A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov.

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Accepted April 25, 1978

TARRAND, J. J., N. R. KRIEG, and J. DÖBEREINER. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Can. J. Microbiol. **24**: 967–980.

Sixty-one strains of the root-associated nitrogen fixer *Spirillum lipoferum* exhibited a similar morphology in peptone – succinate salts medium: vibrioid cells having a diameter of 1.0 μm. When grown in broth the cells had a single polar flagellum, but when grown on agar at 30°C lateral flagella of shorter wavelength were also formed. The DNA base composition was 69–71 mol % guanine + cytosine when determined by thermal denaturation. DNA homology experiments indicated the occurrence of two distinct but related homology groups: 46 strains were in group I and 15 strains were in group II. Group II strains were distinguished by their ability to use glucose as a sole carbon source for growth in nitrogen-free medium, by their production of an acidic reaction in a peptone-based glucose medium, by their requirement for biotin, and by their formation of wider, longer, S-shaped or helical cells in semisolid nitrogen-free malate medium. The results indicate that two species exist, and on the basis of their characteristics it is proposed that they be assigned to a new genus, ***Azospirillum***. Strains belonging to group II are named ***A. lipoferum*** (Beijerinck) comb. nov., while those belonging to group I are named ***A. brasilense*** sp. nov. Strain Sp 59b (ATCC 29707) is proposed as the neotype strain for *A. lipoferum*, and strain Sp 7 (ATCC 29145) is proposed as the type strain for *A. brasilense*.

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Soixante et une espèces de *Spirillum lipoferum* associées aux racines et fixatrices d'azote présentent des morphologies semblables dans les milieux minéraux avec du succinate et la peptone; les cellules ont la forme de vibrion avec un diamètre de 1 μm. Lorsque les cellules se développent dans des bouillons, elles possèdent un seul flagelle polaire; par contre sur la gélose à 30°C elles présentent aussi un flagelle latéral plus court. La composition de l'ADN est de 69 à 71% de guanine + cytosine. Des expériences sur l'homologie de l'ADN indiquent la présence de deux groupes homologues distincts, mais présentant une parenté: 46 espèces ont été classées dans le groupe I et 15 dans le groupe II. Les espèces du groupe II sont différenciées par leur capacité d'utiliser le glucose comme seule source de carbone pour la croissance dans un milieu sans azote, par la production d'une réaction acidique sur un milieu à base de glucose et de peptone, par leur besoin en biotine et par la formation sur un milieu malate semi-solide sans azote, de cellules en forme de S ou d'hélice plus larges et plus longues. Les résultats montrent qu'il existe deux espèces, et d'après leur caractéristiques nous proposons de les classer dans un nouveau genre: ***Azospirillum***. Les espèces appartenant au groupe II sont désignées ***A. lipoferum*** (Beijerinck) comb. nov., tandis que celles appartenant au groupe I sont désignées ***A. brasilense*** sp. nov. L'espèce Sp 59b (ATCC 29707) est désignée comme le néotype de *A. lipoferum* et l'espèce Sp 7 (ATCC 29145) est désignée comme le représentant typique de *A. brasilense*.

[Traduit par le journal]

Introduction

The bacterium known as *Spirillum lipoferum* was first described by Beijerinck (1922, 1925). Except for a few scattered reports (Becking 1963; Schröder 1932; G. Giesberger 1936, Ph.D. Dissertation, Utrecht Univ., Netherlands) relatively little attention was given to this organism until 1974 when Döbereiner and Day (1976) isolated it from the roots of certain tropical grasses in Brazil. The root pieces exhibited nitrogenase activity as indicated by acetylene reduction, and the nitrogen-fixing ability of the plants has since been confirmed by dem-

onstrating incorporation of $^{15}N_2$ into the plant tissue (De-Polli *et al.* 1977). Since 1974 *S. lipoferum* has been isolated from the roots of a variety of forage grasses, legumes, grain crops, and soils (Döbereiner *et al.* 1976; Kumari *et al.* 1976; Von Bülow and Döbereiner 1975). The organism is Gram-negative, motile, generally vibrioid in shape, and contains poly-β-hydroxybutyrate (PHB) granules (Döbereiner and Day 1976; Okon *et al.* 1976*a*, 1976*b*). When growing in N-free media it seems microaerophilic because of a lack of oxygen-protection mechanisms for its nitrogenase (Day and Döbereiner 1976; Döbereiner 1977; Döbereiner and Day 1976; Okon *et al.* 1976*a*). In continuous culture an increased oxygen supply increases nitrogen fixation and growth provided that the oxygen in solution in the medium does not exceed 2.5 μ*M* (M. A. T. Vargas and R. F. Harris 1977. International Symposium on Limitations and Potentials of Biological Nitrogen Fixation in the Tropics. Summary of Papers. Univ. of Brasilia, Brazil, p. 115). When supplied with fixed nitrogen *S. lipoferum* grows as an aerobe (Okon *et al.* 1976*a*). The salts of organic acids such as malate, succinate, lactate, and pyruvate have been found to be satisfactory oxidizable carbon and energy sources (Okon *et al.* 1976*a, b*). Certain strains were found to require low levels of yeast extract for growth in mineral media (Döbereiner *et al.* 1976; Okon *et al.* 1976*a*) and to grow on glucose as the carbon source, whereas strains that did not require yeast extract failed to grow on glucose. Based on these and several other observations the existence of two or possibly three groups of organisms within the species *S. lipoferum* was proposed by several authors (Krieg 1977; Krieg and Tarrand 1978; Neyra *et al.* 1977; Okon *et al.* 1976*a*; M. J. Sampaio, L. de Vasconcellos, and J. Döbereiner. 1976. International Symposium on Environmental Role of Nitrogen-fixing Blue Green Algae and Asymbiotic Bacteria, Uppsala, Sweden; J. J. Tarrand and N. R. Krieg. 1977. Abstk. Annu. Meet. Am. Soc. Microbiol. p. 159; M. E. Tyler and J. R. Milam. 1977. Abst. Annu. Meet. Am. Soc. Microbiol. p. 160; M. E. Tyler, J. R. Milam, and D. A. Zuberer. 1977. International Symposium on Limitations and Potentials of Biological Nitrogen Fixation in the Tropics. Summary of papers. Univ. of Brasilia, Brazil. p. 77).

As indicated by Krieg and Hylemon (1976), the taxonomic status of *S. lipoferum* has been uncertain. Therefore, the objectives of the present report were: (i) to determine by the use of deoxyribonucleic acid (DNA) base composition, and especially by DNA homology experiments, how many species are represented by the various strains of the *S. lipoferum* group; (ii) to learn which phenotypic characteristics can, by correlation with the genetic data, serve to distinguish such species; (iii) to provide a general characterization of the organisms; and (iv) to investigate the problem of which genus would be the most appropriate for the organisms.

Methods and Materials

Strains Used

The various strains studied are listed in Table 1. Upon receipt, each strain was checked for purity by streaking twice on modified peptone – succinate salts (MPSS) agar (Caraway and Krieg 1974). Stock cultures were maintained in the N-free semisolid malate medium of Day and Döbereiner (1976) containing 0.005% yeast extract. Strains requiring yeast extract were transferred biweekly; the remainder were transferred monthly. All stock cultures were grown at 30°C. The strains were also preserved toward the end of the study by suspending the cells in nutrient broth containing 10% dimethyl sulfoxide and freezing in liquid nitrogen.

Morphology

Cell dimensions were determined from photomicrographs taken by phase-contrast microscopy. The magnification was accurately determined from similar photographs of a stage micrometer. The type of flagellation was determined by electron microscopy using preparations shadowed with tungsten oxide.

Acidification of Sugar Media

A medium of the following composition was used (g/ℓ): yeast extract (Difco), 0.05; K_2HPO_4, 0.25; $FeSO_4 \cdot 7H_2O$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.001; $MnSO_4 \cdot H_2O$, 0.002; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.026; $(NH_4)_2SO_4$, 1.0; biotin, 0.0001; brom thymol blue (aqueous), 0.0375; agar (Difco), 15.0. The pH was adjusted to 7.1 with KOH. Each sugar was sterilized by filtration and added aseptically to the sterile basal medium to give a final concentration of 1.0% The sugar media were then added to sterile microtiter plates (flat-bottomed wells, NUNC), about 0.16 ml per well. All of the wells of a given plate contained the same kind of sugar. The method of Wilkins and Walker (1975) was used for filling and inoculating the wells, by use of a replicator constructed of a plastic block with projecting wire brads. The replicator was sterilized by alcohol and by subsequent exposure to an intense source of ultraviolet light; it was then inoculated from a master microtiter plate where each well contained a different strain (0.16 ml of a 48-h-old MPSS broth culture). By pressing the replicator onto the master plate, each wire brad was inoculated, and the replicator was then pressed onto a microtiter plate containing the sugar medium. Thus, each brad would inoculate a particular strain into a particular well of sugar agar. The replicator was returned to the master plate before inoculating another plate of sugar medium. A number of wells located at various positions were not inoculated and served as controls for sterility of the medium and also to indicate that migration of cultures from one well to another did not occur. After inoculation, the plates were covered with sterile lids and incubated at 37°C for 72 h. The pH of each well was then determined with a small pH electrode as described by Wilkins and Walker (1975). The pH of uninoculated wells ranged from 5.7 to 6.2, presumably because of absorption of carbon dioxide. Acidification of the medium by a strain was considered positive if the pH of the well was 3.9–5.0.

TABLE 1. Origin of *D. lipoferum* strains

Strain No.	Country	Place	Plant roots or soil	Isolated by:
Sp 4	Brazil	Rio de Janeiro	*Digitaria*	J. Döbereiner
Sp 7	"	"	"	"
Sp 13	"	"	"	"
Sp 13v	"	"	"	"
Sp 34	"	"	"	"
Sp 35	"	"	"	"
Sp 51e	"	"	Wheat	"
Sp 52	"	"	Sorgham	"
Sp T60	"	"	Wheat	"
Sp 67	"	"	Maize	"
Sp 75	"	"	"	"
Sp 80	"	"	"	"
Sp 81	"	"	"	"
Sp 82	"	"	"	"
Sp P1	Peru		Soil	"
Sp P2	"		"	"
Sp F4	U.S.A.	Gainesville	Millet	S. H. Schank
Sp F6	"	"	"	"
Sp Br 8	Brazil	Brasilia	Soil	J. Döbereiner
Sp Br 11	"	"	Maize	"
Sp Br 13	"	"	Soil	"
Sp Br 14	"	"	Wheat	"
Sp Br 21	"	"	"	"
Sp A2	Nigeria	Ibadan	Maize	"
Sp A7	"	"	Rice	"
Sp A8	"	"	*Panicum*	"
Sp MT 20	Brazil	Mato Grosso	Soil	"
Sp MT 21	"	"	"	"
Sp L69	"	Londrina	Wheat	"
Sp Col 1c	Colombia		Soil	"
Sp RG 8a	Brazil	Passo Fundo	Wheat	J. Döbereiner
Sp RG 16a	"	"	*Lolium*	"
Sp RG 20b	"	"	Wheat	"
JM 6A2	Ecuador		Maize	J. R. Milam
JM 6B2	"		"	"
JM 24B4	"		*Musa* sp.	"
JM 28A2	"		"	"
JM 52B1	Venezuela		*Panicum maximum*	"
JM 73B3	"		Maize	"
JM 73C2B	"		"	"
JM 73C3	"		"	"
JM 75A1	"		*Panicum maximum*	"
JM 82A1	"		Maize	"
JM 119A4	U.S.A.	Gainesville	Millet	"
JM 125A2	"	"	"	"
Cd	"	Riverside	*Cynodon dactylon*	D. L. Eskew
Sp 59b	Brazil	Rio de Janeiro	Wheat	J. Döbereiner
Sp USA 5b	U.S.A.	Pullman	Soil	"
Sp RG 6xx	Brazil	Passo Fundo	Wheat	"
Sp RG 8c	"	"	"	"
Sp RG 9c	"	"	"	"
Sp RG 18b	"	"	"	"
Sp RG 19a	"	"	*Digitaria*	"
Sp RG 20a	"	"	Wheat	"
Sp Col 2b	Columbia	Papayan	Maize	"
Sp Col 3	"	"	*Brachiaria*	"
Sp Col 5	"	"	*Hyparrhenia rufa*	"
Sp Br 10	Brazil	Brasilia	Soil	"
Sp Br 17	"	"	Maize	"
Sp A3a	Senegal	Dakar	Grass	"
Sp S1a	Sweden	Uppsala	Grass	"

Acidification of glucose media was also determined by a tube method using liquid microtiter plate medium, liquid microtiter plate medium with K_2HPO_4 increased to 0.5 g/ℓ, and also a peptone-based medium having the following composition (g/ℓ): peptone (Difco), 2.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; glucose (sterilized by filtration), 10.0; $FeCl_3 \cdot 6H_2O$, 0.002; $MnSO_4 \cdot H_2O$, 0.002; and brom thymol blue (aqueous), 0.025. The pH was adjusted to 7.1 with KOH. The ability to ferment glucose or fructose was tested anaerobically by placing tubes of peptone-based broth or microtiter plate broth in jars containing Gas-Pak catalyst (Bioquest); the jars were then evacuated and refilled once with nitrogen and three times with hydrogen. The jars were incubated at 37°C for 2 weeks. The ability to grow with glucose under anaerobic conditions was also tested by the use of streaked plates of solid media.

Requirement for biotin

Preliminary evidence by an auxanographic method indicated that certain strains required biotin for growth. Subsequently, the following procedure was developed for distinguishing these strains. The medium used had the following composition (g/ℓ): K_2HPO_4, 0.5; succinic acid (free acid), 5.0; $FeSO_4 \cdot 7H_2O$, 0.01; $Na_2MoO_4 \cdot 2H_2O$, 0.002; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.1; $CaCl_2 \cdot 2H_2O$, 0.026; $(NH_4)_2SO_4$, 1.0. The pH was adjusted to 7.0 with KOH. Media with and without biotin (0.0001 g/ℓ) were prepared. Cultures grown in MPSS broth were inoculated into 25 ml of ¼ × nutrient broth (Difco) and incubated at 37°C for 48 h. The cells were harvested by centrifugation, washed twice in 10 ml of sterile distilled water, and suspended in water to a turbidity of 20 Klett units (blue filter, 16-mm cuvettes). One-tenth millilitre of this suspension was used to inoculate each 5-ml volume of medium. Incubation was for 48 h at 37°C. In cases where slight growth occurred in the medium lacking biotin, compared with much heavier growth in the medium with biotin, a second serial transfer (0.1 ml) was made to media with and without biotin to confirm the requirement.

Other Physiological Tests

Physiological tests were performed as described by Hylemon *et al.* (1973) except as indicated below. Tests for nitrate reduction and denitrification were performed as described by Neyra *et al.* (1977). For the indol test, cells were cultured for 48 h in 1.0% tryptone broth (Difco) containing 0.1% tryptophan; *Escherichia coli* was used as a positive control. The ability to use glucose as a sole carbon source for growth in N-free medium was tested by using the N-free semisolid medium of Day and Döbereiner (1976) with the following modifications: brom thymol blue was omitted, malate was replaced by 1% glucose (sterilized by filtration), and 0.00001% biotin was added. The medium was inoculated directly from cultures grown in semisolid malate medium and the growth response at 37°C was observed in 3 days. The following procedure was also used. Cultures grown for 48 h in 25 ml of ¼ × nutrient broth were harvested by centrifugation and suspended in 10 ml of sterile water; 0.05 ml of this suspension was added to the surface of each tube of medium. The medium used was as described above but also contained brom thymol blue (aqueous). The growth response was observed for 2 weeks.

For qualitative determination of sole carbon sources used, a modification of the defined medium described by Hylemon *et al.* (1973) was used. The level of K_2HPO_4 was increased to 0.2%; 0.00001% biotin and 1.5% purified agar (Difco) were added. Before adding the $CaCO_3$ and biotin, the medium was acidified to pH 2.5 with HCl to dissolve precipitates. It was then adjusted to pH 7.0 with KOH. Cells were prepared as for determination of the biotin requirement, except that the final suspension was adjusted to 30 Klett units instead of 20. Two millilitres of suspension were used to seed 20 ml of molten medium (45°C) in a Petri dish. After solidification of the medium, sterile 7-mm discs punched from Beckman electrophoresis filter paper (Cat. No. 319328) were dipped into 5% solutions of carbon sources sterilized by filtration. (Solutions of organic acids were adjusted to pH 7 with KOH before use.) The paper discs were then placed near the periphery of the seeded agar plates (three discs per plate). The plates were incubated at 37°C for 72 h. Any visible zone of turbidity around the discs, as judged with the naked eye by holding the plates against a dark background near a light source, constituted a positive growth response.

DNA Base Composition

DNA used for determination of base composition was prepared as previously described (Hylemon *et al.* 1973). A final concentration of 50 μg/ml was made in 0.025 *M* sodium phosphate buffer (pH 6.8) containing 0.01% sodium dodecyl sulfate. The preparations were dialyzed in this buffer together with the DNA from *E. coli* B. Melting point (Tm) values were determined using a Gilford thermospectrophotometer containing a temperature sensor in the cuvette. The Tm value for *E. coli* B was normalized to 90.5°C, and the Tm values of the test strains were increased by the same factor to give Tm values for 1 × SSC. The moles % guanine + cytosine (mol % G + C) values were calculted by the formula of Marmur and Doty (1962).

DNA Homology Experiments

For some initial DNA homology experiments, the DNA used for binding to membrane filters was prepared by the method of Marmur and Doty (1962); for most experiments, however, the DNA was prepared by the hydroxylapatite (HA) method of Johnson (1978). Comparative DNA homology experiments using both types of preparations indicated that it made little difference which of the two types of DNA was used for binding to filters. Thermal denaturation and binding to filters were done as described by Johnson and Cummins (1972). Filters with bound DNA were incubated before use in the following mixture at 73°C for 30 min to prevent non-specific binding of competitor DNA or radioactive homologous DNA: bovine serum albumin (fraction V), 0.03%; polyvinyl pyrrolidone, 0.02%; Ficol (Pharmacia Fine Chemicals, Uppsala, Sweden), 0.02%.

Competitor DNA was prepared by the HA method and was subjected to brief sonic oscillation before denaturation. The DNA was adjusted to 3 mg/ml in 0.001 *M* HEPES buffer (*N*-2-hydroxyethyl-piperazine-*N'*-(2-ethane)sulfonic acid) containing 0.02 *M* NaCl. NaOH was added to a concentration of 0.25 *M* and the solution was incubated at 45°C for 15 min to allow denaturation of the DNA and also hydrolysis of any residual ribonucleic acid (RNA). The solution was quickly cooled in an ethanol–ice bath and neutralized with an equivalent amount of HCl. The DNA was dialyzed against 2.2 × SSC (1 × SSC = NaCl, 0.15 *M*; sodium citrate, 0.15 *M*; pH = 7.0) and adjusted to a final concentration of 1.5 mg/ml with 2.2 × SSC.

For preparation of radioactive homologous DNA, cultures were grown with ^{32}P or ^{33}P (as orthophosphate). The use of isotopes of phosphorus was necessitated by the failure of strain Sp 7 to incorporate sufficient radioactivity into its DNA when supplied with various tritiated precursors of DNA synthesis (thymine, thymidine, adenine, and deoxyadenosine). The DNA was extracted by the HA method, dialyzed against 0.1 × SSC, and denatured by heating at 100°C for 20 min.

DNA homology experiments were performed as described by Johnson and Cummins (1972). All incubations were performed at 25°C below the Tm of the DNA, i.e., at 73°C.

Results

DNA Base Composition

If significant differences were to occur in DNA base composition among a number of strains, this alone would be strong evidence for the existence of

TABLE 2. Melting points (Tm values) and mol % G + C values for the DNA of 11 strains of the *S. lipoferum* group

Strain	Average Tm, °C[a]	Mol % G + C (± 1%)
Sp 7	98.1	70
Sp 13	98.0	70
Sp 4	97.8	70
Sp 35	98.2	70
Sp 82	98.4	71
Sp 75	98.2	70
Sp 51e	98.2	70
Sp T60	98.4	71
Sp 59b	97.9	70
Sp RGxx	97.8	70
Sp USA 5b	97.6, 97.8[b]	69, 70[b]

[a]Normalized to 1 × SSC values relative to *E. coli* B DNA.
[b]In the case of the second value given the DNA was prepared by the HA method rather than by the method of Marmur and Doty.

more than one species. However, as indicated in Table 2, the mol % G + C values for 11 strains isolated from different regions and different plants were all very close to 70%. This similarity indicated that the strains could possibly, but not necessarily, all belong to a single species.

DNA Homology Experiments

The results of the DNA homology experiments are presented in Table 3. It can be seen that when strain Sp 7 was used as the reference strain, the other strains fell into two distinct but related groups: group I contains the strains with homology values of about 70% or higher, and group II contains the strains with homology values in the 30–50% range. In the reciprocal situation where Sp 59b was used as the reference, those strains (group II) which had exhibited low homology with Sp 7 now were found to have high homology with Sp 59b; conversely, those strains (group I) which had exhibited high homology with Sp 7 were now found to have low homology with Sp 59b.

DNA homology values for strains of various bacterial genera were also determined, using Sp 7 and Sp 59b as references. The bacterial strains used were chosen because they had mol % G + C values and (or) other characteristics similar to those of *S. lipoferum*. As indicated in Table 4, no DNA homology values higher than about 20% were obtained for these strains.

Characteristics Correlated with the Two Homology Groups

Certain characteristics could be correlated with the two DNA homology groups of *S. lipoferum* (Table 5). Only group II strains exhibited a requirement for biotin. Only group II strains were able to grow in semisolid N-free medium with glu-

TABLE 3. DNA homology values for strains of the *S. lipoferum* group

Strain	DNA homology values, % Ref. = Sp 7	Ref. = Sp 59b
Sp 7	100	34
Sp 4	102	38
Sp 13	94	32
Sp 13v	103	47
Sp 34	81	43
Sp 35	74	42
Sp 51e	71	41
Sp 52	85	44
Sp T60	85	30
Sp 67	80	30
Sp 75	71	49
Sp 80	84	52
Sp 81	73	41
Sp 82	73	41
Sp P1	72	46
Sp P2	82	52
Sp F4	80	35
Sp F6	74	40
Sp Br 8	84	37
Sp Br 11	67	38
Sp Br 13	72	41
Sp Br 14	81	38
Sp Br 21	80	ND[a]
Sp A2	92	50
Sp A7	96	33
Sp A8	81	42
Sp MT 20	82	39
Sp MT 21	81	35
Lp L69	83	49
Sp Col 1c	80	34
Sp RG 8a	104	37
Sp RG 16a	77	35
Sp RG 20b	73	32
JM 6A2	78	49
JM 6B2	87	48
JM 24B4	83	48
JM 28A2	91	46
JM 52B1	86	40
JM 73B3	81	45
JM 73C2B	83	49
JM 73C3	79	40
JM 75A1	77	45
JM 82A1	90	45
JM 119A4	91	47
JM 125A2	88	52
Cd	100	49
Sp 59b	31	100
Sp USA 5b	36	70
Sp RG6xx	36	73
Sp RG 8c	34	72
Sp RG 9c	35	73
Sp RG 18b	39	73
Sp RG 19a	28	72
Sp RG 20a	37	76
Sp Col 2b	43	72
Sp Col 3	34	76
Sp Col 5	46	73
Sp Br 10	38	74
Sp Br 17	30	73
Sp A3a	29	70
Sp S1a	34	73

[a]ND not done.

TABLE 4. DNA homology values for bacteria of various genera, with *S. lipoferum* strains used as reference

Genus and species	ATCC No.	DNA homology values, % Ref. = Sp 7	Ref. = Sp 59b
Pseudomonas testosteroni	11996[a]	4	0
P. acidovorans	15668[a]	20	14
	15667	1	1
Aquaspirillum metamorphum	15280[a]	0	13
A. peregrinum[b]	15387[a]	22	11
A. fasciculus[b]	27740[a]	16	11
	29048	5	NT[c]
	29049	11	NT
Comamonas terrigena	8461[a]	0	1
Derxia gummosa[b]	15994[a]	2	0
	15995	0	20
Azomonas insignis[b]	12523	10	1
A. macrocytogenes[b]	12335	6	7
Rhodospirillum rubrum[b]	11170[a]	16	0

[a]Type strain.
[b]Nitrogen fixer.
[c]NT = not tested.

cose as the carbon source. Only group II strains appeared to possess some fermentative ability, as first noted by Dr. Max E. Tyler and Dr. James R. Milam, Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida (personal communication). We found group II strains to be capable of acidifying glucose or fructose media anaerobically, of forming a small amount of gas (less than 5% of the volume of the gas vials), of exhibiting slight growth in sugar broth anaerobically, and of forming minute colonies on sugar plates anaerobically. Yet group II strains grew far better aerobically than anaerobically, and like group I should be considered to have mainly a respiratory type of metabolism.

In peptone-based glucose broth, only group II strains caused acidification, as indicated in a preliminary report (Krieg 1977). By the microtiter plate replicator method, only group II strains produced a pH of 4.5 or less with glucose; however, 2 of 46 group I strains produced a weakly acidic reaction. In liquid microtiter plate medium, 6 of 46 group I strains caused acidification as evidenced by a yellow color; when the level of K_2HPO_4 of this medium was increased to 0.5 g/ℓ, these group I strains produced only a yellow-green color in 48 h, while all group II strains produced a deep yellow color. Consequently, it appeared that group I strains, if capable of oxidizing glucose, produced a weaker reaction. Such group I strains were incapable of causing acidification of glucose media under anaerobic conditions.

In stock cultures of four group II strains (Sp Br 17, Sp RG 9c, Sp RG 20a, and Sp Col 5) variants appeared after continued transfer; these variants were no longer capable of acidifying the peptone-based glucose broth. They could cause acidification of microtiter plate glucose broth, but when the level of phosphate was increased to 0.5 g/ℓ they produced only a yellow-green color in 48 h. Their ability to ferment glucose or fructose was also diminished. The variants still exhibited a requirement for biotin, could use glucose as a sole carbon source for growth in N-free semisolid medium, and exhibited the characteristic morphology of group II strains when cultured in semisolid N-free malate medium + 0.005% yeast extract (see below).

Concerning morphology, both groups I and II had a similar appearance when cultured for 24 h in MPSS broth, namely, short, plump, slightly curved motile rods averaging 1.0 μm in diameter and having a length of 2.1–3.8 μm (see Fig. 1A, B, and C). Many cells had pointed ends, and PHB granules were visible. When a loopful from an MPSS broth culture was transferred to semisolid N-free malate medium + 0.005% yeast extract, group II strains tended to become wider (1.4–1.7 μm), longer (5 μm to over 30 μm), and many S-shaped or helical cells appeared in 24–48 h (see Fig. 1E and F). Group II strains also tended to become non-motile or only sluggishly motile in the semisolid malate medium. The long cells seemed eventually to undergo fragmentation into shorter, ovoid cells; many of these later became large and pleomorphic and were filled with highly refractile granules, probably of PHB. Some of these large cells appeared to be compartmented. In contrast to group II strains, group I strains retained an appearance in semisolid malate medium similar to that in MPSS broth, remaining predominantly short and vibroid (see Fig. 1D); after

TABLE 5. Distinction between DNA homology groups on the basis of phenotypic characters

Test	% of strains positive	
	Homology group I	Homology group II
Glucose used as sole carbon source for growth in N-free semisolid medium	0[a]	100
Acidification of glucose media:		
Microtiter plate method; 72 h	4[b] (pH 5.2–5.8)	100 (pH ≦ 4.5)
Peptone-based glucose broth, 96 h	0	100
Microtiter glucose broth; 48 h	13[c] (deep yellow)	100 (deep yellow)
Microtiter glucose broth with 2 × phosphate; 48 h	13[c] (yellow-green)	100 (deep yellow)
Acidification of mannitol, ribose, and sorbitol (microtiter plate method; 72 h)	0	80[d]
Acid from glucose and fructose anaerobically; slight growth also occurs; 2 weeks	0	87, +13% variable[e]
In semisolid N-free malate medium + 0.005% yeast extract, cells become wider, longer (often S-shaped or helical), and non-motile; 24–48 h	0	100
Sole carbon sources (by paper disc test: 72 h)		
α-Ketoglutarate	0	100
Mannitol	0	80, +7% variable[f]
Sorbitol	0	67, +13% variable[g]
Ribose	0	40, +27% variable[h]
Glucose	9[i]	100

[a]When the medium was inoculated directly from cultures grown in malate medium, some group I strains formed a slight pellicle in the depths of the medium; this initial growth later dispersed. In contrast, group II strains formed a pellicle which within 3 days migrated close to the surface of the medium and became very dense. The difference in response between the two groups was very pronounced. It is possible that carry-over of malate in the inoculum may have been responsible for any slight growth of group I. When washed inocula were used, no group I strain exhibited growth during incubation for 2 weeks. In contrast group II strains formed an initial pellicle generally within a week; once formed, the pellicle migrated close to the surface of the medium and became very dense.
[b]Strains of group I producing the weakly acidic reaction were 34 and F6.
[c]Strains of group I producing acidic reactions were 34, F6, A2, A7, T60, and 82A1.
[d]Negative strains: Br 10, Br 17, and Col 3.
[e]Variable strains: Br 10 and Br 17.
[f]Negative strains: Br 10 and Br 17. Variable strain: Col 3.
[g]Negative strains: Br 10, Br 17, and Col 5. Variable strains: RG 6xx and RG 8c.
[h]Negative strains: Br 10, Br 17, USA 5b, Col 5, and RG 6xx. Variable strains: RG 9c, Col 2b, Col 3, and 59b.
[i]Positive strains: T60, 67, F6,and 34. The growth of these strains appeared to be weaker than for group II strains.

several weeks, however, some S-shaped cells could be observed (see reference by Krieg 1976, Fig. 2) as well as some large, pleomorphic, granule-filled forms. Similar pleomorphic forms were also observed under washout conditions in chemostat cultures. In batch cultures group I strains retained their motility for a month or longer.

Other Characteristics of the Strains

Additional characteristics of the strains of both homology groups are summarized in Table 6. One of the most interesting characteristics concerned the type of flagellation. When cultured in MPSS broth at either 30 or 37°C, the cells exhibited a single polar flagellum. When cultured on MPSS agar at 30°C, however, the cells exhibited numerous lateral flagella in addition to the polar flagellum. The lateral flagella possessed a shorter wavelength than the polar flagellum and they also appeared to be thinner, as judged from the shadowed preparations (see Fig. 2). Although some lateral flagella were also observed on cells cultured on agar at 37°C, the lower temperature appeared to be much more favorable for their formation. The occurrence

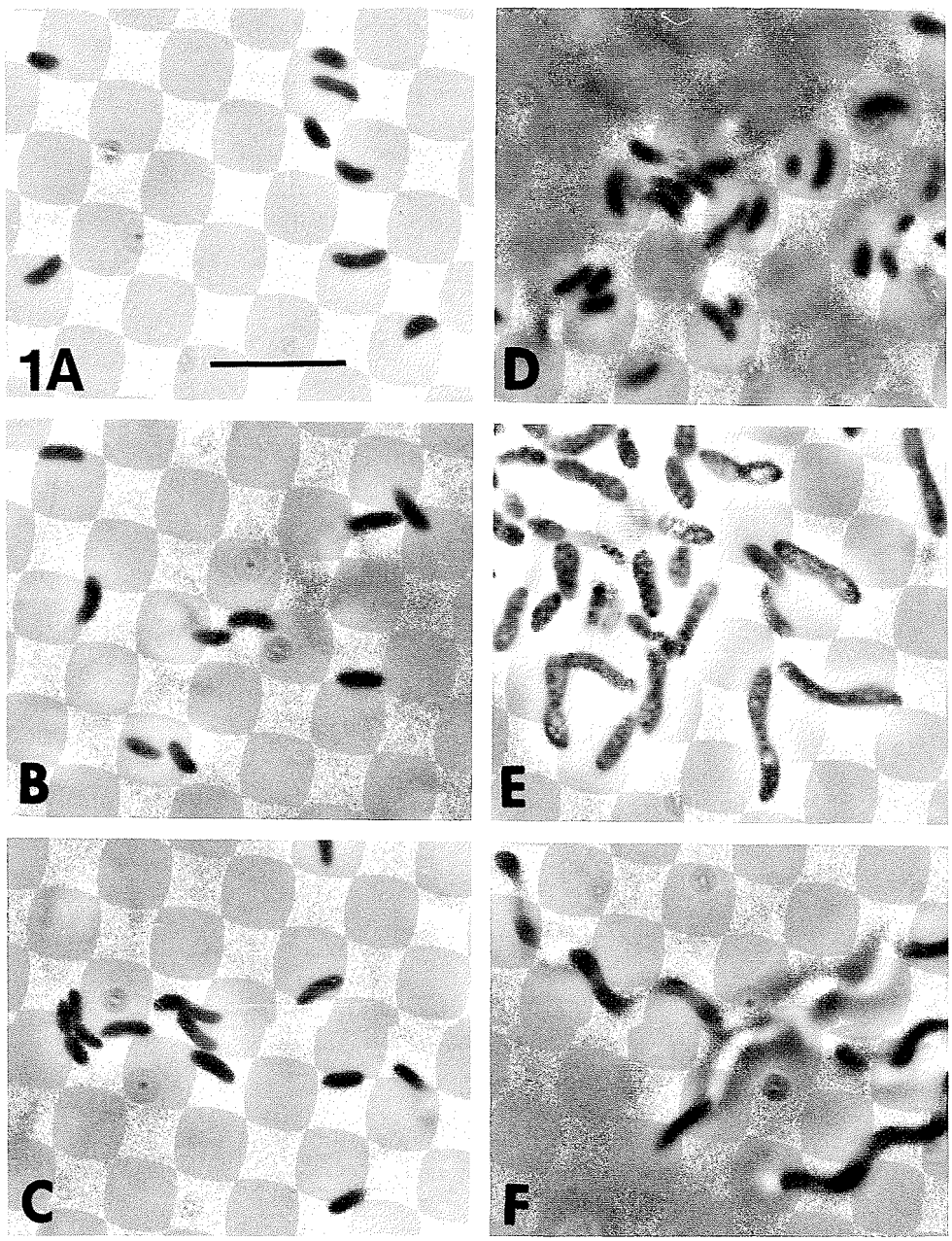

FIG. 1. Appearance of strains of *S. lipoferum* by phase-contrast microscopy. A–C: strains Sp 7, Sp 59b, and Sp RG 20a, respectively, cultured in MPSS broth at 37°C for 24 h. D and E: strains SP 7 and Sp 59b, respectively, cultured in semisolid nitrogen-free malate medium + 0.005% yeast extract for 48 h; E = strain Sp RG 20a cultured similarly but for only 24 h. The bar = 10 μm.

of lateral flagella appeared to be similar to the situation described for *Pseudomonas stutzeri* (Doudoroff and Palleroni 1974), *Vibrio parahaemolyticus* (Shinoda and Okamoto 1977), and *Vibrio alginolyticus* (Boer *et al.* 1975).

As already noted by Okon *et al.* (1976*a*), potato or nutrient agar colonies of *S. lipoferum* tend to develop a light pink pigment. However, certain strains formed very deep pink colonies on MPSS agar: Sp 13v, Sp RG 8a, and Cd. Strains Sp 13v and Sp RG 8a were spontaneous mutants derived from ordinary light pink strains. Strain Cd was isolated

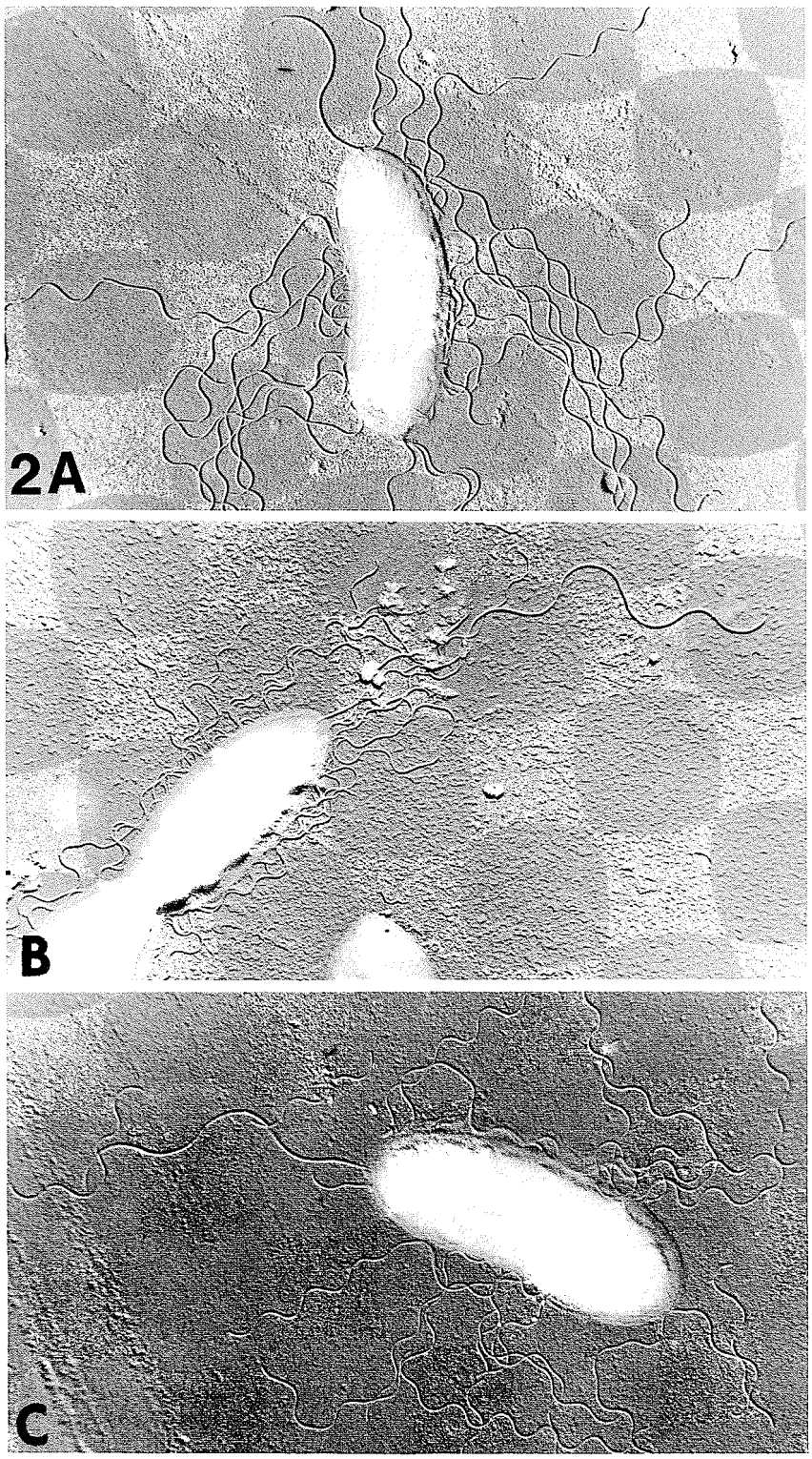

FIG. 2. Electron micrographs of (A) strain Sp 7, (B) strain Sp 59b, and (C) strain BR 17 cultured on MPSS agar at 30°C. Both the single polar flagellum and also numerous lateral flagella of shorter wavelength can be seen. 14 000 ×.

TABLE 6. Additional physiological characteristics of DNA homology groups I and II

Test	% of strains positive	
	Group I	Group II
Oxidase, phosphatase, urease, esculin hydrolysis, anaerobic growth with nitrate	100	100
Starch and gelatin hydrolysis, production of water-soluble fluorescent pigment, indol production	0	0
Acid from fructose	100	100
Acid from:		
arabinose	89, +2% variable[a]	100
galactose	76, +4% variable[b]	93[b]
i-inositol	0	13[c]
xylose	0, +9% variable[d]	80, +7% variable[d]
Acid from lactose, maltose, sucrose, rhamnose, cellobiose, erythritol, dulcitol, and melibiose	0	0
Catalase		
Strong	87	27
Weak	13	40
Negative	0	33[e]
Growth in presence of 1% bile	98[f]	100
Growth in presence of 3% NaCl	51 (weak)	0
Dissimilation of nitrate to nitrite	100	100
Dissimilation of nitrate to gas	79[g]	92[g]
Optimum temperature of 36–38°C	100[h]	100[h]
Sole carbon sources (by disc method)		
Succinate, malate, lactate, oxaloacetate, fumarate, pyruvate, β-hydroxybutyrate, gluconate, glycerol, fructose	100	100
Propionate	98	93, +7% variable
Galactose	65, +15% variable	80, +13% variable
Arabinose	74, +9% variable	67, +27% variable
Citrate	96, +4% variable	100
Malonate	0	0

[a]Negative strains: F6, T60, 34, and P2. Variable strain: 67.
[b]Negative strains: Br 14, T60, 82A1, RG 16a, 51e, 34, 7, 4, P2, 24B4, 28A2, and Col 2b. Variable strains: 67 and F4.
[c]Positive strains: 59b and Col 2b.
[d]In group I, variable strains were 34, Br 11, 28A2, and 82A1. In group II, strains Col 3 and Br 10 were negative and strain Br 17 was variable.
[e]Negative strains: RG 20a, Col 2b, Col 3, Br 10, and Sla. All of these are group II strains.
[f]Strain P2 was negative.
[g]Tested by accumulation of N_2O under C_2H_2 (see reference by Neyra *et al.* 1977). Denitrifying strains of group I were 7, 13, 13v, 51e, T60, 67, P1, Br 8, Br 11, Br 13, MT 21, L 69, Col 1c, RG 20b, 6A2, 6B2, 24B4, 28A2, 52B1, 75A1, 82A1, 119A4, and Cd. Strains 52 and 81 were variable. Denitrifying strains of group II were 59b, USA 5b, RG 6xx, RG 8c, RG 18b, RG 19a, Col 2b, Col 3, Br 17, A3a, and Sla. Strain Col 5 was the only group II strain tested which did not denitrify. The following strains were not tested for denitrification: 4, F6, RG 8a, RG 9c, and Br 10.
[h]Eleven strains were tested: 8 strains from group I and 3 strains from group II.

as a deep pink strain from *Cynodon dactylon* roots by Eskew *et al.* (1977). The three deep pink strains all exhibited nearly 100% homology with the reference strain Sp 7, in which no such deep pink colonies have yet been found. Studies of strain Cd by Eskew *et al.* (1977) indicate that the pigment is probably a *b*- or *c*-type cytochrome.

Discussion

Studies by Johnson (1973) have indicated that in the DNA homology range of 80–100% the major variations that are being measured are differences in genome size and alterations in the linear arrangements of cistrons, whereas in the homology range of 30–50% differences in the base sequences

of the cistrons become increasingly important. DNA homology values of 70% have been considered to represent a sufficient degree of genetic relationship as to warrant consideration of the strains as belonging to a single species (Brenner 1973; Johnson 1973). Consequently, we believe that the results of our DNA homology experiments with *S. lipoferum* strains indicate that two distinct but related species exist, and also that the species belong together within the same genus.

Within group I a continuum of homology values exists from about 70 to 100%, with Sp 7 as the reference strain. In contrast, none of the group II strains exhibited more than 76% homology to the reference strain Sp 59b, suggesting the possibility that group II might consist of two subspecies—one containing Sp 59b, and the other containing the remaining strains. However, whether the latter strains exhibit high homology with each other has not been determined. On the basis of phenotypic characteristics, so far there appears to be no correlation of any particular characteristics with lower or higher homology values within either group II or group I.

Considerations of the Generic Status of the Strains

On the basis of their vibroid and sometimes helical shape, and also their preference for the salts of organic acids as carbon source, *S. lipoferum* was originally assigned to the genus *Spirillum* Ehrenberg by Beijerinck (1925). However, this genus is presently restricted to very large microaerophilic, helical organisms having bipolar tufts of flagella and whose mol % G + C is about 38 (i.e., organisms resembling *Spirillum volutans*) (Hylemon *et al.* 1973). Consequently, the genus *Spirillum* is not appropriate for *S. lipoferum* strains.

If significant DNA homology were to occur between *S. lipoferum* and an established member of a particular genus, this would provide a firm basis for assigning *S. lipoferum* to that genus. As indicated in Table 4, however, none of a number of strains from varous genera having certain similarities to *S. lipoferum* were found to exhibit homology values higher than about 20%, when strains Sp 7 and Sp 59b were the reference strains. Such values are not considered to be significant (Johnson 1973). DNA homology studies are in fact most useful at the species level rather than the genus level of taxonomy, and lack of significant DNA homology would not preclude assignment of an organism to a particular genus. It is likely that ribosomal ribonucleic acid – DNA (rRNA–DNA) studies would be useful at the genus level, since they indicate broader relationships between strains than are evident from DNA–DNA studies (Johnson and Francis 1975; Palleroni *et al.* 1973). In the absence of such studies, however, assignment of *S. lipoferum* strains to an appropriate genus must presently be done on the basis of phenotypic characteristics.

Among the known aerobic nitrogen-fixing bacteria *Derxia gummosa* seems to resemble *S. lipoferum* most closely. It has a mol % G + C of 70, has a cell diameter similar to that for *S. lipoferum*, possesses PHB granules, and has been isolated from tropical soils (Becking 1974*b*). However, *Derxia* is a straight rod, is incapable of reducing nitrate even to nitrite, and does not grow on malate or succinate. It forms a gummy slime or capsule and possesses only a single polar flagellum. For these reasons we believe that *S. lipoferum* should not be assigned to this genus.

Because of the curvature of the cells and also the formation of S-shaped or even spirillum-shaped cells under certain conditions, *S. lipoferum* might resemble members of the genus *Aquaspirillum* (Hylemon *et al.* 1973). However, *S. lipoferum* would be atypical for this genus because it has (i) a higher mol % G + C value (70% compared to 50–65%); (ii) a generally vibroid shape rather than a helical shape; (iii) an ability to grow anaerobically with nitrate and to denitrify; (iv) only a single polar flagellum when grown in broth or lateral flagella when grown on agar; (v) an ability to oxidize a number of carbohydrates; (vi) some fermentative ability (by group II); (vii) a vitamin requirement (by group II); (viii) lack of catalase activity (by some strains of group II); (ix) nitrogenase activity; and (x) an association with plant roots. Although some species of *Aquaspirillum* are not typical of the genus in certain respects (Krieg 1976), the combination of atypical characteristics given above would seem to provide a firm basis for excluding *S. lipoferum* from the genus *Aquaspirillum*.

On the basis of DNA base composition, *S. lipoferum* would be excluded from the nitrogen-fixing genera *Azomonas* and *Beijerinckia* (Becking 1974*a*; Johnstone 1974*b*). The genus *Azobacter* has a mol % G + C of 63–66, however, and one of the species (*A. paspali*) is associated with root surfaces (Johnstone 1974*a*). Yet *Azotobacter* species have respiratory protection for nitrogenase, whereas *S. lipoferum* lacks such protection. Moreover, *Azotobacter* species are large and ovoid in shape, have only peritrichous flagella, and form thick-walled cysts (Johnstone 1974*a*). The genus *Rhizobium* is far more highly specialized for sym-

biotic nitrogen fixation than is *S. lipoferum*. Therefore, both *Azotobacter* and *Rhizobium* would seem to be inappropriate genera for *S. lipoferum*.

On the basis of purely phenotypic characteristics, such as vibrioid shape, ability to reduce nitrate, the occurrence of lateral flagella in addition to a single polar flagellum, and ability to ferment as well as to oxidize sugars, the genus *Vibrio* (Shewan and Véron 1974) might seem to be the most suitable for *S. lipoferum*. However, the mol % G + C for the genus *Vibrio* (40–50%) is far too low to permit inclusion of *S. lipoferum*. Certain oxidative, polar-flagellated, vibrioid bacteria have been assigned to the genus *Comamonas* (Davis and Park 1962), for which the type species is *C. terrigena*. This species is similar to certain members of the genus *Pseudomonas*, especially *P. testosteroni* (Hugh 1965). *Comamonas terrigena* has a mol % G + C of 64 (Sebold and Véron 1963) and is incapable of catabolizing any sugar. Consequently, it would appear that this genus would not be appropriate for *S. lipoferum*.

Members of the genus *Pseudomonas* resemble *S. lipoferum* in certain respects. For example, *P. stutzeri* has a type of flagellation similar to that of *S. lipoferum* and can also denitrify (Doudoroff and Palleroni 1974). The mol % G + C for the genus is 58–70, and vibrioid organisms are not excluded by the definition of the genus (Doudoroff and Palleroni 1974). The occurrence of some fermentative ability in group II of *S. lipoferum*, however, would exclude *S. lipoferum* from the genus. Moreover, unlike plant-associated pseudomonads, *S. lipoferum* is certainly not pathogenic for plants; indeed, it is beneficial.

Some interesting similarities exist between *S. lipoferum* and the polar-flagellated organism *Rhodospirillum rubrum*. For example, *R. rubrum* requires biotin, can grow aerobically in the dark, has a vibrioid shape, and produces a pink pigment. The mol % G + C for *R. rubrum* is 64–66 (Pfennig and Trüper 1974). One of the most interesting similarities is that like *R. rubrum* but unlike other nitrogen-fixing organisms, the Fe protein component of the nitrogenase complex of *S. lipoferum* is almost inactive upon isolation and must be activated by an activating factor that is interchangeable with the factor from *R. rubrum* (Burris *et al.* 1977). Unlike *S. lipoferum*, *R. rubrum* has bipolar tufts of flagella. Moreover, *R. rubrum* is photosynthetic whereas *S. lipoferum* does not appear to be photosynthetic. Strains Sp 7 and 59b were incapable of growing in deep, petrolatum-sealed tubes of peptone – succinate salts agar under illumination. Since all species of *Rhodospirillum* are capable of using succinate as an electron donor for photosynthesis (Pfennig and Trüper 1974), if the two strains of *S. lipoferum* had belonged to this genus they should have been able to grow under these conditions.

In our opinion, the best course to pursue with regard to the generic placement of *S. lipoferum* would be to assign the organisms to a new genus. We believe the name *Azospirillum* would be suitable for this genus for the following reasons: (i) The name *Spirillum lipoferum*, designated by Beijerinck in 1925, has become a familiar one since the work of Döbereiner and Day in 1974, and retention of the term "spirillum" in the new generic name would minimize confusion among those who are working with these organisms. (ii) Although mainly vibrioid, the organisms can exhibit S-shaped or helical cells under certain conditions. (iii) Certain spirilla, such as *Aquaspirillum aquaticum*, *A. delicatum*, *A. metamorphum*, and *Oceanospirillum japonicum*, generally have less than one helical turn, but are nonetheless included with other spirilla (Hylemon *et al.* 1973). (iv) Like spirilla, *S. lipoferum* strains have intracellular PHB granules, have mainly a respiratory type of metabolism, and seem to grow well on the salts of organic acids as carbon sources.

A description of the genus follows:

Azospirillum gen. Nov. (A· zo· spi· ril′ lum. French noun *azote*, nitrogen; Greek noun *spira*, a spiral; *spillum*, a small spiral; *Azospirillum*, a small nitrogen spiral.) Cells generally vibrioid, but may be S-shaped or helical in semisolid N-free malate medium + 0.005% yeast extract. Cell diameter about 1 μm. Motile in liquid media by a single polar flagellum; on solid media at 30°C numerous lateral flagella are formed in addition to the single polar flagellum. Prominent intracellular granules of PHB are present. Slime not formed. Possess mainly a respiratory type of metabolism, but some fermentative ability may also be present. Under severe oxygen limitation nitrate is dissimilated to nitrite, nitrous oxide, or nitrogen gas. Molecular nitrogen is fixed under microaerophilic conditions. Oxidase-positive. Grow well on salts of organic acids such as malate, succinate, pyruvate, or lactate; certain carbohydrates may also serve as carbon sources. Optimum temperature, about 37°C. Isolated from soils and from roots or various plants. The mol % G + C of the DNA is about 70 by thermal denaturation methods. The type species is *Azospirillum lipoferum*.

In the genus *Azospirillum*, two species would presently occur. Although Beijerinck's cultures are no longer extant, it seems likely that his organism

belonged to group II. Beijerinck referred to the development of his spirilla in solutions of glucose or mannitol inoculated with soil, although the spirilla were later displaced by overgrowth with *Azotobacter* and *Clostridium* (Beijerinck 1925). When malate was used as the carbon source, no such displacement occurred. Moreover, Beijerinck (1925) provided drawings of cells cultured in sugar medium. Beijerinck's cells could also exhibit a helical shape. All of this strongly supports the idea that group II organisms were involved. Consequently, we believe that the name *Azospirillum lipoferum* should be applied to group II strains. For group I strains, we propose the name *Azospirillum brasilense*. Descriptions of the two species follow.

Azospirillum lipoferum (Beijerinck) comb. nov. (li·po′ fe· rum. Greek noun *lipus*, fat; Latin verb *fero*, to carry; Modern Latin adjective *lipoferus*, fat-bearing). Capable of using glucose as a sole carbon source for growth in N-free semisolid medium containing biotin. Capable of aerobic growth and acid production in peptone-based glucose medium. Media containing ribose, mannitol, or sorbitol are usually acidified. Acidification and scant growth usually occur in glucose or fructose media under anaerobic conditions. Biotin required for growth. In semisolid N-free malate medium + 0.005% yeast extract, cells tend to become wider (about 1.4–1.7 μm), longer (often S-shaped or helical), and non-motile. The neotype strain is Sp 59b (ATCC 29707). Reference strains Sp RG6xx, Sp Br 17, and Sp RG 20a have been deposited with the American Type Culture Collection (ATCC) under the numbers 29731, 29709, and 29708, respectively.

Azospirillum brasilense sp. nov. (bra· si· len′ se. Pertaining to the country of Brazil, South America). Not capable of using glucose as a sole carbon source for growth in N-free semisolid medium. No acidification of peptone-based glucose medium occurs; acidification of peptone-free media buffered with phosphate may occur, but the reaction is weaker than with *A. lipoferum*. Media containing ribose, mannitol, or sorbitol are not acidified. No fermentative ability for sugars is present. Vitamins are not required for growth. In semisolid N-free malate medium + 0.005% yeast extract, cells tend to remain about 1.0 μm in diameter, short, vibrioid, and motile; S-shaped cells may occur in old cultures. The type strain is Sp 7 (ATCC 29145). Reference strains Sp Cd and Sp 35 have been deposited with the ATCC under the numbers 29710 and 29711, respectively.

Acknowledgements

We are grateful to Dr. John L. Johnson of the Anaerobe Laboratory, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, for teaching us the DNA homology technique and for his help and encouragement. We are also grateful to Mr. Robert H. Lipsky and to Mrs. Connie Alexander for their valuable assistance. We thank Dr. Max E. Tyler, Department of Microbiology and Cell Science, University of Florida, and Dr. Yaacov Okon, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin, for supplying many of the strains. The work was supported by the Rockefeller Foundation, Grants GA AGR 7523 and GA AS 7361.

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