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Emended Description of *Herbaspirillum*; Inclusion of [*Pseudomonas*] *rubrisubalbicans*, a Mild Plant Pathogen, as *Herbaspirillum rubrisubalbicans* comb. nov.; and Classification of a Group of Clinical Isolates (EF Group 1) as *Herbaspirillum* Species 3

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[*Pseudomonas*] *rubrisubalbicans*, a mild plant pathogen, *Herbaspirillum seropedicae*, and EF group 1 strains (clustered by an immunological method) were investigated by a polyphasic approach with DNA-rRNA and DNA-DNA hybridizations and auxanography on 147 substrates. Our results show that they all belong to the genus *Herbaspirillum*. In addition to *H. seropedicae*, two other species are described: *Herbaspirillum rubrisubalbicans* and a new unnamed species, *Herbaspirillum* species 3, containing mainly strains of clinical origin. The three species can be differentiated on the basis of their auxanographic features and DNA-DNA similarities. The type strain of *H. rubrisubalbicans* is NCPPB 1027 (=LMG 2286); representative strains of the third *Herbaspirillum* species are strains CCUG 189 (=LMG 5523), CCUG 10263 (=LMG 5934), and CCUG 11060 (=LMG 5321). It has been confirmed that *H. rubrisubalbicans* is an endophytic diazotroph. It colonizes the roots, the stems, and predominantly the leaves of sugarcane (*Saccharum* spp.), while *Herbaspirillum seropedicae* colonizes in large numbers many different species of the Gramineae. Both diazotrophic *Herbaspirillum* species could be differentiated with *meso*-erythritol and *N*-acetylglucosamine. Oligonucleotide probes based on partial sequences of the 23S rRNA of *H. seropedicae* and *H. rubrisubalbicans* (HS and HR probes, respectively), were constructed and used as diagnostic probes.

Herbaspirillum seropedicae, isolated from the roots and rhizosphere soil samples of various cereals, was originally thought to be a new species of the genus *Azospirillum*, because of its growth behavior in nitrogen-free semisolid medium (2). However, Baldani et al. (3) and Falk et al. (12) found that it belonged in the β-subclass of the *Proteobacteria* and created a new genus, *Herbaspirillum*, with a single species, *H. seropedicae*. Later it was shown that this species belonged to a rRNA cluster also containing [*Pseudomonas*] *rubrisubalbicans*, a group of strains of clinical origin, and *Janthinobacterium lividum* (16). The clinical isolates have been grouped in the so-called EF group 1 on the basis of their immunological relationships (13). *J. lividum* contains strictly aerobic motile bacteria commonly found in soil and water in temperate regions and producing violet colonies (32). [*P.*] *rubrisubalbicans* has been isolated from diseased cane leaves and has been described as the causative organism of mottled stripe disease (18). It has been shown that only some sugarcane genotypes are sensitive to the disease.

In 1991, Pimentel et al. (29) demonstrated that strains of [*P.*] *rubrisubalbicans* were able to grow and fix N_2 in N-free semisolid medium. Later, Baldani et al. (4) used the C_2H_2 reduction tests as well as incorporation of $^{15}N_2$ to confirm that *H. seropedicae* and [*P.*] *rubrisubalbicans* are true diazotrophs. Both species also show a remarkable tolerance of higher sugar concentrations and are able to grow in up to 10% sucrose, although this carbon source is normally not used by *Herbaspirillum* spp. (4).

In this study, we performed a polyphasic taxonomic analysis with a large collection of strains belonging to *H. seropedicae*, [*P.*] *rubrisubalbicans*, *J. lividum*, and EF group 1.

MATERIALS AND METHODS

Media and methods of isolation. For the isolation of the two N_2-fixing *Herbaspirillum* spp., a modified N-free semisolid NFb medium (10) was used at pH 5.8 and with 3× the phosphate concentration of NFb as well as omission of vitamins (4). The new medium (JNFb) was more selective for isolation of *H. seropedicae* and *H. rubrisubalbicans*. Serial dilutions of smashed soil, root, stem, and leaf samples were inoculated into 10-ml vials containing 5 ml of JNFb medium and incubated for 1 week at 32°C. Fine white pellicles, very similar to *Azospirillum* spp. pellicles in classical NFb medium, were observed; cells of the white pellicles were examined under the microscope and were shown to be small, often slightly curved cells which move close to air bubbles. After an additional transfer and 24 to 48 h of growth in new JNFb medium, such cultures were streaked out on solid NFb medium containing 20 mg of yeast extract per liter and 3× the bromothymol blue concentration of normal NFb. On these plates, both diazotrophic *Herbaspirillum* spp. form small moist colonies with blue centers, much different from *Azospirillum* spp. colonies, which are white, dry, and curled. The isolation procedures for *Herbaspirillum* species 3 of mixed origin will be described elsewhere (14).

Strains. The strains used in this study are presented in Table 1. The EF group 1 organisms are of clinical origin and have been grouped before by one of us by an immunological technique (13).

DNA preparation. Crude DNA was prepared by the method of Marmur (23).

DNA base composition. The mean G+C contents were determined by the thermal denaturation method (9) and were calculated by the equation of Marmur and Doty (24), as modified by De Ley (5).

DNA-rRNA hybridizations. High-molecular-mass DNA was further purified by CsCl gradient centrifugation and fixed on cellulose nitrate filters (type SM 11358; Sartorius, Göttingen, Germany) as described previously (8). The amount of filter-fixed DNA was determined chemically (36). ^{3}H-labeled rRNA from *H.*

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TABLE 1. Characteristics of the strains used in this study

Name as received	Taxonomic position[a]	Strain no.[b]	Other strain designation[b]	Received from[b]	Source (yr of isolation)
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	NCPPB 1027^T	LMG 2286^T, CCUG 17697^T	NCPPB	*Saccharum officinarum*, United States (1961)
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	NCPPB 519	LMG 1278, CCUG 17676	NCPPB	*Saccharum officinarum*, Mauritius (1956)
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	ICMP 792	LMG 6415, CCUG 17680	ICMP	*Saccharum officinarum*, Reunion (1960)
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	ICMP 3108	LMG 6417, CCUG 17682	ICMP	*Saccharum officinarum*, Mauritius (1956)
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	ICMP 3109	LMG 6418, CCUG 17683	ICMP	*Saccharum officinarum*, Sri Lanka (1956)
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	ICMP 3110	LMG 6419, CCUG 17684	ICMP	*Saccharum officinarum*, Tanzania (1964)
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	ICMP 5714	LMG 6420, CCUG 17685	ICMP	*Saccharum officinarum*, Jamaica (1961)
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	IBSBF 198	LMG 10463	J. Döbereiner	*Saccharum officinarum*, Mauritius
[*Pseudomonas*] *rubrisubalbicans*	*Herbaspirillum rubrisubalbicans*	IBSBF 175		J. Döbereiner	*Saccharum officinarum*, Mauritius
Herbaspirillum seropedicae	*Herbaspirillum rubrisubalbicans*	HRC 51		J. Döbereiner	Sugarcane, roots, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum rubrisubalbicans*	HCC 103		J. Döbereiner	Sugarcane, stems, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum rubrisubalbicans*	B 4362	LMG 11128	J. Döbereiner	*Saccharum* hybrid, leaves, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum rubrisubalbicans*	HPD 1		J. Döbereiner	Weed plant, roots, Brazil (1992)
[*Pseudomonas rubrisubalbicans*]	*Herbaspirillum seropedicae*	Hayward 0312B	LMG 2284, CCUG 17677	A. Hayward	*Saccharum officinarum*, Australia (1967)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	Z176	LMG 6512	J. Döbereiner	*Zea mays*, roots, Brazil (1982)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	Z176[c]	LMG 10656	J. Döbereiner	*Zea mays*, roots, Brazil (1982)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	Z67^T	LMG 6513^T, ATCC 35892	J. Döbereiner	*Oryza sativa*, roots, Brazil (1982)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	Z67^{Tc}	LMG 10657^T	J. Döbereiner	*Oryza sativa*, roots, Brazil (1982)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	Z78	LMG 6514, ATCC 35893	J. Döbereiner	*Sorghum bicolor*, roots, Brazil (1982)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	Z78[d]	LMG 10658	J. Döbereiner	*Sorghum bicolor*, roots, Brazil (1982)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	ZS 12	LMG 10659	J. Döbereiner	*Sorghum bicolor*, roots, Brazil (1986)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	Z152	LMG 10660	J. Döbereiner	*Zea mays*, roots, Brazil (1986)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	ZAS 74		J. Döbereiner	*Oryza sativa*, roots, Brazil (1986)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HRC 50	LMG 14778	J. Döbereiner	Sugarcane, roots, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HRC 54		J. Döbereiner	Sugarcane, roots, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HCC 100	LMG 14780	J. Döbereiner	Sugarcane, stems, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HCC 102	LMG 14781	J. Döbereiner	Sugarcane, stems, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HPD 5	LMG 14784	J. Döbereiner	Weed plant, roots, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HRL brach.		J. Döbereiner	*Brachiaria decumbens*, roots, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HRL digt.		J. Döbereiner	*Digitaria decumbens*, roots, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HRL gord.		J. Döbereiner	*Melinis minutiflora*, roots, Brazil (1992)
Herbaspirillum seropedicae	*Herbaspirillum seropedicae*	HCC 105		J. Döbereiner	Sugarcane, stems, Brazil (1992)
[*Pseudomonas rubrisubalbicans*]	*Herbaspirillum* species 3	NCPPB 932	LMG 2285, CCUG 17678	NCPPB	*Saccharum officinarum*, Australia (1961)
[*Pseudomonas rubrisubalbicans*]	*Herbaspirillum* species 3	ICMP 6268	LMG 6421, CCUG 17686 NZRCC 10036	ICMP	*Sorghum* sp., New Zealand (1971)
[*Pseudomonas rubrisubalbicans*]	*Herbaspirillum* species 3	ICMP 2850	LMG 6416, CCUG 17681	ICMP	*Zea mays* cv. rugosa, United States (1961)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 11060	LMG 5321	PHLS	Leg wound, Uddevalla, Sweden (1981)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 189	LMG 5523	PHLS	Contaminant, Göteborg, Sweden (1968)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 3105	LMG 6019	U. Berger	Pharynx, Heidelberg, Germany
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 4446	LMG 6030	PHLS	Probably feces, Göteborg, Sweden (1975)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 6888	LMG 6020	PHLS	Wound, Göteborg, Sweden (1980)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 6997	LMG 6021	H. Lomberg	Urine, Göteborg, Sweden (1978)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 8038	LMG 6022	PHLS	Otitis, Göteborg, Sweden (1979)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 10143	LMG 6023	G. Lidin-Jansson	Tibia fracture, Göteborg, Sweden (1980)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 10221	LMG 5522	PHLS	Gastric juice, Göteborg, Sweden (1980)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 10263	LMG 5934	PHLS	Eye, Uddevalla, Sweden (1980)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 12558	LMG 6026	PHLS	Wound, Göteborg, Sweden (1980)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 12633	LMG 6027	PHLS	Respiratory tract, Göteborg, Sweden (1980)

Continued on following page

TABLE 1—*Continued*

Name as received	Taxonomic position[a]	Strain no.[b]	Other strain designation[b]	Received from[b]	Source (yr of isolation)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 12962	LMG 6024	PHLS	Septic arthritis of knee joint Göteborg, Sweden (1982)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 13048	LMG 6025	PHLS	Axillar aspiration, Göteborg, Sweden (1982)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 13403	LMG 6028	PHLS	Ear, Göteborg, Sweden (1983)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 14952	LMG 6029	PHLS	Leg wound, Göteborg, Sweden (1984)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 15235	LMG 5935, CDC F2754	D. Hollis, CDC	Bacteremia, Honolulu, Hawaii (1982)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 17849	LMG 6799	G. Gilardi	United States (1985)
Unclassified EF group 1	*Herbaspirillum* species 3	CCUG 20016	LMG 7680	PHLS	Eye secretion, Göteborg, Sweden (1987)
Janthinobacterium lividum	*Janthinobacterium lividum*	CCUG 15937	LMG 6356, CCM 3355	CCM	Effluent of abattoir
Janthinobacterium lividum	*Janthinobacterium lividum*	NCTC 9796^T	LMG 2892^T (=Sneath HB), CCUG 2344^T	NCTC	Soil, Michigan (1952)
Janthinobacterium lividum	*Janthinobacterium lividum*	CCUG 3359	LMG 6035, NCIB 7917	NCIB	Lake water, United Kingdom
Janthinobacterium lividum	*Janthinobacterium lividum*	NCTC 7150	LMG 3923, CCUG 15726	NCTC	Water
Janthinobacterium lividum	*Janthinobacterium lividum*	Sneath RU	LMG 3934, CCUG 15727, NCIP 9230	P.H.A. Sneath	Soil, United Kingdom
Janthinobacterium lividum	*Janthinobacterium lividum*	Sneath HA	LMG 3931, CCUG 15728, NCIB 9217	P.H.A. Sneath	Soil, Michigan
Janthinobacterium lividum	*Janthinobacterium lividum*	Sneath MB	LMG 3919, CCUG 15729	P.H.A. Sneath	Water of sand filter, United Kingdom
Janthinobacterium lividum	*Janthinobacterium lividum*	ATCC 14487	LMG 3927, CCUG 15730	ATCC	Pasteurized commercial cream
Burkholderia cepacia[d]		ATCC 25609t1	LMG 6981		
Ralstonia pickettii[d]		CCUG 3318^T	LMG 5942^T		
Iodobacter fluviatile[d]		USCC 2237	LMG 6573		
Oligella ureolytica[d]		CCUG 1465A^T	LMG 6519^T		
Comamonas terrigena[d]		NCIB 2581	LMG 1249		
Acidovorax avenae subsp. *avenae*[d]		NCPPB 1011^T	LMG 2117^T		

[a] As proposed in this study.

[b] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md; CCM, Czechoslovak Collection of Microorganisms, Brno, Czech Republic; CCUG, Culture Collection University of Göteborg, Department of Clinical Bacteriology, Göteborg Sweden; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH, Braunschweig, Germany; ICMP, International Collection of Micro-organisms from Plants, Plant Diseases Division, DSIR Mount Albert Research Centre, Auckland, New Zealand; LMG, Culture Collection, Laboratory of Microbiology, Universiteit Gent, Ghent, Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, Hertsfordshire, United Kingdom; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom; NZRCC, New Zealand Reference Culture Collection (Plant and Soil), DSIR Plant Diseases, Auckland, New Zealand; PHLS, Public Health Laboratory, Sweden; USCC, University of Surrey Culture Collection, Department of Microbiology, University of Surrey, Guilford, Surrey, United Kingdom; brach., *Bracharia*; digt., *Digitaria*; gord., from "capim gordura" (*Melinis minuitiflora*).

[c] For some cultures, we separately investigated two subcultures obtained on different dates.

[d] Representative strains from other rRNA branches of the β-subclass of the *Proteobacteria*.

rubrisubalbicans LMG 2286^T was prepared and purified by the method described by De Ley and De Smedt (8). Cells were labeled in vivo by addition of 1.8 mCi of [5,6-^{3}H]uracil (New England Nuclear Research Products, Boston, Mass.) to 100 ml (wt/vol) of the following medium: peptone, 1%; succinate, 0.1%; $(NH_4)_2SO_4$, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.1%; $FeCl_3 \cdot 6H_2O$, 0.0002%; $MnSO_4 \cdot 6H_2O$, 0.0002% (pH 7) with KOH. The activity of the 23S fraction was 30,000 dpm.

DNA-DNA hybridizations. The initial renaturation method was used (7); the optimal renaturation conditions were 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M Na citrate [pH 7.0]) and $T_{OR(2)}$ [$T_{OR(2)}$ is optimal renaturation temperature in 2× SSC] of 71°C.

Species-specific probes. Oligonucleotide probes complementary to a highly variable stretch of helix 55 to 59 (numbering according to reference 19) of the 23S rRNA of *H. seropedicae* (sequenced strain was ATCC 35892^T) and *H. rubrisubalbicans* (sequenced strain was LMG 2286^T) were designed. Probes with 19 base lengths were synthesized (probe HS, 5′-GTC CCG GTT TTT GCA TCG A-3′; probe HR, 5′-TAG TCG GTT TTT GCA TCG A-3′), and species specificity was confirmed by hybridization experiments with the rRNA of 80 different soil- and plant-associated bacteria (data not shown). The bulk nucleic acid of all strains was isolated according to a method described by Oelmuller et al. (25) and transferred to a nylon membrane (Quiagen-plus; Diagen) via spot blotting. The hybridization with radioactive labeled probes was performed for 2 to 12 h at 52°C according to the method of Kirchhof and Hartmann (21). Signals were detected by autoradiography.

Phenotypic tests. API galleries (API 50CH, API 50AO, and API 50AA; bio-Mérieux, Montalieu-Vercieu, France) were used to test the assimilation of 147 organic compounds as sole carbon sources. The experimental procedure we used has been described previously (20). All strains were tested; several of the strains were included in duplicate on separate occasions to verify the reproducibility of the tests. The results of auxanographic tests were scored as described previously (20). Of the 147 features tested, 44 tests (given in a footnote to Table 2) were not included in the numerical analysis because they gave a negative result. The levels of interstrain similarity (S) were calculated with a similarity coefficient derived from the Canberra metric coefficient (d_{CANB}) (33) and the following equation: $S = 100 \times (1 - d_{CANB})$. A cluster analysis was performed with the unweighted average pair group method (33), the Clustan 2.1 program of Wishart (38), and the Siemens model 7570-C computer of the Centraal Digitaal Rekencentrum, Universiteit, Ghent, Belgium.

The ability to catabolize some of these carbon sources was also evaluated with semisolid JNFb medium, in which malate was replaced by the carbon substrates. Growth dependent on nitrogen fixation or NH_4^+ was evaluated by pellicle formation.

RESULTS AND DISCUSSION

Polyphasic taxonomy in the genus *Herbaspirillum*. The fine phenotypic relationships between a number of representative strains of *H. seropedicae*, [*P.*] *rubrisubalbicans*, EF group 1, and

TABLE 2. Auxanographic results

Substrate	Auxanographic result for organism by phenon[a]				
	Herbaspirillum species 3		*H. rubrisubalbicans* (phenon II [n = 8])	*H. seropedicae* (phenon III [n = 7])	*J. lividum* (phenon IV [n = 8])
	Phenon Ia (n = 8)	Phenon Ib (n = 14)			
Arbutin, salicin, D-cellobiose, maltose sucrose, trehalose, inulin, D-tartrate, D-raffinose, starch, L-phenylalanine, DL-5-aminovalerate, betaine, glutarate, L-histidine	−	−	−	−	d
Adonitol, D-galactose, L-fucose, L-arabitol, gluconate, 2-ketogluconate, propionate, isobutyrate, *n*-valerate, isovalerate, D-malate, *meso*-tartrate, butyrate, DL-4-aminobutyrate, aconitate, *p*-hydroxybenzoate, L-proline, D-arabinose	+	+	+	+	d
Ethanolamine	+	+	d	+	−
5-Ketogluconate, acetate, caprate, amylamine, D-fucose, tryptamine	+	+	+	+	−
meso-Erythritol[b]	+	−	+	−	−
L-Rhamnose[b]	−	−	−	+	d
meso-Inositol[b]	−	−	−	+	+
N-Acetylglucosamine[b]	+	+	−	+	d
Suberate[b]	+	+	−	−	+
L-Tartrate[b]	+	+	−	−	−
Sebacate[b]	+	d	−	−	+
Adipate, pimelate, azelate[b]	+	+	−	−	d
Benzoate[b]	+	−	+	d	−
Lactose, glucosamine	d	d	−	−	d
Malonate	d	d	−	−	−
L-Xylose, caprylate	d	d	d	d	−
Glycolate	+	d	−	−	−
Phenylacetate, L-tyrosine, *n*-valerate, L-tryptophan	+	d	+	+	d
o-Hydroxybenzoate	d	−	−	−	−
D-Mandelate	−	−	d	−	−
L-Mandelate, itaconate, mesaconate	d	+	+	+	−
D-α-Alanine	+	d	d	+	+
L-α-Alanine, L-isoleucine	+	d	+	+	+
L-Norleucine	d	d	d	d	d
L-Valine	d	d	d	d	+
Heptanoate	d	−	d	d	−
L-Serine, pelargonate	+	d	+	d	+
L-Cysteine	d	d	+	d	d
β-Alanine	d	d	+	+	−
Butylamine	d	+	d	+	−
DL-3-Aminobutyrate	d	−	+	d	−
Benzylamine	−	−	−	d	−

[a] +, present in 90% or more of the strains; −, present in 10% or less of the strains; d, 11 to 89% of all strains positive. All strains were negative for methyl-xyloside, L-sorbose, dulcitol, methyl-D-mannoside, methyl-D-glucoside, amygdalin, esculin, D-melibiose, D-melezitose, glycogen, β-gentiobiose, D-turanose, D-tagatose, citraconate, levulinate, *m*-hydroxybenzoate, phthalate, isophthalate, terephthalate, *n*-capronate, oxalate, maleate, glycine, DL-norvaline, DL-2-aminobutyrate, L-methionine, D-tryptophan, trigonelline, L-ornithine, L-lysine, L-citrulline, L-arginine, DL-kynurenine, creatine, 2-aminobenzoate, 3-aminobenzoate, 4-aminobenzoate, ureum, acetamide, sarcosine, ethylamine, diaminobutane, spermine, and histamine. All strains were positive for glycerol, L-arabinose, ribose, D-xylose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, xylitol, D-lyxose, D-arabitol, DL-lactate, DL-glycerate, DL-3-hydroxybutyrate, L-malate, pyruvate, citrate, succinate, fumarate, L-leucine, L-threonine, L-aspartate, L-glutamate, and 2-ketoglutarate.

[b] Differentiating substrates.

J. lividum were studied by an auxanographic analysis of 147 substrates. The results are given in Fig. 1. Four phena were delineated. *J. lividum* strains (phenon IV) constitute a separate cluster that is the most divergent from the other three clusters (phena I to III). Phenon I contains all EF group 1 strains and three [*P.*] *rubrisubalbicans* strains, LMG 2285, LMG 6421, and LMG 6416. Within this phenon, two subgroups, a and b, could be differentiated above 85% *S*, with subgroup a containing the three [*P.*] *rubrisubalbicans* strains. Phenon II contains only [*P.*] *rubrisubalbicans* strains, including the type strain, and phenon III consists of nine *H. seropedicae* strains and one [*P.*] *rubrisubalbicans* strain (LMG 2284). The reproducibility of the results varied from 95 to 97%. The auxanographic features of these four phena are given in Table 2; for clarity, the subgroups of phenon I were kept separate. Representative strains of the four phena were used for DNA-rRNA hybridizations in order to study the inter- and intrageneric similarities between the four phena. The $T_{m(e)}$ values (temperature in degrees Celsius at which 50% of the hybrid is denatured under standard conditions [8]) of the DNA-rRNA hybrids with rRNA from [*P.*] *rubrisubalbicans* LMG 2286^T are given in Table 3. [*P.*] *rubrisubalbicans*, *H. seropedicae*, the EF group 1 organisms, and *J. lividum* constitute a separate rRNA cluster and are closely related [$T_{m(e)}$ of 76.2 to 79.7°C]. Within rRNA superfamily III (β-subclass of the *Proteobacteria* [34]), this cluster constitutes a separate rRNA branch with *Burkholderia* and the other members of the former *solanacearum* rRNA complex as its closest neighbors ($T_{m(e)}$ of 73.9 to 74.4°C) (17). The position of the separate rRNA cluster is given in a $T_{m(e)}$ dendrogram in Fig. 2. It has been shown before that members of an rRNA cluster

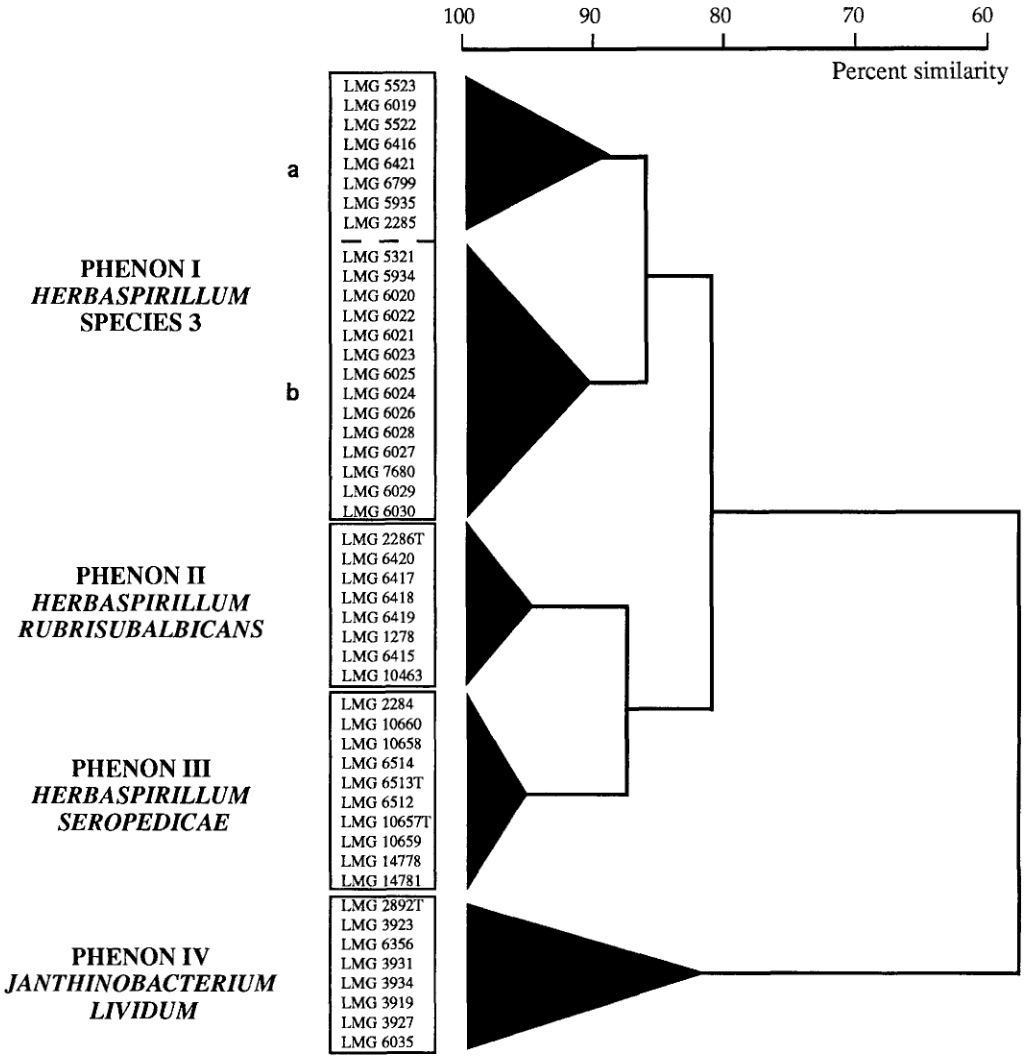

FIG. 1. Dendrogram derived from an unweighted average pair group cluster analysis of similarity coefficients obtained by comparison of the results of 103 auxanographic tests for strains belonging to the *Herbaspirillum* rRNA branch.

with differences (3 to 4°C) in their $T_{m(e)}$, comparable to what we will call the *Herbaspirillum* rRNA cluster, can be considered as belonging to a single genus (6). Because *J. lividum* has the lowest $T_{m(e)}$ value within this rRNA cluster and is phenotypically quite distinct from the other members of this rRNA branch (15, 30, 32), we propose to keep this genus separate and to unify the other members of this rRNA cluster into a single genus. According to the nomenclatural rules (22), the name *Herbaspirillum* has priority because its type species belongs to this rRNA cluster and because the other members of this genus carry no official name or have generically been misnamed according to the present phylogenetic data.

In order to examine the intrageneric relationships within *Herbaspirillum*, we used DNA-DNA hybridizations between members of the different phena (I to III); a representative *J. lividum* strain was included as a control. The results of DNA-DNA hybridizations are given in Fig. 3 and revealed three homology groups (I to III) corresponding to the respective *Herbaspirillum* phena. Within groups II and III, very high values of DNA-DNA hybridization were found (71 to 100%). However, within group I, the percentage of binding varied between 52 and 100%, and two subgroups of phenon I can be differentiated, namely subgroup Ib (89 to 100%) and subgroup Ia (86 to 100%), with 56% DNA binding between both subgroups. Low (≤40%) or nonsignificant values (<30% binding) were found between the different *Herbaspirillum* groups and with a representative *J. lividum* strain. It is clear that the three *Herbaspirillum* DNA groups represent at least three genomic species that do correspond to the three phena found, within the genus *Herbaspirillum*. Although it is recommended that species should share at least 70% DNA homology, together with a maximum 5°C ΔT_m when the S1 nuclease hybridization method is used (37), it is our experience that this species delineation threshold for the initial renaturation method corresponds to percentages of DNA binding above 40%. As a consequence, we conclude that there are at least three species within the genus *Herbaspirillum*. (i) *H. seropedicae* corresponds to phenon III and contains the *H. seropedicae* strains, including the type strain. Strain LMG 2284, which was classified before with the name [*P.*] *rubrisubalbicans*, is, on the basis of phenotypic and genotypic data, certainly a member of *H. seropedicae*. (ii) Also within the genus *Herbaspirillum* are the [*P.*] *rubrisubalbicans* strains of phenon II, including the type strain, which as a consequence should be named *H. rubrisubalbicans*. (iii) Fi-

TABLE 3. $T_{m(e)}$ of DNA-rRNA hybrids with labeled rRNA from *H. rubrisubalbicans* LMG 2286^T and *J. lividum* LMG 2892^T

DNA	% G+C content	$T_{m(e)}$ (°C) of DNA-rRNA hybrid with 23S rRNA from:	
		H. rubrisubalbicans LMG 2286^T	*J. lividum* LMG 2892^T
Herbaspirillum rubrisubalbicans LMG 2286^T	62.5	79.7	
Herbaspirillum seropedicae LMG 6512		77.4	
Herbaspirillum seropedicae LMG 6513^T	64.5	78.3	
Herbaspirillum seropedicae LMG 6514	65	77.1	
Herbaspirillum seropedicae LMG 10659		77.8	
Herbaspirillum seropedicae LMG 2284		77.5	
Herbaspirillum seropedicae LMG 11128		78.3	
Herbaspirillum species 3 LMG 5523	61	79.3	75.8
Herbaspirillum species 3 LMG 5321		79.1	76.1
Herbaspirillum species 3 LMG 2285		77.0	
Janthinobacterium lividum LMG 2892^T	65.5	76.2	78.8
Burkholderia cepacia LMG 6981	69.0	73.9	
Ralstonia pickettii LMG 5942^T		74.4	
Iodobacter fluviatile LMG 6573		69.3	
Oligella ureolytica LMG 6519^T		69.5	
Comamonas terrigena LMG 1249	66	70.0	
Acidovorax avenae subsp. *avenae* LMG 2117^T	62.5	71.4	

nally, most EF group 1 strains should be included, together with three strains that have been named [*P.*] *rubrisubalbicans* and which we refer to as *Herbaspirillum* species 3 until a full proposition with a proper species description can be given (14).

It is not so obvious that nitrogen-fixing bacteria like *H. seropedicae* can also be plant pathogenic and that they are closely related to bacteria isolated from clinical environments. In fact, only intensive DNA-rRNA hybridizations with a wide variety of proteobacteria allowed us to establish the intrageneric relationship between *H. seropedicae*, *H. rubrisubalbicans*, and *Herbaspirillum* sp. 3. We do not have a plausible explanation for such relationships that were also found in such other genera as *Arcobacter* (35) and *Burkholderia* (17).

All *Herbaspirillum* species grown in the API 50CH, 50AO, and 50AA systems used the following substrates: glycerol, D- and L-arabinose, ribose, D-xylose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, xylitol, D-lyxose, DL-lactate, DL-glycerate, DL-3-hydroxybutyrate, pyruvate, citrate, succinate, fumarate, L-leucine, L-threonine, L-aspartate, L-glutamate, 2-ketoglutarate, adonitol, D-galactose, L-fucose, D- and L-arabitol, gluconate, 2-ketogluconate, propionate, isobutyrate, *n*-valerate, isovalerate, D- and L-malate, *meso*-tartrate, butyrate, DL-4-aminobutyrate, aconitate, mesaconate, *p*-hydroxybenzoate, L-proline, D-arabinose, 5-ketogluconate, acetate, caprate, amylamine, D-fucose, and tryptamine.

The following auxanographic features were shown to be discriminative between the three species: *meso*-erythritol, *N*-acetylglucosamine, L-rhamnose, *meso*-inositol, azelate, sebacate, pimelate, suberate, adipate, and L-tartrate.

The endophytic occurrence of *H. seropedicae* and *H. rubrisubalbicans* reported earlier (4, 11) was recently confirmed (27). Neither of the two diazotrophic species could be isolated from soil samples. The occurrence of these diazotrophic bacteria in plants can be explained by their dissemination via the seeds (1). Among a large number of samples of roots, stems, and leaves from 13 species of the family Gramineae collected at various sites, we could not detect *H. seropedicae* only in the species *Paspalum notatum*, *Digitaria insularis*, and *Echinocloa crusgalli*. On the other hand, *H. rubrisubalbicans* could be isolated only from sugarcane (*Saccharum* spp.) and from one sample of a weed plant, *D. insularis*, collected in a sugarcane field. In various samples of weeds collected within maize and sugarcane fields (of the families Compositae, Molluginaceae, Sterculiaceae, Cyperaceae, Portulacaceae, Leguminosae, and Cucurbitaceae), we could not detect either *Herbaspirillum* species. In contrast to *H. seropedicae*, *H. rubrisubalbicans* was not detected in samples of forage grasses grown at different sites of EMBRAPA-CNPAB, Rio de Janeiro, Brazil (27).

Because of the endophytic occurrence of *H. rubrisubalbicans* and *H. seropedicae* (4, 11), it was important to focus on some important but simple features that can distinguish both species from each other and from the other *Herbaspirillum* species. Strains of *H. seropedicae* have one to three flagella at one or both poles, show optimum growth at 34°C, and use *N*-acetylglucosamine but not *meso*-erythritol as a sole carbon source both in API 50CH and in the classical test procedure. In contrast, strains of *H. rubrisubalbicans* have multiple flagella at one pole, show optimum growth at 30°C, and do not use *N*-acetylglucosamine but use *meso*-erythritol as a sole carbon source in API 50CH and in the classical procedure, when performed in the presence of inorganic nitrogen (NH_4Cl) (Table 4). In addition, they cause mottled stripe disease on the sensitive sugarcane variety B-4362 (29). *Herbaspirillum* species 3 strains from subphenon Ia use *meso*-erythritol in the API 50CH system, while the members of subphenon Ib do not. However, in the classical procedure, members of both subphena are positive for *meso*-erythritol. In contrast to *Herbaspirillum* species 3, *H. seropedicae* and *H. rubrisubalbicans* are able to fix dinitrogen. The only exception was *H. seropedicae* LMG 2284, which is unable to fix dinitrogen.

The ability of *Herbaspirillum* species to catabolize *N*-acetyl-

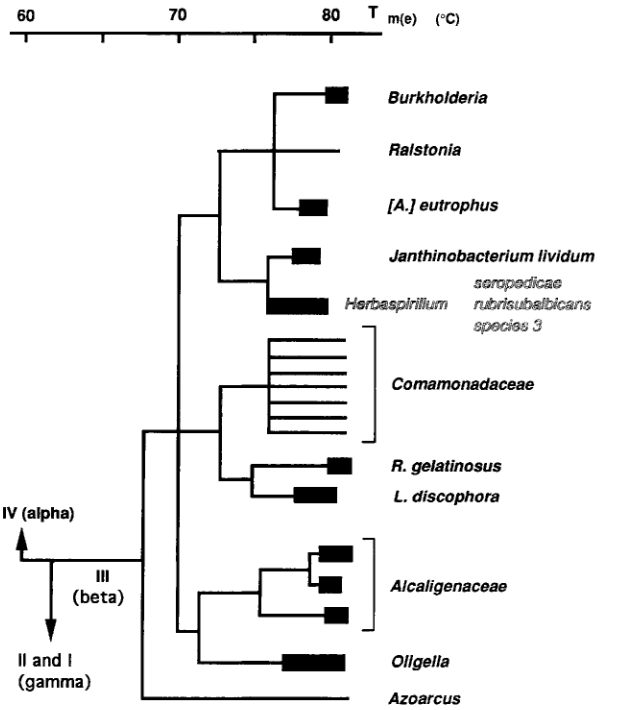

FIG. 2. $T_{m(e)}$ dendrogram showing the position of *Herbaspirillum* within rRNA superfamily III (β-subclass). *A.*, *Alcaligenes*; *L.*, *Leptothrix*; *R.*, *Rhodocyclus*.

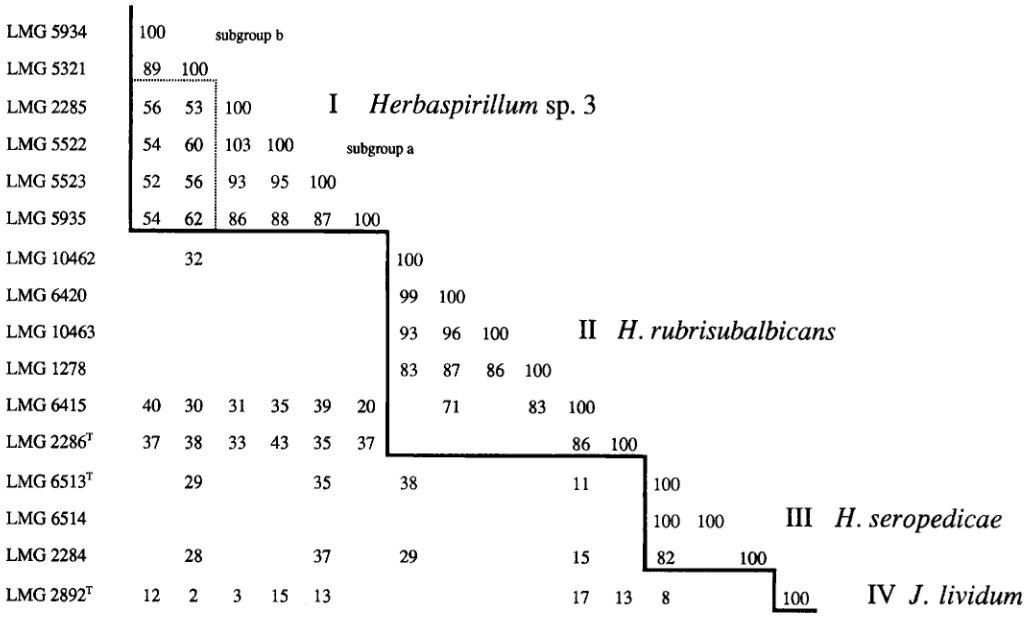

FIG. 3. Results of DNA-DNA hybridization.

glucosamine and/or *meso*-erythritol was confirmed by classical procedures with a larger group of endophytic strains (Table 4). Eight more strains previously classified as *H. seropedicae* used *N*-acetylglucosamine but not *meso*-erythritol (*N*-acetylglucosamine group), while another five strains, previously classified as [*P.*] *rubrisubalbicans*, catabolized *meso*-erythritol in the presence of NH_4Cl but not *N*-acetylglucosamine (*meso*-erythritol group). Both physiological groups (*meso*-erythritol and *N*-acetylglucosamine) were also tested with specific oligonucleotide probes based on highly variable stretches of their 23S rRNA. The results are presented in Table 4. The *H. seropedicae* oligonucleotide probe (HS) could definitely identify all strains of the *N*-acetylglucosamine group as *H. seropedicae* strains. The HR oligonucleotide probe could not distinguish strains of *H. rubrisubalbicans* from strains of *Herbaspirillum* species 3. In addition, sequence comparison of the hypervariable region (helix positions 55 to 59) of the 23S rRNA from *H. rubrisubalbicans* LMG 2286^T and those of strains from *Herbaspirillum* species 3 (LMG 5934, LMG 5523, and LMG 2285, representing both subgroups) showed no sequence variation (data not shown). The two subgroups of *Herbaspirillum* species 3 also could not be distinguished, because all members of the third species showed growth on *meso*-erythritol by conventional tests, while in contrast, in API 50CH, only members of subgroup Ia reacted positively to *meso*-erythritol (Table 2). However, both species of the *meso*-erythritol group could be further differentiated by their ability to fix N_2 and by their growth on adipate, pimelate, suberate, azelate, sebacate, and L-tartrate (Table 2).

Emended description of *Herbaspirillum* (Baldani, Baldani, Seldin, and Döbereiner 1986). *Herbaspirillum* (Her.ba.spi'ril. lum. L. fem. n. *herba*, herbaceous seed-bearing plant that does not produce persistent woody tissue; M.L. dim. neut. n. *spirillum*, small spiral; *Herbaspirillum*, small spiral-shaped bacteria from herbaceous seed-bearing plants).

Cells are gram negative, generally vibroid, and sometimes helical. The cells have one to three or more 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 5.0 μm. The organism has a typical respiratory metabolism, and sugars are not fermented. Some species fix atmospheric N_2 under microaerobic conditions and grow well with N_2 as a sole nitrogen source, even in up to 10% sucrose. The organism is oxidase and urease positive; catalase is weak or variable. Organic acids are the favored carbon sources for growth. All species grow on glycerol, D- and L-arabinose, ribose, D-xylose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, xylitol, D-lyxose, DL-lactate, DL-glycerate, DL-3-hydroxybutyrate, pyruvate, citrate, succinate, fumarate, L-leucine, L-threonine, L-aspartate, L-glutamate, 2-ketoglutarate, adonitol, D-galactose, L-fucose, D- and L-arabitol, gluconate, 2-ketogluconate, propionate, isobutyrate, *n*-valerate, isovalerate, D- and L-malate, *meso*-tartrate, butyrate, DL-4-aminobutyrate, aconitate, mesaconate, *p*-hydroxybenzoate, L-proline, D-arabinose, 5-ketogluconate, acetate, caprate, amylamine, D-fucose, and tryptamine.

The genus constitutes a separate rRNA cluster within the β-subclass of the *Proteobacteria*.

The G+C content of the DNA is 60 to 65% (as determined by thermal denaturation).

The type species is *H. seropedicae*.

Emended description of *H. seropedicae* (Baldani, Baldani, Seldin, and Döbereiner 1986). *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 vibroid and sometimes helical and become very motile when close to an O_2 source. The cells have generally two polar flagella (occasionally one to three flagella) on one or on both poles. On soft nutrient agar at 35°C, pronounced swarming occurs. Most strains fix atmospheric N_2 under microaerobic conditions and grow well with N_2 as a sole nitrogen source. The efficiency of N_2 fixation in semisolid NFb medium is 12 to 15 mg of N_2 per g of DL-malate. Growth in the presence of N_2 is slower than the growth of *Azospirillum* spp., but the growth in the presence of mineral nitrogen or glutamate is much faster. The organism does not grow in the presence of 2% NaCl. Vitamins or growth substances are not required. The organism is susceptible to chloramphenicol, tetracycline, gentamicin, kanamycin, erythromycin, and streptomycin and is resistant to penicillin. The

TABLE 4. Use of *meso*-erythritol and *N*-acetylglucosamine (independent of N_2 fixation) and hybridization with the oligonucleotide probes HR (*H. rubrisubalbicans*) and HS (*H. seropedicae*)

Strain	Use of [a]:		Hybridization with probe:	
	meso-Erythritol	*N*-Acetyl-glucosamine	HR	HS
Herbaspirillum seropedicae				
Z67[T]	−	+	−	+
Z78	−	+	−	+
Z152	−	+	−	+
ZAS74	−	+	−	+
LMG 2284[b]	−	+	−	+
HRC 50	−	+	−	+
HRC 54	−	+	−	+
HCC 100	−	+	−	+
HCC 102	−	+	−	+
HCC 105	−	+	−	+
HPD 5	−	+	−	+
HRL brach.	−	+	−	+
HRL digt.	−	+	−	+
HRL gord.	−	+	−	+
Herbaspirillum rubrisubalbicans				
LMG 2286[T]	+	−	+	−
HRC 51	+	−	+	−
HCC 103	+	−	+	−
HPD 1	+	−	+	−
LMG 1278	+	−	+	−
LMG 6420	+	−	+	−
IBSBF 175	+	−	+	−
IBSBF 198	+	−	+	−
B 4362	+	−	+	−
Herbaspirillum species 3				
LMG 5523	+	−	+	−
LMG 5934	+(−)	−	+	−
LMG 7680	+(−)	−	+	−
LMG 2285	+	−	+	−

[a] As tested by conventional tests and API 50CH. When both systems did not give the same result, the API 50CH result is given in parentheses.

[b] This strain does not fix dinitrogen, as evaluated by $^{15}N_2$ incorporation and C_2H_2 reduction.

optimum temperature for N_2-dependent growth is 34°C; no growth occurs at 22 and 38°C. Starch and gelatin are not hydrolyzed. 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. No NO_3^--dependent anaerobic growth and no visible gas production from NO_3^- occur in solid or semisolid medium. The habitats are roots, stems and leaves of all kinds of members of the family Gramineae (13 species). Members of this species could not be isolated from soil, and when inoculated in large numbers into soil, they cannot be reisolated after 3 weeks, except when sorghum plants are introduced (26). *H. seropedicae* can be differentiated from the other species by its auxanographic features (Table 2) and by the use of the HS DNA probe.

The G+C content of the DNA is 64 to 65%.

The type strain is Z67=ATCC 35892=LMG 6513, with a G+C content of 64.4 mol%.

Description of *H. rubrisubalbicans* (Christopher and Edgerton 1930) comb. nov. (*Pseudomonas rubrisubalbicans Approved Lists 1980*) *H. rubrisubalbicans* (ru.bri. sub.al'bi.cans L. adj. *ruber*, red; L. adj. *subalbicans*, whitish; M.L. adj. *rubrisubalbicans*, red-whitish, referring to the symptoms of mottled stripe disease). The description is based on that of Palleroni et al. (28) for [*P.*] *rubrisubalbicans* and the results of this study. The organism appears as slightly curved rods and is motile by means of several polar flagella. Poly-β-hydroxybutyrate is accumulated. Colonies on 2% glucose–peptone agar are mucoid (not in 2% sucrose–peptone agar). No pigments are produced. Cells do not produce H_2S. There is no hydrolysis of gelatin, starch, and Tween 80. Most strains reduce nitrate to nitrite. Denitrification is negative. Growth occurs at 40°C; the optimal temperature for growth is 30°C. Auxanographic differences from the other *Herbaspirillum* species are given in Table 2.

Acid is produced from glucose, fructose, galactose, arabinose, mannitol, lactose, glycerol, and sorbitol. No acid is produced from sucrose, raffinose, salicin, maltose, cellobiose, or *meso*-inositol. Cells do fix N_2 as efficiently as *H. seropedicae*. The organism causes mottled stripe disease on sugarcane, mainly on plant genotypes from regions in which high-N fertilizer applications were used. *H. rubrisubalbicans* has now been shown to grow abundantly in nitrogen-free semisolid media, with veil-like pellicles, just as *H. seropedicae* does. Nitrogenase activities and efficiency in biological N_2 fixation are the same as those of *H. seropedicae*. In contrast to this species, its occurrence seems limited to sugarcane, but when artificially inoculated by injection, red stripes are also formed on *Sorghum vulgare* and *Pennisetum purpureum* (29). It also could be reisolated from these plants. The optimal growth temperature is 30°C, in contrast to 34°C for *H. seropedicae*.

The G+C content of the DNA is 62 to 63%.

The type strain is NCPPB 1027=LMG 2286. Its G+C content is 62.5%.

Herbaspirillum species 3 combines isolates from various different origins with high levels of DNA relatedness among them, but it is clearly distinguishable from the two species described and is unable to fix dinitrogen. The detailed description of this species will be given elsewhere (14). For additional characteristics of this species, see Table 2.

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