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Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a Root-Associated Nitrogen-Fixing Bacterium

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During a survey of the occurrence of *Azospirillum* spp. in cereal roots, we obtained 119 isolates which could not be identified as members of one of the three previously described *Azospirillum* species. These strains formed a very homogeneous group of N_2-fixing, microaerobic, motile, vibrioid, gram-negative rod-shaped organisms which formed a veillike pellicle in semisolid medium similar to that of *Azospirillum* spp. However, the new isolates differed from *Azospirillum* spp. by their smaller cell width (0.6 to 0.7 μm), variable flagellation (one to three flagella on one or both poles), moist brownish colonies, and broader pH and oxygen tolerance for nitrogenase activity. Organic acids were the preferred carbon sources, but glucose, galactose, L-arabinose, mannitol, sorbitol, and glycerol were also used. The guanine-plus-cytosine content of the deoxyribonucleic acid was slightly lower than the guanine-plus-cytosine contents of *Azospirillum* spp. (66 to 67 mol%). Deoxyribonucleic acid hybridization experiments with 17 strains of the group showed 50 to 100% complementarity, while the levels of hybridization with the type strains of *Azospirillum brasilense*, *Azospirillum lipoferum*, and *Azospirillum amazonense* were 23, 15, and 6%, respectively. For these new isolates we propose a new genus, *Herbaspirillum* (the name refers to the habitat of the organisms, the roots of cereals, which are herbaceous seed-bearing plants). The type species is named *Herbaspirillum seropedicae* after the place where it was first isolated. The type strain is strain Z67, which has been deposited in the American Type Culture Collection as strain ATCC 35892.

During the last decade finding alternative nitrogen supplies for grasses and cereals has been one of the major research challenges in tropical agriculture. While progress in genetic engineering to bring about the incorporation of nitrogen fixation genes and the expression of these genes in higher plants has been slow, investigations of associations of plant roots with various N_2-fixing bacteria have made considerable progress (4, 9, 12, 26, 34; P. J. Dart, Plant Soil, in press). The supply of 15 to 40% of the total nitrogen incorporated by biological fixation by such bacteria has been demonstrated (1; R. M. Boddey and R. L. Victoria, Plant Soil, in press), and the nature of such associations is becoming better understood (27, 33).

Several new N_2-fixing bacteria have been described recently (3, 14, 16, 22, 23, 30), and association of these bacteria with grasses and cereals has been demonstrated. More consistent information is being obtained with *Azospirillum* spp., which are already used in commercial inoculants for cereals (25). The genus *Azospirillum*, which was originally described with two species (32), has been shown to be widely distributed in high numbers, especially in tropical regions (10). Recently, a third species, *Azospirillum amazonense* was added (21), and its status was confirmed by deoxyribonucleic acid (DNA) homology experiments (13). In a brief communication (2) a fourth species, which like the other species occurs in high numbers on and in cereal roots, was proposed. Further characterization of this bacterium and especially very recent data from Falk et al. (13a) led to the proposal of a new genus for this species, *Herbaspirillum*, which we describe here.

MATERIALS AND METHODS

Isolation of bacterial strains. Small vials (7 ml) containing semisolid NFb medium (8) were inoculated with dilutions of root or rhizosphere soil preparations from maize, sorghum, and rice plants grown in three different soils. *Herbospirillum seropedicae* sp. nov. grows as a fine white pellicle very similar to the pellicles of *Azospirillum* species, but very different cell types occur.

As in the isolation of *Azospirillum* spp., the bacteria were transferred to new semisolid NFb medium and after 48 h of incubation at 32°C were streaked onto plates of NFb agar containing 20 mg of yeast extract per liter. After 5 days small (1- to 2-mm) moist colonies with green centers were selected, transferred to new NFb agar-containing vials, and, after growing, streaked onto potato agar (8) for final purification. On this medium small (1- to 4-mm), moist, smooth, raised brownish colonies were formed. For maintaining stock cultures the bacteria were stored in test tubes on potato agar covered with paraffin oil. Isolates identified by colony type and cell shape were obtained from 119 samples of rhizosphere soil or roots (Table 1).

Properties of the strains. A representative group of 17 of the 119 strains was used for further characterization (Table 2). Physiological tests, such as the oxidase, catalase, urease, hydrolysis of gelatin and starch, and oxidation-fermentation tests, were performed by the methods of Smibert and Krieg (31) with all 17 strains. The Gram stain test was done by the Burke method (11). Tolerance to NaCl was examined in semisolid NFb medium supplemented with 0, 2, and 3% NaCl. The biotin requirement was tested in semisolid NFb medium as described by Krieg and Döbereiner (19). Antibiotic resistance tests were performed with type BB2 Sensi-Disks (Becton Dickinson and Co) that were placed on potato agar plates that had been inoculated with 1-ml portions of 24-h cultures grown in liquid NFb medium containing 1 mM glutamate. The levels of resistance were observed after 20 h of growth. These tests were done with two to four strains.

Utilization of carbon substrates. The ability of the bacteria to grow on various carbon substrates was assayed in semisolid NFb medium lacking malate but containing NH_4Cl (0.5 g/liter) or N_2 as a nitrogen source. All carbon substrates were dissolved in 3 mM phosphate buffer, and the preparations

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TABLE 1. Sources of 119 *H. seropedicae* strains isolated during counting of *Azospirillum* spp.[a]

Location	Sample	No. of isolates from:[b]						Soil C (maize)[c]
		Maize		Sorghum		Rice		
		Soil A[c]	Soil B[c]	Soil A[c]	Soil B[c]	Soil A[c]	Soil B[c]	
Rio de Janeiro	Rhizosphere soil	1	0	0	2	3	1	
	Washed roots	4	10	2	3	3	10	
	Short-time root sterilization[d]	7	6	3	2	2	15	
	Long-time root sterilization[e]	4	10	6	5	6	4	
Brasilia	Long-time root sterilization[f]							10

[a] Baldani, M.S. thesis.
[b] Isolates were obtained from semisolid NFb medium inoculated with root or soil dilutions (10^{-3} to 10^{-6}).
[c] Soil A, Gray hydromorphic soil; soil B, reddish yellow podzolic soil; soil C, dark red latosol.
[d] Root surfaces were sterilized with 1% chloramine t for 5, 5, and 1 min for the maize, sorghum, and rice samples, respectively.
[e] Root surfaces were sterilized with 1% chlroramine t for 60, 30, and 15 min for the maize, sorghum, and rice samples, respectively.
[f] Root surfaces were sterilized with 1% chloramine t for 30 min.

were adjusted to pH 6.5, sterilized by filtration, and added aseptically to the vials along with autoclaved medium at a final carbon substrate concentration of 5 g/liter.

The vials were inoculated with 0.1-ml portions of inocula and incubated for 32 h. Growth was considered positive when the bacteria formed a thick pellicle near the surface of a vial, as observed in NFb medium containing malate. The tests were conducted in duplicate vials with 10 strains of *H. seropedicae* (strains Z67^T [T = type strain], Z176, ZA95, ZS64, ZA113, Z78, ZM141, ZA110, ZS57, and ZA80) and *Azospirillum lipoferum* Sp59^T (= ATCC 29707^T) as a control.

Oxygen effects on nitrogenase activity. *H. seropedicae* Z67^T was compared with *Azospirillum brasilense* Sp7^T (= ATCC 29145^T) to determine the optimum oxygen concentration for nitrogenase activity. The bacteria were grown in liquid NFbHP-glutamate medium (28) under an air atmosphere (100 ml in 300-ml conical flasks 120 rpm, 32°C) until the optical density reached 0.87. Samples of cells (3 ml) were transferred carefully with a Cornwall syringe pipette under an N_2 atmosphere to 60-ml flasks closed with Suba-Seals which had previously been filled with N_2. Different concentrations of oxygen and 10% (vol/vol) C_2H_2 were injected. Acetylene reduction rates were determined after 1 h. Three vials were used for each treatment.

pH and temperature optima. The effect of temperature was evaluated by measuring the nitrogenase activities of three strains of *H. seropedicae* (strains Z67^T, Z78, and Z176) in semisolid NFb medium. The inocula were prepared by growing the strains in liquid NFb medium containing 5 mM glutamate for 24 h at 32°C. Vials were inoculated with 0.1-ml portions of the inocula and incubated for 43 h at different temperatures. Nitrogenase activity was determined by the C_2H_2 reduction method after 1 h of incubation. During the C_2H_2 reduction assay the vials were maintained at the same temperature at which the bacteria had been grown. Three vials were used for each treatment.

The pH effect was evaluated in semisolid NFb medium containing 30 mM phosphate. The medium was autoclaved at pH 6.2 and then adjusted to different pH values with sterilized KOH or H_2SO_4. Triplicate vials were inoculated with 0.1-ml portions of the inocula grown as described above for the temperature experiment and were incubated for 22 h at 32°C. Nitrogenase activity was determined as described above for the temperature experiment after 1 h of incubation. Two strains of *H. seropedicae* (strains Z67^T and Z78) were used, and *Azospirillum brasilense* Sp7^T was included as a control.

Nitrate reduction, denitrification, and anaerobic NO_3^--dependent growth. Disappearance of NO_3^-, accumulation and disappearance of NO_2^-, and derepression of nitrogenase were observed in semisolid NFb medium (pH 7.0) lacking indicator but containing 2 mM KNO_3. Three vials were inoculated with 0.1-ml portions of inocula grown in liquid NFb medium supplemented with 5 mM glutamate for 24 h at 32°C. Nitrate was determined by the method of Cataldo et al. (5), and NO_2^- was determined by the method of Neyra and van Berkum (24). Nitrogenase activity was evaluated by the C_2H_2 reduction method, using additional cultures inoculated and grown in the same way. Three strains of *H. seropedicae* (strains Z67^T, Z78, and Z176) were used, and *Azospirillum brasilense* Sp7^T was used as a control. In a second test NO_2^- accumulation was confirmed qualitatively with all 17 strains.

Denitrification was evaluated in semisolid NFb medium (pH 7.0) supplemented with 2 mM KNO_3. Duplicate vials were inoculated with 0.1-ml portions of 24-h cultures in liquid NFb medium supplemented with 5 mM glutamate and incubated for 72 h at 32°C under 10% (vol/vol) C_2H_2. N_2O production was evaluated by the method of Yoshinari and Knowles (35). All 17 *H. seropedicae* strains were used, and *Azospirillum lipoferum* Sp59^T was used as a control. Anaerobic NO_3^--dependent growth and gas production were assayed in potato agar medium (7 g of agar per liter) with and without 10 mM KNO_3. Duplicate large test tubes were inoculated at the bottom with 0.2-ml portions of inocula grown in liquid NFb medium supplemented with 5 mM glutamate and then filled with potato agar kept at 50°C. The tubes were closed with Suba-Seals and incubated for 1 week at 32°C. All 17 *H. seropedicae* strains were used, and *Azospirillum lipoferum* Sp59^T was used as a control.

Efficiency of N_2 fixation. Two experiments were conducted in semisolid NFb medium containing 0.05% DL-malate or 0.05% mannitol as a carbon source. The low carbon source concentrations were used to ensure carbon source exhaustion before other limiting factors could affect N_2 fixation or growth. Quadruplicate vials were inoculated with 0.1-ml portions of 24-h liquid cultures in NFb medium supplemented with 5 mM glutamate and were incubated at 32°C. Vials inoculated with heat-killed inoculum were also incubated and used as controls. The total amount of nitrogen fixed was determined by a micro-Kjeldahl analysis when all

TABLE 2. Origins of the strains used for characterization of *H. seropedicae*

Soil	Plant	Strains isolated from:		
		Rhizosphere soil	Washed roots	Surface sterilized roots[a]
Gray hydromorphic	Maize	ZM141		ZM139
	Sorghum			Z78
	Rice	ZA76		ZA69, ZA83
Reddish yellow podzolic	Maize		ZM145	Z176
	Sorghum		ZS64	ZS57, ZS12
	Rice	ZA113	ZA80, ZA95	ZA94, ZA110, Z67^T

[a] Treated with 1% cloramine t.

TABLE 3. Growth of *H. seropedicae* on various carbon substrates in semisolid NFb medium

Carbon source(s)	% of strains positive with the following nitrogen sources:[a]	
	NH_4Cl	N_2
Malate, succinate, citrate, α-keto-glutarate, fumarate, pyruvate, *trans*-aconitate	100	100
Malonate	0	0
Mannitol, glycerol, sorbitol	100	100
Glucose, galactose, L-arabinose	100	100
Fructose	100	0
Acid from L-arabinose	—[b]	100
Acid from glucose, galactose	—[b]	0

[a] Strains Z67[T], Z176, ZA95, ZS64, ZA113, Z78, ZM141, ZA110, ZS57, ZA80 were tested.

[b] Acid reactions accompanying growth in medium supplemented with NH_4Cl occurred with all sugars and alcohols and could not be distinguished from acid production.

of the malate or mannitol was used (when no further C_2H_2 reduction could be detected). *H. seropedicae* strains Z67[T], Z78, and Z176 were used, and *Azospirillum brasilense* Sp7[T] and *Azospirillum lipoferum* Sp 59[T] were used as controls.

Swarming experiments. Swarming was observed on soft nutrient agar plates (0.8% agar), which were inoculated in the center with one loopful of a 24-h liquid culture in NFb medium containing 0.5 g of yeast extract and were incubated at 35°C. The diameter of surface growth was measured daily.

DNA hybridization experiments: isolation of chromosomal DNA. The cells from 40-ml cultures grown in nutrient broth (5 g of Bacto-Peptone [Difco Laboratories], 5 g of NaCl, 2 g of yeast extract, 1 g of meat extract, 1,000 ml of distilled water, pH 7.0) were concentrated in 5-ml portions of lysis buffer [0.05 M tris (hydroxymethyl)aminomethane, 0.05 M ethylenediaminetetraacetate, 0.1 M NaCl, pH 8.0] and treated with 1 mg of lysozyme per ml for 30 min on ice. *Azospirillum amazonense* was grown in liquid LGI broth medium supplemented with 2 mM KNO_3 (pH 6.0). Sodium dodecyl sulfate was added to a final concentration of 1%, and the preparations were incubated for 30 min at 65°C. The

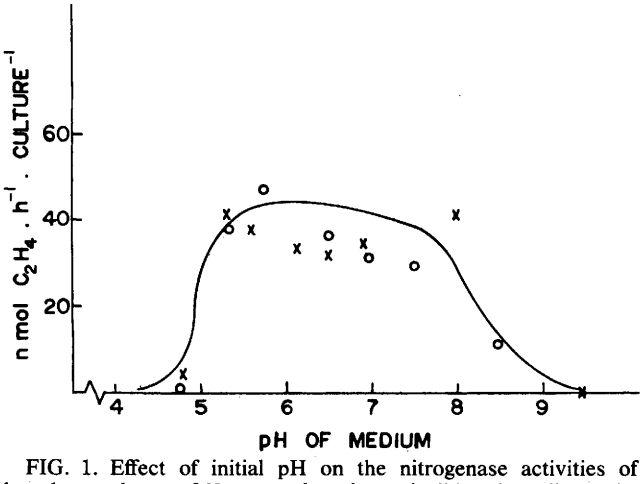

FIG. 1. Effect of initial pH on the nitrogenase activities of log-phase cultures of *H. seropedicae* in semisolid NFb medium. The data are from two experiments (× and ○) done with triplicate cultures.

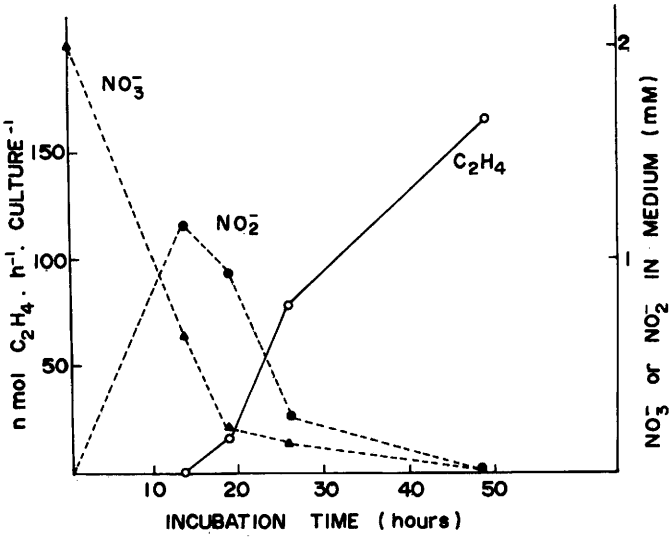

FIG. 2. Effect of NO_3^- and NO_2^- on the derepression of nitrogenase of *H. seropedicae* in semisolid NFb medium. The data points are means of triplicate cultures.

DNA was purified as described by Seldin and Dubnau (29). *Bacillus polymyxa* DNA (29) was used as a negative control. All preparations were kept at 4°C and were treated with a few drops of chloroform.

Preparation of DNA probes. Chromosomal DNA of *H. seropedicae* Z67[T] was used as a probe in hybridization experiments. This DNA was nick translated as described by Davis et al. (6). DNA probes were radiolabeled with [^{32}P]deoxyadenosine triphosphate to a radioactivity of approximately 4×10^7 cpm/μg of DNA.

DNA hybridization. Samples containing 0.5 μg of DNA (quantified by using diphenylamine as described by Johnson [17]) were denaturated and loaded onto nitrocellulose membranes by using a slot blot system. The denaturation procedure and the hybridization experiments were performed as previously described by Seldin and Dubnau (29). All experiments were repeated three times in order to minimize differences in DNA immobilization on the membranes.

Quantification of the DNA homology. After exposure to X-ray film, the individual slots were cut from the nitrocel-

TABLE 4. Denitrification by *H. seropedicae* in semisolid NFb medium[a]

Strain(s)	Denitrification (nmol of N_2O per h per culture)
H. seropedicae	
Seven strains with > 70% DNA complementarity with strain Z67[T][b]	8.7 ± 0.6
Nine strains with < 70% DNA complementarity with strain Z67[T]	9.7 ± 1.7
Strain ZA69	1.0
Azospirillum lipoferum Sp59[T]	256.1

[a] Flasks containing 5 ml of semisolid NFb medium supplemented with KNO_3 2 mM were inoculated with 0.1-ml portions of 24-h liquid cultures, closed, and incubated at 32°C for 72 h under an atmosphere containing 10% (vol/vol) C_2H_2 in air.

[b] See Table 8 for the levels of complementarity of various strains.

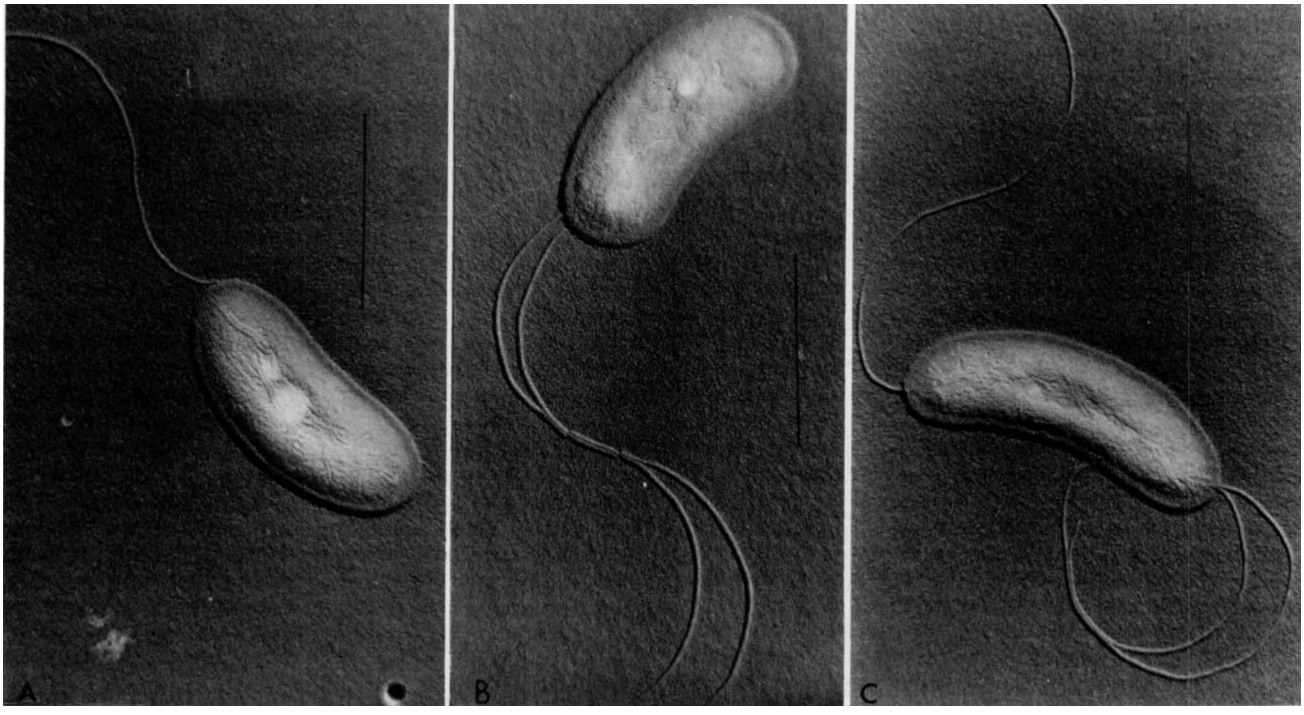

FIG. 3. Transmission electron micrographs of *H. seropedicae* cells grown in nutrient agar. (A, B, and C) One, two, and three polar flagella on one or both poles, respectively. Bars = 1 μm.

lulose membranes and placed in scintillation vials; 2.5 ml of scintillation solution containing 4.5 g of PPO (2,5-diphenyloxazole) and 0.1 g of POPOP [1,4-bis(5-phenyloxazolyl)benzene] in 1,000 ml of toluene was added to each vial, and the radioactivity was measured with a scintillation counter. The percentage of complementarity was calculated by using the following relationship: percentage of complementarity = (counts per minute with heterologous DNA/counts per minute with homolgous DNA) × 100.

Electron microscopy. Strain Z67^T was cultured on nutrient agar (0.75% agar) for 48 h at 30°C. Formalin (0.2 ml) was added to 100 ml of the culture, and the cells were harvested by centrifugation after 30 min. The cells were washed once in distilled water, suspended in water, placed on carbon-stabilized, Parlodion-coated grids, shadowed with tungsten oxide, and examined by transmission electron microscopy.

RESULTS AND DISCUSSION

During counting of *Azospirillum* spp. in cereal roots in Rio de Janeiro, Brazil (J. I. Baldani, M.S. thesis, Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Brazil, 1984), the frequent occurrence of small white colonies with greenish centers on plates containing NFb medium was noticed. When these colonies were transferred to semisolid NFb medium, they formed the typical veillike pellicles of *Azospirillum* species and demonstrated nitrogenase (C_2H_2) activity. However, the cells were much smaller and less motile than the cells of the previously described *Azospirillum* spp.

According to these characteristics, 119 isolates were obtained from three areas, from soil and especially from roots of maize, sorghum, and rice (Table 1); these isolates could be distinguished from the three previously described *Azospirillum* spp. by their colony type, cell shape, and size. They were microaerobic, motile, vibrioid, gram-negative, rod-shaped organisms and therefore could be included in section 2 of *Bergey's Manual of Systematic Bacteriology* (18). Based on the guanine-plus-cytosine (G+C) contents of their DNAs, the new isolates could not be included in any of the genera of this section except *Azospirillum* and possibly *Aquaspirillum*. DNA-ribosomal ribonucleic acid (RNA) hybridizations have indicated possible relatedness between *Aquaspirillum* and *Azospirillum* (7).

A brief proposal that the new isolates should be considered a fourth *Azospirillum* species was made by Baldani et al. (2). However, based on more detailed studies and especially on very recent data from Falk et al. (13a), who

TABLE 5. Swarming of *H. seropedicae* and *Azospirillum* spp. on soft nutrient agar[a]

Strain	Diam (mm) after:		
	20 h	48 h	72 h
H. seropedicae			
Z67^T	25	33	69
Z78	32	40	
Z152	17	23	72
Z175	32	43	
ZS12	27	36	
Azospirillum brasilense			
Sp7^T	7	8	19
Sp245	19	38	
Azospirillum lipoferum			
Sp59^T	8	10	
SpBr17	8	14	
Azospirillum amazonense			
Am14^T	5	7	7
Am23	6	6	6

[a] Nutrient agar (0.8% agar) was inoculated in the center with one loopful of a liquid culture and incubated at 35°C.

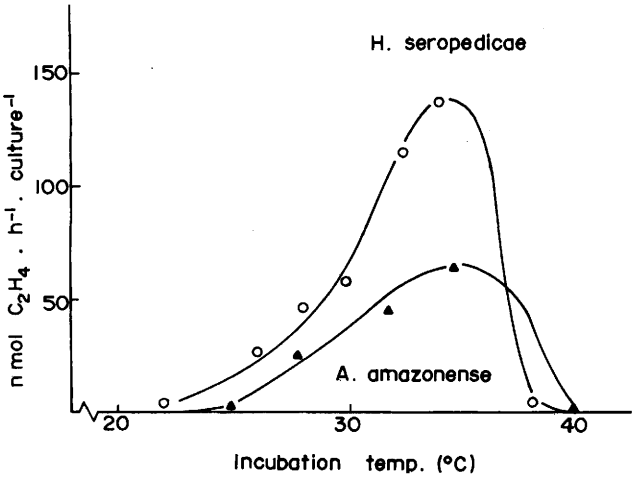

FIG. 4. Effect of incubation temperature on N_2-dependent growth of *H. seropedicae* and *Azospirillum amazonense*. Cultures were evaluated by determining C_2H_2 reduction rates at log phase (43 h for *H. seropedicae* and 72 h for *Azospirillum amazonense* in semisolid NFb medium and LGI medium, respectively). The data points are means of triplicate cultures.

performed RNA-RNA hybridization experiments with two of our isolates, we concluded that the new isolates belong to neither *Azospirillum* nor *Aquaspirillum*. Therefore, we propose a new genus, *Herbaspirillum*. A detailed description of this new genus is given below.

Herbaspirillum gen. nov. *Herbaspirillum* (Her.ba.spi'ril. lum. L. fem. n. *herba* herbaceous seed-bearing plant that does not produce persistent woody tissue; M.L. dimin. neut. n. *spirillum* small spiral; *Herbaspirillum* small spiral-shaped bacteria from herbaceous seed-bearing plants) cells are gram negative, generally vibrioid, sometimes helical. The cells have one to three polar flagella, on one or both poles. The cell diameter is 0.6 to 0.7 μm, and the cell length varies with the medium from 1.5 to 5 μm. Has a typical respiratory metabolism, and sugars are not fermented. Fixes atmospheric N_2 under microaerobic conditions and grows well with N_2 as a sole nitrogen source. Oxidase, catalase, and urease positive. Organic acids, such as malate, fumarate, succinate, pyruvate, citrate, and *trans*-aconitate are the favored carbon substrates for both NH_4^+- and N_2-dependent growth (Table 3). Mannitol, sorbitol, glycerol, and several sugars (glucose, galactose, and L-arabinose) are oxidized (Table 3). Starch and gelatin are not hydrolyzed. The optimum pH range for N_2-dependent growth is much broader than the optimum pH range for *Azospirillum* species (Fig. 1). Equally good growth is observed between pH 5.3 and 8.0. Nitrate is assimilated or dissimilated or both to NO_2^- under O_2 limitation (Fig. 2). No NO_3^--dependent anaerobic growth and no visible gas production from NO_3^- occur in solid or semisolid medium. Small amounts of N_2O are formed under an C_2H_2 atmosphere (Table 4). The habitat of *Herbaspirillum* strains is soil and the roots of members of the Gramineae. The type species is *H. seropedicae*.

***Herbaspirillum seropedicae* sp. nov.** *H. seropedicae* (se.ro.ped'i.cae. L. gen. n. *seropedicae* of Seropédica, Rio de Janeiro, Brazil, where the species was first isolated) cells are vibrioid and sometimes helical and become very motile when they are close to an O_2 source. The cells generally have two polar flagella (occasionally one to three flagella) on one or both poles (Fig. 3). The cell size is as described above for the genus. On soft nutrient agar at 35°C pronounced swarming occurs (Table 5), but lateral flagella as described previously for *Azospirillum* spp. (15) have not been observed. The efficiency of N_2 fixation in semisolid NFb medium is 12 to 15 mg of N^- per g of DL-malate or 13 mg of N^- per g of mannitol. Growth in the presence of N_2 is slower than the growth of *Azospirillum* spp. even though growth in the presence of mineral nitrogen or glutamate is much faster. Does not grow in the presence of 2% NaCl. Vitamins or growth substances are not required. Susceptible to chloramphenicol, tetracycline, gentamicin, kanamycin, erythromycin, and streptomycin and resistant to penicillin. The optimum temperature for N_2-dependent growth is 34°C; no growth occurs at 22 and 38°C (Fig. 4). Other physiological characteristics are as described above for the genus. The G+C content of the DNA is 67 ± 0.5 mol%. Strain Z67 (= ATCC 35892) is the type strain, and strains Z78 (= ATCC 35893) and Z152 (= ATCC 35894) are additional isolates.

TABLE 6. Comparison of *H. seropedicae* with similar N_2-fixing bacteria[a]

Characteristic	*H. seropedicae*	*Aquaspirillum itersonii* and *Aquaspirillum peregrinum*	*Xanthobacter*	*Pseudomonas* sp.[b]	*R. rubrum* and *R. tenue*	*Bradyrhizobium*
Cell shape						
Vibroid	+	−	−	−	−	−
Helical	−	+	−	−	+	−
Predominant type of flagellation[c]	PF	BT	−	SP	PF	SSP
Veillike pellicle in semisolid media	+	−	−	−	−	−
Growth on N_2 as a sole N source	+	−	+	−	+	−
Anaerobic growth with NO_3^-	−	±[d]	−	−	−	+
H_2O-soluble pigments	−	+	−	+	+	−
Use of sugars	+	−[e]	+	+	−[e]	+
Photoautotrophic	−	−	−	−	+	−
Associated with plant roots	+	−	−	+	−	+
G+C content of DNA (mol %)	66–67	60–66	65–70	64	62–66	59–64

[a] Bacteria with DNA G+C contents DNA less than 50 mol % were not included, even if they seemed phenotypically similar. See reference 19.
[b] Because the description of the genus *Pseudomonas* is so heterogeneous, we included only the characteristics of the *Pseudomonas* sp. described by Barraquio et al. (3), a root-associated N_2-fixing bacterium.
[c] PF, One to three polar flagella; SP, single polar flagellum; BT, bipolar tuft; SSP, single subpolar flagellum.
[d] *Aquaspirillum itersonii* only.
[e] Only fructose was used by *Aquaspirillum* spp. and *R. rubrum*.

TABLE 7. Characteristics which differentiate *H. seropedicae* from *Azospirillum* spp.[a]

Characteristic	*H. seropedicae*	*Azospirillum amazonense*	*Azospirillum lipoferum*	*Azospirillum brasilense*
Cell width (μm)	0.6–0.7	0.8–1.0	1.0–1.5	1.0–1.2
Flagellation	One to three flagella, mono- or bipolar	One flagellum, monpolar	One flagellum, monopolar	One flagellum, monopolar
Polymorphic cells in alkaline media	−	−	+	−
Colony type on potato agar	Brownish, small, raised, smooth	White, flat, with raised margin	Pink, raised, curled	Pink, raised, curled
Dissimilation of:				
NO_3^- to NO_2^-	+[b]	∓	+	+
NO_2^- to N_2O	∓[c]	−	∓	∓
NO_3^--dependent anaerobic growth	−	−	+	+
Biotin requirement	−	−	+	−
Use of glucose	+	+	+	−
Use of sucrose	−	+	−	−
Use of keto-glutarate	+	−	+	−
Use of citrate	+	−	+	+
pH range for good growth	5.3–8.0	5.7–6.5	5.7–6.8	6.0–7.3
DNA G+C content, (mol%)	66–67	67–68	69–70	69–70

[a] See references 2, 21, and 32.
[b] +, All strains positive; ∓, several strains positive but the majority of strains negative; −, all strains negative.
[c] All 17 strains tested showed very weak N_2O production.

A comparison of the new genus (represented by *H. seropedicae*) with seven N_2-fixing genera other than *Azospirillum* (Table 6) is not easy because the species in most of these genera are either very variable or not well defined. The most closely related organisms seem to be some *Aquaspirillum* species, especially *Aquaspirillum itersonii* and *Aquaspirillum peregrinum*. Both of these species contain strains which can fix N_2 under microaerobic conditions (20). *H. seropedicae* can be distinguished from them by its vibrioid cell shape, by its double flagella, usually on one pole, by its capacity to use several sugars, and by its occurrence in soil and roots (*Aquaspirillum* species typically occur in freshwater). Furthermore, *Aquaspirillum itersonii* contains no urease and can grow anaerobically on NO_3^-. Coccoid bodies which are formed in old cultures of *Aquaspirillum* species and water-soluble pigments which diffuse into the medium have never been observed in *H. seropedicae*. The RNA-RNA hybridization experiments of Falk et al. (13a) confirm the very remote (if any) relatedness between *H. seropedicae* and *Aquaspirillum itersonii* (15%

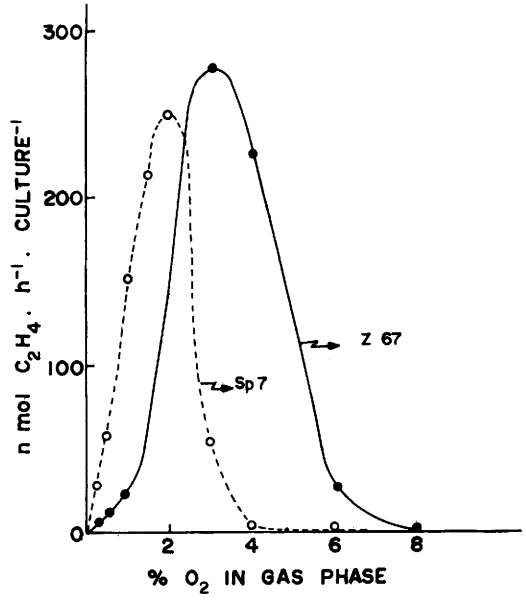

FIG. 5. Effect of oxygen concentration in the gas phase on the nitrogenase activities of *H. seropedicae* Z67^T and *Azospirillum brasilense* Sp7^T. Cells were grown under air to an optical density of 0.87 in liquid NFb medium supplemented with glutamate and after derepression of nitrogenase exposed to various partial O_2 pressures. The data points are means of triplicate cultures.

TABLE 8. Levels of DNA hybridization of *H. seropedicae* and *Azospirillum* spp. strains when *H. seropedicae* Z67^T was used as the reference strain

Strain	Origin of strain	Relative binding (%)
H. seropedicae		
Z67^T	Rice roots	100
Z78	Sorghum roots	56 ± 2[a]
Z176	Maize roots	100
ZM139	Maize roots	84 ± 5
ZM141	Maize rhizosphere	50 ± 1
ZM145	Maize roots	58 ± 8
ZA69	Rice roots	58 ± 4
ZA76	Rice rhizosphere	73 ± 3
ZA80	Rice roots	56 ± 5
ZA85	Rice roots	56 ± 5
ZA94	Rice roots	61 ± 8
ZA95	Rice roots	85 ± 9
ZA110	Rice roots	54 ± 14
ZA113	Rice rhizosphere	86 ± 6
ZS12	Sorghum roots	53 ± 5
ZS57	Sorghum roots	57 ± 2
ZS64	Sorghum roots	91 ± 6
Azospirillum brasilense Sp7^T (= ATCC 29145^T)	*Digitaria* rhizosphere	23 ± 7
Azospirillum lipoferum Sp59^T (= ATCC 29707^T)	Wheat rhizosphere	15 ± 8
Azospirillum amazonense Am14^T (= ATCC 35119^T)	*Digitaria* roots	6 ± 4
Bacillus polymyxa NCTC 10343		5 ± 5

[a] Mean ± standard deviation.

complementarity) or between *H. seropedicae* and some other N_2-fixing genera (level of complementarity with *Rhodospirillum rubrum*, 15%; level of complementarity with *Rhodospirillum tenue*, 47%; level of complementarity with *Beijerinckia indica*, 17%).

A comparison of *H. seropedicae* with the three *Azospirillum* species is shown in Table 7. The most prominent differences between the new genus and *Azospirillum* spp. are the smaller cell size, the presence of more than one flagellum, the distinct colony type, and the slightly lower G+C content of the DNA of *H. seropedicae*. Other differences are the broader pH tolerance in the acid and alkaline range (Fig. 1) and the higher tolerance of nitrogenase activity to O_2 (Fig. 5).

The quantification of DNA complementarity shown in Table 8 confirms the differences between *H. seropedicae* and the three *Azospirillum* species. The 17 *H. seropedicae* strains showed 50 to 100% DNA complementarity, indicating possible subspecies. For this reason all phenotypic characteristics were studied by using strains with high and low levels of DNA complementarity, but no differences were detected between strains showing levels of DNA complementarity higher than 70% and strains showing levels of complementarity lower than 70%. A slight indication of this type seemed to occur in denitrification (Table 4), but the difference was not significant. DNA complementarity of more than 50% have been considered insufficient to distinguish groups within species (13). In fact, *H. seropedicae* seems to represent a very stable and uniform group of strains as judged from all other physiological characteristics. Even the utilization of carbon substrates was identical for all of the strains tested.

The differences in the characteristics of *H. seropedicae* and *Azospirillum* spp. shown in Table 7 might not justify on their own the creation of a new genus. However, recent results of RNA-RNA hybridization experiments comparing strains Z67^T and Z78 with the three *Azospirillum* species revealed very low levels (less than 25%) of RNA complementarity (13a). Therefore, *H. seropedicae* could not be included in the genus *Azospirillum* or the genus *Aquaspirillum*.

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