

## Deoxyribonucleic Acid Homology of *Azospirillum amazonense* Magalhães et al. 1984 and Emendation of the Description of the Genus *Azospirillum*

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**The results of deoxyribonucleic acid homology experiments with the type strains of *Azospirillum lipoferum*, *Azospirillum brasilense*, and *Azospirillum amazonense* and 19 additional strains of *A. amazonense* confirmed that *A. amazonense* is a distinct new species. The description of the genus *Azospirillum* is emended to accommodate *A. amazonense*.**

In 1983 Magalhães et al. (6) reported the isolation of 35 strains of a microaerophilic, nitrogen-fixing *Azospirillum*-like organism from soils and roots of various members of the Gramineae and other plants in the Amazon region and Rio de Janeiro state, Brazil. In shape, size, characteristic spinning motility, and microaerophilic nitrogen-fixing ability, this organism resembled the previously described species of the genus *Azospirillum* (viz., *Azospirillum brasilense* and *Azospirillum lipoferum*) (5, 8); moreover, the guanine-plus-cytosine content of the deoxyribonucleic acid (DNA) was 67 to 68 mol%, only slightly lower than the value of 69 to 70 mol% reported for azospirilla (8). However, several phenotypic differences were described that differentiated the new organism from previously described azospirilla (Table 1). On the basis of these differences, it was proposed that the new organism be assigned to a new species, *Azospirillum amazonense*, and this name has since been validly published (2). Strain ATCC 35119 (= strain Am14 of Magalhães et al.) was designated the type strain. In this paper we describe the results of DNA homology experiments in which we used the type strains of *A. brasilense*, *A. lipoferum*, and *A. amazonense* and 19 additional strains of *A. amazonense* described by Magalhães et al. (6). We also provide an emended description of the genus *Azospirillum* to accommodate the new species.

DNA was extracted and purified by the hydroxylapatite procedure described by Johnson (3). Portions of the DNA preparations from the type strains of *A. brasilense*, *A. lipoferum*, and *A. amazonense* were labeled by in vitro iodination. The  $\text{TiCl}_3$  iodination procedure of Selin et al. (7) was modified by the addition of sodium perchlorate to a final concentration of 5.0 M in the reaction mixture to ensure single-stranded DNA (1). The S1 nuclease procedure described by Johnson (4) was used for the DNA homology experiments. The reassociation mixtures consisted of 10  $\mu\text{l}$  of labeled DNA (0.03  $\mu\text{g}$ ), 50  $\mu\text{l}$  of unlabeled DNA (20  $\mu\text{g}$ ) or 0.1 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M trisodium citrate), 25  $\mu\text{l}$  of 5.28 M NaCl-1.0 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 25  $\mu\text{l}$  of formamide. The mixtures were reassociated at 65°C (25°C below the thermal melting point in this buffer system) for 24 h. The S1-resistant duplexes and 60  $\mu\text{g}$  of sheared salmon sperm DNA were coprecipitated by adding 0.25 volume of a mixture containing 1 N HCl, 1% sodium pyrophosphate, and 1%  $\text{NaH}_2\text{PO}_4$ . After refrigeration for 1 h at 4°C, the precip-

itates were collected on glass fiber filters (Whatman GF/F). Radioactivity was measured with a Beckman model 5500 gamma scintillation counter.

The results of the DNA homology experiments are shown in Table 2. All of the new strains tested had high levels of homology (>56%) to the type strain of *A. amazonense* but only very low levels of homology (2 to 6%) to the type

TABLE 1. Characteristics differentiating *A. amazonense* from *A. lipoferum* and *A. brasilense*<sup>a</sup>

Characteristic	<i>A. amazonense</i>	<i>A. lipoferum</i>	<i>A. brasilense</i>
Cell width ( $\mu\text{m}$ )	0.9-1.0	1.0-1.5	1.0-1.2
Flagellar arrangement			
Monotrichous	+ <sup>b</sup>	+	+
Peritrichous	-	+ <sup>c</sup>	+ <sup>c</sup>
Enlarged, pleomorphic cells develop in alkaline media	-	+	-
Biotin required	-	+	-
Growth at pH:			
>6.8	W	+	+
6.0	+	+	+ or W
Pigmentation on potato agar			
White	+	-	-
Pink	-	+	+
Dissimilation of:			
$\text{NO}_3^- \rightarrow \text{NO}_2^-$	d <sup>d</sup>	+	+
$\text{NO}_2^- \rightarrow \text{N}_2\text{O}$	-	d	d
Anaerobic growth on nutrient agar in the presence of $\text{NO}_3^-$	-	+	+
Sole carbon sources for $\text{N}_2$ fixation			
Sucrose	+	-	-
Fructose	-	+	+
Glucose	+	+	-
Guanine-plus-cytosine content of DNA (mol%) <sup>e</sup>	67-68	69-70	70-71

<sup>a</sup>The characteristics of *A. amazonense* are described in reference 6; the characteristics of *A. lipoferum* and *A. brasilense* are described in references 5 and 6.

<sup>b</sup> +, Positive in more than 90% of the strains; d, positive in 11 to 89% of the strains; -, negative in all strains; W, scant growth.

<sup>c</sup> In liquid media the cells possess only a single polar flagellum; on nutrient agar at 30°C numerous lateral flagella of shorter wavelength occur in addition to the polar flagellum.

<sup>d</sup> Only 4 of the 35 strains described by Magalhães et al. (6) produced  $\text{NO}_2^-$ , which was afterward assimilated (strains Am17, Am22, Am27, and Am31).

<sup>e</sup> As determined by the thermal denaturation method.

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TABLE 2. DNA homology among *Azospirillum* strains

Unlabeled DNA from strain:	% Homology to DNA from reference strain:		
	<i>A. lipoferum</i> ATCC 29707 <sup>T</sup>	<i>A. brasilense</i> ATCC 29145 <sup>T</sup>	<i>A. amazonense</i> ATCC 35119 <sup>T</sup>
<i>A. lipoferum</i> ATCC 29707 <sup>T</sup>	100.0	16.9	2.9
<i>A. brasilense</i> ATCC 29145 <sup>T</sup>	14.1	100.0	7.8
<i>A. amazonense</i> ATCC 35119 <sup>T</sup>	3.3	2.5	100.0
Am15	2.5	2.5	67.6
Am16	3.9	4.1	56.2
Am17	3.4	3.5	66.8
ATCC 35120(=Am18)	3.0	2.1	88.0
Am19	2.5	3.0	87.3
Am20	3.7	4.0	66.5
Am21	2.5	2.9	70.1
Am22	4.0	6.0	62.8
Am24	2.6	2.3	57.3
Am25	2.3	3.7	60.4
Am26	3.2	4.8	58.4
Am27	5.8	5.2	67.4
Am28	3.5	4.2	65.7
Am29	3.6	4.8	68.6
Am31	2.0	5.0	67.1
Am32	2.9	4.4	64.5
Am33	2.6	4.8	59.0
Am34	3.0	3.3	67.4
Am35	5.3	5.3	72.4

strains of *A. brasilense* and *A. lipoferum*. These data confirm that *A. amazonense* is a distinct species. *A. lipoferum* ATCC 29707<sup>T</sup> (T = type strain) and *A. brasilense* ATCC 29145<sup>T</sup> exhibited a lower degree of homology (14 to 17%) toward each other than the previous values of 31 to 34% reported by Tarrand et al. (8); this is due to use of the S1 nuclease method rather than the membrane filter competition method used previously.

Inclusion of *A. amazonense* in the genus *Azospirillum* requires a modification of the genus description, as given below.

**Emended description of the genus *Azospirillum* Tarrand et al. 1978.** Plump, vibrioid, or straight rods, often with pointed ends. Gram negative to gram variable. Intracellular granules of poly- $\beta$ -hydroxybutyrate are present. Motile with a characteristic corkscrew-like or vibratory motion in liquid media by means of polar flagella. Lateral flagella may also be formed by some strains when cells are cultured on solid media at 30°C. The colonies of some strains are pigmented (light or dark pink) on potato agar. Optimum growth temper-

ature, 34 to 37°C. Some strains grow well at pH 7; others prefer more acidic conditions. Nitrogen fixers, exhibiting N<sub>2</sub>-dependent growth under microaerobic conditions. Grow well under an air atmosphere in the presence of a source of fixed nitrogen, such as an ammonium salt. Possess mainly a respiratory type of metabolism with O<sub>2</sub> and, with some strains, NO<sub>3</sub><sup>-</sup> as terminal electron acceptors. Weak fermentative ability may also occur. Under severe O<sub>2</sub> limitation some strains may dissimilate nitrate to NO<sub>2</sub><sup>-</sup> or to N<sub>2</sub>O and N<sub>2</sub>. Oxidase positive. Chemoorganotrophic; some strains are facultative hydrogen autotrophs. Grow well on the salts of organic acids, such as malate, succinate, lactate, and pyruvate; certain carbohydrates may also serve as carbon sources. Some strains require biotin. Occur free-living in soil or in association with the roots of cereal crops, grasses, and tuber plants. Root nodules are not induced. The guanine-plus-cytosine content of the DNA is 67 to 71 mol% (thermal denaturation method).

Type species: *Azospirillum lipoferum* (Beijerinck) Tarrand et al. 1978.

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