* Sp7^T (four genomes available), *Azospirillum* sp. Sp245^T (three genomes available), and other *Azospirillum* spp. type strains were retrieved from the NCBI Assembly database (Table 2). *A. formosense* CC-Nfb-7^T, *A. melinis* TMCY0552^T, and strains BR 11017, BR 11019, BR 11649 were sequenced in this work. Total DNA was extracted as described by previous work [21]. Libraries were constructed using Nextera XT kit and sequenced with MiSeq Reagent Kit V3 (2×300 bp) in an Illumina Miseq platform. The genome of strain Sp245^T (BR 11005), despite being available, was re-sequenced in order to check if we had in our collection exactly the same strain as the first sequenced strain. This strain together with BR 12001 and BR 11002 were sequenced at the DOE-Joint Genome Institute (JGI) as part of the Genomic Encyclopaedia of Type Strains, Phase IV (KMG-V): Genome sequencing to study the core and pangenomes of soil and plant-associated prokaryotes (https://gold.jgi.doe.gov/studies?id=Gs0129091), using the Illumina NovaSeq 6000 platform. Draft genomes for the new strains were assembled using SPAdes version 3.13 (k-mer values equal to 21, 33, 55, 77, 99, 127) [22], and they were annotated using the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP), which combines Hidden Markov Model (HMM)-based gene prediction methods with homology-based methods [23]. The quality of assemblies was evaluated with Blobtools 1.1.1 [24] Quast v. 2.3 [25] and Checkm v. 0.9.6 [26]. The genome features are presented in the Table 2.

ANI values based on BLAST and Mummer alignments from all pairwise genome comparisons were calculated using Pyani version 0.2.7, a python module for calculating genomic metrics (https://github.com/widdowquinn/pyani [27]), gANI (ANI based on whole genome), and AF (alignment fraction) based on the Microbial Species Identifier (MiSI) method [28]. Digital DNA–DNA hybridization (dDDH) was estimated using GGDC (Genome Distance Calculator for Genome; http://ggdc.dsmz.de/ggdc.php) and OrthoANI were calculated using OAT version 1.30 (www.ezbiocloud.net/tools/orthoani [29]).

Ortholog protein groups were defined using bidirectional best hits algorithm implemented in Get_homologues build 20170609 following [30]. Briefly, the core-proteome was compiled using minimum BLAST searches and clusters with inparalogs were excluded. Each of the single-copy proteins was aligned with MUSCLE using default parameters and the alignments were concatenated with MegaX (https://pubmed.ncbi.nlm.nih.gov/29722887/). Subsequently, the phylogenetic tree of the core-proteome was reconstructed using Neighbour-Joining approach with Jones-Taylor-Thornton substitution model, deleting positions containing gaps.

We employed a clustering method using the genomic relatedness values to better visualize the groups of strains. Procedures with the same goal were described before [28, 31]. Here, the genomes were grouped according to a connection criterion of ≥95% of identity with ≥70% of alignment coverage for ANIb, ANIm and OrthoANI analysis; and ≥96.5% of gANI with ≥0.6 of AF. We use an approach similar to the procedure of community detection from the network science. For ANIm and ANIb approaches that generate an output of a two way calculation, we generate a triangular form of the coverage and

Table 2. Characteristics of the studied genomes

Species	Strain	Genbank (Ac. no.)	RefSeq assembly accession	Genome size (Mbp)	Contigs	Protein	GC (mol%)	Coverage	Reference
A. baldaniorum	Sp245^T	NZ_CP022253	GCF_003119195.2	768239	7	6703	68,4	145.0	unpublished
	BR 12001	NZ_VITE01000000	GCF_007827765.1	73645	61	6536	68,6	204.0	this study
	Vi22	VSRJ00000000	GCF_013341015.1	7440708	301	6642	68,5	57	this study
A. brasilense	Sp7^T	NZ_CP033312	GCF_008274945.1	710024	6	6270	68,4	100.0	unpublished
	BR 11017	WFKA00000000	GCF_013340975.1	729369	314	6506	68,2	67	this study
	BR 11019	WFKB00000000	GCF_013340985.1	7073989	1640	6384	68,5	15	this study
	BR 11649	WFKD00000000	GCF_013340915.1	7287852	512	6554	68,2	23	this study
	BR 11002	NZ_VITX01000000	GCF_007828115.1	696097	42	6184	68,4	215.0	unpublished
	MTCC4038	NZ_CP032339	GCF_005222145.1	713417	6	6214	68,3	100.0	unpublished
	SR80	NZ_QXHE01000000	GCF_003584185.1	714659	231	6318	68,3	59.0	unpublished
A. formosense	CC-Nfb-7^T	WHOR00000000	GCF_013340925.1	6161078	569	5702	68,6	17	this study
A. melinis	TMCY0552^T	WHOS00000000	GCF_013340935.1	7970174	160	6920	67,7	107	this study
A. lipoferum	59b^T	NZ_VTTN01000000	GCF_008364955.1	798718	77	6880	67,3	127.0	unpublished
A. halopraeferens	DSM 3675^T	NZ_AUCF01000000	GCF_000429625.1	6512380	56	5872	70,7	unknown	unpublished
A. doebereinerae	GSF71^T	NZ_RZIJ01000000	GCF_003989665.1	700006	115	6000	68,9	250.0	unpublished
A. oryzae	COC8^T	NZ_VTTM01000000	GCF_008364795.1	67552	42	5852	67,4	154.0	unpublished
A. thiophilum	DSM 21654^T	NZ_LAEL01000000	GCF_000960825.1	763752	11	6516	68,2	108.39	[48]
A. palustre	B2^T	NZ_PDKW01000000	GCF_002573965.1	799749	99	6887	67,8	30.0	[49]
A. humicireducens	SgZ-5^T	NZ_CP015285	GCF_001639105.2	68627	29	5853	67,4	259.0	[50]
Rhodospirillum centenum	ATCC51521^T	NC_011420	GCF_000016185.1	435554	1	3847	70.5	unknown	[51]

Table 3. Genomic metrics of *A. baldaniorum* (Sp245^T), *A. brasilense* (Sp7^T) and CC-Nfb-7^T with related strains

Strain	Sp245^T					Sp7^T				
	ANIm (%)	ANIb (%)	OrthoANI (%)	gANI (%)	dDDH (mol%)	ANIm (%)	ANIb (%)	OrthoANI (%)	gANI (%)	dDDH (mol%)
BR 12001	**98.63**	**98.55**	**98.63**	**98.76**	**87.50**	94.58	94.22	94.55	95.2	56.80
Vi22	**98.65**	**98.54**	**98.65**	**98.79**	**87.80**	94.60	94.23	94.48	95.2	57.00
BR 11017	94.59	94.25	94.43	95.12	56.70	**98.62**	**98.44**	**98.61**	**98.73**	**87.10**
BR 11019	94.40	93.73	94.26	94.26	54.80	**98.18**	**97.68**	**98.25**	**98.39**	**81.50**
BR 11649	94.59	94.23	94.39	95.13	56.70	**98.60**	**98.35**	**98.59**	**98.72**	**87.20**
BR 11002	94.61	94.35	94.60	95.17	57.00	**99.98**	**99.90**	**99.97**	**99.98**	**99.60**
MTCC4038	94.62	94.38	94.50	95.16	57.10	**99.97**	**99.85**	**99.96**	**99.98**	**99.50**
SR80	94.59	94.32	94.45	95.11	56.70	**98.62**	**98.47**	**98.61**	**98.74**	**87.50**
CC-Nfb-7^T	94.45	94.17	94.44	95.12	56.00	94.76	94.52	94.69	95.40	57.70
TMCY0552^T	85.45	79.63	79.20	80.94	23.80	85.29	79.30	79.04	80.68	23.30
59b ^{T}	85.41	79.38	79.00	80.79	23.40	85.33	79.20	78.82	80.53	23.20
DSM 3675^T	84.29	77.94	77.09	81.67	21.80	84.26	77.87	77.05	81.23	21.60
GSF71^T	85.52	80.29	79.92	81.61	23.60	85.45	80.12	79.79	81.48	23.40
COC8^T	85.40	79.73	79.30	80.98	23.40	85.27	79.52	79.14	80.77	23.20
DSM 21654^T	85.35	79.62	79.26	80.88	23.50	85.32	79.57	79.27	80.92	23.40
B2^T	85.51	79.67	79.46	81.16	23.80	85.41	79.39	70.14	80.91	23.60
SgZ-5^T	85.43	79.58	79.22	80.98	23.40	85.38	79.45	79.08	80.78	23.20

Bold, values within the species circumscription limits. Species circumscription thresholds, ANIm, ANIb, OrthoANI and gANI (96%), dDDH (70%). Access number of reference strain deposited in NCBI genome database: Sp245^T(GCF_003119195.2), Sp7^T(GCF_008274945.1) and CC-Nfb-7^T(GCF_013340925.1).

identity matrices considering the higher value and the mean value from the two way calculation, respectively. Then, for all different ANI methods, identity and coverage/AF matrices had their values above the respective connecting criterion replaced by 1, otherwise they were replaced by 0. The two matrices generated for each method were then multiplied and the distances were obtained. Nodes were connected using the graph_from_adjacency_matrix function from igraph R package [32] and visualized with forceNetwork function from networkD3 R package [33]. The formed groups have nodes corresponding to genomes and edges corresponding to ANI values above the cutoff for species delineation with a coverage or AF value reflecting a reliable alignment between the set of genomes. A detailed tutorial for the clustering steps on R can be found at https://osf.io/h25wv/wiki/home/?view_only=58afccd2aa004d19884c5102c8b92e95.

The G+C% content of the strains Sp245^T, BR 12001 and Vi22 varied from 68.4 to 68.6 (Table 2). These values are similar to *A. brasilense* Sp7^T and *A. formosense* CC-Nfb-7^T. ANI (ANIm, ANIb, OrthoANI, gANI) or dDDH were first estimated for the new genome that was sequenced for strain Sp245^T (assembly GCF_007827915.1) with the two already deposited in NCBI (assembly GCF_000237365.1 and GCF_003119195.2). We observed all values close to 100% confirming that the different assemblies came from the same strain. Therefore, in the subsequent analyses, we used GCF_003119195.2, because it was the most finalised assembly. Similarly, we compared different assemblies available for Sp7^T and we verified that they also came from the same strain, since the genomes available (GCF_008274945.1, GCF_001315015.1, GCF_007827425.1, GCA_002027385.1) were close to 100% in ANI values.

All ANI calculations returned values above 98.54 and 98.79%, respectively, between strains inside the Sp245^T-cluster (i.e. Sp245^T, BR 12001 and Vi22) and 98.18% a 99.98% for the Sp7^T-cluster (i.e. Sp7^T, BR 11017, BR 11019, BR 11649, BR 11002, MTCC4038 and SR80) (Table 3). Moreover, dDDH calculations showed values above 81.0 inside each cluster, proving the strains in each cluster represented the same species (Table 3). Thereafter, we compared strain Sp245^T with strains from the Sp7^T-cluster and vice versa, and both strains Sp245^T and Sp7^T against CC-NFb-7^T (Table 3). OGRI estimate with ANIm, ANIb, OrthoANI between Sp245^T and Sp7^T-cluster or with CC-NFb-7^T were less than 94.60; similarly, Sp7^T against Sp245^T-cluster or CC-NFb-7^T were less than 94.76 (Table 3). Calculations with gANI proved to be more conservative, showing values slightly above 95.0 in all comparison (Table 3). dDDH estimation were always below 60.0 when we compared Sp245^T with the Sp7^T-cluster or vice versa and both Sp245 and Sp7^T with CC-NFb-7^T (Table 3).

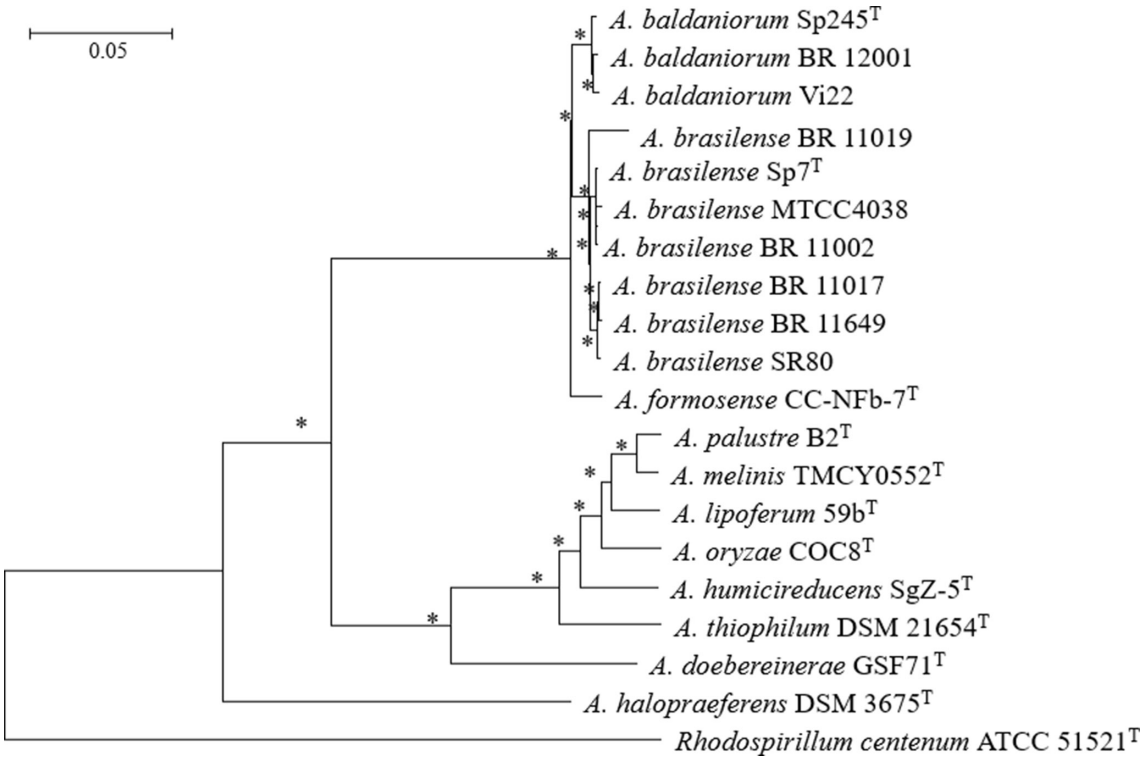

Fig. 3. Core-proteome phylogeny of *Azospirillum* species. The core-proteome rooted tree was constructed using the Neighbour-Joining method. *Bootstrap values 100%. Bar indicates the number of nucleotide substitution.

Complete genome comparison has been shown to be reproducible, reliable and informative to infer phylogenetic relationships among prokaryotes to replace the classical wet-lab-methods, like DNA hybridization, leading to a taxonomy subcommittee to establish the genome publication as a mandatory for species description [34]. Different ANI calculation methods based on pairwise genome analysis have been proposed, such as ANIm, ANIb, OrthoANI, and gANI, or even other metrices to compare genomes, like dDDH. Details regarding ANI methods and requirement of gDNA for the calculation were recently published [35, 36]. ANI value of 95–96% and dDDH 70% have been recommended as threshold as these values correlate well with 70% similarity estimated by classical DNA–DNA method and with 5 °C melting temperature differences based on denaturation method [36–38]. Considering that the ANI values between different *Azospirillum* species are around the grey zone (94–96%), in order to have more confidence on the species groups, we established an ANIb value of 96% as species circumscription limit. In a recently published paper, it was concluded that more than one ANI method must be used to stablish the cut-off, not only use 95–96% arbitrarily [36]. Therefore, based on different methods applied we have the confirmation that the strain Sp245^T, together BR 12001 and Vi22, represents a new species of the *Azospirillum* genus with *A. brasilense* Sp7^T and *A. formosense* CC-NFb-7^T as the closest neighbours.

In order to get a better resolution of the genome analysis we also used the core-proteome analysis to infer the phylogenetic position of the new species. The size of the proteomes utilized in this analysis varied between 3847 to 6920 proteins (median 6351). The core-proteome was composed of 1079 proteins, and the concatenated alignment contained 338791 positions. The phylogenetic reconstruction of the core-proteome demonstrated that the strains Sp245^T, Vi22, and BR 12001 form a distinct group, separate from the *A. brasilense* Sp7^T-cluster and the strain *A. formosense* CC-NFb-7^T (Fig. 3).

Finally, the genome clustering analysis corroborated the findings of the phylogenetic reconstruction of the core-proteome and housekeeping phylogeny, i.e. strain Sp245^T was grouped together to BR 12001 and Vi22, while *A. brasilense* Sp7^T was grouped with BR 11017, BR 11019, BR 11649, BR 11002, MTCC4038, and SR80 (Fig. 4). *A. formosense* CC-NFb-7^T did not cluster with any of the genome clusters where the groups Sp245^T and Sp7^T were located (Fig. 4).

PHENOTYPE AND PHYSIOLOGY OF NEW SPECIES

Strain Sp245^T is one of the most studied plant-growth-promoting bacteria worldwide, especially considering its potential as plant inoculant [12]. The knowledge accumulated over the years lead this strain to be considered a

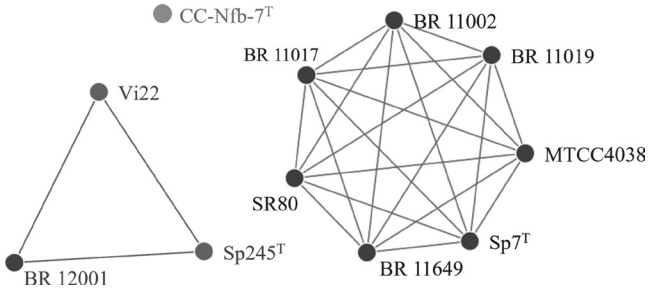

Fig. 4. Genome clustering analysis of the ANIb values. Species clusters were defined according to a linkage criterion of ≥95% of ANIb with ≥70% of alignment coverage.

model for studies on plant-bacteria interactions [3, 39, 40]. Cells of strains Sp245^T are Gram-negative, slightly curved rods, spiral, 1.6–2.1 µm long and 0.5–0.7 µm wide. Motile with long polar flagellum and several lateral ones shorter in length [41, 42]. The strain Sp245^T is able to grow between 20 and 38 °C with an optimum temperature around 30 °C depending of the culture medium [43]. As for *A. brasilense* Sp7^T, the closest species, it has been demonstrated that Sp245^T is a nitrogen-fixing bacterium, and produces high number of plant-growth-regulators, such as IAA and nitric oxide [3, 21, 39, 40, 44]. Sp245^T is able to denitrify and possess the entire pathway for the denitrification process, i.e. *nap*, *nir*, *nor* and *nos* genes [44, 45].

We additionally investigated some carbon source utilization of the strains Sp245^T and BR 12001 (both new species), Sp7^T (*A. brasilense*) and CC-NFb-7^T (*A. formosense*), as well as the enzyme activity using Biolog GN II and API 20NE test kit following method previously described [46]. We also tested the resistance to antibiotic, using impregnated discs (Oxoid). For that, the strains were grown on Dyg's liquid medium [47], and after 24 h the cultures (100 µl) were inoculated over solid Dyg's medium in Petri dishes, and the antibiotic discs were laid on the agar in three repetitions.

The strains Sp245^T and BR 12001 were able to grow using the same carbon source, but not α-keto valeric acid and D-serine (Table 4). In general, these strains present more ability to growth in the carbon source in Biolog GNII kit than the closest strains Sp7^T and CC-NFb-7^T (Tables 4 and Table S1). Sp245^T and BR 12001 differentiate from CC-NFb-7^T in the usage of five C-source and from Sp7^T in the usage of 15 different sources; L-rhamnose was only used by the strains Sp245^T and BR 12001 (Tables 4 and S1). All the four strains tested presented sensitivity to gentamicin (10 µg per disc), kanamycin (30), rifampicin (30), streptomycin (25) and tetracycline (30), and only BR 12001 was resistance/tolerant to chloramphenicol (30) and erythromycin (15). Enzymatic tests using API 20NE showed that Sp7^T and CC-NFb-7^T, as well as Sp245^T and BR 12001 are able to reduce nitrate and nitrite; urease is negative in Sp245, BR 12001 and CC-NFb-7^T, but active in Sp7^T.

Table 4. Differential characteristics of strains *A. baldaniorum* (Sp245^T and BR 12001) and related species of the genus *Azospirillum: A. brasilense* (Sp7^T) and *A. formosense* (CC-NFb-7^T)

Characteristics	Strains			
	Sp245^T	BR 12001	Sp7^T	CC-NFb-7^T
L-Arabinose	+	+	+	+
D-Arabitol	+	+	-	+
D-Fructose	+	+	-	+
L-Fucose	+	+	-	+
D-Galactose	+	+	-	+
Gentioniose	+	+	-	+
α-D-Glucose	+	+	-	+
D-Mannitol	+	+	+	+
D-Mannose	+	+	+	+
Melibiose	+	+	-	+
D-Picose	+	+	-	+
L-Rhamnose	+	+	-	-
Formic Acid	+	+	-	+
D-Galactonic Acid Lactone	+	+	-	+
D-Galacturonic Acid	+	+	-	+
D-Glucosaminic Acid	+	+	+	-
α-Keto butyric	+	+	+	-
α-Keto valeric	+	–	–	+
Glucuronamide	+	+	-	+
D-Serine	+	–	+	+
Thymidine	+	+	+	-
Phenyethylamine	+	+	+	-
Glucose-6-Phosphate	+	+	-	+
Nitrate and nitrite reduction	+	+	+	+
Indol production	+	+	-	+
Urease	-	–	–	–
Protease	-	+	+	–

All phenotypic characteristics were determined in this study. +, Positive; –, Negative.

DESCRIPTION OF *AZOSPIRILLUM BALDANIORUM* SP. NOV.

Azopirillum baldaniorum (bal.da.ni.o'rum. N.L. gen. pl. n. *baldaniorum* of the Baldanis's, named in honour of Dr. José Ivo Baldani and Dr. Vera Divan Baldani, Brazilian

microbiologists, for their pioneering contributions to *Azospirillum* research.

Cells are slightly curved rods, spiral, 1.6–2.1 µm long and 0.5–0.7 µm wide. Motile with long polar flagellum and several lateral ones shorter in length. Positive for nitrogen fixation in the environment and cells can grow on nitrogen-free medium. Grows at 20 and 38 °C with a maximum around 30 °C depending on the culture medium. With Biolog GN II, the strains grown in a variety of carbon source, differentiating from the closest related strain Sp7^T by using: D-arabitol, D-fructose, L-fucose, D-galactose, gentioniose, α-D-glucose, melibiose, D-picose, L-rhamnose, formic acid, D-galactonic acid lactone, D-galacturonic acid, α-keto valeric, glucuronamide, glucose-6-phosphate; and from CC-NFb-7^T by using L-rhamnose, D-glucosaminic acid, α-keto butyric, α-keto valeric, D-serine, thymidine, and phenylethylamine. With API 20NE, the strains were clearly positive to nitrate and nitrite reduction, indole production, β-glucosidase, β-galactosidase, but not to urease and protease. The average nucleotide identity between strains of the new species is above 98.6%. The type strain is Sp245^T (=BR 11005^T=IBPPM 219^T), a diazotrophic plant growth-promoting bacterium isolated from surface-disinfected wheat roots in Brazil. The DNA G+C content of the type strain based on genome is 68.4%.

16S rRNA AND GENOME NCBI ACCESSION

Accession numbers for 16S rRNA – Sp7^T (AB681745), CC-Nfb-7^T (GU256444), LMG 23617^T (HM636063), IMMIB AFH-6^T (AM419042), ww 10^T (EU747318), DSM 13131^T (AJ238567), CC-HIH038^T (KR296799), CFH 70021^T (MH265951), L-25–5 w-1^T (MH997485), BV-S (EU678791), IMMIB TAR-3^T (AM922283), SgZ-5^T (JX274435), ATCC 29707^T (FJ871055), N7^T (DQ682470), M2T2B2^T (GQ246693), COC8^T (AB185396), LMG 24250^T (GU256442), ACM 2041^T (X90759), B2^T (DQ787330), CC-LY788^T (KC297124), DSM 3675^T (Z29618), CC-LY743^T (JX843282), BR11142^T (Z29616)

Accession numbers for Genome - Sp245^T (NZ_CP022253, GCF_003119195.2), BR 12001 (NZ_VITE01000000, GCF_007827765.1), Vi22 (VSRJ00000000, GCF_013341015.1), Sp7^T (NZ_CP033312, GCF_008274945.1), BR 11017 (WFKA-00000000, GCF_013340975.1), BR 11019 (WFKB00000000, GCF_013340985.1), BR 11649 (WFKD00000000, GCF_013340915.1), BR 11002 (NZ_VITX00000000, GCF_007828115.1), MTCC4038 (NZ_CP032339, GCF_005222145.1), SR80 (NZ_QXHE01000000, GCF_003584185.1), CC-Nfb-7^T (WHOR00000000, GCF_013340925.1), TMCY0552^T (WHOS-00000000, GCF_013340935.1), 59b^T (NZ_VTTN01000000, GCF_008364955.1), DSM 3675^T (NZ_AUCF01000000, GCF_000429625.1), GSF71 (NZ_RZIJ01000000, GCF_003989665.1), COC8 (NZ_VTTM01000000, GCF_008364795.1), DSM 21654^T (NZ_LAEL01000000, GCF_000960825.1), B2^T (NZ_PDKW01000000, GCF_002573965.1), SgZ-5^T (NZ_CP015285, GCF_001639105.2), ATCC51521^T (NC_011420, GCF_000016185.1).

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Author contributions
V. M. R., L. M. P. P., E. M. S. and A. H., (Conceptualization); J. E. Z., V. M. R., E. d. S., L. M. P. P. and A. H., (Funding acquisition); J. Z., N. S. F., F. H. S., A. A., C. G. V. and M. R., (Methodology); J. E. Z. and F. H. S., (Supervision); N. S. F., J.E. Z. and F. H. S., (Writing – original draft); all authors (review and editing).

Conflicts of interest
The authors declare that there are no conflicts of interest.

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