Intra- and Intergeneric Relationships of the Genus *Aquaspirillum*: *Prolinoborus*, a New Genus for *Aquaspirillum fasciculus*, with the Species *Prolinoborus fasciculus* comb. nov.

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A comprehensive study of the intra- and intergeneric relationships of *Aquaspirillum* species in which we used DNA-rRNA hybridization is described. Radioactively labeled rRNAs from *Aquaspirillum anulus*, *A. serpens* biovar bengal, *A. delicatum*, *A. gracile*, *A. metamorphum*, *A. sinuosum*, and several other organisms were hybridized with filter-fixed DNAs from representative strains of the genus *Aquaspirillum* and many other possibly related genera of gram-negative bacteria. Our results show that the genus *Aquaspirillum* is very heterogeneous and should be restricted to *A. serpens*, the type species, including *A. serpens* biovar *serpens* and *A. serpens* biovar bengal. DNA-DNA hybridization data showed that *A. serpens* biovar bengal exhibits only 55 to 60% binding with *A. serpens* biovar *serpens* strains. *A. fasciculus*, the only rod-shaped organism of the genus, is the only other *Aquaspirillum* species located on the *A. serpens* rRNA branch. With differences in the midpoint temperatures of thermal denaturation curves of the DNA-rRNA hybrids of more than 6°C, differences in genomic DNA G+C contents of 11 to 16 mol%, and a phenotype which differs from the phenotypes of all other *Aquaspirillum* species, *A. fasciculus* is a member of a different genus, for which we propose the name *Prolinoborus fasciculus* gen. nov., comb. nov. *A. anulus*, *A. aquaticum*, *A. autotrophicum*, *A. delicatum*, *A. dispar*, *A. giesbergeri*, *A. gracile*, *A. itersonii*, *A. metamorphum*, *A. peregrinum*, *A. polymorphum*, *A. psychrophilum*, *A. putridiconchylum*, and *A. sinuosum* should also be removed from the genus *Aquaspirillum* on the basis of phylogenetic data; these organisms form at least 10 distinct groups that are intermingled phylogenetically with the gram-negative bacteria belonging to rRNA superfamilies III and IV. Although DNA-rRNA hybridization results clearly reveal that the genus *Aquaspirillum* is a very heterogeneous taxon, no phenotypic information is available to differentiate the various phylogenetic branches of the *aquaspirilla*. Moreover, most of the species descriptions were based on only one or a few strains. Therefore, we will not propose nomenclatural changes until (i) sufficient readily determinable phenotypic characteristics are available and (ii) more strains have been isolated and investigated.

The early taxonomy of the spirilla has been investigated by several authors (23, 36, 40, 43, 56–58, 67–69). In *Bergey's Manual of Determinative Bacteriology*, 8th ed. (29), the genus *Spirillum* was included with the genus *Campylobacter* in the family *Spirillaceae* Ehrenberg 1832. This taxon was merely a family of convenience, as the genotypic relationships between these genera were not known (29). At that time, the genus *Spirillum* contained 19 species that were based mainly on the 17 serological groups of spirilla (marine and freshwater spirilla) found by McElroy and Krieg (40). Serological identification of most *Spirillum* species was possible by cross-agglutination with specific antisera prepared against *Spirillum* thermolabile antigens. The subsequent description of new species (1b, 5, 34, 38, 53, 58) and the use of improved tools for the taxonomic study of the spirilla have resulted in other identification routines.

On the basis of DNA base composition and a limited number of physiological characteristics, the genus *Spirillum* was divided into the genera *Aquaspirillum*, *Oceanospirillum*, and *Spirillum* (26, 30). Large obligately microaerophilic spirilla having large bipolar tufts of flagella and a DNA base composition of 38 mol% G+C constituted the redefined genus *Spirillum*, containing one species, *Spirillum volutans*, which was the type species of the original genus *Spirillum* Ehrenberg 1832 (21). All of the marine spirilla, which required seawater for growth and had G+C contents of 42 to

48 mol%, were included in the new genus *Oceanospirillum*, which contained five species. The genus *Aquaspirillum*, with 13 species, was created for all of the aerobic freshwater spirilla, which had low salt tolerances and G+C contents of 50 to 65 mol%. Later, the genus *Aquaspirillum* was expanded by the addition of six new species (1b, 5, 34, 38, 53, 58). As mentioned by Krieg (30, 31), this was an improvement over the previous classification, but it was still not entirely satisfactory. Indeed, on the basis of phenotypic characteristics, there seems to be a continuum between the spirilla and a variety of other oxidative, gram-negative bacteria (32, 33), resulting in rather vague generic definitions. The descriptions of the genera *Spirillum*, *Aquaspirillum*, and *Oceanospirillum* in *Bergey's Manual of Systematic Bacteriology* (32) are based mainly on nutritional, morphological, and physiological data, as well as a limited amount of genotypic data (DNA base composition). The numbers of exceptions listed in the general descriptions of these taxa (e.g., the genus *Aquaspirillum*) are large. This allows much variation in morphological features (cells can be helical, curved, vibrioid, or straight rods; flagellation is polar or bipolar in tufts or the cells are monotrichous), as well as physiological characteristics (catalase, phosphatase, and nitrogenase can be present or absent; aerobic, microaerophilic, or facultatively anaerobic), nutritional properties (carbohydrates usually are not catabolized; vitamins usually are not required), and genotypic data (DNA base compositions range from 49 to 66 mol%). From these and other observations (16, 46, 71, 72, 74) we concluded that the

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genera *Aquaspirillum* and *Oceanospirillum* needed a thorough genotypic analysis in order to reveal the different subgroups that are probably present in these genera. The genus *Oceanospirillum* has already been emended (48), and some of our results for the genus *Aquaspirillum* are presented in this paper.

As the DNA-rRNA hybridization technique has proven to be a powerful tool in the study of the relationships among bacteria at the generic and suprageneric levels (13, 18–20, 49, 50, 60, 66), it was the method of choice for the study of the genus *Aquaspirillum*. If necessary, the finer relationships were investigated by using DNA-DNA hybridization or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell proteins.

MATERIALS AND METHODS

Bacterial strains and growth media. Table 1 shows the bacterial strains which we used along with their sources of isolation. All *Aquaspirillum* strains were grown and maintained by weekly transfers on peptone succinate salt medium (PSS) containing (per liter of distilled water) 10.0 g of neutralized bacteriological peptone (type L34; Oxoid Ltd., Basingstoke, United Kingdom), 1.0 g of succinic acid, 1.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.0 g of $(\text{NH}_4)_2\text{SO}_4$, and 2.0 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. The pH was adjusted to 6.8 with KOH rather than NaOH to avoid possible inhibitory levels of Na^+ .

Most of the other strains were grown on nutrient agar, which contained (per liter of distilled water) 5 g of bacteriological peptone, 2 g of yeast extract, 1 g of beef extract, and 5 g of NaCl (pH 7.4); *Leptothrix*, *Rubrivivax*, *Sphaerotilus*, and *Xylophilus* strains were grown on PYG medium, medium 27, PYG medium, and GYCA medium, respectively, as described previously (64).

The bacteriological purity of the strains was checked by regular plating and microscopic examination of living and Gram-stained cells. Mass cultures for extraction of DNA were grown in Roux flasks on the appropriate media as described by De Smedt and De Ley (15).

Preparation of high-molecular-weight DNA. For DNA preparation, purification, and fixation on nitrocellulose filters, the procedures of Gillis and De Ley (24) were used for most organisms; the exceptions were *Aquaspirillum anulus*, *Aquaspirillum delicatum*, *Aquaspirillum giesbergeri*, *Aquaspirillum gracile*, and *Aquaspirillum putridiconchylum*, for which the isolation method was modified as described below. The NaClO_4 step, which often resulted in fragmentation of the DNA, was replaced by treatment with 50 μg of proteinase K per ml for 2 h at 37°C immediately after lysis. The chloroform-isoamyl mixture was replaced by a mixture containing chloroform and phenol (1:1, vol/vol) to which 1.5 g of hydroxyquinoline per liter was added, and saline EDTA buffer (150 mM NaCl, 100 mM EDTA; pH 8.0) was replaced by TES buffer (150 mM NaCl, 50 mM EDTA, 20 mM Tris; pH 7.9). Whenever possible, the procedures were carried out at 4°C.

Chemical determination of DNA concentration. For DNA-DNA hybridizations, the concentration of DNA was determined by using the method of Burton (4). The amount of filter-fixed DNA was determined by using the method of Van Landschoot and De Ley (60).

DNA base composition. The average G+C contents of the DNAs were determined by thermal denaturation (14) and were calculated by using the equation of Marmur and Doty (39), as modified by De Ley (8).

Preparation of labeled rRNA. Using the in vivo labeling method of De Ley and De Smedt (11), we prepared 23S [^3H]rRNAs from the following strains: *Aquaspirillum serpens* biovar bengal ATCC 27641^T (T = type strain) (the cells were grown in 150 ml of medium 717 [American Type Culture Collection, Rockville, Md.] [1] supplemented with 3 mCi of [^3H]uracil [National Instituut voor Radio-elementen, Brussels, Belgium]; the specific activity of the labeled rRNA was 150×10^3 dpm $\cdot \mu\text{g}^{-1}$); *A. delicatum* NCIB 9419^T (cells were grown in 150 ml of PSS containing 1.5 mCi of [$^5,6\text{-}^3\text{H}$]uracil [New England Nuclear Research Products, Boston, Mass.]; the specific activity of the labeled rRNA was 250×10^3 dpm $\cdot \mu\text{g}^{-1}$); *A. gracile* ATCC 19624^T (the cells were grown in two batches containing 150 ml of medium 233 [1a] supplemented with 0.3% peptone and 1 mCi of [$^2,8\text{-}^3\text{H}$]adenine [New England Nuclear Research Products, Boston, Mass.]; the specific activity of the labeled rRNA was 900×10^3 dpm $\cdot \mu\text{g}^{-1}$); *Aquaspirillum metamorphum* NCIB 9509^T (the cells were grown in 150 ml of PSS supplemented with 3% peptone and 1.5 mCi of [$^2,8\text{-}^3\text{H}$]adenine [New England Nuclear Research Products, Boston, Mass.]; the specific activity of the labeled rRNA was 120×10^3 dpm $\cdot \mu\text{g}^{-1}$); and *Aquaspirillum sinuosum* ATCC 9786^T (the cells were grown in 150 ml of PSS containing 2 mCi of [$^5\text{-}^3\text{H}$]orotate [New England Nuclear Research Products, Boston, Mass.]; the specific activity of the labeled rRNA was 80×10^3 dpm $\cdot \mu\text{g}^{-1}$). 23S [^{14}C]rRNA was prepared from *A. anulus* NCIB 9012^T (the cells were grown in 200 ml of PSS containing 0.12 mCi of [^{14}C]orotate [Nationaal Instituut voor Radio-elementen, Brussels, Belgium]; the specific activity of the labeled rRNA was 2×10^3 dpm $\cdot \mu\text{g}^{-1}$).

New batches of 23S [^3H]rRNA were prepared from the following organisms: *Comamonas acidovorans* Stanier 14^T (the cells were grown in 100 ml of Moore-McCarthy medium [41] containing 2 mCi of [$^5\text{-}^3\text{H}$]uracil [Nationaal Instituut voor Radio-elementen, Brussels, Belgium]; the specific activity of the labeled rRNA was 62×10^3 dpm $\cdot \mu\text{g}^{-1}$); *Chromobacterium violaceum* NCTC 9757^T (the cells were grown in 100 ml of Moore-McCarthy medium supplemented with 0.6% glucose, 0.5% yeast extract, and 2 mCi of [$^5\text{-}^3\text{H}$]uracil [Nationaal Instituut voor Radio-elementen, Brussels, Belgium]; the specific activity of the labeled rRNA was 130×10^3 dpm $\cdot \mu\text{g}^{-1}$); and from *Janthinobacterium lividum* Sneath HB^T (the cells were grown in 100 ml of Moore-McCarthy medium supplemented with 0.6% glucose, 0.5% yeast extract, and 2 mCi of [$^5\text{-}^3\text{H}$]uracil [Nationaal Instituut voor Radio-elementen, Brussels, Belgium]; the specific activity of the labeled rRNA was 28×10^3 dpm $\cdot \mu\text{g}^{-1}$) as described previously (12, 17).

Labeled 16S and 23S rRNA fractions were separated as described previously (15, 41). To minimize RNase activity, rRNAs were prepared and stored in the presence of 0.2% bentonite.

Purified 23S [^3H]rRNAs from *Acidovorax facilis* ATCC 11228^T, *Comamonas terrigena* NCIB 8193^T, *Hydrogenophaga palleronii* Stanier 3621^T, "*Leptothrix discophora*" SP-6(s), *Neisseria flavescens* ATCC 13120^T, and *Rubrivivax gelatinosus* NCIB 8290^T, 16S [^{14}C]rRNAs from *Variovorax paradoxus* ATCC 17713t1^T, *Pseudomonas solanacearum* NCPPB 325^T, and *Alcaligenes xylooxidans* subsp. *denitrificans* ATCC 15173^T, and 23S [^{14}C]rRNAs from *Hydrogenophaga pseudoflava* GA3^T and *Xylophilus ampelinus* NCPPB 2217^T (specific activities ranging from 2×10^3 to $1,120 \times 10^3$ dpm $\cdot \mu\text{g}^{-1}$) were available from members of our research group (13, 51, 61, 63–66).

TABLE 1. Strains used

Name	Strain designation as received ^a	Other designation(s) ^a	Source, place, and/or year of isolation
<i>Aquaspirillum</i> strains			
<i>Aquaspirillum</i> <i>serpens</i> biovar bengal	ATCC 27641 ^T	LMG 6234 ^T	Freshwater pond, West Bengal, India, 1974
<i>Aquaspirillum</i> <i>serpens</i> biovar <i>serpens</i>	ATCC 12638 ^T	LMG 3734 ^T , CCUG 2092 ^T , CIP 63.19 ^T , NCIB 8658 ^T , NCTC 10593 ^T , NRRL B-2045 ^T	Freshwater, 1936
<i>Aquaspirillum</i> <i>serpens</i> biovar <i>serpens</i>	ATCC 27050	LMG 4346, CCUG 13729, DSM 50911	
<i>Aquaspirillum</i> strains	ATCC 15278	LMG 4345, IFO 12014, NCIB 9510	Putrid infusion of freshwater shellfish, 1962
[<i>Aquaspirillum</i>] <i>serpens</i> var. <i>azotum</i>	NCIB 9011 ^T	LMG 4343 ^T , ATCC 11335 ^T , NCMB 57 ^T	Freshwater, United States, 1957
[<i>Aquaspirillum</i>] <i>anulus</i>	NCIB 9012 ^T	LMG 5404 ^T , ATCC 11879 ^T , ATCC 35958 ^T	Pond water, United States, 1957
[<i>Aquaspirillum</i>] <i>aquaticum</i>	ATCC 11330 ^T	LMG 2370 ^T , CCUG 15845 ^T	Freshwater, small lake, United States, 1957
[<i>Aquaspirillum</i>] <i>autotrophicum</i>	ATCC 29984 ^T	LMG 4326 ^T , CCUG 12808 ^T , DSM 732 ^T	Eutrophic lake, Switzerland, 1978
[<i>Aquaspirillum</i>] <i>autotrophicum</i>	DSM 733	LMG 7377, CCUG 19112	Eutrophic lake, Switzerland, 1978
[<i>Aquaspirillum</i>] <i>delicatum</i>	NCIB 9419 ^T	LMG 4327 ^T , ATCC 14667 ^T , CCUG 15846 ^T	Distilled water, 1962
[<i>Aquaspirillum</i>] <i>dispar</i>	ATCC 14667 ^T	LMG 4328 ^T , NCIB 9419 ^T , CCUG 15846 ^T	Distilled water, 1962
[<i>Aquaspirillum</i>] <i>dispar</i>	ATCC 27510 ^{Tb}	LMG 4329 ^T , CCUG 13793 ^T , DSM 736 ^T	Freshwater, Woods Hole, Mass., 1973
[<i>Aquaspirillum</i>] <i>dispar</i>	ATCC 27650	LMG 4330	Freshwater, Woods Hole, Mass., 1973
[<i>Aquaspirillum</i>] <i>giesbergeri</i>	ATCC 11334 ^T	LMG 4332 ^T	Pond water, United States, 1957
[<i>Aquaspirillum</i>] <i>giesbergeri</i>	NCIB 9073 ^T	LMG 4331 ^T , CCUG 15844 ^T , ATCC 11334 ^T	Pond water, United States, 1957
[<i>Aquaspirillum</i>] <i>gracile</i>	ATCC 19624 ^T	LMG 4333 ^T , LMG 8201 ^T	Pond water, Massachusetts, 1966
[<i>Aquaspirillum</i>] <i>gracile</i>	ATCC 19625	LMG 4334	Pond water, Massachusetts, 1966
[<i>Aquaspirillum</i>] <i>gracile</i>	ATCC 19626	LMG 4335, CCUG 15843	Pond water, Massachusetts, 1966
[<i>Aquaspirillum</i>] <i>itersonii</i>	ATCC 12639 ^T	LMG 4337 ^T , CCUG 13727 ^T , NCIB 9070 ^T	Freshwater, 1936
[<i>Aquaspirillum</i>] <i>metamorphum</i>	NCIB 9509 ^T	LMG 4338 ^T , ATCC 15280 ^T , CCUG 13794 ^T	Putrid infusion of freshwater shellfish, 1961
[<i>Aquaspirillum</i>] <i>peregrinum</i>	NCIB 9435 ^T	LMG 4340 ^T , ATCC 15387 ^T , CCUG 13795 ^T	Primary oxidation pond, 1963
[<i>Aquaspirillum</i>] <i>polymorphum</i>	ATCC 11332 ^T	LMG 6235 ^T , CIP 63.15 ^T , NCIB 9072 ^T	Pond water, United States, 1957
[<i>Aquaspirillum</i>] <i>psychrophilum</i>	IFO 13611 ^T	LMG 5408 ^T , ATCC 33335 ^T	<i>Ceratodon purpureus</i> , Antarctic, 1964
[<i>Aquaspirillum</i>] <i>putridiconchylum</i>	ATCC 15279 ^T	LMG 4342 ^T , IFO 12013 ^T , NCIB 9508 ^T	Putrid infusion of freshwater shellfish, 1958
[<i>Aquaspirillum</i>] <i>sinuosum</i>	ATCC 9786 ^T	LMG 4347 ^T , CCUG 13728 ^T , CIP 63.18 ^T	Freshwater, United States, 1957
<i>Prolinoborus fasciculus</i>	ATCC 27740 ^T	LMG 6233 ^T	Pond water, Blacksburg, Va., 1971
Reference strains			
<i>Acidovorax delafieldii</i>	CCUG 1779 ^T	LMG 5943 ^T	Soil enriched with PHB as sole carbon source ^c
<i>Acidovorax delafieldii</i>	ATCC 17505 ^T	LMG 6832 ^T	Soil enriched with PHB as sole carbon source
<i>Acidovorax delafieldii</i>	Stanier 134	LMG 179212, ATCC 1750612, CCUG 14277	Soil enriched with PHB as sole carbon source
<i>Acidovorax facilis</i>	ATCC 11228 ^T	LMG 2193 ^T , CCUG 2113 ^T	Lawn soil, United States
<i>Acidovorax temperans</i>	Popoff Alc29	LMG 3332, CIP 239.74	Blood culture, France
<i>Acidovorax temperans</i>	Popoff Alc30	LMG 3334, CIP 471.74	Unknown
<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i>	ATCC 15173 ^T	LMG 1231 ^T , CCUG 407 ^T	Soil, 1954
[<i>Alcaligenes</i>] <i>latus</i>	Palleroni H-4 ^T	LMG 3321 ^T , ATCC 29712 ^T , CCUG 10983 ^T	Soil, California
<i>Variovorax paradoxus</i>	ATCC 177131 ^T	LMG 179711 ^T , CCUG 1777 ^T	Soil in mineral medium under an atmosphere containing 91% H ₂ , 4% O ₂ , and 5% CO ₂
<i>Comamonas acidovorans</i>	Stanier 14 ^T	LMG 1226 ^T , ATCC 15668 ^T , CCUG 14481 ^T	Soil enriched with acetamide, Delft, The Netherlands, 1926
<i>Comamonas terrigena</i>	NCIB 8193 ^T	LMG 1253 ^T , ATCC 8461 ^T , CCUG 15327 ^T , LMG 5929 ^T , CCUG 2185 ^T	Hay infusion filtrate, United States
<i>Comamonas terrigena</i>	NCIB 2581	LMG 1249, CCUG 2474	Soil

<i>Comamonas testosteroni</i>	NCTC 10698 ^T	LMG 1786 ^T , ATCC 11996 ^T , CCUG 1426 ^T	Soil, Berkeley, Calif.
<i>Chromobacterium violaceum</i>	NCTC 9757 ^T	LMG 1267 ^T , ATCC 12472 ^T , DSM 30191 ^T	Freshwater, Mentekab, Malaya, 1952
<i>Herbaspirillum seropedicace</i>	Döbereiner Z67 ^T	LMG 6513 ^T	Surface-sterilized roots, <i>Grysa sativa</i> , Brazil
<i>Herbaspirillum seropedicace</i>	Döbereiner Z78	LMG 6514	Surface-sterilized roots, <i>Sorghum bicolor</i> , Brazil
<i>Hydrogenophaga flava</i>	DSM 619 ^T	LMG 2185 ^T , ATCC 33667 ^T	Mud from ditch, 1942
<i>Hydrogenophaga palleronii</i>	Stanier 36211 ^T	LMG 23661 ^T , ATCC 177241 ^T , CCUG 1780 ^T	Water enriched for hydrogen bacteria in an atmosphere containing 6% O ₂ , United States
<i>Hydrogenophaga pseudoflava</i>	GA3 ^T	LMG 5945 ^T , ATCC 33668 ^T , CCUG 13799 ^T	Water, River Weende, Göttingen, Germany
<i>Hydrogenophaga taeniospiralis</i>	DSM 2082 ^T	LMG 7170 ^T , CCUG 15921 ^T	Soil, Spain
<i>Iodobacter fluvialis</i>	USCC 2237	LMG 6573, NCTC 11160, CCM 3309	River water, United Kingdom
<i>Iodobacter fluvialis</i>	USCC 2238	LMG 6574, NCTC 11161	River water, United Kingdom
<i>Janthinobacterium lividum</i>	Sneath HB ^T	LMG 2892 ^T , ATCC 12473 ^T , CCM 160 ^T , NCIB 9130 ^T , NCTC 9796 ^T	Soil, Michigan
<i>Leptothrix cholodnii</i>	CCM 1827	LMG 9467	Active sludge
" <i>Leptothrix discophora</i> "	SP-6(s)	LMG 8142	Artificial iron seep in laboratory, Ithaca, N. Y.
<i>Neisseria flavescens</i>	ATCC 13120 ^T	LMG 5319 ^T , CCUG 345 ^T , NCTC 8263 ^T	Spinal fluid, case of fatal meningitis
<i>Neisseria lactamica</i>	NCTC 10617 ^T		
<i>Neisseria flavescens</i>	NCTC 10025		
<i>[Pseudomonas] avenae</i>	NCPPB 1011 ^T	LMG 2117 ^T , ATCC 19860 ^T , CCUG 15838 ^T	<i>Zea mays</i> , United States, 1958
<i>[Pseudomonas] rubrisubalbicans</i>	NCPPB 1027 ^T	LMG 2286 ^T , ATCC 19308 ^T , CCUG 17392 ^T , CCUG 17679 ^T , PDDCC 5777 ^T	<i>Saccharum officinarum</i> , United States
<i>[Pseudomonas] rubrisubalbicans</i>	NCPPB 932	LMG 2285, PDDCC 793	<i>Saccharum officinarum</i> cv. Q67 leaf, Australia, 1961
<i>[Pseudomonas] rubrisubalbicans</i>	PDDCC 792	LMG 6415, NCPPB 904	<i>Saccharum officinarum</i> cv. M134-32 leaf
<i>[Pseudomonas] saccharophila</i>	ATCC 15946 ^T	LMG 2256 ^T , LMG 7831 ^T	Mud from a stagnant pool
<i>[Pseudomonas] solanacearum</i>	NCPPB 325 ^T	LMG 2299 ^T , ATCC 11696 ^T , CCUG 14272 ^T	<i>Lycopersicon lycopersicum</i> , United States, 1953
<i>Rubrivivax gelatinosus</i>	NCIB 8290 ^T	LMG 4311 ^T , ATCC 17011 ^T , CCUG 15841 ^T	Acetate enrichment, pH 6.6
<i>Xylophilus ampelinus</i>	NCPPB 2217 ^T	LMG 5856 ^T , ATCC 33914 ^T , CCUG 21976 ^T	<i>Vitis vinifera</i> var. <i>sultana</i> , Crete, 1966

^a Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; CCUG, Culture Collection of the University of Göteborg, Department of Clinical Bacteriology, University of Göteborg, Sweden; CIP, Collection de l'Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; IFO, Institute for Fermentation Osaka, Osaka, Japan; LMG, Culture Collection, Laboratorium voor Microbiologie, Rijksuniversiteit Gent, Ghent, Belgium; NCIB, National Collection of Industrial Bacteria, Torrey Research Station, Aberdeen, Scotland; NCMB, National Collection of Marine Bacteria, Torrey Research Station, Aberdeen, Scotland; NCPPB, National Collection of Plant Pathogenic Bacteria, Hatching Green, England; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, England; NRRL, Agricultural Research Service Culture Collection, Peoria, Ill.; PDDCC, Culture Collection of the Plant Disease Division, New Zealand Department of Scientific and Industrial Research, Auckland, New Zealand; USCC, University of Surrey Culture Collection, Department of Microbiology, University of Surrey, Guildford, Surrey, United Kingdom; VPI, Virginia Polytechnic Institute and State University, Blacksburg.

^b We isolated two stable colony types from the original culture and labeled them t1 and t2. If both types had almost identical protein patterns, we used only one type.

^c PHB, poly-β-hydroxybutyrate.

TABLE 2. $T_{m(e)}$ values of the DNA-rRNA hybrids obtained with reference rRNAs from members of rRNA superfamily III^a

DNA from strain:	Mean G+C content (mol%)	$T_{m(e)}$ value (°C) with [³ H]rRNA from:					$T_{m(e)}$ value (°C) with [¹⁴ C]rRNA from [<i>Pseudomonas</i>] <i>solanacearum</i> NCPBP 325 ^T
		<i>Aquaspirillum</i> <i>serpens</i> bio- var bengal ATCC 27641 ^T	<i>Chromo- bacterium</i> <i>violaceum</i> NCTC 9757 ^T	<i>Neisseria</i> <i>flavescens</i> ATCC 13120 ^T	<i>Janthino- bacterium</i> <i>lividum</i> Sneath HB ^T	<i>Alcaligenes</i> <i>xylosoxi- dans</i> subsp. <i>denitrificans</i> ATCC 15173 ^T	
<i>Aquaspirillum</i> species							
<i>Aquaspirillum serpens</i> biovar bengal ATCC 27641 ^T	51	80.2	73.1	69.4	67.9		
<i>Aquaspirillum serpens</i> biovar serpens ATCC 12638 ^T	50	78.9	71.3		68.8	69.4	69.8
<i>Aquaspirillum serpens</i> biovar serpens ATCC 15278	50	80.3	71.2				
<i>Aquaspirillum serpens</i> biovar serpens ATCC 27050	50	79.5	71.7				
[<i>Aquaspirillum</i>] species							
<i>Prolinoborus fasciculus</i> ATCC 27740 ^T	62	74.1	71.8	71.2	68.7	68.9	
[<i>Aquaspirillum</i>] <i>dispar</i> ATCC 27510 ^T	65	72.1	74.2		69.4	68.4	
[<i>Aquaspirillum</i>] <i>dispar</i> ATCC 27650	63	71.8	73.2	70.2		69.1	
[<i>Aquaspirillum</i>] <i>putridiconchylum</i> ATCC 15279 ^T	52	72.3	72.8	70.0	69.2	68.8	71.1
[<i>Aquaspirillum</i>] <i>serpens</i> var. <i>azotum</i> NCIB 9011 ^T	51	69.3	68.3	66.6	71.5	71.7	
[<i>Aquaspirillum</i>] <i>autotrophicum</i> ATCC 29984 ^T	62	68.6	67.3		75.6	68.5	71.0
[<i>Aquaspirillum</i>] <i>autotrophicum</i> DSM 733	60				75.7		
Reference strains							
<i>Chromobacterium violaceum</i> NCTC 9757 ^T	67	69.9	80.9	70.9	70.2		72.0
<i>Neisseria meningitidis</i> NCTC 10025 ^T	53	71.0	72.4	78.5	68.6	67.3	
<i>Neisseria flavescens</i> ATCC 13120 ^T	50		72.6	80.6			
<i>Neisseria lactamica</i> NCTC 10617 ^T	53	70.0				67.0	70.1
<i>Iodobacter fluvialis</i> USCC 2237	50	70.0	70.1	66.1	66.4		
<i>Iodobacter fluvialis</i> USCC 2238	50	70.1		67.0			
<i>Janthinobacterium lividum</i> Sneath HB ^T	66	67.9	70.4	67.9	78.8		72.5
<i>Comamonas acidovorans</i> Stanier 14 ^T	67		67.5	66.0			70.5
<i>Comamonas testosteroni</i> NCTC 10698 ^T	62						72.0
<i>Acidovorax delafieldii</i> ATCC 17506t2	64						72.0
[<i>Pseudomonas</i>] <i>saccharophila</i> ATCC 15946 ^T	69						71.0
[<i>Pseudomonas</i>] <i>rubrisubalbicans</i> NCPBP 932	63				75.6		
[<i>Pseudomonas</i>] <i>rubrisubalbicans</i> NCPBP 1027 ^T	62				75.9		75.0
[<i>Pseudomonas</i>] <i>rubrisubalbicans</i> PDDCC 792	63				75.8		
[<i>Pseudomonas</i>] <i>solanacearum</i> NCPBP 325 ^T	66		70.6				81.5
<i>Herbaspirillum seropedicae</i> Döbereiner Z67 ^T	64	67.4					
<i>Herbaspirillum seropedicae</i> Döbereiner Z78	65	67.9	70.4				
<i>Variovorax paradoxus</i> ATCC 17713t1 ^T	67						71.0
<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> ATCC 15173 ^T	69					81.5	
<i>Hydrogenophaga palleronii</i> Stanier 362t1 ^T	67						72.5
<i>Hydrogenophaga flava</i> DSM 619 ^T	66						69.0

^a Some of the data (including G+C values) are from references 1, 12, 13, 17–19, 26, 27, 34, 51, 53, 61, 63, 64, and 66.

DNA-rRNA hybridization and thermal stability of DNA-rRNA hybrids. Saturation hybridizations between labeled 16S or 23S rRNAs and filter-fixed DNAs were carried out as described by De Ley and De Smedt (11). From the melting curves of the DNA-rRNA hybrids, we calculated two hybridization parameters, the midpoint temperature of the thermal denaturation curve [$T_{m(e)}$] and the percentage of rRNA binding (determined from the amount [in micrograms] of labeled rRNA duplexed to 100 µg of filter-fixed DNA after RNase treatment under standard conditions).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by using the method of Laemmli (35), as modified by Kiredjian et al. (28).

DNA-DNA hybridization. The degree of binding (expressed as a percentage), which is a quantitative measurement of the level of DNA homology between a pair of DNAs, was determined spectrophotometrically from the initial renaturation rates by using the method of De Ley et al. (10). Degrees of binding of 30% or less are not significant. The total DNA concentration was ca. 50 µg · ml⁻¹, and the

optimal renaturation temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) was calculated by using the method of De Ley et al. (10). A model 2600 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a thermostatically controlled cuvette chamber and a model 7225A plotter (Hewlett-Packard Co., Palo Alto, Calif.) was used.

RESULTS

Purity of strains. For *Aquaspirillum dispar* ATCC 27510^T, we identified two stable colony types, which were designated t1 and t2. DNAs were prepared from both types, and DNA-DNA hybridization experiments revealed a mean value for degree of binding of 97%. Both DNAs were used for DNA-rRNA hybridizations (Table 2).

Preparation of labeled rRNA. The type strain of *A. serpens* biovar serpens, strain ATCC 12638, produced a very low cell yield at the normal peptone concentration in PSS (70 Klett units at 600 nm at the end of the log phase). A peptone concentration of 3% resulted in an increase in the growth

yield (120 Klett units), but under these conditions uptake of the radioactive precursor decreased to less than 5×10^{-3} mCi · g of wet cells⁻¹ for [5-³H]orotate and to less than 4×10^{-3} mCi · g of wet cells⁻¹ for [5-³H]uracil. These amounts were insufficient for the preparation of labeled rRNA. As a close relationship was expected between *A. serpens* biovar bengal and *A. serpens* biovar serpens (3, 74), growth and uptake tests were performed also with the type strain of *A. serpens* biovar bengal, strain ATCC 27641. The best uptake was observed with [5,6-³H]uracil (0.1 mCi · g of wet cells⁻¹) in medium 717 containing 1% peptone. The uptake of [5-³H]orotate was less than 0.01 mCi · g of wet cells⁻¹ under comparable conditions.

The distributions after sucrose gradient centrifugation of the labeled rRNAs from *Chromobacterium violaceum* NCTC 9757^T, *J. lividum* Sneath HB^T, and *Comamonas acidovorans* Stanier 14^T were very similar to the profiles obtained previously (12, 17).

DNA-rRNA hybridization. Table 2 shows the DNA-rRNA hybridization results [$T_{m(e)}$ values] obtained with the labeled reference rRNAs from members of rRNA superfamily III. Tables 3 and 4 show the $T_{m(e)}$ values obtained for members of the acidovorans rRNA complex. As pointed out previously (15), $T_{m(e)}$ is a parameter which allows valuable taxonomic conclusions at the suprageneric level. This parameter was used to draw the dendrograms in Fig. 1 and 2. Figure 1 shows the results obtained for a part of rRNA superfamily III with links to rRNA superfamilies I, II, and IV. Figure 2 shows a more detailed picture of the phylogenetic structure of the acidovorans rRNA complex. The $T_{m(e)}$ values which we used were from this study, from references 13, 51, 61, 63–66, and from unpublished data obtained from members of our group.

The reproducibility of the $T_{m(e)}$ values was found to be 0.5 to 1.0°C for the $T_{m(e)}$ range from 73 to 81°C and 1.0 to 2.0°C for the lower $T_{m(e)}$ values.

The variations observed at some $T_{m(e)}$ levels are indicated in Fig. 1 and 2.

DNA base composition and DNA-DNA hybridization. The average G+C values for the strains which we used are shown in Tables 2 through 4. As the preparation of large amounts of pure, high-molecular-weight DNA was often difficult for species belonging to the genus *Aquaspirillum*, DNA-DNA hybridizations were not performed systematically. The DNA binding values which we determined are shown in Fig. 3 or are mentioned below.

DISCUSSION

Using the DNA-rRNA hybridization results, workers have delineated a number of large groups of gram-negative bacteria. These groups were named rRNA superfamilies (9) and were confirmed by using 16S rRNA cataloging and sequencing data. There is excellent correlation within the gamma (rRNA superfamily I + II), beta (rRNA superfamily III), and alpha (rRNA superfamily IV) subclasses (72–74) of the *Proteobacteria* as defined by Stackebrandt et al. (52) and obtained by partial sequencing of 16S rRNAs. Each rRNA superfamily contains a number of rRNA branches, which frequently represent a genus or a group of genera. We hybridized the DNAs of representative species of the genus *Aquaspirillum* with representative rRNAs from members of the different rRNA superfamilies and found that these organisms are members of rRNA superfamily III (most of the species) and rRNA superfamily IV (three species) (Fig. 1).

For the sake of clarity, below we use brackets to indicate that organisms are misnamed taxonomically.

***Aquaspirillum* species located on the *A. serpens* rRNA branch and [A.] *serpens* var. *azotum*.** According to the data in Table 2 and Fig. 1, the genotypic nucleus of the genus *Aquaspirillum* consists of only the type species *A. serpens*, including *A. serpens* biovar serpens and *A. serpens* biovar bengal [$T_{m(e)}$ values versus rRNA from *A. serpens* biovar bengal, 79.5 to 80.3°C]. The *A. serpens* rRNA branch is a separate rRNA branch in rRNA superfamily III that is equidistantly related to the family *Neisseriaceae* and the genus *Chromobacterium* (Table 2 and Fig. 1); the latter rRNA branches form a separate cluster in rRNA superfamily III. Because *A. serpens* is the type species of the genus *Aquaspirillum*, the authentic aquaspirilla should be restricted to members of this rRNA branch.

Our DNA-rRNA hybridization results (Table 2), as well as our DNA-DNA hybridization results (Fig. 3), agree very well with the high DNA-DNA homology values found by Boivin et al. (3) for *A. serpens* biovar bengal and *A. serpens* biovar serpens. These authors detected 59 to 73% homology by using slot blot hybridization, values which are roughly the same as the values of 55 to 60% which we determined by using the optical renaturation method (Fig. 3).

[A.] *serpens* var. *azotum* was described by Williams and Rittenberg (69). [A.] *serpens* var. *azotum* differs from *A. serpens* biovar serpens and *A. serpens* biovar bengal by its ability to reduce nitrate to nitrite. McElroy and Krieg (40) found that [A.] *serpens* var. *azotum* ATCC 11335 is serologically related to *A. serpens* VH of R. G. E. Murray (a strain not included in this study) and to [A.] *putridiconchylum* ATCC 15279^T. No relatedness was found between strain ATCC 11335 and *A. serpens* ATCC 12638^T or *A. serpens* ATCC 15278, which represented two separate serogroups. In their large comprehensive study of the taxonomy of the spirilla, Hylemon et al. (26) concluded that despite the serological differences, the many similarities that they observed between strain ATCC 11335 and nine other strains of *A. serpens* justified the inclusion of this strain in the species *A. serpens*. This was confirmed by the results of the numerical taxonomy study of Carney et al. (7), who investigated 175 morphological, physiological, biochemical, and nutritional characteristics. A phenotypic similarity level of about 86% was calculated for [A.] *serpens* ATCC 11335 and *A. serpens* ATCC 12638^T; this level was within the range found for other species included in the study. However, Carney et al. (7) reported a DNA base composition for [A.] *serpens* var. *azotum* ATCC 11335 of 63.4 mol%, in contrast to the value of 51 mol% reported by Hylemon et al. (26), indicating that different strains may have been used. Table 2 shows that [A.] *serpens* var. *azotum* NCIB 9011 (identical to strain ATCC 11335 [43a]) is phylogenetically not closely related to *A. serpens* biovar serpens or to *A. serpens* biovar bengal. The mean $T_{m(e)}$ values for this strain versus reference rRNAs from members of rRNA superfamily III do not completely exclude a relationship with [A.] *putridiconchylum* ATCC 15279^T. Such a relationship would be in agreement with (i) serological data (40), (ii) phenotypic data ([A.] *putridiconchylum* differs from *A. serpens* only by its weak catalase activity and its ability to use a wide variety of compounds as sole carbon sources [26]), and (iii) the DNA base compositions given by Hylemon et al. (26). It is obvious that strains VH, ATCC 11335, NCIB 9011, and ATCC 15279^T ought to be studied further by using DNA-DNA hybridization or by using methods which reveal similarities on a comparable taxonomic level.

TABLE 3. $T_{m(e)}$ values of the DNA-rRNA hybrids obtained with reference rRNAs from members of the acidovorans rRNA complex^a

DNA from strain:	Mean G+C content (mol%)	$T_{m(e)}$ value (°C) with [³ H]rRNA from:					$T_{m(e)}$ value (°C) with [³ H]rRNA from:		$T_{m(e)}$ value (°C) with [¹⁴ C]rRNA from Xylophilus ampelinus NCPPB 2217 [†]	
		Comamonas acidovorans Stanier 14 [†]	Comamonas terrigena NCIB 8193 [†]	Acidovorax facilis ATCC 11228 [†]	Hydrogenophaga palleronii Stanier 3621 [†]	Hydrogenophaga pseudoflava GA3 [†]	$T_{m(e)}$ value (°C) with [³ H]rRNA from:			
							Rubrivivax gelatinosus NCIB 8290 [†]	“Leptothrix discophora” SP-6(s)		
[Aquaspirillum] species belonging to the acidovorans rRNA complex										
[Aquaspirillum aquaticum] ATCC 11330 [†]	64	76.3	76.6	75.8	76.3	75.6	75.1	72.4	69.8	75.1
[Aquaspirillum] anulus NCIB 9012 [†]	58	77.6	75.5	76.6	74.8	75.0	74.6	73.2		75.9
[Aquaspirillum] delicatum NCIB 9419 [†]	64	75.1	75.1	76.8	76.6	75.2	76.4	73.4	72.7	77.0
[Aquaspirillum] delicatum ATCC 14667 [†]	63	76.0								
[Aquaspirillum] giesbergeri ATCC 11334 [†]	57	77.5	74.4	76.1	74.8	73.7	75.4			76.4
[Aquaspirillum] giesbergeri NCIB 9073 [†]	57	76.6								
[Aquaspirillum] gracile ATCC 19624 [†]	66	75.8								
[Aquaspirillum] gracile ATCC 19625	65	76.2	75.8	76.6	75.8	76.8	75.5	73.6	72.2	77.2
[Aquaspirillum] gracile ATCC 19626	65	74.7								75.8
[Aquaspirillum] metamorphum NCIB 9509 [†]	63	75.4	76.2	77.6	76.2	77.0	75.8	72.6		77.1
[Aquaspirillum] psychrophilum IFO 13611 [†]	63	76.6	76.8	74.8	72.4	73.0	73.2	73.6		76.6
[Aquaspirillum] sinuosum ATCC 9786 [†]	58	77.2	75.2	76.4	75.3	75.7	77.0	71.1	70.6	76.6
Reference strains belonging to the acidovorans rRNA complex										
Acidovorax delafieldii CCUG 1779 [†]	66	77.2	75.1	78.3	75.6	75.7	76.6	72.3		77.4
Acidovorax facilis ATCC 11228 [†]	65	77.0	75.0	79.8	76.3	76.8	77.5	73.2	71.6	77.3
Acidovorax temperans Popoff ALC29	63	76.9	77.7	78.4	76.6	76.7	75.8	73.3		76.7
Acidovorax temperans Popoff ALC30	63	76.8	77.7	79.3						
[Alcaligenes] latus Palleroni H-4 [†]	70	74.5	71.6	73.0	73.4	70.2	73.2	75.8	75.5	72.2
Variovorax paradoxus ATCC 17713 [†]	67	76.5	74.8	75.4	76.0	76.0	81.0	72.6	73.4	78.1
Comamonas acidovorans Stanier 14 [†]	67	80.6	75.0	76.6	73.6	73.6	77.0	73.6	72.5	76.2
Comamonas terrigena NCIB 8193 [†]	64	75.9	81.1	75.5	73.9	74.2	75.5	72.1	72.5	76.0
Comamonas terrigena NCIB 2581	66	75.5	79.4				76.0			76.6
Comamonas testosteroni NCTC 10698 [†]	62	76.5	76.7	75.9	75.6	74.5	76.5	72.3	71.2	75.7
Hydrogenophaga flava DSM 619 [†]	67	75.5		75.1	78.2	79.6	76.0	73.1	75.7	76.3
Hydrogenophaga palleronii Stanier 3621 [†]	67	76.0	73.4	75.2	80.4	78.1	77.5	75.2		76.7
Hydrogenophaga pseudoflava GA3 [†]	66	76.0	75.2	75.0	77.4	80.8		74.6	72.5	76.2
Hydrogenophaga taeniospiralis DSM 2082 [†]	65	75.0		75.2	79.0	78.5		72.9		76.6
Leptothrix cholodnii CCM 1827	70	72.2	75.3		73.3		73.2	74.6	79.0	
“Leptothrix discophora” SP-6(s)	69	73.4						74.3	80.3	
[Pseudomonas] avenae NCPPB 1011 [†]	70	76.6	75.7	76.6	75.6	76.8	76.5	73.5		76.4
[Pseudomonas] saccharophila ATCC 15946 [†]	69	72.6	71.8	74.2	73.4	72.5	73.4	75.8	74.9	73.1
Rubrivivax gelatinosus NCIB 8290 [†]	72	74.5	73.5	73.4	71.9	70.8	71.0	81.4	74.0	71.4
Xylophilus ampelinus NCPPB 2217 [†]	68	76.0	75.6	76.8	75.8	75.5	76.2	73.1		80.3
Other Aquaspirillum and [Aquaspirillum] species										
[Aquaspirillum] autotrophicum ATCC 29984 [†]	62	69.2								
Proteinoborus fasciculus ATCC 27740 [†]	62	67.4								
[Aquaspirillum] dispar ATCC 27510 [†]	65	66.5								
[Aquaspirillum] iersonii ATCC 12639 [†]	62	59.0								
[Aquaspirillum] polymorphum ATCC 11332 [†]	62	59.0								
[Aquaspirillum] peregrinum NCIB 9435 [†]	62	58.8								
[Aquaspirillum] putridiconchylum ATCC 15279 [†]	52	68.0								
Aquaspirillum serpens biovar serpens ATCC 12638 [†]	50	68.5								
Aquaspirillum serpens biovar serpens ATCC 15278	50	68.3								
Other reference organisms										
Chromobacterium violaceum NCTC 9757 [†]	67	67.6								
[Pseudomonas] rubrisulbalbicans NCPPB 1027 [†]	62	71.0								
[Pseudomonas] solanacearum NCPPB 325 [†]	66	70.3								

^a Some of the data (including G+C values) are from references 1, 13, 17–19, 25, 26, 53, 58, 61, 63, 64, and 66.

TABLE 4. $T_{m(e)}$ values of the DNA-rRNA hybrids obtained with rRNAs isolated from [*Aquaspirillum*] species belonging to the acidovorans rRNA complex^a

DNA from strain:	Mean G+C content (mol %)	$T_{m(e)}$ value (°C) with [¹⁴ C]rRNA from [<i>Aquaspirillum</i>] <i>anulus</i> NCIB 9012 ^T	$T_{m(e)}$ value (°C) with [³ H]rRNA from:				
			[<i>Aquaspirillum</i>] <i>delicatum</i> NCIB 9419 ^T	[<i>Aquaspirillum</i>] <i>gracile</i> ATCC 19624 ^T	[<i>Aquaspirillum</i>] <i>metamorphum</i> NCIB 9509 ^T	[<i>Aquaspirillum</i>] <i>sinuosum</i> ATCC 9786 ^T	[<i>Aquaspirillum</i>] <i>aquaticum</i> ATCC 11330 ^T
[<i>Aquaspirillum</i>] species belonging to the acidovorans rRNA complex							
[<i>Aquaspirillum aquaticum</i>] ATCC 11330 ^T	64	74.2	75.9	75.2	76.0	75.1	80.0
[<i>Aquaspirillum anulus</i> NCIB 9012 ^T	58	79.3	76.2	75.6	77.1	75.4	74.3
[<i>Aquaspirillum delicatum</i> NCIB 9419 ^T	64	74.0	80.3	76.6	75.7	73.6	75.5
[<i>Aquaspirillum delicatum</i> ATCC 14667 ^T	63	74.3	79.2				
[<i>Aquaspirillum giesbergii</i> ATCC 11334 ^T	57	75.9	77.4				
[<i>Aquaspirillum gracile</i> ATCC 19624 ^T	66			76.3	77.4	78.7	75.3
[<i>Aquaspirillum gracile</i> ATCC 19625 ^T	62			80.2	76.2	74.0	
[<i>Aquaspirillum gracile</i> ATCC 19626 ^T	65	74.6	76.6	80.8	75.7	73.6	76.2
[<i>Aquaspirillum metamorphum</i> NCIB 9509 ^T	63			80.3	75.8	73.0	
[<i>Aquaspirillum psychrophilum</i> IFO 13611 ^T	63	76.1	75.8	75.6	80.1	75.1	77.1
[<i>Aquaspirillum sinuosum</i> ATCC 9786 ^T	58	76.7	77.6	75.5	77.4	75.6	76.2
Reference strains belonging to the acidovorans rRNA complex							76.0
<i>Acidovorax delafieldii</i> ATCC 17505 ^T	65	76.1			75.8		77.4
<i>Acidovorax facilis</i> ATCC 11228 ^T	65	75.5			76.9		76.8
<i>Acidovorax temperans</i> Popoff Alc29	63		77.0	76.3		74.2	
<i>Acidovorax temperans</i> Popoff Alc30	63		77.5				77.3
[<i>Alcaligenes</i>] <i>latus</i> Palleroni H-4 ^T	70		77.2				76.8
<i>Variovorax paradoxus</i> ATCC 17713 ^{1T}	67	72.4	75.4				75.0
<i>Comamonas acidovorans</i> Stanier 14 ^T	67	74.8	77.4	76.7	77.3	75.7	75.4
<i>Comamonas acidovorans</i> Stanier 14 ^T	67	75.6	76.1	76.6	76.5	75.2	75.4
<i>Comamonas terrigena</i> NCIB 8193 ^T	64	73.0	75.0	75.5	76.8	75.1	76.4
<i>Comamonas terrigena</i> NCIB 2581	66	74.3					77.0
<i>Comamonas testosteroni</i> NCTC 10698 ^T	62	75.1	76.0	75.2	76.4	76.0	76.8
<i>Hydrogenophaga flava</i> DSM 619 ^T	67	75.1			74.3		77.1
<i>Hydrogenophaga palleroni</i> Stanier 36211 ^T	67	73.6	77.2	76.1	75.7	74.1	76.0
<i>Hydrogenophaga pseudoflava</i> GA3 ^T	66	76.9	75.7	76.4	75.9	73.8	77.5
<i>Hydrogenophaga taeniospiralis</i> DSM 2082 ^T	65				75.3		
<i>Leptothrix cholodnii</i> CCM 1827	70	73.0	72.0				72.4
“ <i>Leptothrix discophora</i> ” SP-6(s)	70			73.6			
[<i>Pseudomonas</i>] <i>avenae</i> NCPPB 1011 ^T	70	75.6	77.2	76.0	76.2	74.8	75.4
[<i>Pseudomonas</i>] <i>saccharophila</i> ATCC 15946 ^T	69	72.2	75.4				72.4
<i>Rubrivivax gelatinosus</i> NCIB 8290 ^T	72	70.9	75.2		70.9	70.0	73.0
<i>Xylophilus ampepinus</i> NCPPB 2217 ^T	68	73.7	76.7	75.6	75.6	74.9	75.6
Other <i>Aquaspirillum</i> and [<i>Aquaspirillum</i>] species							
<i>Aquaspirillum serpens</i> biovar <i>serpens</i> ATCC 15278	50						68.7
[<i>Aquaspirillum autotrophicum</i> ATCC 29984 ^T	62						71.9

^a Some of the data (including G+C values) are from references 1, 13, 17–19, 25, 26, 53, 58, 61, 63, 64, and 66.

FIG. 1. Simplified rRNA cistron similarity dendrogram of rRNA superfamily III. The $T_{m(e)}$ values are from Table 2 and from references 12, 13, 17, 46, 49, 59, and 60. The solid bars represent $T_{m(e)}$ ranges within individual rRNA branches. The dashed line represents a subbranch from which labeled rRNA is not yet available. The acidovorans rRNA complex, which was recently described as the family *Comamonadaceae* (62), is indicated by a solid triangle and is represented in more detail in Fig. 2. The Roman numerals indicate the roots of the rRNA superfamilies sensu De Ley (9); superfamilies III and IV correspond to the beta and alpha subclasses, respectively, and superfamily I + II is part of the gamma subclass of the *Proteobacteria* (52). A., *Aquaspirillum*; L., *Leptothrix*; PS., *Pseudomonas*; R., *Rubrivivax*. For some $T_{m(e)}$ levels, the mean $T_{m(e)}$ value is given along with the amount of variation and the number of experiments performed (in parentheses).

Of all previously described *Aquaspirillum* species (except "*Aquaspirillum arcticum*," which was not investigated, and *Aquaspirillum magnetotacticum*, which was recently placed in the alpha group of the proteobacteria [corresponding to rRNA superfamily IV] [20a]), [*Aquaspirillum*] *fasciculus* is the only species that is located on the *A. serpens* rRNA branch [$T_{m(e)}$ with rRNA from *A. serpens* biovar bengal ATCC 27641^T of 74.1°C, compared with values of 71.8 and 71.2°C with rRNAs from *Chromobacterium violaceum* and *N. flavescens* respectively) (Table 2). As a rod-shaped organism with nitrogenase activity, a DNA base composition of 62 to 65 mol% (compared with 49 to 52 mol% for *A. serpens*), and a remarkable type of locomotion (53, 54), [*A.*] *fasciculus* can be differentiated easily from the other *Aquaspirillum* species. The phenotypic relationships of this organism with *Pseudomonas*, *Zoogloea*, and *Azomonas* species have been discussed previously (53). From the results presented here it is also evident that a close relationship with the genus *Comamonas* (19, 62) can be excluded (Fig. 1). The remote relationships with other nitrogen-fixing species belonging to the genus *Aquaspirillum* are evident from the DNA-rRNA data in Table 2. [*Aquaspirillum*] *pere-*

grinum and [*Aquaspirillum*] *itersonii*, which fix nitrogen (32, 53), both belong to rRNA superfamily IV (see below). No other *Aquaspirillum* species in rRNA superfamily III is known to exhibit nitrogenase activity. The presence of a polar membrane is a feature that is present in all of the genera belonging to rRNA superfamilies II (*Oceanospirillum*) (48), III (the aquaspirilla in general), IV (phylogenetically misnamed *Aquaspirillum* species [see below] and *Rhodospirillum*), and VI (*Campylobacter*) (59). Within the genus *Aquaspirillum*, broadly defined, the formation of coccoid bodies has been observed only in [*A.*] *fasciculus* and in the [*Aquaspirillum*] species belonging to rRNA superfamily IV.

As [*A.*] *fasciculus* exhibits a difference in $T_{m(e)}$ of more than 6°C with its closest relative, *A. serpens*, it definitely is a member of a different taxonomic unit. As a $T_{m(e)}$ difference of 6°C or more clearly separates genera (51), [*A.*] *fasciculus* is a member of a separate genus, for which we propose the name *Prolinoborus*.

Description of *Prolinoborus* gen. nov. *Prolinoborus* (Pro.li.no'bo.rus. L. N. *prolina*, the amino acid proline; Gr. adj. *boros*, voracious; M. L. masc. n. *Prolinoborus* [bacteria] that readily consume proline). The description below is

FIG. 2. Simplified rRNA cistron similarity dendrogram of the members of the *Comamonadaceae* in rRNA superfamily III. The $T_{m(e)}$ values are from Tables 3 and 4 and from references 19, 61, and 63–66. The solid bars represent $T_{m(e)}$ ranges within individual rRNA branches. The dashed line represents a subbranch from which labeled rRNA is not yet available. The Roman numerals indicate the roots of the rRNA superfamilies sensu De Ley (9); superfamilies III and IV correspond to the beta and alpha subclasses, respectively, and superfamily I + II is part of the gamma subclass of the *Proteobacteria* (52). A., *Aquaspirillum*; C., *Comamonas*; PS., *Pseudomonas*. For the baseline of the dendrogram, the mean $T_{m(e)}$ value is given along with the amount of variation and the number of experiments performed (in parentheses).

based on data from references 32 and 53. The gram-negative cells are straight rods with diameters of 0.7 to 0.9 μm (mean values). The cells are approximately 5 to 10 μm long, but old cultures may contain cells up to 42 μm long. After prolonged serial transfer, curved or S-shaped variants may occur (53). Poly- β -hydroxybutyrate is formed. A polar membrane underlies the cytoplasmic membrane. Coccoid bodies are formed in old cultures. Initial isolation cannot be achieved by ordinary streaking onto agar media and can be achieved only after prolonged enrichment by the pour plate method, using L-proline as the sole carbon and nitrogen source. Cells occur in flocs upon initial isolation. After several serial transfers, the floc-forming ability can gradually be lost, and eventually the strains exhibit homogeneous, turbid growth. Flagellation is bipolar with the flagella occurring in fascicles and is clearly visible by phase-contrast or dark-field microscopy. The flagella exhibit an unusual and distinctive behavior when cells are suspended in ordinary nonviscous media. The flagella exhibit helical wave propagation and basal bending and have the ability to coil up like springs. The

behaviors of the fascicles at the opposite poles are not coordinated. Free cells or cells at the periphery of flocs in ordinary media are not motile or their motility is irregular over short distances. Within the flocs or in media having greater viscosity (10 to 200 cP, obtained by using, e.g., methylcellulose 400 cP), the cells move in a steady straight-line pattern. The ability to swim only in viscous media may represent an adaptation to the viscous conditions that occur within cell flocs.

Chemoorganotrophic with a respiratory type of metabolism and with oxygen as the terminal electron acceptor. Optimum temperature, 30°C. No growth occurs at 20 or 40°C. Cells grow anaerobically with nitrate. Gelatin, esculin, hippurate, casein, and starch are not attacked, and indole is not formed. NaCl and glycine (1%) are not tolerated. The enzymes catalase, oxidase, and phosphatase are present. When cells are cultured in semisolid test medium lacking $(\text{NH}_4)_2\text{SO}_4$, nitrogenase activity occurs. The sole nitrogen sources utilized include nitrate, ammonium salts, and 10 amino acids, with L-proline and L-alanine being the most effective. Nitrate is reduced to nitrite. Carbohydrates are not catabolized, and tricarboxylic acid cycle intermediates can be used as sole carbon sources with $(\text{NH}_4)_2\text{SO}_4$ as the sole nitrogen source. Pyruvate and proline are the most effective sole carbon sources. Isolated from pond water. The DNA base content ranges from 62 to 65 mol% G+C. As determined by DNA-rRNA hybridization experiments, the genus *Prolinoborus* belongs to rRNA superfamily III, with a difference in $T_{m(e)}$ value of more than 6°C versus rRNA from *A. serpens* biovar bengal ATCC 27641^T. The type species is *Prolinoborus fasciculus*, the only species at present. The type strain is strain ATCC 27740 (= LMG 6233).

Strain investigated	Strain number	%Binding			
<i>Aquaspirillum serpens</i>	ATCC 12638 T	100			
<i>A. serpens</i>	ATCC 27050	72	100		
<i>A. serpens</i>	ATCC 15278	72		100	
<i>A. serpens</i> biovar bengal	ATCC 27641	59	55	60	100
<i>A. serpens</i> var. azotum	NCIB 9011	20	23		100

FIG. 3. Results of DNA-DNA hybridizations performed with five strains of *A. serpens*, including the type strain. The degrees of binding (expressed as percentages) are the means of at least two experiments. The separate position of *A. serpens* biovar bengal and the aberrant position of *A. serpens* var. azotum are obvious.

The description of the species *P. fasciculus* is as given by Strength et al. (53).

Other [Aquaspirillum] species belonging to rRNA superfamily III. (i) *[A.] putridiconchylum*, *[A.] dispar*, and *[A.] autotrophicum*. *[A.] putridiconchylum* is equidistant from the *A. serpens* rRNA branch, the *Chromobacterium* rRNA branch (12) (Fig. 1), and the *Neisseriaceae* rRNA cluster (51) and should be removed from the taxon containing the authentic aquaspirilla. As explained above, the serological, phenotypic, and genotypic similarities observed (26, 32) with strain *A. serpens* var. *azotum* ATCC 11335 (=NCIB 9011 in Table 2) warrant further taxonomic research. The possibility of a close relationship to *Iodobacter fluvialis* (37) can be eliminated on the basis of $T_{m(e)}$ values [mean $T_{m(e)}$ value of 68.7°C versus rRNAs from members of the *Aquaspirillum-Chromobacterium-Neisseriaceae* group in rRNA superfamily III (Table 2)] and on the basis of phenotypic characteristics.

[A.] dispar ATCC 27510^T and ATCC 27650 (which have a degree of binding value of 100% [1b]) exhibit the highest levels of rRNA cistron similarity with *Chromobacterium violaceum* NCTC 9757^T rRNA [$T_{m(e)}$ values of 74.2 and 73.2°C, respectively (Table 2)]. These $T_{m(e)}$ values are distinctly higher than those obtained with rRNAs from *A. serpens* and *N. flavescens*. Woese et al. (74) found that *[A.] dispar* is more closely related to *A. serpens* (S_{AB} , 0.71) than to *Chromobacterium violaceum* (S_{AB} , 0.59). *A. serpens* and *Chromobacterium violaceum* were found to have an S_{AB} value of 0.62. Because of a $T_{m(e)}$ difference of 6°C or more and the many phenotypic differences, *[A.] dispar* cannot be included in the genus *Chromobacterium*; a separate genus should be created for organisms belonging to this species.

[A.] dispar and *[A.] putridiconchylum* cannot be differentiated unambiguously from the other aquaspirilla by using the available phenotypic data; in agreement with the report of the Ad Hoc Committee on Approaches to Taxonomy within the Proteobacteria (42), we prefer not to propose nomenclatural changes until more differentiating phenotypic features have been found.

With a $T_{m(e)}$ of 75.6°C versus rRNA from *J. lividum* Sneath HB^T [$T_{m(e)}$ difference, 3.2°C], *[Aquaspirillum] autotrophicum* ATCC 29984^T is closely related to the genus *Janthinobacterium*. According to Gillis et al. (25), the following other taxa exhibit similar $T_{m(e)}$ values versus this rRNA: (i) an unnamed group containing various clinical isolates that have a characteristic corkscrewlike type of motility (EF group 1 [22]); (ii) *Pseudomonas rubrisubalbicans*, a generically misnamed plant-pathogenic *Pseudomonas* species; and (iii) *Herbaspirillum seropedicae*, a root-associated nitrogen-fixing bacterium (2). Detailed results, including the exact taxonomic position of *[A.] autotrophicum*, will be presented elsewhere.

(ii) **[Aquaspirillum] species belonging to the acidovorans rRNA complex.** The [*Pseudomonas*] *acidovorans* rRNA group (45) is one of at least five groups that were delineated within the phenotypically defined genus *Pseudomonas* (44) by using DNA-rRNA hybridization data (45). The separate nature of this group was confirmed by using several other techniques (6, 70). The five groups are only very remotely related (17, 70) and deserve at least separate genera (17). By using DNA-rRNA hybridization (17) it was shown that the [*Pseudomonas*] *acidovorans* rRNA group is part of a much larger acidovorans rRNA complex, which comprises a wide variety of bacterial species belonging to several genera ([*Alcaligenes*], *Comamonas*, [*Pseudomonas*], and many others) (17–19). Most of these taxa should be generically re-

named according to phylogenetic data (61, 63). For many of them an improved classification has already been proposed (19, 55, 61, 63–66), including the description of the family *Comamonadaceae* for this rRNA complex (62). From Table 3 and Figure 2 it is evident that the following species of the genus [*Aquaspirillum*] belong to the *Comamonadaceae* in rRNA superfamily III: *[A.] anulus*, *[A.] aquaticum*, *[A.] delicatum*, *[A.] giesbergeri*, *[A.] gracile*, *[A.] metamorphum*, *[A.] sinuosum*, and *[A.] psychrophilum*. These organisms are only distantly related to the type species of the genus *Aquaspirillum*, *A. serpens*, and therefore phylogenetically are not authentic aquaspirilla. This finding was not completely unexpected, as Krieg and Hylemon (33) previously noticed certain similarities between species of the genus *Aquaspirillum* (in casu *[A.] delicatum*) and species of the genera *Comamonas* and *Pseudomonas*.

The relationships of these eight species were investigated by using DNA-rRNA hybridization and the available rRNAs from members of the *Comamonadaceae* (*Acidovorax facilis*, *Variovorax paradoxus*, *Comamonas acidovorans*, *Comamonas terrigena*, *Hydrogenophaga palleronii*, *Hydrogenophaga pseudoflava*, and *Xylophilus ampelinus*) and by using hybridization with newly prepared labeled rRNAs from the type strains of several of the [*Aquaspirillum*] species (*[A.] anulus*, *[A.] aquaticum*, *[A.] delicatum*, *[A.] gracile*, *[A.] metamorphum*, and *[A.] sinuosum*).

[A.] aquaticum belongs in the genus *Comamonas* (19); it is closely related to *Comamonas terrigena* and to another group of unnamed clinical isolates (EF group 10 [22]) which exhibit a characteristic corkscrewlike type of motility. According to 16S rRNA cataloging data (74), *[A.] aquaticum* and *Comamonas terrigena* are very closely related. Other [*Aquaspirillum*] species differ from members of the genus *Comamonas* in their helical shape, their inability to grow on nutrient agar, and their relatively poor and slow growth. Additional genotypic and phenotypic data support these hybridization data, as discussed in detail by Willems et al. (66).

Reciprocal hybridizations with all of the rRNAs from members of the *Comamonadaceae* (Tables 3 and 4) revealed that at least the following five [*Aquaspirillum*] species are members of separate rRNA branches in the *Comamonadaceae*: *[A.] anulus*, *[A.] delicatum*, *[A.] gracile*, *[A.] metamorphum*, and *[A.] sinuosum*. These organisms are related to each other at $T_{m(e)}$ values of $76.0 \pm 1.1^\circ\text{C}$ (mean of 370 reciprocal hybridizations between members of the different branches of the *Comamonadaceae*; data from Tables 3 and 4 and references 61, 63, 65, 66 and unpublished data from our laboratory). The results for the few strains of the *Comamonadaceae* that have been included in 16S rRNA cataloging experiments (71, 74) are in very good agreement with our results; according to the cataloging data, *[A.] gracile* can be placed on a single branch with *Comamonas terrigena*, *Comamonas acidovorans*, and *Comamonas testosteroni*. DNA-DNA hybridizations between the type strains of *[A.] delicatum*, *[A.] gracile*, and *[A.] sinuosum*, which represent three separate rRNA branches within the *Comamonadaceae*, revealed no significant levels of DNA binding (4 to 15%), confirming that each of the separate [*Aquaspirillum*] rRNA branches in the *Comamonadaceae* definitely constitutes a separate taxon with the rank of species or higher (Fig. 2).

[A.] psychrophilum (Tables 3 and 4) has mean $T_{m(e)}$ values of $75.4 \pm 1.6^\circ\text{C}$ versus all members of the branches belonging to the *Comamonadaceae* and therefore probably represents a sixth rRNA branch that is equidistantly related to the

FIG. 4. Normalized protein electropherograms of *[A.] sinuosum* ATCC 9786^T and *[A.] giesbergeri* ATCC 11334^T. The positions of the molecular weight markers are indicated in lane MWM; the molecular weight markers were (from left to right) lysozyme (molecular weight, 14,200), trypsin inhibitor (20,100), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), egg albumin (45,000), bovine albumin (66,000), and β -galactosidase (116,000).

other five *[Aquaspirillum]* rRNA branches of the *Comamonadaceae* (Fig. 2). The separate position of this species is not unexpected, since it has several phenotypic features (e.g., psychrophilic growth, denitification) that differentiate it from the other *[Aquaspirillum]* species in the *Comamonadaceae*.

On the basis of G+C values (57 to 58 mol%) (Tables 3 and 4), a possible relationship could be expected among *[A.] anulus*, *[A.] giesbergeri*, and *[A.] sinuosum*. Our results clearly show that *[A.] giesbergeri* belongs to the *[A.] sinuosum* rRNA branch, with a $T_{m(e)}$ value of 78.7°C (Table 3 and Fig. 2). These two species have several phenotypic features in common (32). In a serological study, McElroy and Krieg (40) placed *Spirillum giesbergeri* and *Spirillum sinuosum* in a separate group together with *Spirillum graniferum*. The latter species was later incorporated into *[A.] giesbergeri* (26). The electrophoretic protein profiles of the type strains of *[A.] giesbergeri* and *[A.] sinuosum* are shown in Fig. 4. These two species have very similar protein patterns, which exhibit some small differences at molecular weights of approximately 40,000 to 70,000 (Fig. 4). They definitely belong in one genus and may be related at the species level, but this should be confirmed by DNA-DNA hybridization. In the phenotypic study of Carney et al. (7), *[A.] sinuosum* was found to be slightly more closely related to *[A.] anulus* than to *[A.] giesbergeri*.

Despite their separate genotypic positions we prefer to not rename the *[Aquaspirillum]* species discussed above generically because (i) each branch is composed of very few strains and (ii) more phenotypic data seem to be needed to clearly differentiate these branches from all of the other aquaspirilla.

***[Aquaspirillum]* species that do not belong to rRNA superfamily III.** *[Aquaspirillum] polymorphum*, *[A.] peregrinum*, and *[A.] itersonii* have $T_{m(e)}$ values of 57.5 to 60.6°C versus rRNAs from members of rRNA superfamily III (Table 3) (47). These organisms are in fact members of rRNA superfamily IV (15, 47). As the type species, *A. serpens*, belongs to rRNA superfamily III, we concluded that *[A.] polymorphum*, *[A.] peregrinum*, and *[A.] itersonii* are not related phylogenetically to the authentic aquaspirilla. Moreover, these bacteria can be differentiated phenotypically from the authentic aquaspirilla and from the other *[Aquaspirillum]* species. Their transfer and proper nomenclatural proposals will be discussed in a forthcoming paper on the taxonomy of the spirilla belonging to rRNA superfamily IV (47).

We concluded that the taxonomy of *Aquaspirillum* sensu lato needed a more genotypically oriented approach to reveal the true phylogenetic relationships with and within this heterogeneous group of organisms. Although we agree with Murray et al. (42) that for practical purposes a proper

differentiating phenotypic description is required, we cannot neglect the importance of genotypic parameters (DNA base composition [26], 16S rRNA catalog and sequence [71–74], stability of DNA-rRNA hybrids and DNA-DNA hybridization [48; this paper]) as essential and useful criteria to use with the sometimes limited number of differentiating phenotypic features for unambiguous description of new taxonomic units.

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