

A TAXONOMIC STUDY OF THE GENUS SPIRILLUM EHRENBERG,
WITH SPECIAL REFERENCE TO NUTRITION AND CARBOHYDRATE CATABOLISM

by

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INTRODUCTION

Members of the genus Spirillum consists of helical, polarly flagellated (Canale-Parola, Rosenthal, and Kupfer; 1966, Terasaki; 1961, Watanabe; 1959, Pretorius; 1963, Williams; 1960, Williams and Rittenberg; 1957), gram negative rods which are found in both fresh and marine waters. In hay-infusion and pond-water, most members of this genus are very actively motile, and one can easily become fascinated by their characteristic rotating helices during motion.

Although the genus is one of the oldest in bacteriology (Ehrenberg, 1832), study of the spirilla has not been extensive because of three factors. (1) Lack of medical, industrial, or agricultural significance. Only one species, Spirillum minor is known to be pathogenic (Carter, 1888; Robertson, 1924; Beeson, 1943) and this has never been isolated. Other spirilla may possibly have more medical importance than is presently suspected; for example, the only bacterial species present in a recently studied aborted bovine fetus was found to be a Spirillum (Smibert, 1970). This isolate was initially confused with Campylobacter fetus because of a similar morphology and a failure to employ flagellar staining. (2) Difficulty of isolation. The difficulty of isolating spirilla from enrichment cultures has been commented on by nearly everyone who has attempted to isolate these organisms from natural sources (Williams and Rittenberg, 1957). For example, a laborious capillary tube method must be used even today for isolation of Spirillum volutans (Rittenberg and Rittenberg, 1962). Moreover, this species was not isolated and grown in pure culture until 1965 (Krieg and Wells, 1965), even though it was first described in

1832 by Ehrenberg. Spirillum gracile can be isolated only by passage through a membrane filter into agar (Canale-Parola, Rosenthal, and Kupfer, 1966). A selective procedure recently reported by Cody (1968) involving 5-fluorouracil may prove to be useful in this regard. (3)

Difficulty of preservation. Many species of spirilla could not be preserved in a dormant state prior to development of liquid nitrogen techniques (Williams, 1959).

Most investigators agree the accumulation of large quantities of information concerning the nutritional, physiological, biochemical, and serological traits of a group of bacteria has made it possible to establish classification schemes for these bacteria which probably indicate their relationships with considerable validity. In contrast, the paucity of information concerning genus Spirillum has made it impossible to develop such a satisfactory classification scheme.

Several morphological taxonomic schemes for spirilla were devised before 1936 (reviewed by Williams, 1959 a); but Giesberger (1936) in a classic work was the first investigator to use certain physiological characteristics of spirilla. In 1957, Williams and Rittenberg attempted a more up-to-date systematization of the genus.

- In order to develop further a comprehensive characterization and classification of spirilla as begun by Giesberger and by Williams and Rittenberg, and in view of several reports of isolation of new species since 1957, I carried out the taxonomic study described in this report. Moreover, the characterization of genus Spirillum was recently approached by two other investigators, whose work complements my study. The treatment by Wells (1970) was concerned primarily with the

physiological and biochemical characterization of the members of genus Spirillum, while the investigation by McElroy (1970) was concerned with the serological relationships of spirilla. In contrast, the present investigation deals with the nutrition, certain biochemical traits, and the carbohydrate catabolism of members of this genus. The type strains of 20 species of the genus Spirillum plus additional strains of S. serpens, S. itersonii, S. volutans, and S. gracile have been used for nutritional and physiological studies. In addition, three new isolates have been obtained from a near-by pond and these have been included in this investigation. Since all characteristics of a bacterium are important in its taxonomy, the combined results of the three independent investigations should make it possible to provide the most complete "picture" of the genus Spirillum to date. Furthermore, I have defined standardized procedures for the characterization of strains to provide a more satisfactory basis of comparison for spirilla than has been previously available.

REVIEW OF LITERATURE

The early taxonomic investigations of genus Spirillum were entirely morphological and the early history of the genus has been discussed by Williams and Rittenberg (1957) and Williams (1959 a). Although mention will be made of earlier work, the present review will be primarily concerned with the investigations of Giesberger (1936), Williams and Rittenberg (1957), and some of the more recent investigations.

Giesberger (1936) was the first investigator to use certain physiological characteristics of spirilla. He indicated that by either nutritional or morphological criteria the same four distinct groups were found with the available isolates. The four groups described by Giesberger (1936) included Spirillum itersonii, Spirillum serpens, Spirillum tenue, and Spirillum undula. Furthermore, he recognized nine species based on his new isolates as well as those which could be identified by the literature descriptions. These species included S. undula, S. serpens, S. volutans, S. tenue, S. minus, S. kutscheri, S. itersonii, S. virginianum, and S. cardiopyrogenes.

Williams and Rittenberg (1957), in a more recent attempt to systematize the genus, similarly found that all the cultures of a single morphological group had very similar nutritional characteristics; however, they also reported that isolates belonging to distinctly different morphological groups did, in certain cases, demonstrate the same nutritional pattern. Moreover, they suggested that if a large variety of nutritives were to be employed, differences in the nutritional patterns among strains within the same morphological groups would probably occur. Williams and Rittenberg used morphology primarily and

nutrition secondarily to divide genus Spirillum into 19 species. These species were based on 1) their new isolates, 2) the species included by Giesberger, 3) those species found in Bergey's Manual (6th ed.), and 4) S. manucuniense, described by Cayton and Preston (1955) as being catalase negative.

In 1959, Watanabe isolated and described four new species of spirilla from marine shellfish. These new species included S. japonicum, S. halophilum, S. minutulum, and S. maritimum. The descriptions of these four species were based on morphological, cultural, and physiological characteristics. A nutritional description was not included in this report.

Terasaki (1961) isolated and described three new species of spirilla: S. putridiconchylum, S. metamorphum, and S. crassum from a putrid infusion of fresh water snails. Morphological, physiological, nutritional, and cultural characteristics were presented; these indicated differences from known species.

In 1962, Leifson isolated and described a new species, S. delicatum, from distilled water. However, his descriptions were based primarily on morphological, cultural, and physiological characteristics. He indicated this species may be more properly placed in genus Vibrio because of a similar morphology and a single polar flagellum. Furthermore, since isolation, electron micrographs have shown this species to possess a tuft of flagella at each pole (Wells, personal communication).

While doing a systematic study of spirilla in oxidation ponds, Pretorius (1963) isolated and described a new species of spirilla,

S. peregrinum. He included morphological, physiological, nutritional, and cultural descriptions of this new species.

Canale-Parola, Rosenthal, and Kupfer (1966) isolated spirilla measuring 0.25 to 0.3 microns in diameter, by passing them through a cellulose ester filter into agar. A complete description of this species was included in their report, including morphological, physiological, nutritional, and cultural descriptions.

In 1962, Rittenberg and Rittenberg obtained for the first time Spirillum volutans in pure culture. They employed the capillary tube technique, and exploited the rapid motility of this organism in order to separate it from contaminating bacteria. Pure cultures were achieved by growing the isolated spirilla inside of dialysis bags which were suspended in mixed cultures.

Wells and Krieg (1965), using the capillary tube technique of Rittenberg and Rittenberg, isolated S. volutans and cultured this organism in dialysis bags. Wet mounts indicated a narrow band of spirilla formed near the edge of the cover-slip, indicating a micro-aerophilic response. They found S. volutans could be cultured indefinitely in pure culture provided that the atmospheric oxygen concentration was reduced to 3 per cent.

Media Employed in Nutritional Characterization

In regard to the history of the nutrition of the genus Spirillum, Giesberger (1936) used a basal salts medium of the following composition: MgSO_4 , 0.05%; K_2HPO_4 , 0.05%; CaCl_2 , 0.05%; NH_4Cl , 0.1%; carbon source to be tested 0.2-1.0%; and distilled water.

Williams and Rittenberg (1957) used Giesberger's basal salts medium and a carbon source concentration of 0.05% (except for fatty acids

where 0.02% was employed). Moreover, only the supernatant was used as the "defined medium" and the voluminous precipitate that formed upon autoclaving was discarded.

Terasaki (1961) isolated and described three new species of spirilla; however, the methods for the nutritional characterization were not specified.

Watanabe (1959) isolated and described four new halophilic species of spirilla, but a nutritional characterization was not performed.

Canale-Parola, Rosenthal, and Kupfer (1966) isolated and characterized a new species, S. gracile. The nutritional characterization of this species was investigated by using a basal medium which contained the following components (g/100 ml of distilled water): K_2HPO_4 , $MgCl_2 \cdot 6 H_2O$, 10^{-2} each; $MnSO_4 \cdot H_2O$, $5 \cdot 10^{-3}$; $FeSO_4 \cdot H_2O$, $2 \cdot 10^{-4}$; thiamine HCl, nicotinic acid, calcium pantothenate, 10^{-5} each; pyridoxine HCl, riboflavin, vitamin B_{12} , 10^{-6} each; biotin, folic acid, $5 \cdot 10^{-7}$ each. Various carbon sources (0.1% w/v) were added to this basal medium.

Pretorius (1963) used the methods of Williams and Rittenberg and Giesberger's basal salts medium. Carbon sources were added at a concentration of 0.2% (w/v).

Inoculum Used in Nutritional Investigation

Giesberger (1936) did not specify the type of inoculum employed for his nutritional characterization of spirilla. Williams and Rittenberg (1957) employed a standard inoculum of 0.1 ml of a 48 hr. broth culture which was washed twice in basal salts solution and resuspended

in the original volume. Pretorius (1936) apparently used the same inoculum as Williams and Rittenberg, since he reported using their methods. Canale-Parola, Rosenthal, and Kupfer (1966) employed an inoculum of 10^6 cells. Other recent investigators of spirilla, e.g. Terasaki (1961) and Watanabe (1959), did not specify the type of inoculum employed in their nutritional investigations.

Methods of Sterilizing Media

Giesberger (1936) did not specify his method of sterilization, however, it was presumably by autoclaving. Williams and Rittenberg (1957) sterilized all media by autoclaving at 15 lbs/in² for 15 minutes. Pretorius (1963) sterilized the salts of organic acids separately by autoclaving momentarily at 111 C before adding to basal medium. Other recent investigators did not specify the method(s) used for sterilizing media.

Definition of Growth

In Giesberger's nutritional investigation of spirilla, a definition of growth was not employed. Moreover, Williams and Rittenberg (1957) apparently did not employ a definition of growth in their nutritional investigation, although growth was measured turbidimetrically. Canale-Parola, Rosenthal, and Kupfer (1966) recorded the number of cells per ml; however, a definition of growth was not specified. Other recent investigators also did not specify a definition of growth.

Composition of Artificial Sea Water

Williams and Rittenberg (1957) did not indicate the ingredients

of their artificial sea water.

Watanabe (1959) used a sea water medium of the following composition for isolation and cultivation of spirilla: (g/L) NaCl, 25; MgCl₂, 3; MgSO₄, 1.0; CaSO₄, 1.0; K₂SO₄, 1.0; and CaCO₃, 0.5.

MATERIALS AND METHODS

Origin of the strains. The strains of spirilla used in this investigation were obtained from the American Type Culture Collection (ATCC), the National Collection of Industrial Bacteria (NCIB), and from private collections. A listing of these strains follows:

1. Strain 104. Dr. H. W. Jannasch, Woods Hole Oceanographic Institution, 1967. Isolated from fresh-water sources.
2. S. gracile. Dr. E. Canale-Parola et. al. (1966). Isolated from pond water. ATCC 19625.
3. S. gracile type strain. Same as source 2. ATCC 19624.
4. S. gracile. Same as source 2. ATCC 19626.
5. S. delicatum type strain. Dr. E. Leifson (1962). Isolated from a distilled water specimen. ATCC 14667.
6. Strain 103. Same as source 1. Isolated from fresh-water sources.
7. S. metamorphum type strain. Dr. Y. Terasaki (1961). Isolated from a putrid infusion of a fresh-water shellfish. ATCC 15280.
8. S. lunatum type strain. Williams and Rittenberg (1957). Isolated from coastal sea water. ATCC 11337.
9. S. polymorphum type strain. Same as source 8. Isolated from fresh-water sources. ATCC 11332.
10. S. peregrinum type strain. Dr. W. A. Pretorius (1963). Isolated from water of a primary oxidation pond. ATCC 15387.
11. S. itersonii. Same as source 8. Isolated from fresh-water sources. ATCC 11331.

12. S. itersonii (unnumbered strain). Dr. S. C. Rittenberg, Department of Bacteriology, University of California, Los Angeles, 1967. Isolated from pond water.
13. S. itersonii type strain. Same as source 8. ATCC 12639.
14. Strain 204. Same as source 1. Isolated from sea water.
15. S. giesbergeri type strain. Same as source 8. Isolated from fresh-water sources. ATCC 11334.
16. S. anulus type strain. Same as source 8. Isolated from fresh-water sources. ATCC 19259.
17. S. graniferum type strain. Same as source 8. Isolated from fresh-water sources. NCIB 8230.
18. S. sinuosum type strain. Same as source 8. Isolated from fresh-water sources. ATCC 9786.
19. S. putridiconchylum type strain. Same as source 7. Isolated from a putrid infusion of a fresh-water shellfish. ATCC 15279.
20. S. serpens. Same as source 8. Isolated from fresh-water sources. ATCC 11330.
21. S. serpens. Same as source 8. Isolated from fresh-water sources. ATCC 12289.
22. S. serpens. Same as source 8. Isolated from fresh-water sources. ATCC 11335.
23. S. serpens St. Rhodes strain. Dr. R. G. E. Murray, Department of Bacteriology and Immunology, the University of Western Ontario.
24. S. serpens strain VHL. Same as source 23.

25. S. serpens strain VH. Same as source 23.
26. S. serpens. Same as source 8. ATCC 15278.
27. S. serpens strain VHA. Same as source 23.
28. S. serpens Victoria strain. Same as source 12.
29. S. serpens type strain. Same as source 8. ATCC 12638.
30. S. serpens strain VHS. Same as source 23.
31. S. serpens (unnumbered strain). Same as source 12.
32. S. linum type strain. Same as source 8. Isolated from coastal sea water. ATCC 11336.
33. S. atlanticum type strain. Same as source 8. Isolated from coastal sea water. ATCC 12753.
34. S. beijerinckii type strain. Same as source 8. Isolated from coastal sea water. ATCC 12754.
35. Strain 101. Same as source 1. Isolated from sea water.
36. Strain 102. Same as source 1. Isolated from sea water.
37. Strain 206. Same as source 1. Isolated from sea water.
38. S. japonicum type strain. Dr. N. Watanabe (1959). Isolated from marine shellfish at Samugawa Beach. ATCC 19191.
39. S. halophilum type strain. Same as source 38. Isolated from marine shellfish at Samugawa Beach. ATCC 19192.
40. S. minutulum type strain. Same as source 38. Isolated from marine shellfish at Samugawa Beach. ATCC 19193.
41. S. volutans Wells strain. (1965). Isolated from pond water hay-infusion. ATCC 19554.
42. S. volutans type strain. Obtained in mixed culture by Dr. E. G. Pringsheim from a cooling tower vat of a sugar beet

refinery in England in 1951. Isolated by Rittenberg and Rittenberg (1962). ATCC 19553.

43. S. itersonii strain 1. Isolated from pond water by Mr. B. H. Caraway in 1970. Biology Department, Virginia Polytechnic Institute and State University, Blacksburg, Va.
44. S. itersonii strain 6. Same as source 43. Isolated from pond water.

Cleaning of Glassware. All glassware was washed in 7X detergent and distilled water rinsed for one minute by means of a Heinicke glassware-washer. In addition, glassware used in nutritional work was rinsed 5 times in distilled water and 3 times in ion-free water. The glassware was then inverted and dried at 125° and capped with aluminum foil to prevent dust contamination. Screw caps were cleaned by boiling three times in ion-free water and dried at 125° in paper towel lined baskets.

Maintenance of Strains. All cultures were maintained in semi-solid (0.15% agar) peptone-succinate-salts (PPS) medium, with serial transfers being made weekly. All stock cultures were stored in liquid nitrogen in PSS broth medium containing 15% glycerol.

Media and Chemicals. Adenine, cis-Aconitic acid, D-arabinose, D-aspartic acid, L-asparagine, BES(N₁N BIS)(2 hydroxyethyl) 2 Amino-ethane sulfonic acid, capric acid, caprylic acid, casein hydrolysate, citric acid, L-cysteine, D-erythrose, D-fructose, galactose-6-phosphate, D-galactose, glucose-6-phosphate dehydrogenase (yeast), glutathione, D-glutamic acid, glycerol, guanosine-5-triphosphate

(GTP), L-histidine, inosine-5'-triphosphate (ITP), DL-isocitrate (trisodium), 2-ketogluconic acid (Ca), lactose, L-malic acid, malonic acid, maltose, D-mannitol, D-mannose, L-rhamnose, D-ribose, sedoheptulose, L-serine, sucrose, spermine, spermidine, sodium pyruvate, D-tartaric acid, L-tartaric acid, L-threonine, triphosphopyridine nucleotide (TPN), uridine-5'-triphosphate (UTP), and L-valine were all obtained in the highest commercially available purity from Nutritional Biochemical Corporation, Cleveland, Ohio.

Trans-aconitic acid, L-alanine, L-arginine, L-aspartic acid, para-aminobenzoic acid, fumaric acid, glycine, L-hydroxy-proline, L-histidine, L-lactic acid, L-leucine, L-lysine, alpha-ketoglutaric acid, L-methionine, 2-mecaptoethanol, hemin, L-phenylalanine, oxaloacetic acid (cis-enol), L-serine, tri(hydroxy methyl) amino methane (TRIS), L-tryptophan, and L-tyrosine were obtained in the highest commercially available purity from Mann Research Lab., New York, N.Y.

Alpha-glycerophosphate dehydrogenase/triose phosphate isomerase (alpha-GDH-TPI), adenosine 3'-5'-cyclic phosphate, adenosine-5'-monophosphate (AMP), cis-aconitic acid, catalase (bovine liver), L-citrulline, diphosphopyridine nucleotide (NAD), diphosphopyridine nucleotide (reduced NADH), fructose 1, 6-diphosphate (tetracyclohexylammonium salt), D-fructose-6-phosphate, glycylglycine hydrochloride, lactic acid dehydrogenase (yeast), L-ornithine, triphosphopyridine nucleotide (TPN), and urea were obtained in the highest commercially available purity from Calbiochem, Los Angeles, California.

D-gluconic acid (Na salt, practical grade), L-glutamine, L-histidine, and D-raffinose, were obtained in the highest commercially

available purity from Eastman Organic Chemicals, Rochester, New York.

Acetic acid, L-ascorbic acid, butanol, catechol, ethylene-diaminetetraacetic acid (EDTA), formic acid, ferric chloride, magnesium sulfate, maltose, potassium chloride, potassium nitrate, propanol, propionic acid, sodium carbonate, sodium chloride, sodium bicarbonate, sodium sulfite, succinic acid, and sucrose were obtained in reagent grade from Fisher Scientific Co., Fair Lawn, New Jersey.

D-glucose, lactose, D-mannitol, and purified agar were obtained from Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Md.

Ammonium chloride, ammonium sulfate, boric acid, calcium chloride, cobalt chloride, copper sulfate, ferrous sulfate, manganous sulfate, magnesium chloride, potassium hydroxide, potassium phosphate (K_2HPO_4), selenium chloride, sodium fluoride, sodium metasilicate, and zinc sulfate were obtained in reagent grade from the J. T. Baker Chemical Co., Phillipsburg, N.J.

Peptone (Bacto) and casamino acids (vitamin-free, Bacto) were obtained from Difco Laboratories, Detroit, Mich.

Para-hydroxybenzoic acid was obtained from the Chemistry Department, at Virginia Polytechnic Institute and State University, Blacksburg, Virginia.

2-keto-3-deoxy-6-phosphogluconate (KDPG) was a gift of Dr. Mark Roseman, Department of Biochemistry, Michigan State University, East Lansing, Michigan.

✓ Composition of Peptone-Succinate-Salts Medium. Peptone-succinate-

salts medium (PSS) had the following composition (g/l): peptone, 5.0; succinic acid, 1.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.002; and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.002. The pH was adjusted to 7.0 with 2 N KOH. Semi-solid media were prepared by adding 0.15% agar to PSS broth. PSS agar media were prepared by adding 1.5% agar to PSS broth. For the cultivation and characterization of marine spirilla, artificial sea water (Zobell, 1946) was employed in preparation of media. S. lunatum grew in the presence or absence of sea water; therefore, this organism was tested under both conditions.

Composition of Nutritional Media (carbon sources). The standard basal medium (SBM) to which carbon sources were added (0.1%) had the following composition (g/l): $(\text{NH}_4)_2\text{SO}_4$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; K_2HPO_4 , 0.5; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.0047; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.0025; CaCO_3 , 0.001; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00072; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.000125; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00014; H_3BO_3 , 0.000031; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.000245; and distilled water passed through a Bantam standard ion-exchange resin cartridge. The pH was adjusted to 7.0 with 2 N KOH. Media were sterilized by autoclaving at 112° (7.5 lb/in^2) for 10 minutes. Sugars and thermolabile compounds were sterilized by filtration and added aseptically. For the cultivation of marine spirilla, sea water (Zobell, 1946) was substituted for ion-free water and the media were sterilized by filtration.

Composition of Nutritional Media (nitrogen sources). For determination of sole nitrogen sources, a combination of succinate plus malate (0.1% each) was added and $(\text{NH}_4)_2\text{SO}_4$ omitted.

Composition of Media Used for Determination of NaCl Tolerance.

The medium used for determining NaCl tolerance contained SBM plus (0.1% each) succinic acid, pyruvic acid, and L-glutamic acid. In addition, a second medium was employed for strains which failed to grow on the defined medium. This medium contained SBM plus (0.1% each) succinic acid and casamino acids (Difco). The pH was adjusted to 7.0 with 2 N KOH. Sodium chloride concentrations were tested at 0%, 0.1%, 0.3%, 0.5%, 0.7%, 1.0%, 1.5%, and 2.0%. The media were autoclaved at 112° (7.5 lb/in²) for 10 minutes.

Medium Used for Determination of Calcium Ion Effects on Growth.

The medium used for determining the effects of calcium ions on growth contained SBM plus 0.05% calcium chloride. The media were sterilized by filtration.

Preparation of Inoculum and Definition of Growth. The standard inoculum for 10 ml of defined medium was 0.1 ml of a 100-klett unit saline (for fresh-water spirilla) or sea water (for marine spirilla) suspension of cells previously grown 24-36 hours in PSS broth and washed once in saline or sea water. All nutritional experiments were incubated at 30°. Inoculum for the second transfer was 0.1 ml of the 72 hour growth from the initial culture. A positive growth response was defined as the production of at least 10 klett units of turbidity in the second serial transfer after 72 hours, using a blue (420 nm) filter and 16 mm cuvettes. All nutritional experiments were run in duplicate and the turbidities averaged.

Detection of Fluorescent Pigment Production. Fluorescent pigment production on PSS agar plates was tested at 48-72 hours with a 253.7 nm ultra-violet light source.

Urease Activity as Measured by Synthetic Medium. Urease activity was assayed by using a synthetic medium with the following composition (g/l): BES (N_1N -BIS)(2 hydroxyethyl) 2 Aminoethane sulfonic acid, 1.065; Urea, 20.0; and Bacto phenol red, 0.01. The pH was adjusted to 7.0 with 2 N KOH. Cells were cultured in PSS broth medium, centrifuged and resuspended in distilled water; 0.5 ml of this suspension was then added to 2.0 ml of urease medium and incubated at 30° for 24 hours before readings were taken. A positive test was indicated by a distinct purple color.

Preparation of Specimens for Electron Microscopy of Flagella. The growth from 24 hour PSS broth cultures was suspended in distilled water (0.3% formalin and 0.01% bovine serum albumin were incorporated). Suspensions were mixed with potassium phosphotungstate (pH 7.0) at a final concentration of 0.7% for negative staining and placed on parlodion coated 200 mesh copper grids for examination with a Jeolco 100 B electron microscope.

Enzyme Assays. Cells were cultured on defined basal medium containing 0.05% casamino acids (Difco) and also containing the desired sugar (1.0%) or succinic acid (0.2%). Log phase cells were harvested by centrifugation, washed once in 0.1 M-tris-HCl buffer (pH 7.8) and broken by sonic oscillation in a Raytheon sonic oscillator for 2 minutes at 10 kc. at 0-4°. The crude extract was then centrifuged at 12,000g for 30 minutes at 0-4°. The clear supernate was used in all enzyme assays.

Enzyme assays were carried out spectrophotometrically by using standard techniques (Colowick and Kaplan, 1966). Specific activities

were determined for the following: hexokinase (EC 2.7.11); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); phosphogluconate dehydrogenase (EC 1.1.1.44); phosphogluconate dehydrase (EC 4.2.1.12); 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (EC 4.1.2.14); fructose 1,6-diphosphate aldolase (EC 4.1.2.13); and mannitol dehydrogenase (EC 1.1.1.67). The reaction mixture for hexokinase included excess exogenous glucose-6-phosphate dehydrogenase. KDPG aldolase was assayed by using KDPG as the substrate. Phosphogluconate dehydrase was measured with 6-phosphogluconate as substrate by coupling the reaction with endogenous KDPG aldolase. Fructose 1,6-diphosphate aldolase was assayed by coupling its activity with that of excess added alpha-glycerophosphate dehydrogenase/triose phosphate isomerase. Mannitol dehydrogenase was assayed as described by Phibbs and Eagon (1970).

The rates of NADH disappearance were corrected for nonspecific oxidation by subtracting the rate in assay mixtures containing no substrate. Enzyme activities were expressed as enzyme units (EU)/mg of protein in the centrifuged extract. One EU was defined as that quantity of the enzyme which converted 1 μ mole of substrate/minute/mg protein at 25° under the specified conditions. The method of Lowry (1951) was used for protein estimation. For more detailed information regarding enzyme assay procedures see Appendix.

RESULTS

Compounds Utilized as Sole Carbon and Energy Sources

Tables I and II indicate various compounds which can serve as sole carbon and energy sources for spirilla. The strains are arranged in descending order of the guanosine plus cytosine content of their DNA (65 to 38), except strains 43 and 44 which are new isolates of Spirillum itersonii. It is apparent from Tables I and II that a variety of compounds can serve as sole carbon and energy sources; e.g. most strains can utilize tricarboxylic acid cycle intermediates and amino acids, with fatty acids, alcohols, and amines being utilized to a lesser degree. Moreover, sugars are utilized by only a few strains, and where this occurs the kinds of sugars utilized are rather restricted, e.g. strains 8, 10, 12, 13, 43, and 44. Strain 14, which can utilize a variety of sugars, is probably not a Spirillum species for reasons I will discuss later. The results indicate that strains with high per cent guanosine plus cytosine (60-65%) are, in general, more nutritionally versatile than strains with lower guanosine plus cytosine content of their DNA (38-58%). For example, strains 1-14 and strains 43-44 grew on a larger variety of compounds than strains 15-42. The only obvious exceptions are strains 2, 3, and 4 which appear to have more complex growth requirements. Furthermore, the ability to utilize sugars, alcohols, aromatic amino acids, L-ornithine, L-citrulline, L-arginine, L-lysine, L-cysteine, glycine, L-leucine, L-hydroxyproline, L-isoleucine, L-valine, and putrescine is also highly correlated with strains possessing a high guanosine plus cytosine content of the DNA (62-65%). Strains 2, 3, 4, 15, 16,

Table I. Sole carbon and energy sources for Spirillum strains 1 - 22.

Strain	Citrate	Aconitate	Isocitrate	α -Ketoglutarate	Succinate	Fumarate	L-malate	Oxaloacetate	Pyruvate	L-Lactate	Malonate	Acetate	Propionate	Caproate	β -Hydroxybutyrate	Ethanol	n-Propanol	n-Butanol	Glycerol	L-Histidine	L-Tyrosine	L-Phenylalanine	L-Alanine	L-Glutamate	L-Aspartate	L-Glutamine	L-Asparagine	L-Proline	L-Hydroxyproline	L-Ornithine	L-Citrulline	L-Arginine	L-Lysine	Putrescine	L-Methionine	L-Serine	L-Cysteine	Glycine	L-Leucine	L-Isoleucine	L-Valine	D-Glucose	D-Gluconate	D-Mannitol	D-Fructose	D-Galactose	D-Mannose			
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■ = Abundant growth (> 35 klett units); ■ = Moderate growth (20 - 35 klett units);
 □ = Slight growth (10 - 20 klett units); Lack of symbol = No growth (< 10 klett units).

Table II. Sole carbon and energy sources for Spirillum strains 23 - 44.

Strain	Citrate	Aconitate	Isocitrate	-Ketoglutarate	Succinate	Fumarate	L-Malate	Oxaloacetate	Pyruvate	L-Lactate	Malonate	Acetate	Propionate	Caproate	-Hydroxybutyrate	Ethanol	n-Propanol	n-Butanol	Glycerol	L-Histidine	L-Tyrosine	L-Phenylalanine	L-Alanine	L-Glutamate	L-Aspartate	L-Glutamine	L-Asparagine	L-Proline	L-Hydroxyproline	L-Ornithine	L-Citrulline	L-Arginine	L-Lysine	Putrescine	L-Methionine	L-Serine	L-Cysteine	Glycine	L-Leucine	L-Isoleucine	L-Valine	D-Glucose	D-Gluconate	D-Mannitol	D-Fructose	D-Galactose	D-Mannose			
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44	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣	▣

▣ = Abundant growth (> 35 klett units); ▣ = Moderate growth (20 - 35 klett units);
 ▣ = Slight growth (10 - 20 klett units); Lack of symbol = No growth (< 10 klett units).

17, 18, 20, 21, 32, 33, 41, and 42 appear to have complex growth requirements, in that all are somewhat nutritionally limited. For example, strains 2, 3, 4, 20, 21, 33, 41, and 42 grew on none of the 67 different compounds tested as sole carbon and energy sources. Strains 15, 16, 17, and 18 and 32 gave only a slight growth response on a limited number of compounds, perhaps indicating that the standard conditions may not be optimal growth conditions for these organisms. In addition, the following compounds were found to yield a negative growth response for all Spirillum strains tested: formate, L-tartrate, D-tartrate, anthranilate, benzoate, para-hydroxybenzoate, tertiary-butyl alcohol, D-ribose, D-xylose, 2-keto-gluconate, D-galactose, lactose, sucrose, maltose, sedoheptulose, D-raffinose, L-tryptophan, L-methionine, and L-threonine.

The compounds which were most readily utilized included: ~~alpha-keto-glutarate, succinate, fumarate, malate, malonate, oxaloacetate, pyruvate, lactate, L-glutamate, L-glutamine, L-aspartate, L-asparagine, L-alanine, and L-proline.~~ Other amino acids, fatty acids, alcohols, amine, and sugars were used to a lesser degree (Tables I and II).

Compounds Utilized as Sole Nitrogen Source

Tables III and IV indicate compounds which can serve as sole nitrogen sources by Spirillum strains. It is apparent from Tables III and IV that most amino acids and ammonium ions can serve as sole nitrogen sources, but not potassium nitrate or urea. In fact, only five strains (5, 8, 11, 12, and 14) can utilize potassium nitrate and three strains (8, 10, and 17) can utilize urea as a sole nitrogen source under standard conditions.

Table III. Sole nitrogen sources used by
Spirillum strains 23-44.

Strain	L-Alanine	L-Glutamate	L-Aspartate	L-Glutamine	L-Asparagine	L-Proline	L-Hydroxyproline	L-Histidine	L-Tyrosine	L-Tryptophan	L-Phenylalanine	L-Ornithine	L-Citrulline	L-Lysine	L-Arginine	L-Methionine	L-Serine	L-Cysteine	Glycine	L-Leucine	L-Isoleucine	L-Valine	L-Threonine	Urea	Ammonium ions	Nitrate
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■ = Abundant growth (>35 klett units); ■ = Moderate growth (20 - 35 klett units); □ = Slight growth (10-20 klett units); Lack of symbol = No growth (<10 klett units).

Table IV. Sole nitrogen sources used by Spirillum strains 1-22.

Strain	L-Alanine	L-Glutamate	L-Aspartate	L-Glutamine	L-Asparagine	L-Proline	L-Hydroxyproline	L-Histidine	L-Tyrosine	L-Tryptophan	L-Phenylalanine	L-Ornithine	L-Citrulline	L-Lysine	L-Arginine	L-Methionine	L-Serine	L-Cysteine	Glycine	L-Leucine	L-Isoleucine	L-Valine	L-Threonine	Urea	Ammonium ions	Nitrate
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 = Abundant growth (> 35 klett units);
  = Moderate growth (20 - 35 klett units);
  = Slight growth (10-20 klett units);
 Lack of symbol = No growth (< 10 klett units).

It is interesting to note that strains 32 and 33 appear to be highly stimulated by L-methionine and therefore may require this compound for optimal growth. In addition, strains 2, 3, 4, 15, 18, 20, 21, 34, 41, and 42 failed to grow on any of the 30 compounds tested as a sole nitrogen source and hence appear to have more complex growth requirements.

Urease Test

The following Spirillum strains yielded a positive urease reaction in synthetic urease medium (see methods): 2, 3, 4, 10, 14, 15, 17, 18, and 28. Moreover, only strains 10, 14, and 28 yielded a positive urease reaction in Difco urease medium under comparable conditions.

NaCl Tolerance of Fresh Water Spirillum Strains

Table V shows the effects of NaCl on Spirillum strains. As is apparent from Table V, most Spirillum strains can grow at 1-1.5% NaCl, except strains 15, 16, 17, and 18 which appear to be very sensitive to NaCl ions. For example, strain 16 is completely inhibited by 0.3% NaCl while strain 15 is completely inhibited by 0.5% NaCl. Strains 1, 6, 10, 43, and 44 grew at a NaCl concentration of 2.0%.

Effects of Calcium Ions on Growth of Spirillum Strains

Table VI shows the effects of 0.05% calcium chloride on the growth of Spirillum strains. The results indicate no major changes in the spectrum of carbon source utilization as reported by Giesberger (1936). Moreover, standard basal medium (SBM) in almost all cases proved to be a better culturing medium than SBM plus 0.05% calcium chloride.

Table V. NaCl Tolerance of Fresh-water Spirilla

Strain	NaCl concentration							
	0.0%	0.1%	0.3%	0.5%	0.7%	1.0%	1.5%	2.0%
<u>Defined medium:</u>								
24	17	90	70	84	70	64	20	0
25	7	50	20	30	30	30	10	0
26	120	120	125	90	45	45	20	0
27	70	70	74	77	61	55	15	0
28	70	27	18	53	30	28	15	0
1	100	90	100	100	70	60	45	25
6	80	70	87	95	75	63	35	10
7	24	20	18	15	12	10	0	0
10	35	28	40	33	30	29	20	14
12	40	40	80	40	35	34	20	0
13	55	80	80	70	60	48	12	0
43	100	85	65	45	45	50	35	9
44	105	103	75	60	55	40	20	15
45	33	40	47	42	40	40	5	0
19	60	60	65	55	48	38	10	0
<u>Semi-defined medium:</u>								
15	60	93	12	0	0	0	0	0
16	28	17	0	0	0	0	0	0
17	35	34	28	28	23	0	0	0
18	65	90	70	26	0	0	0	0

^a Numbers indicate turbidity at 420 nm at 72 hr. growth.

Table VI. Effects of Calcium Ions on Growth of Spirillum Strains

Strain	D-xylose	D-fructose	D-glucose	D-arabinose	D-ribose	maltose	succinate	L-lactate	acetate	L-glutamate
25	0	0	0	0	0	0	0	0	0	24
	0	0	0	0	0	0	0	0	0	45
26	0	0	0	0	0	0	0	0	0	10
	0	0	0	0	0	0	0	0	0	20
12	0	25	0	0	0	0	20	25	0	18
	0	35	0	0	0	0	27	21	0	70
43	0	37	0	0	0	0	24	23	0	45
	0	40	0	0	0	0	30	40	0	67
44	0	10	0	0	0	0	28	33	0	31
	0	21	0	0	0	0	45	39	0	50
16	0	0	0	0	0	0	0	0	0	10
	0	0	0	0	0	0	0	0	0	21
10	0	0	0	0	0	0	0	0	0	10
	0	8	0	0	0	0	10	15	10	21
7	0	0	0	0	0	0	8	0	0	10
	0	0	0	0	0	0	16	21	0	20
19	0	0	0	0	0	0	0	0	10	10
	0	0	0	0	0	0	24	25	20	28
1	0	0	0	0	0	0	15	18	10	10
	0	0	0	0	0	0	43	40	30	29

^a Numbers in upper rows indicate turbidities at 420 nm in SBM plus 0.05% CaCl₂.

^b Numbers in lower rows indicate turbidities at 420 nm in SBM.

^c SBM = Standard Basal Medium.

Production of Fluorescent Pigment

Strains 1, 4, 6-14, 19-21, 23-33 and 43-44 produce a yellowish-green water-soluble fluorescent pigment when observed under a 253.7 nm light source. All other strains were negative for this characteristic.

Enzyme Assay Results of Strain 8 (*Spirillum lunatum* ATCC 11337)

The results of enzyme assays for *Spirillum lunatum* are summarized in Table VII. The results show high levels of activities of hexokinase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrase, and 2-keto-3-deoxy-6-phosphogluconate aldolase and very low levels of 6-phosphogluconate dehydrogenase and fructose 1,6-diphosphate aldolase when cultured on D-glucose/casamino acids or D-gluconate/casamino acids. Only basal levels of activity are found when *S. lunatum* is cultured on succinate/casamino acids. Furthermore, when *S. lunatum* is cultured on D-mannitol/casamino acids, a mannitol dehydrogenase is induced which is specific for NAD^+ and non-reversible with fructose-1-phosphate or fructose-6-phosphate; however, mannitol dehydrogenase is reversible with D-fructose.

Table VIII shows the results of the effects of various inhibitors on glucose-6-phosphate dehydrogenase from *S. lunatum*. This enzyme is apparently specifically inhibited by ATP (56%) at a concentration of 3.33 millimolar. Furthermore, glucose-6-phosphate dehydrogenase is non-specific for NAD^+ or NADP^+ , with either serving approximately equally well. Inhibitor studies also revealed that this enzyme can be inhibited (50%) by CuCl_2 ($1.16 \cdot 10^{-3}$ molar) and iodoacetate ($5 \cdot 10^{-4}$ molar).

Table VII. Enzyme Assays of Strain 8 (Spirillum lunatum ATCC 11337)

Enzyme	Specific Activity (10 ⁻⁴ EU/mg of protein)			
	Succinate/ casamino acids	Gluconate/ casamino acids	Glucose/ casamino acids	Mannitol/ casamino acids
Hexokinase	<1	129	76	
Mannitol dehydrogenase ..	<1	<1	<1	34
Glucose-6-phosphate dehydrogenase	5	1100	520	
Phosphogluconate dehydrogenase	<1	<1	<1	
Phosphogluconate dehydrase	<1	74	42	
KDPG aldolase	8	540	217	
Fructose Diphosphate aldolase	<1	<1	<1	

^aOne EU is defined as that quantity of the enzyme which converts 1 μ mole of substrate per min. under the conditions specified.

^bKDPG = 2-keto-3-deoxy-6-phosphogluconate.

^cGlucose-6-phosphate dehydrogenase was assayed by using NAD⁺ as cofactor.

Table VIII. Glucose-6-phosphate Dehydrogenase Activities of
Strain 8 (Spirillum lunatum ATCC 11337)

Compound	% Activity
NAD ⁺ (1.78 mM)	100
NADP ⁺ (1.78 mM)	89
ATP	56
GTP	89
CTP	100
UTP	100
ITP	100
AMP	100
3'-5'-cyclic AMP	95
Orthophosphate	100
Pyrophosphate	100
Sodium fluoride	100
No magnesium chloride	105
Cupric chloride (1.16 mM)	50
Iodoacetate (0.5 mM)	50

^aConcentrations unless specified are 3.33 mM.

^bNAD⁺ was used in all inhibition studies.

Enzyme Assay Results of Strain 13

(Spirillum itersonii ATCC 12639)

Table IX indicates the enzyme assay analysis of S. itersonii. The table shows activities of hexokinase, glucose-6-phosphate dehydrogenase, phosphogluconate dehydrase, 2-keto-3-deoxy-6-phosphogluconate aldolase, and fructose 1,6-diphosphate aldolase and very low levels of activity of 6-phosphogluconate dehydrogenase when S. itersonii is cultured on D-fructose/casamino acids. When this organism is cultured on succinate/casamino acids the specific activities of all enzymes assayed fall to a basal level.

Enzyme Assay Results of Strain 10

(Spirillum peregrinum ATCC 15387)

Table X shows the results of the enzyme assay analysis of S. peregrinum. As is apparent from Table X only hexokinase and fructose 1,6-diphosphate aldolase are detectable when this organism is cultured on D-fructose/casamino acids. Upon culturing S. peregrinum in succinate/casamino acid medium, one detects only basal levels of activity.

Table IX. Enzyme Assays of Strain 13

(Spirillum itersonii ATCC 12639)

Enzyme	Specific Activity (10 ⁻⁴ EU/mg of protein)	
	Fructose/ casamino acids	Succinate/ casamino acids
Hexokinase	26	<1
Glucose-6-phosphate dehydrogenase	40	<1
Phosphogluconate dehydrogenase	<1	<1
Phosphogluconate dehydrase	11	<1
KDPG aldolase	61	4
Fructose Diphosphate aldolase	29	4

^aOne EU is defined as that quantity of the enzyme which converts 1 μ mole of substrate per min. under the conditions specified.

^bKDPG = 2-keto-3-deoxy-6-phosphogluconate.

Table X. Enzyme Assays of Strain 10

(Spirillum peregrinum ATCC 15387)

Enzyme	Specific Activity (10 ⁻⁴ EU/mg of protein)	
	Fructose/ casamino acids	Succinate/ casamino acids
Hexokinase	74	<1
Glucose-6-phosphate dehydrogenase	<1	<1
Phosphogluconate dehydrogenase	<1	<1
Phosphogluconate dehydrase	<1	<1
KDPG aldolase	11	<1
Fructose Diphosphate aldolase	34	3

^aOne EU is defined as that quantity of the enzyme which converts 1 μ mole of substrate per min. under the conditions specified.

^bKDPG = 2-keto-3-deoxy-6-phosphogluconate.

DISCUSSION

Many recent classification schemes for bacteria have been based on the use of a large number of characteristics (biochemical, morphological, nutritional, physiological, and serological) to give as complete a picture as possible of each organism. For examples, one may cite the taxonomic scheme of Edwards and Ewing (1962) for the members of the family Enterobacteriaceae, and the scheme of Stanier, Baumann, and Doudoroff (1968) for the genus Moraxella. Numerical taxonomy is based on the concept that each characteristic is to be assigned unit weight. Even where there is disagreement with this concept, there is little disagreement with the fundamental goals of numerical taxonomy, namely, to provide objectivity in bacterial classification. If one first holds the view that all characteristics of an organism are important in its taxonomy, the old arguments of whether or not morphology of spirilla should hold precedence over physiological characteristics of spirilla become irrelevant for the classification of spirilla. Here the broader view is that the more that one knows about all of the various characteristics of spirilla, the more satisfactory will be attempts to classify them. This is the view to which I have subscribed in the present work. Such a view also implies that any new classification scheme reflects only what is currently known about a group of organisms, and since such knowledge is continually increasing, so the taxonomy of a genus should continually be improved. It follows that the classification scheme presented in this manuscript is not to be considered in any sense final, but only a progress report which will hopefully lead to a more satisfactory

classification scheme in the future. The latter philosophy is in fact one of the major reasons for the popularity of Bergey's Manual of Determinative Bacteriology, which is continually undergoing revision as new information accumulates.

As more strains of the various species are described, the more certain becomes the establishment of those few characteristics to be used in diagnostic schemes for identification of the species and genus as well as those characteristics which may be variable within a given species. A good diagnostic character may be defined as one which is positive (or negative) for at least 95-100% of the strains tested, is highly correlated with a large number of other characteristics, is highly reproducible, and is easy to perform. An example of a poor diagnostic characteristic might be the determination of the per cent guanosine plus cytosine content of the DNA, in that it requires special equipment to perform. An example of a good diagnostic characteristic is the phenylalanine deaminase test for the Proteus-Providence group of the family Enterobacteriaceae because it is positive for 100% of the strains, is easy to perform, is highly reproducible, and is highly correlated with a large number of other characteristics.

Sometimes it is the case in bacterial taxonomy that a few characteristics can be highly correlated with, and can thus represent a large number of other characteristics. Because their determination may be difficult, these few traits may not be useful for diagnostic purposes. However, they may be highly useful for classification purposes as this trait provides a very simple basis for grouping organisms. For example, Sebald and Veron (1963) separated the genus Vibrio

into several genera, primarily on the basis of DNA base composition. This was not a good diagnostic characteristic but was very useful for classification purposes. As arbitrary as this trait may seem, vibrios with low guanosine plus cytosine content (30-34 GC%) do not ferment a large variety of carbohydrates, are microaerophilic, have mono-trichous or multi-trichous polar flagella, and share a large number of other characteristics. Note that this is in contrast to vibrios with a higher guanosine plus cytosine content of the DNA (47 GC%), which ferment a large variety of carbohydrates, are motile by a single polar flagella, are strictly or facultatively anaerobic, and have a large number of other characteristics in common. As another example, Orla-Jensen in 1909 presented a bacterial classification scheme based on the type of flagellation, and his scheme has since generally remained unchanged. He separated bacteria with polar flagellation from those with peritrichous flagellation. This may seem to be an arbitrary choice; however, upon closer examination one finds polarly flagellated bacteria are usually non-pathogenic, are saprophytic, have simple nutrition, and are oxidizers. This is in marked contrast to peritrichously flagellated bacteria. Though there are exceptions, as a general rule this classification scheme is probably a good "natural" one because Orla-Jensen chose a characteristic which is highly correlated with a large number of other traits.

With reference to the considerations expressed in the preceeding paragraphs, it was unfortunate that many of the species in the present study dealt with only one isolate - the type strain, thus making it difficult to determine the extent of variation within different species.

However, in a few cases more than one strain was available, and it is encouraging to note that the number of similar characteristics is very high. As an outstanding example, the two strains of S. volutans seem to be identical in every way, even though they were isolated twelve years apart and were from a different continent. The present study has great value, because, for the first time, all available strains have been subjected to a large number of characterization tests using standard methodology to provide a valid basis of comparison. Regarding species represented currently by only one strain, it is hoped that future investigations will determine the amount of variation of characteristics within each species and genus.

Importance of Utilizing Standard Methods

It is apparent from the literature review that a number of different methods have been employed in previous investigations for the determination of compounds which can serve as sole carbon and energy sources for spirilla. I have defined all methods in this study, and in many cases the methodology represents an improvement over similar methods used by other investigators. For example, (1) sugars, asparagine, glutamine, urea, and other thermolabile compounds were sterilized separately by filtration and added aseptically. (2) To prevent a large precipitate from forming in the defined media, I employed a basal medium containing a low concentration of calcium ions. A precipitate is undesirable because it makes turbidimetric growth determinations very difficult to quantitate. In the nutritional characterization of spirilla by Williams and Rittenberg (1957), turbidimetric growth quantitation was achieved by using only the supernatant

as the "defined media." Since the complete contents of the precipitate were not known, the media used for the nutritional characterization cannot be considered defined. Moreover, the elimination of a large concentration of calcium ions from the defined media appeared to be justified because the data in Table VI indicates that a high concentration of calcium ions are not required for optimal growth of spirilla. In Giesberger's (1936) comparison of the utilization of calcium salts as opposed to sodium salts of organic acids, he found much better growth with the former. He inferred that calcium ions were stimulatory. It would appear from Table VI that a more satisfactory interpretation is that the poorer growth was not due to calcium ion stimulation but to sodium ion toxicity. (3) All sea water media were sterilized by filtration to prevent the formation of a large precipitate which results from autoclaving. (4) Ion-free water and a mixture of trace metals were used throughout this study in order to standardize the types and amounts of trace metals, since it is possible that these may vary in distilled water from one geographical location to another. (5) A standard inoculum was employed throughout this investigation (see methods). Initially, I had several possibilities in regard to establishing a standard inoculum: (a) use of dry weight measurements; (b) use of total (Kjeldahl) nitrogen determinations; (c) determination of cell number per milliliter; (d) determination of the amount of DNA in the inoculum; or (e) use of standard turbidity. As a matter of convenience for future investigators, I used a standard turbidity as an inoculum. By doing so, I realize that the number of cells and the protoplasmic mass will vary from strain to strain in this

system; this is largely because of the differences in cell size and presence of highly refractile intracellular granules such as poly-beta-hydroxybutyrate. However, with a standard reference point in terms of a specific number of klett units, the results are easily reproducible for a given strain. (6) A definition of growth was employed throughout the study (see methods). Although technically speaking, one cell division, indicates growth, the term positive growth has many meanings for different investigators. Thus in an attempt to avoid subjective error, I chose to include a definition of growth.

Discrepancies Arising from a Comparison of the Present Data
with Earlier Results

Generally, I found that the results obtained in the present study coincided with those in the literature, but there are several cases in which there were discrepancies. For example, Giesberger (1936) reported S. serpens utilized acetate, succinate, lactate, and pyruvate as sole carbon and energy sources with ammonium chloride as the sole nitrogen source. My results were negative for these compounds. In fact, it appears that 80% of the available strains of S. serpens require organic nitrogen (Tables I and II). On the other hand, of the 67 carbon sources I tested, the results are in contrast with only the four cases indicated above. Giesberger (1936) found S. itersonii to use acetate, citrate, xylose, arabinose, glucose, mannose, galactose, maltose, lactose, and sucrose as sole carbon and energy sources. My results show all these to yield a negative growth response. The only sugar I found to be utilized as a sole carbon and energy source

was D-fructose, also reported positive by Giesberger. It is interesting to note when Giesberger used these compounds for respirometric studies, he obtained oxygen uptake only on fructose and glucose. It is difficult to understand how an obligately aerobic organism would grow on a compound as a sole carbon and energy source without oxidizing it in some manner. In further support of my nutritional results, Wells (1970) tested 32 different carbohydrates for the production of acid in complex media with only D-fructose and glycerol yielding a positive reaction.

Williams and Rittenberg (1957) reported that S. serpens could use acetate, propionate, pyruvate, succinate, fumarate, malate, and lactate as sole carbon and energy sources. In contrast, I found all of these compounds to yield a negative growth response. In regard to the nutrition of S. itersonii, Williams and Rittenberg (1957) reported this species to utilize glucose, acetate and citrate as sole carbon and energy source. However, my results indicate a negative growth response with these compounds. In the study of compounds which might serve as sole nitrogen sources, Williams and Rittenberg (1957) reported S. itersonii to use urea, nitrate, and asparagine; however, my results indicate only L-asparagine to be a suitable nitrogen source. Williams and Rittenberg (1957) reported that S. annulus and S. sinuosum used pyruvate, succinate, fumarate, malate, and lactate as sole carbon and energy sources. My results showed S. annulus to yield a positive growth response only on pyruvate and L-glutamate; whereas, S. sinuosum utilized only pyruvate, oxaloacetate, and L-malate. With regard to S. graniferum and S. giesbergeri, Williams and Rittenberg (1957) reported

these species to use pyruvate, succinate, fumarate, malate, lactate, and malonate as sole carbon and energy sources. In contrast, my results indicated that S. graniferum uses only pyruvate and fumarate; in addition, S. giesbergeri uses only pyruvate under standard conditions. Furthermore, Williams and Rittenberg (1957) reported S. polymorphum to utilize ethanol, glycerol, pyruvate, succinate, fumarate, malate, lactate, citrate, and malonate as sole carbon and energy sources; whereas, I found a positive growth response only with acetate, malonate, L-glutamate, L-aspartate, L-glutamine, and L-proline as sole carbon and energy source (Table I).

In the investigation of S. putridiconchylum, Terasaki (1961) reported this species to utilize the salts of succinic, fumaric, pyruvic, and malic acids in synthetic media, but not propionate, butyrate, citrate, malonate, glucose, fructose, glycerol, and ethanol. In comparison, my results (Table I) indicate that this species can use those reported positive by Terasaki, but acetate and malonate as well. S. metamorphum was reported by Terasaki (1961) to be unable to grow on synthetic medium containing succinate, fumarate, malate, lactate, acetate, propionate, citrate, malonate, glucose, fructose, glycerol, and ethanol as sole carbon source. Furthermore, ammonium salts, nitrate, urea, and asparagine could not serve as sole nitrogen sources. My results (Table I) shows this species can grow on alpha-ketoglutarate, succinate, fumarate, L-malate, oxaloacetate, pyruvate, L-lactate, malonate, L-glutamate, L-aspartate, L-glutamine, and L-asparagine as sole carbon and energy sources. In reference to sole nitrogen sources, I found S. metamorphum to utilize several amino

acids, but not potassium nitrate, urea, or ammonium sulfate under standard conditions.

Pretorius (1963) reported S. peregrinum to use citrate and ethanol as sole carbon and energy sources. In contrast, my results (Table I) indicate a negative growth response on these compounds. Nevertheless, these are the only two cases where our results differ.

Canale-Parola, Rosenthal, and Kupfer (1966) reported S. gracile to utilize L-glutamate and potassium pyruvate in a defined medium containing a mixture of vitamins. On the other hand, my results were uniformly negative for all compounds tested (Table I); however, this species appears to require vitamins as evidenced by the above investigators inability to obtain growth in their absence.

Possible Explanations for Discrepancies

It should be noted that some of the discrepancies in the results may be due to one or more of the following factors. (1) The present strains of spirilla may have changed as a result of continued transfer and storage since isolation (Rogosa and Hansen, 1971). For example, S. lunatum ATCC 11337 was first reported to require sea water for growth; however, it now grows in either fresh-water or sea water. On the other hand, it is impossible to determine if strains have changed because they are no longer fresh isolates. Continued transfer may have selected for mutants which require one or more growth factors found in the culture medium. As another example, S. atlanticum was reported by Williams and Rittenberg (1957) to use a number of compounds as sole carbon and energy sources; however, the present strain appears to require L-methionine for growth (Table II). Again I have no way of

knowing if the original strain has changed. (2) To my knowledge, a definition of a positive growth response seems to be absent in all investigations of spirilla. (3) Autoclaving thermolabile compounds may affect one's conclusions about an organism's ability to use a compound as a sole carbon and energy source or a sole nitrogen source. For example, Williams and Rittenberg (1957) reported all of their isolates of spirilla to use urea as a sole nitrogen source. In comparison, my results indicate only a few strains of spirilla can use urea as a sole nitrogen source (Table III and IV). My results are reinforced by the fact that only a very few strains are urease positive. (4) The purity of commercial biochemicals is probably better today than 25-30 years ago (Rogosa and Hansen, 1971). This seems to be especially true if we consider that new techniques for purification, such as chromatography, have come into widespread use only in the last one or two decades.

Guanosine plus Cytosine Content of DNA for genus *Spirillum*

According to Wells (1970) the per cent guanosine plus cytosine content of the DNA in the genus *Spirillum* spans from 38% in the case of strains 41 and 42 to 65% in the cases of strains 1-4 and strain 20. There exist an almost continuous spectrum of base composition values for different species between these two extremes, with the widest gap being only 5%. It is, therefore, difficult to believe that all of these bacteria had a common ancestry or at least a relatively recent one.

Obligate Microaerophilism of Strains 41 and 42

Strains 41 and 42 differ from all other *Spirillum* strains in being obligately microaerophilic (Krieg and Wells, 1965; Wells, 1970).

As noted above, these two strains also possess the lowest % G + C values of all the strains studied. The fundamental nature of obligate microaerophilism is not yet known. One possibility has been suggested by Cole and Rittenberg (1971). These authors suggest the inability of S. volutans to synthesize sufficient amounts of succinic dehydrogenase may impair the rapid utilization of oxygen via the terminal respiratory chain and result in toxic levels of oxygen intracellularly. A second possibility might be the inability of this organism to synthesize sufficient amounts of catalase to break down H_2O_2 which forms in the growth medium, as a result of oxidative amino acid deamination. This possibility is supported by the fact that increased growth yields occur when catalase is added exogenously. However, intracellular H_2O_2 might be high enough to be toxic. A third possibility might be that abnormally high levels of ATP are formed in the presence of atmospheric oxygen concentrations. Abnormally high levels of ATP may result in an "upset" of intracellular control mechanisms and hence in a failure of this organism to grow. Although it is hard to access the number of genes associated with obligate microaerophilism, the nutritional, physiological, and morphological evidence (Table II and Wells, 1970) suggest this may be an important taxonomic characteristic.

Morphology of Strains 41 and 42

In the study by Wells (1970), strains 41 and 42 were found to possess the greatest cell diameter (1.4-1.7 microns) and cell length (14-60 microns) of any of the spirilla strains studied. These dimensions can be correlated with the low % G + C value and obligately

microaerophilic nature of these strains.

Fresh-water vs Marine Strains of Spirilla

Strains 41 and 42 are fresh-water strains of spirilla, but are distinguished from all other fresh-water strains by the characteristics cited above. The remaining fresh-water strains (strains 1-7, 9-13, 15-31, and 43-44) collectively have a range of cell widths from 0.2-1.4 microns and a range of % G + C contents of their DNA from 50 to 65 (Wells, 1970). More details from Wells' study of morphology and base composition are presented in Table XI.

The marine strains studied are strains 8, 14, and 32-40 (Wells, 1970). Strain 14 in my judgment is not a spirillum, because as noted by Wells (1970) it is not helical and it typically is found in pairs (quite unlike any of the other strains studied). Moreover, strain 14 produces urease activity and is unable to form coccoid bodies, both characteristics differ from all other marine strains studied. With the elimination of strain 14 from further consideration, the remaining marine strains fall into two clear-cut categories with respect to % G + C content of their DNA (Wells, 1970). Strain 8 (% G + C = 63) and strains 32-40 (% G + C = 42-48). More details on G + C ratios are presented in Table XII. It should be noted that these values differ from the aerobic fresh-water strains (% G + C = 50-65) and the obligately microaerophilic freshwater strain (% G + C = 38). The range of cell width for strains 32-40 is 0.3-0.8 microns (Table XI). Although this range overlaps that for the aerobic freshwater strains (0.2-1.4 microns), those fresh-water strains of cell with 0.2-0.8 microns

possess a % G + C content of 57-65 (with the majority falling between 62 and 65). These % G + C values sharply differ from the 42-48 % range of the marine strains of similar cell width.

Strain 8 possesses a cell diameter of 0.8-1.0 microns (Wells, 1970), similar to that of several aerobic fresh-water strains. Moreover, its % G + C value of 63 falls within the 50-65 % range for aerobic fresh-water spirilla. Nevertheless, strain 8 differs from the latter, besides possessing a marine habitat, in that it (a) produces an acid reaction from a large variety of carbohydrates (Wells, 1970), and (b) can use a large variety of carbohydrates as sole carbon and energy sources (Table I). It is true that certain fresh-water strains also produce acid reactions from carbohydrates and can use carbohydrates as sole carbon sources, but the variety of carbohydrates is much more restricted and the pathway of catabolism involved would appear to be different. Table VII indicates the enzyme activities found for strain 8; these activities should be compared to those activities for strain 13 (Table IX) and strain 10 (Table X). Although a more comprehensive discussion of the enzymatic activities is presented later in this discussion, it can be seen that the array of activities found in strain 8 is characteristic of only the Entner-Doudoroff (ED) pathway, that of strain 10 is characteristic of the Embden-Meyerhof-Parnas (EMP), and that of strain 13 is characteristic for both the ED and EMP pathways. It is also noteworthy that the activities found for enzymes of the ED pathway in strain 8 are 25-30 times higher than those found in strain 13. The nutrition of strain 8 is different from fresh-water spirilla. Strain 8 could grow on D-mannitol, D-glucose, D-gluconate, L-tyrosine,

and glycine as a sole carbon and energy source (Tables I and II). This is in contrast to all fresh-water spirilla. Another contrast between strain 8 and fresh-water spirilla is that strain 8 can use any of 3 nitrogen sources (urea, ammonium ions, and nitrate) as a sole nitrogen source (Tables III and IV), whereas, fresh-water spirilla can use at the most only two of these.

All marine spirilla can grow in the presence of 1% glycine, which is in sharp contrast to the fresh-water spirilla. Among the latter, only strains 1 and 6 could tolerate 1% glycine (Wells, 1970). Glycine is an agent that has been employed for the preparation of sphaeroplasts of various gram-negative bacteria (McQuillen, 1960). Many species of gram-negative bacteria can be converted to sphaeroplasts by inclusion of 1-5% glycine in their growth medium. Consequently, the ability of marine spirilla to grow in the presence of 1% glycine may well reflect a difference in cell wall structure between marine and fresh-water spirilla.

All of the marine spirilla can grow in the presence of 7% added NaCl; whereas, none of the fresh-water strains can tolerate this concentration of salt (Wells, 1970). Indeed, the fresh-water strains can tolerate only 3% added NaCl at the most, and most strains can tolerate only 2% or less (Table V). The tolerance of all the marine strains of spirilla must not be assumed to be a consequence of the fact that these spirilla live in the sea. MacLeod (1965) noted considerable variation in salt tolerance (with growth-limiting concentrations anywhere from 4.7% to 15.2% NaCl) among various marine bacteria studied. He noted that, in contrast, many terrestrial species, among them organisms not

classified as halophiles, can tolerate much higher concentration of salt than the marine bacteria studied. Therefore, the tolerance of marine spirilla to 7% added NaCl (or a total NaCl concentration of about 9.6%) cannot be attributed to the marine origin of these strains; in a similar manner, the sensitivity of fresh-water spirilla cannot be attributed merely to the fact that these strains are not marine in origin.

The fundamental difference between a marine bacterium versus a fresh-water bacterium is not yet known. According to Stanier (1941) the central problem of marine microbiology is the question of the existence of specific marine bacteria, and, until this problem is settled, work on marine bacteria apart from studies on gross transformations of matter would have very little point. MacLeod (1965) summarized a review of the question of the existence of specific marine bacteria by noting that marine bacteria have special requirements for inorganic ions, partly to supply the needs of the organism for growth and metabolism, and partly to maintain the integrity of the cells. They have a high specific need for Na^+ for growth, which has been shown in two species of bacteria to reflect the presence of a Na^+ - dependent mechanism for transporting substrates into the cells. The possession of a requirement for Na^+ for growth distinguishes marine bacteria from most non-marine species. The distinction is not absolute, however, since a few cases of non-marine sodium-requiring bacteria have been reported. Another difference between marine and terrestrial bacteria is that the need of marine bacteria for a combination of Mg^{++} and Ca^{++} exceeds that of most terrestrial species. For

some marine bacteria, the effect of salts in maintaining the integrity of the cells has been shown to be due entirely to the capacity of the salts to interact directly with the cell envelopes to stabilize them and prevent cell lysis.

MacLeod (1965) concluded his review by stating that "although the marine bacteria examined have a number of characteristics in common the only one which clearly distinguishes them from bacteria in other habitats is a capacity to survive and grow in the sea. In this respect, then, marine bacteria are unique." This view was further developed by Baumann, Baumann, and Mandel (1971), who recognized two ecologically distinct groups in the genus Vibrio: straight and curved rods of marine origin, and V. cholerae and related strains which are inhabitants of fresh-water and the human intestines. These investigators felt that the ecological distinctions between marine and fresh-water strains may reflect difference in the cell wall and permease systems, and may therefore reflect profound biochemical dissimilarity between the two groups. This view, supported by phenotypic differences and lack of DNA hybridization, led to the removal of Vibrio parahemolyticus from genus Vibrio to the genus Beneckea (a genus containing only marine bacteria).

The above considerations appear to be directly applicable to the question of the distinction between fresh-water and marine spirilla. In the case of strains 32-40, I believe that, on the basis of ecological differences, re-inforced by consideration of glycine tolerance, salt tolerance, and difference in % G + C content of the DNA, strains 32-40 represent a group distinct from obligately microaerophilic

fresh-water spirilla and from the aerobic fresh-water spirilla. Similar arguments, except for % G + C content of the DNA, compel me to separate strain 8 from the fresh-water spirilla.

Within the group of marine spirilla, a further distinction of strain 8 from strains 32-40 can be argued. The difference in % G + C content of the DNA has already been mentioned (strain 8 = 63 %; 32-40 = 42-48%). Another important consideration is that strain 8 produces an acid reaction from, and can utilize as sole carbon sources, a large variety of carbohydrates. None of the strains 32-40 can attack any carbohydrates. Another difference is that strain 8 can grow anaerobically in the presence of nitrate (and therefore must possess a respiratory nitrate reductase), whereas none of the strains 32-40 can so grow. In regard to nutrition, in addition to the various carbohydrates used by strain 8 as sole carbon sources, strain 8 can also use citrate, aconitate, isocitrate, malonate, beta-hydroxy-butyrate, n-propanol, glycerol, L-tyrosine, and glycine, whereas, none of the strains 32-40 can utilize these compounds as sole carbon sources under standard conditions. Another difference concerns sole nitrogen sources: strain 8 can use urea, ammonium ions, or nitrate, whereas strains 38 and 40 can use only ammonium ions, and strains 32-37 and 39 can use none of these sources under standard conditions. All of these differences compel me to distinguish strain 8 from all of the other marine spirilla studied. In the course of my investigation, I found myself thinking more and more frequently of 4 major groups of spirilla. (i) The very large, obligately microaerophilic fresh-water spirilla having a % G + C content of 38; (ii) the aerobic, fresh-water

Table XI. Morphological and Physiological Characteristics of Fresh water Spirillum Strains,
from Wells, 1970

Strain	guanosine plus cytosine content of the DNA (in moles per cent, T _m)	cell diameter (microns)	helix diameter (microns)	wavelength (microns)	cell length (microns)	gelatin hydrolysis	urease*	nitrate reduction	selenite reduction	RNAase	DNAase	phosphatase	acid reaction from su- gars in complex media	anaerobic growth in the presence of nitrate	fluorescent pigment production*	coccoid bodies in older cultures
22-31	50-51	0.7-1.0	1.4-3.0	6.3-10.0	5.0-21.0	d	d	-	-	d	d	+	-	-	d	-
19	52	1.0-1.2	1.8-2.0	6.0-7.0	4.0-22.0	-	-	-	-	+	-	+	-	-	+	-
16	58	1.4-1.5	2.8-3.0	4.9-7.0	7.0-15.0	-	-	-	-	+	+	+	-	-	-	-
15	58	1.1-1.4	2.0-2.5	<1	4.0-10.0	-	+	-	-	+	+	+	-	-	-	-
17	57	1.0-1.2	3.5-4.2	7.0-8.4	7.0-17.0	-	+	-	-	+	+	+	-	-	-	-
18	57	0.5-0.7	3.5-4.2	8.5-10.0	5.0-20.0	-	+	-	-	-	+	+	-	-	-	-
21	58	0.9-1.0	2.8-4.2	4.2-5.0	4.0-22.0	-	-	-	-	-	-	+	-	-	+	-
7	63	1.1-1.3	2.8-3.0	<1	4.0-9.0	+	-	-	-	-	-	+	-	-	+	-
11-13, 43-44	62	0.4-0.6	1.2-1.6	3.0-3.5	2.0-6.0	-	-	+	d	+	+	+	+	+	+	+
10	62	0.5-0.7	1.4-1.6	3.5-4.0	2.0-22.0	-	+	-	+	-	-	+	+	-	+	+
1, 6	63-65	0.5-0.8	1.0-2.1	2.0-3.5	2.0-6.0	-	-	+	+	-	-	+	-	-	+	-
9	62	0.3-0.5	0.5-0.8	<1	3.0-8.0	-	-	+	+	-	+	-	-	+	+	+
5	63	0.3-0.4	0.4-0.7	<1	3.0-5.0	-	-	+	-	+	-	+	-	-	-	-
20	65	0.5-0.6	0.8-1.0	2.0-2.8	2.0-7.0	-	-	+	+	+	+	+	-	-	+	-
2-4	65	0.2-0.3	0.5-2.1	2.8-3.5	3.0-14.0	-	+	+	-	+	d	+	+	-	d	-

+ = All strains positive. - = All strains negative.

d = Some strains positive and others negative.

*Results obtained in the present investigation.

Table XII. Genus Aquaspirillum (Nutritional Traits)

Strain	Citrate	Aconitate	Isocitrate	α -Ketoglutarate	Succinate	Fumarate	L-malate	Oxaloacetate	Pyruvate	L-lactate	Malonate	Acetate	Propionate	Caproate	β -Hydroxy-butyrate	Ethanol	n-Propanol	n-Butanol	Glycerol	L-Histidine	L-Phenylalanine	L-Alanine	L-Glutamate	L-Aspartate	L-Glutamine	L-Asparagine	L-Proline	L-Hydroxyproline	L-Ornithine	L-Citrulline	L-Arginine	L-Lysine	Putrescine	L-Serine	L-Cysteine	L-Leucine	D-Fructose	
22-31	-	-	-	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-
19	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	-	-	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
11-13,43-44	-	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	-	+	-	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+
1,6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ = All strains positive.

- = All strains negative.

d = Some strains positive and others negative.

Table XIII

Spirillum Strains Producing an Acid Reaction from
Carbohydrates or Utilizing Carbohydrates as Sole Carbon Sources

Strain	Acid Reaction from*	Sole Carbon Sources
11	D-fructose, Glycerol	Glycerol
12	D-fructose, Glycerol	Glycerol, D-fructose
13	D-fructose, Glycerol	D-fructose
2-4	D-glucose, L-arabinose and D-galactose	none
10	D-fructose	D-fructose
8	D-fructose, D-mannose D-glucose, D-galactose D-mannitol, D-gluconate, and Dextrin.	D-glucose, D-gluconate D-mannitol

*Results of Wells (1970)

spirilla not tolerant to 7% salt or 1% glycine and possessing a % G + C of 50-65; (iii) the marine spirilla which could not attack carbohydrates, which could not grow anaerobically with nitrate, which could not use urea or nitrate as sole nitrogen sources, and which possessed a % G + C content of 42-48; and (iv) the marine spirilla which would attack a variety of carbohydrates, which could grow anaerobically with nitrate, which could use urea or nitrate as sole nitrogen source, and which possessed a % G + C content of 63. These 4 groups of spirilla represented to me a useful way in which some order could be brought out of the chaos that was represented by the genus Spirillum as a whole, and eventually led to my decision to designate these groups as 4 genera, retaining the name Spirillum for that genus containing the type species of the original genus, S. volutans.

I ranked the 4 groups as genera rather than as species for two major reasons. 1.) One of the most important characteristics of a genus is the span of % G + C content of the DNA of its species. For most genera for which such information is available, the span of % G + C values is fairly narrow. For example, Sober and Harte (1970) compiled a list of values of the base composition for the species of a large number of bacterial genera. Of 75 genera consisting of more than 2 species, the average span of % G + C values per genus can be calculated to be 6.7%, with only 6 genera having a span of more than 15%. The highest spans occurring were those for Ramibacterium (21%) and Desulfovibrio (20%). Even with genera containing a large number of species, relatively narrow spans occurred; e.g., Pseudomonas species had a span of 12%; Bacillus species, 15%; Vibrio species, 8%;

Clostridium species, 7%; Moraxella species, 9%; Xanthomonas, 6%. The entire Family Enterobacteriaceae had a span of only 21%, while the type genus Escherichia had a span of 5%. Such spans should be contrasted with the span found by Wells (1970) for genus Spirillum, whose species possessed mole % G + C values from 38 to 65 (a span of 27%). My division of this genus into four genera, each possessing a much smaller span of % G + C values, therefore appeared to me to be supported by these considerations. 2.) Another important consideration for a genus is the cell diameter of its species. For example, Breed et al. (1957) reported in Bergey's Manual the genus Beneckea to have a range in cell diameter from 0.2-0.9 microns, for genus Erwinia 0.3-1.0 microns, for genus Serratia 0.5-0.8 microns, for genus Bacillus 0.5-1.5 microns, and genus Pseudomonas 0.2-1.2 microns. The span of values for cell diameter for a given bacterial genus usually did not exceed 1.0 micron. This is in contrast to the genus Spirillum which ranges from 0.2-1.7 microns in cell diameter (Wells, 1970).

In the following description of genera and species, the following data are taken from the study by Wells (1970): cell morphology and dimensions, cultural characteristics, % G + C content of the DNA, and the following physiological tests- oxidase; catalase; phosphatase; H₂S from cysteine; hydrolysis of gelatin, casein, starch, and aesculin; indol production; reduction of nitrate, nitrite, and selenite; DNAase and RNAase activity; acid production from sugars in complex peptone media; growth in the presence of 1 per cent bile and glycine; growth in the presence of various concentrations of salt in PSS broth; growth in triple-sugar-iron agar (TSI), eosin-methylene blue agar (EMB), and

MacConkey agar, and in litmus milk, MRVP broth, and Sella's slants; phenylalanine, tyrosine, and tryptophan deaminase activity and pigment production from these amino acids.

I propose the establishment of these genera as follows:

Genus I. Spirillum (Ehrenberg, 1832. 58)

Very large rigid helical cells, 1.4-1.7 microns in diameter. Obligately microaerophilic. Motile with bipolar polytrichous flagella. Gram-negative. Chemoorganotrophic, having a strictly respiratory metabolism for growth, with oxygen as the terminal electron acceptor. Intracellular poly-beta-hydroxybutyrate granules present. Oxidase and catalase positive, however, catalase may be weak. Indol negative. Hydrolysis of casein, starch, and aesculin negative.

Only one species, the type species (Spirillum volutans) is included at the present time; this species has a G + C content of its DNA of 38 moles per cent.

Genus II. Aquaspirillum gen. nov.

Aq . ua . spi . ril' . lum. L. adj. aqua water, spirillum. Gr. noun spira a spiral; M. L. dim. neut. n. spirillum a small spiral. Aquaspirillum a small water spiral.

Rigid helical cells, 0.2-1.4 microns in diameter, ranging from less than one turn to many turns. Motile with bipolar poly-trichous flagella. Gram-negative. Aerobic. Chemoorganotrophic, having a strictly respiratory metabolism for growth with oxygen as the terminal electron acceptor. Indol negative. Hydrolysis of casein, starch, and aesculin negative. Oxidase and catalase positive. Found in fresh-water. G + C content of the DNA ranges from 50-65 moles per cent.

Type species: Aquaspirillum serpens

Genus III. Oceanospirillum gen. nov.

O' . ce . on . o . spi . ril . lum. N. L. oceanus, ocean, spirillum. Gr. Noun spira a spiral; M. L. dim. neut. N. Spirillum a small spiral. Oceanospirillum a small ocean spiral.

Rigid helical cells, 0.3-0.8 microns in diameter, ranging from less than one turn to many turns. Motile with bipolar poly-trichous flagella. Gram-negative. Aerobic. Chemoorganotrophic, having a strictly respiratory metabolism for growth with oxygen as the terminal electron acceptor. Indol negative. Hydrolysis of casein, starch, and aesculin negative. Oxidase and catalase positive. Can grow in 1% glycine or 7% added NaCl. Found in marine waters. G + C content of the DNA ranges from 42-48 moles per cent.

Type strain: Oceanospirillum atlanticum

Genus IV. Pseudospirillum gen. nov.

Pseu . do' . spi . ril . lum. Gr. pseudos false; Gr. noun spira; M. L. dim. neut. n. spirillum a small spiral. Pseudospirillum a small false spiral.

Rigid helical cells, 0.8-1.0 microns in diameter, ranging from less than one turn to many turns. Motile with bipolar poly-trichous flagella. Gram-negative. Aerobic. Chemoorganotrophic, having a strictly respiratory metabolism for growth with oxygen as the terminal electron acceptor. Indol negative. Hydrolysis of casein and starch negative. Oxidase and catalase positive. Can grow in 7% NaCl. Can oxidize several hexoses. Found in marine waters. G + C content of

Table XIV. Characteristics of the genera: Spirillum, Aquaspirillum, Oceanospirillum, and Pseudospirillum

Genus	guanosine plus cytosine content of the DNA (in moles per cent, T _m)	obligately microaerophilic	cell diameter (microns)	sea water required for growth	acid reaction from sugars in complex media	anaerobic growth in the presence of nitrate	glycine and tyrosine used as sole carbon source	growth in 7 per cent NaCl in complex media	urease	gelatin hydrolysis	growth in 1% glycine	sero-groups	glucose, gluconate, and mannitol used as sole carbon and energy source	coccoid bodies in older cultures
<u>Spirillum</u>	38	+	1.4-1.7	-	-	-	-	-	-	-	-	not yet defined but unlike other species	-	-
<u>Aquaspirillum</u>	50-65	-	0.2-1.4	-	d	d	-	-	d	d	-	2, 5, 6, 7, 8 9, 10, 12, 13, 15, 17	-	d
<u>Oceanospirillum</u>	42-48	-	0.3-0.8	+	-	-	-	+	-	-	+	1, 3, 11, 14	-	+
<u>Pseudospirillum</u>	63	-	0.8-1.0	+	+	+	+	+	-	-	+	4	+	+

+ = All species positive. - = All species negative.
d = Some species positive and others negative.

the DNA is about 63 moles per cent.

Type species: Pseudospirillum lunatum.

Genus I. Spirillum (Ehrenberg, 1832, 58)

1. Spirillum volutans: (Ehrenberg, 1832, 58)

Strains which fit the generic description of genus Spirillum include strains 41 and 42. The largest of the spirilla, probably first seen by Müller. Helical cells, 1.4 to 1.7 microns in diameter. Wavelength, 16 to 28 microns; diameter of helix, 5 to 8 microns; length of cells, 14 to 60 microns; number of turns, from less than 1 to a maximum of 5. Exceptionally prominent dark granules of polybetahydroxybutyrate in cytoplasm. Motile, possessing a tuft of about 75 flagella at each pole. The polar flagellar fascicles are easily seen in living cells by phase or darkfield. Front and rear fascicles appear to rotate at high speed, forming oriented cones of revolution. Reversal of orientation results in reversal of direction of cell motion. The orientation of the front and rear fascicles is coordinated, and the coordination can be affected reversibly by a variety of compounds (Krieg and I, 1967). Coccoid bodies not predominant in older cultures. Gram negative.

Isolation is difficult, and as yet may be accomplished only by the capillary tube procedure of Rittenberg and Rittenberg (1962).

Nutrient broth: scanty growth. PSS broth: abundant, cloudy growth with flocculation. PSS agar (0.7 per cent agar): pinpoint colonies with fimbriated edge. Water-soluble fluorescent pigment not formed.

Nutrition: Abundant growth occurs in the following medium (g per l): Vitamin-free, salt-free acid hydrolyzed casein (Nutritional Biochemicals Corporation), 6.0; succinic acid, 1.0; $(\text{NH}_4)_2\text{SO}_4$, 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.002; and $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.002. Synthetic casein hydrolysate fails to substitute for the acid hydrolysate, even when supplemented with vitamins, synthetic peptides, or trace minerals. Growth fails to occur in SBM supplemented with any of a variety of sole carbon sources.

Positive tests: oxidase; catalase (weakly); phosphatase; H_2S from cysteine.

Negative tests: urease; hydrolysis of gelatin and aesculin; reduction of nitrate and selenite; DNAase and RNAase; acid reaction from sugars in complex peptone media; growth in the presence of 1 per cent bile or glycine; growth on TSI, EMB, and MacConkey agars, litmus milk, MRVP broth, and Sella's slants; phenylalanine, tyrosine, and tryptophan deaminase; pigment production from these amino acids.

In PSS broth, growth is inhibited by concentrations of added phosphate greater than 0.0029 M (Wells, 1966).

Obligately microaerophilic, requiring an atmosphere of 1 to 9 per cent oxygen (Wells and Krieg, 1965). Growth can be obtained without such conditions by stratifying liquid media with 0.15 percent agar. Microaerotactic. No growth anaerobically with nitrate. Optimum temperature, 30 C. Scanty growth at 25 and 37 C; no growth at 10 or 42 C.

Source: isolated from stagnant pond water in Virginia and also from a mixed culture originally derived from water from the cooling

tower of a sugar beet refinery in England.

The G + C content of the DNA is 38 moles per cent (T_m).

Type strain: ATCC No. 19553. Strain ATCC No. 19554 (Wells strain) is identical in its characteristics even though isolated 14 years later from a different continent.

According to Bergey's Manual of Determinative Bacteriology, 7th ed., and according to the list of type species in Index Bergeyana, Spirillum undula was designated as the type species by Ehrenberg (1832). I believe this is incorrect.

Dr. R. E. Buchanan, Chairman of the Bergey's Manual Board of Trustees, 1968, gives his analysis of the problem as follows: "If you will note on Page 1038 of Index Bergeyana, the following synonyms of Spirillum volutans Ehrenberg are listed: Vibrio spirillum Müller, Spirillum Ehrenbergii Matzschita and Dicrospirillum volutans (Ehrenberg) Enderlein."

"You will note in Index Bergeyana, page 1038, that Spirillum volutans Ehrenberg is based upon the older name Vibrio spirillum Müller 1786, 49. Stiles (1905) in the International Code of Zoological Nomenclature as Applied to Medicine, Bull. Hygienic Laboratory, Washington, D.C. 24, discusses the problem of type species from the standpoint that both Vibrio and Spirillum were proposed as generic names in zoology. He says: "Judged from the zoological point of view, Vibrio spirillum is the type species." Later he says: "If Spirillum is retained in bacteriology, consistency calls for the rejection of the name S. volutans Ehrenberg in favor of Spirillum spirillum Müller."

"This next is a quotation from Buchanan (1925, General Systematic Bacteriology, page 474): "Stiles is in error in holding that the type species should be called Spirillum spirillum, for Article 55 of the Brussels Code reads: "Specific names must be rejected. . . . when they merely repeat the generic name." The opposite is true in zoology. This older reference was apparently overlooked in the preparation of Index Bergeyana and on page 1031 Spirillum undula (Müller) Ehrenberg 1832, 38. (Basionym: Vibrio undula Müller 1773, 43.) is given as the type species. This should have been replaced by Spirillum volutans Ehrenberg. One must recall that in 1925 bacteriology was operating under the botanical code, would suggest you replace your statement on page 2, "Spirillum undula" by Spirillum volutans Ehrenberg as designated by Stiles in 1905." (Dr. R. E. Buchanan, personal communication.)

Aquaspirillum gen. nov.

Strains which fit the generic description of genus Aquaspirillum include strains 1-7, 9-13, 15-31, and 43-44. Table XI shows strains 22-31 are all morphologically similar. For example, the cell diameter of these strains ranges from 0.7-1.0 microns. Furthermore, the guanosine plus cytosine content of the DNA for these ten strains ranges from 50-51 moles per cent (T_m). With the exception of strain 19, this is 6% lower than for any other Aquaspirillum strains. Table II shows these ten strains to be very similar in regard to their nutritional requirements. E.g. all strains utilized L-glutamate and L-aspartate as a sole carbon and energy source. Moreover, 90 per cent of the strains utilized L-alanine as a sole carbon and energy source.

These strains were also very similar in that a very large spectrum of carbon sources were not utilized (Tables I and II). In this regard, only two strains (26 and 28) gave a positive growth response on tri-carboxylic acid (TCA) cycle intermediates and related compounds. The physiological and biochemical study by Wells (1970) also showed these ten strains to be very similar. For instance, 8 of the 10 strains hydrolyzed gelatin, which is in contrast to all other Aquaspirillum strains except strain 7. None of the strains reduced nitrate or selenite, produced an acid reaction from carbohydrates, formed coccoid bodies, or grew anaerobically in the presence of nitrate. Furthermore, the study by McElroy (1970) showed strains of this group to be serologically similar. It is my opinion that all of these similarities justify the placing of these 10 strains together in a single species, A. serpens.

Strains 7, 15-17, 19, and 21 all have large cell diameters (0.9-1.5 microns). Strain 19 is apparently different from strains 15-17 in having a low % G + C content (Table XI), and in being more nutritionally versatile: growth occurs on all TCA cycle intermediates (except citrate), L-lactate, malonate, acetate, beta-hydroxybutyrate, L-histidine, L-alanine, L-glutamate, L-aspartate, L-glutamine, L-asparagine, and L-proline (Tables I and II). Strain 7 is different from strains 15-17 and 21 in having a high % G + C (63% compared to 57-57% for strains 15-17 and 21), and its nutrition differs in that growth occurs on most TCA cycle intermediates and several amino acids (Table I). Furthermore, strain 7 differs from strains 15-17, 19, and 21 in being able to hydrolyze gelatin (Table XI). Table XI shows

strain 7 differs from strain 19 in regard to its per cent G + C (63 moles % for strain 7 as compared to 52 moles % for strain 19), and also in regard to its nutrition: strain 7 fails to yield a positive growth response on aconitate, isocitrate, acetate, beta-hydroxybutyrate, L-histidine, L-alanine, and L-proline (Table I). In my opinion the above considerations appear to justify consideration of strain 7 and strain 19 as belonging to separate species, A. metamorphum and A. putridiconchylum respectively.

Strains 15-17 are very similar in G + C content of the DNA (which ranges from 57-58 moles per cent), DNAase production, phosphatase production, sensitivity to a low concentration of NaCl (Table V), and in being negative for a number of physiological tests including gelatin hydrolysis, nitrate and selenite reduction, acid reaction from carbohydrates, anaerobic growth in the presence of nitrate, fluorescent pigment production, and formation of coccoid bodies (Table XI). Furthermore, although strain 18 is smaller (0.5-0.7 microns in diameter compared to 1.0-1.5 microns for the other three strains), the G + C content of the DNA (57 moles %) is similar to strains 15-17 (57-58 moles % G + C). Moreover, a variety of physiological tests show these four strains to be quite similar (Table XI). Differences occur, however; strain 16 is the only strain to be urease negative, and strain 18 is the only strain to be RNAase negative. The four strains appear to be almost identical with regard to sole carbon sources (Table I), but strain 17 is the only strain to use fumarate, and 16 is the only strain to use glutamate. The serological study by McElroy (1970) showed strains 15, 17, and 18

to belong to the same sero-group. Moreover, he also reported a reciprocal cross-agglutination reaction to occur between strains 16 and 18, thereby indicating these two strains to share similar antigens. It is tempting to combine these four strains into one or two species on the basis of their numerous similarities, but many of the similarities can be seen to be based on a common failure to yield positive results in physiological or nutritional tests. For example, the four strains fail to use most of the sole carbon sources provided (Table I). Stanier et al. (1966) have noted that the inability of a strain to use a given compound as a source of carbon and energy for growth is subject to several possible interpretations. A strain may simply be sensitive to the toxic effects of the compound at the concentration used even though it may possess the complete complement of enzymes necessary for utilization. A strain may similarly possess the enzymes required for utilization, but may lack a single gene controlling the permease for the substrate. Lastly, failure to grow on a compound may be caused by an inability to synthesize one or more of the enzymes necessary for metabolism. This latter could be determined only by a biochemical analysis of the enzymatic activities of the cells. In view of such considerations Stanier et al. concluded that the determination of nutritional spectra, useful as this may be for practical taxonomic purposes, represents only a first approximation (and a very crude one) towards characterizing the phenotype of an organism. Because of this, I would prefer to await the results of DNA hybridization studies before considering the four strains of spirilla in question to be related closely enough to warrant their combination into one or two

species. For the present, I propose that they be considered to belong to separate species. I assign strain 15 to the species A. giesbergeri, strain 16 to A. annulus, strain 17 to A. graniferum, and strain 18 to A. sinuosum.

Strain 21 is apparently different from strains 15, and 17-18 in that it fails to produce urease, but is similar to strain 16 in this respect. Unlike strains 15-18, strain 21 does not exhibit DNAase activity. Further, strain 21 produces a water-soluble fluorescent pigment which is in contrast to strains 15-18. Strain 21 differs from strains 15-17 in that it does not exhibit RNAase activity. The % G + C content of the DNA of strain 21 is 58, closely similar to the values for strains 15-18. The investigation by McElroy (1970) showed strain 21 to be serologically different from strains 15-18.

Strain 21 was designated by the American Type Culture Collection as Spirillum serpens ATCC strain 12289, but it seems clear that it is not closely related to strains 22-31 which I have placed together in the species A. serpens. For example, the strains of A. serpens have a % G + C content of their DNA of 50-51, whereas strain 21 has a value of 58%. Such a difference (7%) in my opinion is too great to warrant inclusion with the strains of A. serpens, and would almost certainly preclude a satisfactory degree of DNA homology (Johnson, 1971). Furthermore, strain 21 differs from the A. serpens strains in that it fails to use any sole carbon source tested. Strain 21 would appear to be more closely related to strains 15-18 than to A. serpens strains; however, I feel compelled to wait for additional DNA hybridization results before deciding the degree of relationship. For the

present, I prefer simply to designate this strain as Aquaspirillum Group A.

The remaining strains of Aquaspirillum all have cell diameters which range from 0.2-0.8 microns (Table XI). Furthermore, the G + C content of this group ranges from 62-65 moles per cent. Strains 2-4 are very similar in regard to cell diameter (0.2-0.3 microns), G + C ratio (65 moles per cent), acid production from the same spectrum of carbohydrates (Table XIII), similar physiologically (Table XI), and nutritionally (Tables I-IV). In my opinion, these numerous similarities seem to justify placing these three strains together in a single species, A. gracile.

Strains 11-13 and 43-44 are all similar in regard to cell diameter (0.4-0.6 microns), G + C ratio (62 moles per cent), nitrate reduction, RNAase, DNAase, phosphatase, acid reaction from the same spectrum of carbohydrates (Table XIII), anaerobic growth in the presence of nitrate, fluorescent pigment production, formation of coccoid bodies in older cultures, and the spectrum of compounds which can be utilized as sole carbon and energy sources (Tables I-IV). Further, McElroy (1971) found all five strains to belong to the same sero-group. In my opinion, these five strains should all belong to the same species, A. itersonii, because of their many similarities.

Although strain 10 is similar morphologically to A. itersonii, it differs in regard to urease production, nitrate reduction, production of RNAase, DNAase, anaerobic growth in the presence of nitrate, formation of coccoid bodies, the spectrum of carbohydrates which can be oxidized, and pigment production in the presence of 0.2% L-phenylalanine (Tables I-II). Moreover, Tables I and II show distinct

differences in the nutritional pattern of this strain as compared to all other Aquaspirillum species. In my opinion, strain 10 should remain separated from the other aquaspirilla in a distinct species, A. peregrinum, because of these extensive differences.

Strains 5 and 9 are very similar morphologically (Table XI). However, Table XI also shows marked physiological and biochemical dissimilarities between these two strains. For example, these two strains differ in regard to selenite reduction, RNAase and DNAase production, phosphatase production, anaerobic growth in the presence of nitrate, fluorescent pigment production, coccoid body formation, and growth in the presence of 1% glycine. Moreover, Table XII shows marked differences in the nutritional patterns of these two strains. In my opinion, strains 5 and 9 should be retained in distinct species, A. delicatum and A. polymorphum, respectively, because of numerous differences that distinguish them from each other and from other aquaspirilla.

Strains 1 and 6 are new isolates of spirilla which are very similar to each other in regard to all morphological and physiological traits (Wells, 1970). The nutritional patterns of these two strains are very similar (Table I). In regard to compounds which can be used as sole carbon and energy sources, ethanol and L-glutamate are the only compounds which were variable. Moreover, Tables I and II show these two strains to be nutritionally different from all other Aquaspirillum species. For example, strains 1 and 6 were the only strains of Aquaspirillum to use L-ornithine and L-citrilline as a sole carbon and energy source. The study by Wells (1970) similarly showed these

two strains to be different from other Aquaspirillum species in regard to the pattern of their physiological reaction (Table XI). In my opinion, these two strains should be designated as a new species, A. mobile.

Strain 20 is apparently different in its pattern of physiological reactions from other Aquaspirillum strains (Table XI). In regard to cell diameter and % G + C content, strain 20 resembles strains 10-13, 43-44, 1, and 6. Unlike strain 10-13 and 43-44, strain 20 fails to produce acid reactions from sugars. It differs from all of the above strains in that it fails to use any sole carbon sources tested (Tables I and II). McElroy (1970) also found that strain 20 differed serologically from all other strains tested. This strain is designated by the American Type Culture Collection as Spirillum serpens ATCC 11330, but is clearly distinct from the other strains of A. serpens (strains 22-31). E.g., its % G + C content of the DNA is 65, compared to 50-51 for strains of A. serpens. Other differences can be found by inspection of Table XI. I suggest that this strain be designated as Aquaspirillum Group B for the present.

1. Aquaspirillum serpens (Müller Winter 1884, 63) comb. nov.

ser' . pens L. v. serno to crawl or creep; L. part. adj. serpens creeping.

Helical cells, 0.6 to 1.0 microns in diameter. Wavelength, 3.5 to 12 microns; diameter of helix, 1.2 to 2.8 microns; length of cells, 5 to 35 microns; number of turns, from less than one to two. Dark granules of polyhydroxybutyrate present in cytoplasm. Motile, possessing a tuft of flagella at each pole. By electron microscopy, the

basal structure of the flagella appears to be similar to that of peritrichous flagella in Proteus vulgaris and other gram-negative organisms. Each flagellum originates within the protoplast in an individual distinct vesicle and has a separate insertion site in the cell wall. Visibility of flagellar tufts with darkfield differs among strains. Coccoid bodies not predominant in older cultures. The cell wall is composed of an inner peptidoglyson layer, a protein granule layer, a lipopolysaccharide layer, and an external lipoprotein layer. The peptidoglycan contains alanine, glutamate, and meso-diaminopimelate, and is tightly knit, with 54 per cent of the DAP molecules involved in cross-linkage between tetrapeptides.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-succinate-salts agar: circular, convex, white colonies, 1.0 to 2.2 mm. Some strains produce a yellow-green water-soluble fluorescent pigment.

Standard sole carbon sources: From 21 to 79 per cent of strains use glutamate, aspartate, alanine, glutamine, proline, serine, and asparagine. Other Krebs cycle acids, other amino acids, glycerol, lactate, pyruvate, acetate, ethanol, butanol, and sugars not utilized. Standard sole nitrogen sources: glutamate. From 21 to 79 per cent of strains use aspartate, alanine, glutamine, histidine, proline, arginine, leucine, valine, lysine, serine, asparagine, ornithine, citrulline, threonine, and cysteine. Other amino acids nitrate, urea, putrescine, and ammonium ions not used.

Positive tests: oxidase; catalase; phosphatase; RNAase and DNAase; H₂S from cysteine; growth on TSI agar (no change) and EMB agar, and in the presence of 1 per cent bile or 1 per cent glycine.

Some strains grow on MacConkey agar, MRVP broth, and on Seller's slants (when positive, slant only is alkaline, with greenish colonies on surface). Some strains hydrolyze gelatin.

Negative tests: urease (except strain 28); hydrolysis of aesculin; reduction of selenite and nitrate; phenylalanine, tyrosine, and tryptophan deaminase; growth in litmus milk or in the presence of 3 per cent salt; acid reaction from sugars in complex peptone media.

Strictly aerobic. Anerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: fresh water; aborted bovine fetuses (initially mistaken for Campylobacter fetus).

The G + C content of the DNA ranges from 50-51 moles per cent (T_m).

Type strain: ATCC No. 12638.

2. Aquaspirillum putridiconchylum (Terasaki 1961, 79) comb. nov.
pu' . tri . di . con . chy . li . um. L. noun n. putridi-
conchylum decayed shellfish.

Helical cells, 1.0 to 1.2 microns in diameter. Wavelength, 6 to 7 microns; diameter of helix, 1.8 to 2.0 microns; length of cells, 4 to 22 microns; number of turns, from less than one to a maximum of four, but usually three. Dark granules present in cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagellar tufts are straight and easily seen with living cells by darkfield but not phase. Coccoid bodies not predominant in older cultures. Gram negative.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-

succinate-salts agar: circular, convex, white colonies, 1.5 to 2.0 mm. A yellow-green water-soluble fluorescent pigment is weakly produced.

Standard sole carbon sources are ketoglutarate, succinate, fumarate, malate, oxaloacetate, isocitrate, pyruvate, lactate, acetate, malonate, glutamate, aspartate, alanine, glutamine, histidine, beta-hydroxybutyrate, proline, and asparagine. Other Krebs cycle acids, other amino acids, sugars, propionate, glycerol, ethanol, propanol, and butanol not utilized. Standard sole nitrogen sources: ammonium ions, glutamate, aspartate, alanine, glutamine, histidine, proline, asparagine, cysteine, methionine, threonine, valine, and citrulline. Nitrate, urea, and other amino acids not utilized.

Positive tests: oxidase; phosphatase, catalase; H_2S from cysteine; RNAase; growth in the presence of 1 per cent bile and on TSI (no change) and EMB agars.

Negative tests: urease; hydrolysis of gelatin and aesculin; reduction of nitrate and selenite; DNAase; phenylalanine, tyrosine, and tryptophan deaminase; acid reactions from sugars in complex peptone media; growth on MacConkey agar, litmus milk, Sella's slants, and in the presence of 1 per cent glycine or 3 per cent salt.

Strictly aerobic. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; scanty growth at 42 C; no growth at 10 or 45 C.

Source: isolated from putrid infusion of a fresh-water shellfish.

The G + C content of the DNA of the type strain is 52 moles per cent (T_m).

Type strain: ATCC No. 15279.

3. Aquaspirillum giesbergeri (Williams and Rittenberg 1957, 88)
comb. nov.

gies' . ber . ger . i. M.L. gen. noun. giesbergeri of Giesberger,
the first investigator of the genus to define certain physiological
characteristics useful in identification.

Helical cells, 1.1 to 1.4 microns in diameter. Diameter of
helix, 2.0 to 2.5 microns; length of cells, 4 to 10 microns; number
of turns, less than one. Exceptionally prominent dark granules in
cytoplasm. Motile, possessing a tuft of flagella at each pole; tufts
are helical and can be seen with living cells by darkfield but not
phase. Coccoid bodies not predominant in older cultures. Gram
negative.

Peptone-succinate-salts broth: moderate growth, with bottom sed-
iment and clear supernatant. Slow-growing. When 0.15 per cent agar is
incorporated, a microaerophilic band of growth forms a few millimeters
below the surface of the medium; as growth increases, this band eventu-
ally moves to the surface. Peptone-succinate-salts agar: circular,
convex, white colonies, 1.0 mm. Water-soluble fluorescent pigment not
produced.

Nutrition: growth does not occur in defined succinate-ammonium
sulfate-salts medium; however, upon incorporation of 0.15 per cent
agar, a microaerophilic band forms 3 to 6 mm below the surface of the
medium; even after continued growth, this band fails to migrate to the
surface. The nutrition of this species has not yet been studied under
microaerophilic conditions, and the use of defined media under aerobic
conditions has yielded scanty growth and conflicting results.

Positive tests: oxidase; catalase; phosphatase; H_2S from cysteine; RNAase and DNAase; growth in the presence of 1 per cent bile; growth in MRVP broth; RNAase and DNAase.

Negative tests: urease; hydrolysis of gelatin and aesculin; reduction of nitrate and selenite; phenylalanine, tyrosine, and tryptophan deaminase; growth on TSI, EMB, and MacConkey agars, litmus milk, or in the presence of 1 per cent glycine or 3 per cent salt; acid reactions from sugars in complex peptone media.

Facultative microaerophile. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 C; scanty growth at 37 C; no growth at 10 or 42 C.

The G + C content of the DNA of the type strain is 58 moles per cent (T_m).

Type strain: ATCC No. 11334.

4. Aquaspirillum graniferum (Williams and Rittenberg 1957, 92)
comb. nov.

gra . ni' . fer . um. L. adj. granifer grain-bearing.

Helical cells, 1.0 to 1.2 microns in diameter. Wavelength, 7.0 to 8.4 microns; diameter of helix, 3.5 to 4.2 microns; length of cells, 7 to 17 microns; number of turns, one to three. Dark granules present in cytoplasm. Motile, possessing a tuft of flagella at each pole; tufts can be easily seen with living cells by darkfield but not phase. Coccoid bodies not predominant in older cultures. Gram negative.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-succinate-salts agar: circular, convex, translucent colonies, 1.0 mm. Water-soluble fluorescent pigment not produced.

Standard sole carbon sources: fumarate and pyruvate. Other Krebs cycle acids, amino acids, sugars, malonate, acetate, propionate, propanol, butanol, ethanol, lactate, and glycerol not utilized.

Standard sole nitrogen sources: L-glutamate, L-aspartate, L-glutamine, L-asparagine, and urea. Other amino acids, putrescine, and nitrate are not utilized. It should be noted that when fumarate or malate are used singly as carbon sources, ammonium ions can serve as a sole nitrogen source.

Positive tests: oxidase, catalase; phosphatase; RNAase; DNAase; growth in the presence of 1 per cent bile and in MRVP broth.

Negative tests: urease; hydrolysis of gelatin and aesculin; reduction of nitrate and selenite; H_2S from cysteine; growth in the presence of 1 per cent glycine or 3 per cent salt; growth on TSI, EMB, and MacConkey agars, and litmus milk; acid reaction from sugars in complex peptone media.

Strictly aerobic. Growth does not occur anaerobically in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: fresh water.

The G + C content of the DNA of the type strain is 57 moles per cent (T_m).

Type strain: NCIB No. 8230.

5. Aquaspirillum anulus (Williams and Rittenberg 1957, 86) comb. nov.

an' . u . lus. L. noun m. anulus a ring.

Helical cells, 1.4 to 1.5 microns in diameter. Wavelength, 4.9 to

7.0 microns; diameter of helix, 2.8 to 3.0 microns; length of cells, 7 to 15 microns; number of turns, one to three. Dark intracellular granules of polyhydroxybutyrate present. Motile, possessing a tuft of flagella at each pole; tufts are visible by darkfield with living cells. Coccoid bodies not predominant in older cultures. Gram negative.

Peptone-succinate-salts broth: abundant, cloudy growth.

Peptone-succinate-salts agar: pinpoint, circular, white colonies.

Water-soluble fluorescent pigment not formed.

Sole carbon sources: pyruvate and L-glutamate. Sole nitrogen source: L-glutamate.

Positive tests: oxidase; catalase; phosphatase; H_2S from cysteine; RNAase; DNAase.

Negative tests: urease; hydrolysis of gelatin and aesculin; reduction of nitrate and selenite; phenylalanine, tyrosine, and tryptophan deaminase; acid reactions from sugars in complex peptone media; growth in the presence of 1 per cent bile or glycine, or of 3 per cent salt; growth on TSI, EMB, or MacConkey agars, litmus milk, MRVP broth, and Sella's slants.

Strictly aerobic. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: isolated from pond water.

The G + C content of the DNA of the type strain is 58 moles per cent (T_m).

Type strain: ATCC No. 19259.

6. Aquaspirillum sinuosum (Williams and Rittenberg, 1957) comb. nov.

Helical cells, 0.5 to 0.7 microns in diameter. Wavelength, 8.5 to 10.5 microns; diameter of helix, 1.4 to 3.5 microns; length of cells, 5 to 20 microns; number of turns, 1 to 2. Dark granules are present in the cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagellar tufts cannot be seen with darkfield microscopy. Coccoid bodies are not predominant in older cultures. Gram negative.

Peptone-succinate-salts broth: abundant, cloudy growth.

Peptone-succinate-salts agar: circular, convex, white colonies, 1.0 mm. Water-soluble fluorescent pigment not produced.

Standard sole carbon sources: pyruvate. Other Krebs cycle acids, acetate, propionate, ethanol, propanol, butanol, glycerol, amino acids, and sugars cannot be utilized. Standard sole nitrogen sources: none has been found to be effective, as yet.

Positive tests: oxidase; phosphatase; catalase; DNAase; H_2S from cysteine; growth in the presence of 1 per cent bile.

Negative tests: urease; hydrolysis of gelatin and aesculin; RNAase; reduction of selenite and nitrate; phenylalanine, tyrosine, and tryptophan deaminase; acid reaction from sugars in complex peptone media; growth on TSI, EMB, and MacConkey agars, MRVP broth, litmus milk, Sella's slants, or in the presence of 1 per cent glycine or 3 per cent salt.

Strictly aerobic. No growth anaerobically in the presence of nitrate. Optimum temperature: 30 to 32 C. Moderate growth at 25 C; scanty growth at 37 C; no growth at 10 or 42 C.

Source: fresh water.

The G + C content of the DNA of the type strain is 57 moles per

cent (T_m).

Type strain: ATCC No. 9786.

7. Aquaspirillum Group A.

Helical cells, 0.9-1.0 microns in diameter. Wavelength, 4.2-5.0 microns; diameter of helix, 2.8-4.2 microns; length of cells 4.0-22.0 microns. Dark granules in cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagella tufts can be seen with darkfield microscopy. Gram-negative.

Peptone-succinate-salts broth: moderate, cloudy growth. Peptone-succinate-salts agar: circular, convex, white, colonies 1.0 mm. A yellow-green fluorescent pigment is produced.

Nutrition: The nutritional requirements of this strain have not been defined. However, no growth occurred on any of the 67 compounds tested as sole carbon and energy sources.

Positive tests: catalase; oxidase; phosphatase; and H_2S from cysteine. All other tests negative.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Slight growth at 25 and 37 C; no growth at 10 or 42 C.

Source: fresh water

The G + C content of the DNA of this strain is 58 moles per cent (T_m).

Type strain: ATCC No. 12289.

8. Aquaspirillum peregrinum (Pretorius, 1965. 407). comb. nov.
per . e . gri' . num. L. adj. peregrinum strange, foreign.

Helical cells, 0.5 to 0.7 microns in diameter. Wavelength, 3.5 to 4.0 microns; diameter of helix, 1.4 to 1.6 microns; length of cells, 5 to 22 microns; number of turns, 4 to 5. Dark granules in cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagellar tufts cannot be seen with darkfield microscopy. Coccoid bodies not predominant in older cultures. Gram negative.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-succinate-salts agar: circular, convex, white colonies, 1.0 to 1.5 mm. A yellow-green water-soluble fluorescent pigment is produced.

Standard sole carbon sources: succinate, ketoglutarate, fumarate, malate, pyruvate, lactate, malonate, acetate, glutamate, aspartate, alanine, beta-hydroxybutyrate, glutamine, proline, arginine, hydroxyproline, asparagine, ornithine, citrulline, and fructose. Other Krebs cycle acids, other amino acids, other sugars, glycerol, propionate, ethanol, propanol, and butanol not utilized. Standard sole nitrogen sources: ammonium ions, glutamate, aspartate, alanine, glutamine, histidine, proline, arginine, hydroxyproline, lysine, asparagine, ornithine, citrulline, leucine, cysteine, methionine, threonine, and urea. Other amino acids, and nitrate not utilized.

Positive tests: oxidase, catalase; phosphatase; H_2S from cysteine; urease; reduction of selenite; hydrolysis of aesculin; growth in the presence of 1 per cent bile; growth on EMB agar, TSI agar (no change), litmus milk (no change), and Sella's slants (surface growth only, greenish colonies, alkaline reaction only in butt); yellow water-soluble pigment production in the presence of 0.2 per cent

phenylalanine: acid reaction from fructose (but no other sugar) in complex peptone media.

Negative tests: hydrolysis of gelatin; RNAase and DNAase; reduction of nitrate; phenylalanine, tyrosine, and tryptophan deaminase; growth on MacConkey agar, MRVP broth, or in the presence of 1 per cent glycine or 3 per cent salt.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: water from a primary oxidation pond.

The G + C content of the DNA of the type strain is 62 moles per cent (T_m).

Type strain: ATCC No. 15387.

9. Aquaspirillum itersonii (Giesberger 1936. 68) comb. nov.

i . ter . so' . ni . i. M. L. gen. noun itersonii of Itersoni named for G. Van Iterson, A Dutch bacteriologist.

Helical cells, 0.4 to 0.6 microns in diameter. Wavelength, 3.0 to 3.5 microns; diameter of helix, 1.2 to 1.6 microns; length of cells, 2.0 to 7 microns; number of turns, from less than one to two. Dark granules of polyhydroxybutyrate present in cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagellar tufts cannot be seen with darkfield microscopy. Coccoid bodies not predominant in older cultures. Gram negative.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-succinate-salts agar: circular, convex, white colonies, 0.8 to 1.5 mm. A yellow-green water-soluble fluorescent pigment is produced.

Standard sole carbon sources: succinate, ketoglutarate, fumarate, malate, glutamate, aspartate, glutamine, histidine, proline, lysine, phenylalanine, asparagine, ornithine, citrulline, propanol, and ethanol. From 21 to 79 per cent of strains use oxaloacetate, pyruvate, lactate, malonate, propionate, alanine, arginine, hydroxyproline, cystine, leucine, valine, butanol, and fructose. Other Krebs cycle acids, other amino acids, other sugars, acetate, and glycerol are not utilized. Standard sole nitrogen sources: ammonium ions, glutamate, aspartate, alanine, glutamine, proline, arginine, hydroxyproline, methionine, cystine, leucine, lysine, histidine, phenylalanine, asparagine, cysteine. From 21 to 79 per cent of strains use nitrate and serine. Remaining amino acids, urea, and putrescine are not utilized.

Positive tests: oxidase; phosphatase; catalase; H_2S from cysteine, hydrolysis of aesculin; RNAase and DNAase; reduction of nitrate to ammonia or nitrogen; growth in the presence of 1 per cent bile; growth on TSI (no change), EMB, and MacConkey agars, and Sella's slants (growth and alkaline reaction in butt and slant, greenish colonies on slant); yellow water-soluble pigment production in the presence of 0.2 per cent tryptophan; brown water-soluble pigment production in the presence of 0.2 per cent tyrosine. Some strains grow in MRVP broth and reduce selenite.

Negative tests: urease; hydrolysis of gelatin; phenylalanine deaminase; growth in the presence of 1 per cent glycine or 3 per cent salt; growth in litmus milk.

Strictly aerobic; however, anaerobic growth occurs in the

presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: pond water.

The G + C content of the DNA is 62 moles per cent (T_m).

Type strain: ATCC No. 12639.

10. Aquaspirillum metamorphum (Terasaki 1961. 220).

me . ta . morph' . um. Gr. adj. metamorphum changing.

Helical cells, 1.1 to 1.3 microns in diameter. Diameter of helix, 2.8 to 3.0 microns; length of cells 4 to 9 microns; number of turns, less than one. Dark granules present in cytoplasm. Motile, possessing a tuft of flagella at each pole; tufts are not seen by darkfield. Coccoid bodies not predominant in older cultures. Gram negative.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-succinate-salts agar: circular, convex, translucent colonies, 2.0 mm. A yellow-green water-soluble fluorescent pigment is produced.

Standard sole carbon sources: succinate, ketoglutarate, fumarate, malate, malonate, pyruvate, lactate, glutamate, aspartate, and glutamine. Other Krebs cycle acids, other amino acids, sugars, acetate, propionate, glycerol, ethanol, propanol, and butanol not utilized. Standard sole nitrogen sources: aspartate, leucine, phenylalanine, tyrosine, cysteine, and serine. Other amino acids, ammonium ions, nitrate, and urea are not utilized.

Positive tests: oxidase; catalase; phosphatase; H_2S from cysteine; hydrolysis of gelatin (when tubes of 12 per cent gelatin are employed); growth in the presence of 1 per cent bile; growth on TSI

(no change) and EMB agars, MRVP broth, and on Seller's slants (growth on slant only, greenish colonies, alkaline reaction in slant only).

Negative tests: urease; hydrolysis of aesculin; reduction of nitrate and selenite; RNAase; DNAase; phenylalanine, tyrosine, and tryptophan deaminase; growth on MacConkey agar, litmus milk, or in the presence of 3 per cent salt or 1 per cent glycine; acid reactions from sugars in complex peptone media.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; scanty growth at 42 C; no growth at 10 or 45 C.

Source: putrid infusion of a fresh-water shellfish.

The G + C content of the DNA of the type strain is 63 moles per cent (T_m).

Type strain: ATCC No. 15280.

11. Aquaspirillum polymorphum (Williams and Rittenberg, 1957.
85) comb. nov.

po . ly . mor' . phum. Gr. adj. polymorphus multiform

Helical cells, 0.3 to 0.5 microns in diameter. Diameter of helix, 0.5 to 0.8 microns; length of cells, 3.5 to 8.4 microns; number of turns, less than one Dark granules are present in cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagellar tufts cannot be seen by darkfield microscopy. Coccoid bodies predominate in older cultures. Gram negative.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-succinate-salts agar: circular, convex, translucent, pinpoint colonies. Yellow-green water-soluble fluorescent pigment produced.

Standard sole carbon sources: ketoglutarate, acetate, malonate, glutamate, aspartate, glutamine, and asparagine. Other Krebs cycle acids, other amino acids, sugars, pyruvate, lactate, glycerol, propionate, ethanol, propanol, and butanol not used. Standard sole nitrogen sources: glutamate, ammonium ions, aspartate, alanine, glutamine, proline, serine, asparagine, threonine, citrulline, and cysteine. Other amino acids, urea, and nitrate, utilized; however, when standard sole carbon sources are used singly, ammonium ions can serve as a sole nitrogen source.

Positive tests: oxidase; catalase; H_2S from cysteine; hydrolysis of aesculin; reduction of nitrate to nitrite; reduction of selenite (weakly); DNAase (weakly); growth in the presence of 1 per cent bile and on EMB and MacConkey agars.

Negative tests: phosphatase; urease; hydrolysis of gelatin; RNAase; phenylalanine, tyrosine, and tryptophan deaminase; growth in the presence of 1 per cent glycine and of 3 per cent salt; growth on TSI agar, MRVP broth, litmus milk, and Sella's slants; acid reactions from sugars in complex peptone media.

Strict aerobe. Anaerobic growth fails to occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: pond water

The G + C content of the DNA of the type strain is 62 moles per cent (T_m).

Type strain: ATCC No. 11332 or NCIB 9072.

12. Aquaspirillum delicatum (Leifson, 1962. 164) comb. nov.

Helical cells, 0.3 to 0.4 microns in diameter. Diameter of helix, 0.4 to 0.7 microns; length of cells, 3 to 5 microns; number of turns, less than one. Cytoplasmic granules not evident, probably because of small cell diameter. Motile, said to possess predominantly a single polar flagellum. Leifson places this species provisionally in genus Spirillum but suggests that it might be included more appropriately with the non-fermentative vibrios, i.e., Campylobacter. Flagella cannot be seen with darkfield microscopy. Coccoid bodies not predominant in older cultures.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-succinate-salts agar: small, circular, white colonies, ca. 0.5 mm. Water-soluble fluorescent pigment not produced.

Standard sole carbon sources: ketoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, lactate, malonate, acetate, asparagine, leucine, and putrescine. Other Krebs cycle acids, other amino acids, sugars, propionate, propanol, ethanol, butanol, and glycerol not utilized. Standard sole nitrogen sources: valine, methionine, threonine, hydroxyproline, glutamate, leucine, phenylalanine, cysteine, and putrescine. Other amino acids, nitrate, ammonium ions, and urea are not utilized; however, when standard carbon sources are used singly, ammonium ions can serve as a sole nitrogen source.

Positive tests: oxidase; phosphatase; catalase; RNAase; H₂S from cysteine; reduction of nitrates to nitrites; growth on TSI agar (no change) and in MRVP broth.

Negative tests: urease; hydrolysis of gelatin and aesculin; reduction of selenite; DNAase; phenylalanine, tyrosine, and tryptophan

deaminase; acid reaction from sugars in complex peptone media; growth in the presence of 1 per cent bile, 1 per cent glycine, or 3 per cent salt; growth on EMB and MacConkey agars, litmus milk, and Sella's slants.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: isolated from distilled water.

The G + C content of the DNA of the type strain is 63 moles per cent (T_m).

Type strain: ATCC No. 14667.

13. Aquaspirillum gracile (Canale-Parole, Rosenthal, and Kupfer 1966, 113) comb. nov.

gr. cil' . e. L. adj. gracile slender or thin.

Helical cells, 0.25 to 0.3 microns in diameter. Wavelength, 2.8 to 3.5 microns; diameter of helix, 0.5 to 2.0 microns; length of cells, 4 to 14 microns; number of turns, 3 to 4. Cytoplasmic granules not evident, probably because of small cell diameter. Motile, possessing a tuft of flagella at each pole. Flagellar tufts cannot be seen with darkfield. Coccoid bodies not predominant in older cultures. Gram negative.

Peptone-succinate-salts broth: moderate, cloudy growth. Peptone-succinate-salts agar: white, pinpoint colonies, with a large part of the colony embedded in the agar. When originally isolated, Canale-Parole, Rosenthal, and Kupfer noted that all strains formed subsurface, spreading, semi-transparent non-pigmented colonies, the spreading

occurring within the agar. They noted that after prolonged sub-culturing, some of the spirilla in each strain lost the ability to diffuse through agar and formed smaller, non-spreading, well-defined colonies, similar to those observed in recent analysis of these strains. Some strains produce a yellow-green water-soluble fluorescent pigment.

Standard sole carbon sources: on recent analysis, no compound has been found to serve as sole carbon source when a large variety of Krebs cycle acids, monocarboxylic acids, sugars, alcohols, and amino acids were tested. This is contrasted with the report of Canale-Parole, Rosenthal, and Kupfer, who indicated that glutamate, aspartate, and cysteine could serve as sole carbon and nitrogen sources in a defined mineral salts medium containing vitamins.

Positive tests: oxidase; phosphatase; catalase; H_2S from cysteine; RNAase; acid reaction from glucose, galactose, and arabinose in complex peptone media (but from no other sugars); growth in the presence of 1 per cent bile and also on EMB agar (after 7 days). Nitrates are reduced to nitrites (in contrast to the original report of Canale-Parole, Rosenthal, and Kupfer). DNAase is positive for some strains.

Negative tests: urease; hydrolysis of gelatin and aesculin; reduction of selenite; phenylalanine, tyrosine, and tryptophan deaminase; growth in the presence of 1 per cent glycine or 3 per cent salt; growth on TSI and MacConkey agars, litmus milk, MRVP broth, and Sella's slants.

Although recent analysis indicates that this species is strictly

aerobic and does not form microaerophilic bands in semi-solid peptone-succinate-salts agar, Canale-Parole, Rosenthal, and Kupfer reported that the spirilla developed a few mm below the surface of semi-solid media, forming one to five growth discs. The thickness of the discs varied from a fraction of a mm to several mm, and their distance from the surface of the medium changed during incubation. Sometimes a growth disc was present immediately under the surface. This behavior, together with the tendency of the spirilla to form subsurface colonies, indicates that low oxygen pressures are optimal for the growth of the spirilla. It is likely that available isolates have undergone some change in their oxygen requirements during intervening years.

No growth anaerobically in the presence of nitrate. Optimum temperature, 30 to 32 C. Slight growth at 25 and 37 C; no growth at 10 or 42 C.

Source: isolated from surface waters by penetration of a membrane filter into underlying agar.

The G + C content of the DNA is 65 moles per cent (T_m).

Type strain: ATCC No. 19624.

14. Aquaspirillum mobile sp. nov.

mo' . bi . le. L. adj. mobilis; movable, motile.

Helical cells, 0.5-0.8 microns in diameter. Diameter of helix, 1.0-21. microns; length of cells, 2.0-6.0 microns; number of turns, less than one to 3 turns. Dark granules of poly-beta-hydroxybutyrate present in cytoplasm. Motile, possessing a tuft of flagella at each pole. Electron microscopy indicates this species has 10-12 flagella

at each pole. Gram negative.

Peptone-succinate-salts broth; abundant, cloudy growth. Peptone-succinate-salts agar: circular, convex, translucent colonies, 2.0 mm. A yellowish-green water-soluble fluorescent pigment is produced.

Standard sole carbon sources: citrate, aconitate, isocitrate, alpha-ketoglutarate, succinate, fumarate, L-malate, oxaloacetate, pyruvate, L-lactate, malonate, acetate, propionate, caproate, beta-hydroxybutyrate, L-alanine, L-aspartate, L-proline, L-citrulline. Ethanol and L-glutamate was found to be variable among strains of this species. Standard sole nitrogen sources: L-alanine, L-glutamate, L-aspartate, L-glutamine, L-asparagine, L-proline, L-ornithine, L-histidine, L-citrulline, L-arginine, L-leucine, and ammonium ions. L-phenylalanine, L-serine, L-valine, and L-threonine were found to be variable among strains of this species.

Positive tests: oxidase; catalase; phosphatase; H_2S from cysteine; growth in the presence of 1% bile and 3% NaCl; growth on TSI (no change); EMB agars; MRVP broth; and on Sella's slants; reduction of nitrate and selenite; growth in 1% glycine.

Negative tests: urease; hydrolysis of aesculin; RNAase; DNAase; gelatin hydrolysis; phenylalanine, tyrosine, and tryptophan deaminase; and acid reaction from carbohydrates in complex media.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 45 C.

Source: isolated from fresh-water sources.

The G + C content of the DNA ranges from 63-65 moles per cent (T_m).

Type strain: Strain 104.

15. Aquaspirillum Group B

Helical cells, 0.5-0.6 microns in diameter. Wavelength, 2.0-2.8 microns, diameter of helix, 0.8-1.0 microns; length of cells, 2.0-7.0 microns; number of turns, from one to three. Dark granules present in cytoplasm. Motile, possessing a tuft of flagella at each pole. Electron micrographs indicate this strain to have from 6-15 flagella at each pole. Gram-negative.

Peptone-succinate-salts broth: abundant, cloudy growth. Peptone-succinate-salts agar: circular, convex, translucent, pinpoint colonies. Yellowish-green water-soluble fluorescent pigment produced.

Nutrition: the nutrition of this strain has not been defined. Growth does not occur on any of the standard sole carbon and nitrogen sources.

Positive tests: oxidase; catalase; H_2S from cysteine; phosphatase; reduction of nitrate to nitrite; selenite reduction; RNAase; DNAase; growth in 1% bile; TSI agars (no change); MRVP broth; EMB and MacConkey agars. A water-soluble fluorescent pigment is produced.

Negative tests: urease; hydrolysis of gelatin and aesculin; phenylalanine, tyrosine, and tryptophan deaminase; growth in the presence of 1% glycine and 3% NaCl; acid reaction from sugars in complex media.

Strict aerobe. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37; no growth at 10 or 42 C.

Source: fresh-water.

The G + C content of the DNA of the type strain is 65 moles per cent (T_m).

Type strain: ATCC No. 11330.

Oceanospirillum gen. nov.

Strains 32-40 fit the generic description of the genus Oceanospirillum. Strains 39 and 40 differ from other oceanospirilla by having the lowest G + C ratio (42%) (Table XIV), by being able to reduce nitrate, and by growing on succinate, alpha-ketoglutarate, fumarate, and L-proline. Moreover, these two strains are serologically different from other Oceanospirillum strains (McElroy, 1971). McElroy (1971) also reported strains 39 and 40 to be serologically identical to each other. In my opinion, strains 39 and 40 should be considered as belonging to the same species, O. minutulum.

Strains 32 and 33 are apparently different from all other Oceanospirillum in that both strains require L-methionine as a sole nitrogen source (Table IV). Furthermore, McElroy (1971) reported these two species to be identical in regard to their serological cross-reactivity and different from all other Oceanospirillum strains, in this regard. The study by Wells (1970) showed these two strains to have the highest % G + C content of any oceanospirilla (both were 48%). I recommend these two strains be designated as belonging to the same species, O. atlanticum.

Strain 38 is the only Oceanospirillum species which had a tuft of flagella which can be seen under darkfield (Wells, 1970). This strain also has the largest cell diameter (0.7-0.8 microns) of any Oceanospirillum strain. Furthermore, Table XV shows this strain to

have a unique nutritional spectrum. McElroy (1971) reported this strain to be serologically different from other Oceanospirillum strains. I suggest this strain be retained in a separate species, O. japonicum.

Strain 34 has the same cell diameter as strains 35-37. However, Table XV shows strain 34 to be different from strains 35-37 in regard to RNAase, DNAase, and phosphatase production. Table XVI shows the nutritional patterns of strains 34 differs from strains 35-37. McElroy (1970) reported this species to differ serologically from all other Oceanospirillum strains. I suggest this strain be retained as belonging to a separate species, O. beijerinckii.

Strains 35-37 are new isolates of Oceanospirillum. These three strains are similar to each other in regard to their % G + C content of the DNA (45-46 moles %), cell diameters (0.5-0.7 microns), most physiological tests (Table XV), and most nutritional tests (Table XVI). These three strains appear to be most similar to strains 34 and 38. However, the differences occurring between strain 34 and 35-37 have already been discussed above. Strains 35-37 differs from strain 38 in regard to cell diameter (Table XV), and physiologically in that strains 35-37 is phosphatase negative and strain 38 is phosphatase positive. Moreover, strains 35-37 differs in the spectrum of compounds which can serve as a sole carbon and energy source (Table XVI). For example, strain 38 can use pyruvate, L-lactate, acetate, L-alanine, and L-glutamine as a sole carbon and energy source which is in contrast to strains 35-37. I suggest these three strains be designated as a new species, Oceanospirillum maris.

Table XV: Morphological and Physiological Characteristics of Marine Strains of Spirillum, from Wells, 1970

Species	guanosine plus cytosine content of the DNA (in moles per cent, T _m)	cell diameter (microns)	helix diameter (microns)	wavelength (microns)	cell length (microns)	nitrate reduction	selenite reduction	RNAase	DNAase	phosphatase	*fluorescent pigment production
39 & 40	42	0.3-0.5	0.6-1.5	2.0-2.8	3.0-8.0	+	+	-	-	-	-
38	45	0.7-0.8	1.0-3.0	8.0-20.0	20.0-75.0	-	+	-	-	+	-
35-37	45-46	0.5-0.7	1.4-2.8	3.0-7.0	7.0-21.0	-	d	-	-	-	d
34	47	0.5-0.7	1.0-1.6	6.0-7.2	7.0-14.0	-	-	+	+	+	-
32-33	48	0.4-0.6	0.8-1.4	2.0-3.5	4.0-30.0	-	+	d	-	+	+
8	63	0.8-1.0	1.0-1.3	2.8-3.0	2.4-5.4	+	+	+	+	+	+

+ = All strains positive. - = All strains negative.

d = Some strains positive and others negative.

*Results obtained in the present investigation.

Table XVI: Genus Oceanospirillum (Nutritional and Physiological Traits)

Strain	alpha-ketoglutarate	succinate	L-malate	oxaloacetate	pyruvate	L-lactate	acetate	fumarate	L-alanine	L-glutamate	L-glutamine	L-proline	hydrogen sulfide from cysteine*	growth on EMB agar*	growth in MRVP broth*	growth in 1% bile*
39 & 40	d	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
38	-	-	+	+	+	+	+	-	+	+	+	-	-	-	+	+
35-37	-	-	d	+	-	-	-	-	-	+	-	-	+	d	+	+
34	-	-	+	-	+	-	-	-	-	-	-	-	+	-	+	+
32-33	-	-	-	-	-	-	-	-	-	-	-	-	d	-	+	+

+ = All strains positive. - = All strains negative.

d = Some strains positive and others negative.

*Results obtained by Wells (1970).

1. Oceanospirillum minutulum (Watanabe, 1959. 83) comb. nov.

mi . nu' . tu . lum. L. adj. dim. minutulus very little

Helical cells, 0.3 to 0.4 microns in diameter. Wavelength, 2.0 to 2.3 microns; diameter of helix 0.6 to 0.8 microns; length of cells, 3 to 8 microns; number of turns, 1 to 2. Dark granules are present in cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagellar tufts cannot be seen by darkfield microscopy. Coccoid bodies are predominant in older cultures. Gram negative.

Peptone-succinate-salts-sea water broth: abundant, cloudy growth. Peptone-succinate-salts-sea water agar: circular, convex, translucent colonies, 0.5 mm. Water-soluble fluorescent pigment not produced.

Dependent on sea water, natural or artificial. Standard sole carbon sources: ketoglutarate, succinate, fumarate, malate, oxaloacetate, pyruvate, lactate, glutamate, glutamine, proline, and acetate. Other Krebs cycle acids, other amino acids, sugars, ethanol, propanol, butanol, glycerol, propionate, malonate, and putrescine not utilized. Standard sole nitrogen sources: ammonium ions, phenylalanine, alanine, aspartate, glutamine, asparagine, histidine, proline, leucine, and valine. Nitrate, urea, and other amino acids not utilized.

Positive tests: oxidase; catalase; reduction of selenite; nitrate reduced to nitrite; growth in the presence of 1 per cent bile or glycine, and of 7 per cent added salt (but not 10 per cent); growth on TSI agar (no change).

Negative tests: urease; H_2S from cysteine; phosphatase; hydrolysis of gelatin and aesculin; RNAase; DNAase; phenylalanine, tyrosine, and tryptophan deaminase; growth on EMB and MacConkey agars and in

MRVP broth; acid reaction from sugars in complex peptone media.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 5, 25, and 37 C; no growth at 42 C.

Source: Marine shellfish at Samugawa Beach.

The G + C content of the DNA of the type strain is 42 moles per cent (T_m).

Type strain: ATCC No. 19193.

The type strain of Oceanospirillum halophilum Watanabe 1959, 80, (ATCC No. 19192) has been found to be nearly identical to the type strain of O. minutulum on the basis of morphology, biochemical tests, nutrition, serology, and DNA base composition. Differences occur with regard to pigment production in the presence of 0.2% tryptophan, growth on alpha-ketoglutarate, and L-glutamate. I feel that these differences are not sufficient to warrant species distinction and the two species have been combined in this manuscript.

2. Aquaspirillum japonicum (Watanabe, 1959) comb. nov.

ja . pon' . i . cum. M. L. adj. japonicus pertaining to Japan.

Helical cells, 0.7 to 0.8 microns in diameter; wavelength, 8 to 20 microns; diameter of helix, 1 to 3 microns; length of cells, 20 to 75 microns; number of turns, from less than one to one. Dark granules present in cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagellar tufts can be seen by darkfield microscopy. Coccoid bodies predominant in older cultures. Gram negative.

Peptone-succinate-salts-sea water broth: abundant, cloudy growth.
Peptone-succinate-salts-sea water agar: circular, convex, white

colonies, less than 1.0 mm. Water-soluble fluorescent pigment not produced.

Dependent on sea water, natural or artificial. Standard sole carbon sources: malate, oxaloacetate, pyruvate, lactate, acetate, glutamate, glutamine, and alanine. Other Krebs cycle acids, other amino acids, sugars, ethanol, propanol, butanol, glycerol, malonate, acetate, and putrescine not utilized. Standard sole nitrogen sources: ammonium ions, alanine, glutamate, glutamine, asparagine, histidine, cysteine, glycine, leucine, phenylalanine, and citrulline. Nitrate, urea, and other amino acids not utilized.

Positive tests: oxidase; phosphatase; catalase (weakly); reduction of selenite (weakly); growth in presence of 1 per cent bile or glycine, or of 7 per cent salt (but not 10 per cent).

Negative tests: urease; H_2S from cysteine; hydrolysis of gelatin and aesculin; reduction of nitrate; RNAase; DNAase; phenylalanine, tyrosine, and tryptophan deaminase; growth on TSI, EMB, and MacConkey agars and in MRVP broth; acid reactions from sugars in complex peptone media.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 5 or 42 C.

Source: Marine shellfish at Samugawa Beach.

The G + C content of the DNA of the type strain is 45 moles per cent (T_m).

Type strain: ATCC No. 19191.

3. Oceanospirillum atlanticum (Williams and Rittenberg, 1957)

comb. nov.

at . lan' . ti . cum. M. L. adj. atlanticus pertaining to the Atlantic Ocean.

Helical cells, 0.4 to 0.6 microns in diameter. Wavelength, 3.2 to 3.5 microns; diameter of helix, 1.2 to 1.4 microns; length of cells, 4 to 14 microns; number of turns, 2 to 3. Dark granules present in cytoplasm. Motile, possessing a tuft of flagella at each pole. Flagellar tufts cannot be seen by darkfield microscopy. Coccoid bodies predominate in older cultures. Gram negative.

Peptone-succinate-salts-sea water broth: abundant, cloudy growth. Peptone-succinate-salts-sea water agar: circular, convex, translucent colonies, 0.5 mm. Water-soluble fluorescent pigment not produced.

Dependent of sea water, natural or artificial. Standard sole carbon sources: none yet to be found. Krebs cycle acids, amino acids, sugars, ethanol, propanol, butanol, glycerol, lactate, pyruvate, propionate, acetate, malonate, and putrescine not utilized. Standard sole nitrogen sources: methionine. Other amino acids, ammonium ions, nitrate, and urea not utilized.

Positive tests: oxidase; phosphatase; catalase (weakly); RNAase; reduction of selenite; growth in the presence of 1 per cent bile or glycine, or 7 per cent added salt (but not 10 per cent); growth on TSI agar (no change) and MRVP broth.

Negative tests: urease; H_2S from cysteine; hydrolysis of gelatin and aesculin; reduction of nitrate; DNAase; growth on EMB and MacConkey agars; phenylalanine, tyrosine, and tryptophan deaminase.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; scanty growth at 5 C; no growth at 42 C.

Source: isolated from coastal sea water of Long Island Sound.

The G + C content of the DNA of the type strain is 48 moles per cent (T_m).

Type strain: ATCC No. 12753.

Comment: the type strain of Oceanospirillum linum Williams and Rittenberg 1957, 82, (ATCC No. 11336) has been found to be nearly identical to the type strain of O. atlanticum with regard to cell diameter, DNA base composition, serology, nutrition, and most biochemical tests. Differences occur in cell length and number of helical turns, RNAase activity, H_2S production from cysteine, and pigment production from aromatic amino acids. The type strain of O. linum differs from that of O. atlanticum in that it produces a slight growth response with acetate as a sole carbon source. These differences do not appear to warrant species distinction, and the two species have been combined in this manuscript.

4. Oceanospirillum beijerinckii (Williams and Rittenberg, 1957)
comb. nov.

bei . jer . inck' . i . i. M. L. gen. noun. beijerinckii of Beijerinck; named for Prof. M. W. Beijerinck of Delft, Holland.

Helical cells, 0.5 to 0.7 microns in diameter. Wavelength, 6.3 to 7.2 microns; diameter of helix, 1.5 to 1.6 microns; length of cells, 7 to 14 microns; number of turns, one to two. Dark granules are present in cytoplasm. Motile, possessing a tuft of flagella at each pole.

Flagellar tufts cannot be seen with darkfield microscopy. Coccoid bodies predominate in older cultures. Gram negative.

Peptone-succinate-salts-sea water broth: abundant, cloudy growth. Peptone-succinate-salts-sea water agar: circular, convex, translucent colonies, less than 0.5 mm. Water-soluble fluorescent pigment not produced.

Dependent on sea water, natural or artificial. Standard sole carbon sources: malate and pyruvate. Remaining Krebs cycle acids, amino acids, sugars, ethanol, propanol, butanol, glycerol, lactate, pyruvate, propionate, acetate, malonate, and putrescine not utilized. Standard sole nitrogen sources: none has been found to be effective.

Positive tests: oxidase; phosphatase; catalase (weakly); RNAase; DNAase; H_2S from cysteine. A deep brown water-soluble pigment is produced in the presence of 0.2 per cent tyrosine. Growth occurs in the presence of 1 per cent bile and of 5 per cent added salt (but not 7 per cent).

Negative tests: urease; hydrolysis of gelatin and aesculin; phenylalanine and tryptophan deaminase; reduction of nitrate and selenite. No growth in the presence of 1 per cent glycine or on TSI, EMB, or MacConkey agars, or in MRVP broth.

Strictly aerobic. Anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: isolated from coastal sea water of Long Island Sound.

The G + C content of the DNA of the type strain is 47 moles per cent (T_m).

Type strain: ATCC No. 12754.

5. Oceanospirillum maris sp. nov.

Maris. mar' . is. L. noun. mare the sea; L. gen. maris of the sea.

Helical cells, 0.5 to 0.7 microns in diameter. Wavelength, 3.0 to 7.0 microns; diameter of helix, 1.4 to 2.8 microns; length of cells, 3.0 to 7.0 microns. Motile, possessing a tuft of flagella at each pole. Electron micrographs indicate each tuft has from 2-6 flagella. Gram-negative.

Peptone-succinate-salts-sea water broth: abundant, cloudy growth. Peptone-succinate-salts-sea water agar: circular, convex, translucent colonies, 0.5 mm. Water-soluble fluorescent pigment produce by 1 of 3 strains;

Dependent on sea water, natural or artificial. Standard sole carbon sources: oxaloacetate and L-glutamate with L-malate being variable. Standard sole nitrogen sources: (see Table III).

Positive tests: oxidase; catalase; growth in the presence of 1 per cent bile or glycine, and of 7 per cent added salt (but not 10 per cent); and H_2S from cysteine.

Negative tests: nitrate reduction; phosphatase; hydrolysis of gelatin and aesculin; RNAase; DNAase; phenylalanine, tyrosine, and tryptophan deaminase; acid reaction from sugars in complex media; and urease production.

Strictly aerobic; anaerobic growth does not occur in the presence of nitrate. Optimum temperature, 30 to 32 C. Moderate growth at 25 and 37 C, no growth at 10 or 42 C.

Source: isolated from marine waters.

The G + C content of the DNA ranges from 45-46 moles per cent (T_m).

Type strain: Strain 101.

Pseudospirillum gen. nov.

1. Pseudospirillum lunatum (strain 8) (Williams and Rittenberg, 1957) comb. nov.

The characteristics of this genus are summarized in Table XII.

Positive tests: oxidase; catalase; phosphatase; reduction of selenite; reduction of nitrate to NH_3 or N_2 ; acid reaction from glucose, fructose, galactose, mannose, mannitol, and dextrin (but from no other carbohydrates) in complex peptone media; H_2S from cysteine; dark brown water-soluble pigment produced in the presence of 0.2 per cent tryptophan; light brown pigment produced in the presence of 0.2 per cent tyrosine; growth in the presence of 1 per cent bile; growth with 10 per cent added salt (but not 20 per cent); growth on TSI (no change), EMB, and McConkey arags, MRVP broth, and on Seller's slants (growth in butt and slant, alkaline slant, slightly alkaline butt, greenish colonies on slant); RNAase and DNAase (both weakly).

Negative tests: urease; hydrolysis of gelatin; and aesculin; growth in the presence of 1 per cent glycine; phenylalanine deaminase; pigment production from phenylalanine.

Nutrition: the following compounds can serve as sole carbon and energy source: all tricarboxylic acid cycle intermediates and related compounds, acetate, beta-hydroxybutyrate, n-propanol, glycerol, L-tyrosine, L-alanine, L-glutamate, L-glutamine, L-asparagine, L-proline,

glycine, D-glucose, D-gluconate, and D-mannitol (Table I). Moreover, a variety of amino acids, ammonium sulfate, urea, and potassium nitrate can serve as a sole nitrogen source (Table IV).

The apparent pathway of carbohydrate catabolism is the Entner-Doudoroff Pathway (Table VII).

Strictly aerobic; however, growth does occur anaerobically in the presence of nitrate. Optimum temperature, 30 C. Moderate growth at 25 and 37 C; no growth at 10 or 42 C.

Source: isolated from coastal sea water.

Type strain: ATCC No. 11337.

Species for Which No Cultures are Available

Several species of spirilla which are listed in Bergey's Manual of Determinative Bacteriology (7th ed.) or have been described in the literature since the publication of this manual have no known available representative isolates. If these species should become available in the future, they should be subjected to the same standardized tests as all the presently available species. Previously described species for which no available isolates exist include:

- 1) Spirillum undula (Müller, 1786) Ehrenberg 1830, 58.

The description of Spirillum undula given in Bergey's Manual (7th ed.) is, in my opinion, too vague to conclusively identify this species even if it became available. For example, S. undula was reported to be 0.9 microns in diameter; this falls, however, approximately within the size range of four species of spirilla (Table XIII). S. undula was reported to liquify gelatin; but S. serpens, which is the same size, can also liquify gelatin, thus making it very difficult to distinguish

S. undula from S. serpens. On the other hand, the information reported in Giesberger's Thesis (1936) may be helpful in distinguishing S. undula from S. serpens. For example, Giesberger (1936) reported S. undula to use ethyl, n-propyl, and n-butyl alcohols as sole carbon and energy sources. This is in contrast to S. serpens.

- 2) Spirillum tenue Ehrenberg 1838, 84.
- 3) Spirillum virginianum Dimitoroff 1926, 19.
- 4) Spirillum minor Carter 1888, 47.
- 5) Spirillum kutscheri Migula 1900, 1026.
- 6) Spirillum curvatum Williams and Rittenberg 1957, 84.
- 7) Spirillum manconiense Cayton and Preston 1955, 519.
- 8) Spirillum crassum Terasaki 1961, 223.
- 9) Spirillum maritimum Watanabe 1959, 82.

Key to the genera: Spirillum, Aquaspirillum,
Oceanospirillum, and Pseudospirillum

I. Sea water not required for growth:

- A. Cell diameter 1.4-1.7 microns; cell length 14 to 60 microns;
obligately microaerophilic.

1) Spirillum

- B. Cell diameter 0.2-1.4 microns, aerobic.

2) Aquaspirillum

II. Sea water required for growth:

- A. Acid reactions produced in dilute peptone media from glucose,
fructose, galactose, mannose, mannitol, and dextrin.

3) Pseudospirillum

- B. Acid reactions not produced in dilute peptone media from

glucose, fructose, galactose, mannose, mannitol, and dextrin.

4) Oceanospirillum

Key to the Species of Spirillum

Since Spirillum volutans is the only species in this genus, the key is the same as the genus.

Key to the Species of Aquaspirillum

A. Cell diameter 1.4 to 1.5 microns; cell length 7 to 15 microns;
no growth with 0.3 per cent NaCl.

1) Aquaspirillum anulus

B. Average cell diameter between 1.0 to 1.4 microns.

1) Succinate used as sole carbon source; urease negative.

a) Gelatin liquefied.

2) Aquaspirillum
metamorphum

aa) Gelatin not liquefied.

3) Aquaspirillum
putridiconchylum

2) Succinate not used as sole carbon source; urease positive.

a) Cells have less than one
helical turns; H₂S
produced from cysteine.

4) Aquaspirillum
giesbergeri

aa) Cells have 2 to 4
helical turns; H₂S not
produced from cysteine.

5) Aquaspirillum
graniferum

3) Tricarboxylic acid cycle intermediates or amino acids not
used as sole carbon and energy source; urease negative.

6) Aquaspirillum
Group A

C. Average cell diameter 0.5 to 0.9 microns.

1) Anaerobic growth in the presence of nitrate.

7) Aquaspirillum
itersonii

2) No growth anaerobically with nitrate.

- a) Acid reaction produced in dilute peptone media from D-fructose. 8) Aquaspirillum peregrinum
- aa) No acid reaction from sugars.
- b) Nitrate reduced to nitrite; selenite reduced.
- c) L-ornithine, L-citrulline, caproate, and propionate used as sole carbon and energy sources. 9) Aquaspirillum mobile
- cc) L-ornithine, L-citrulline, caproate, and propionate not used as sole carbon and energy sources. 10) Aquaspirillum Group B
- bb) Nitrate not reduced to nitrite; selenite not reduced.
- c) Glutamate and aspartate utilized as sole carbon and energy source. 11) Aquaspirillum serpens
- cc) Glutamate and aspartate not utilized as sole carbon and energy source. 12) Aquaspirillum sinuosum

D. Average cell diameter between 0.25 and 0.4 microns.

- 1) Acid reaction produced in dilute peptone media from glucose, galactose, and arabinose.

13) Aquaspirillum gracile

2) No acid reaction from sugars.

- | | | | |
|-----|---|-----|----------------------------------|
| a) | Phosphatase negative; krebs cycle acids not utilized as sole carbon sources. | 14) | <u>Aquaspirillum polymorphum</u> |
| aa) | Phosphatase positive; a variety of tricarboxylic acid cycle intermediates used as sole carbon and energy sources. | 15) | <u>Aquaspirillum delicatum</u> |

Key to the Species of Oceanospirillum

A. Phosphatase negative.

- | | | | |
|-----|--|----|----------------------------------|
| a) | Nitrate reduced to nitrite; H ₂ S not produced from cysteine. | 1) | <u>Oceanospirillum minutulum</u> |
| aa) | Nitrate not reduced to nitrite; H ₂ S produced from cysteine. | 2) | <u>Oceanospirillum maris</u> |

B. Phosphatase positive; nitrate not reduced to nitrite.

- | | | | |
|-----|--|----|-------------------------------------|
| a) | Selenite not reduced. | 3) | <u>Oceanospirillum Beijerinckii</u> |
| aa) | Selenite reduced. | | |
| b) | Tricarboxylic acid cycle intermediates not used as sole carbon and energy sources. | 4) | <u>Oceanospirillum atlanticum</u> |
| bb) | A variety of tricarboxylic acid cycle intermediates used as sole carbon and energy source. | 5) | <u>Oceanospirillum japonicum</u> |

Key to the Species of Pseudospirillum

Since P. lunatum is the only species of this genus, the key is the same as the genus.

Nutritional Spectra of Spirilla: Taxonomic Utility,

Physiological Interpretation, and Limitations

The fact that a particular strain of spirilla can use an organic compound as a sole carbon and energy source demonstrates it has a special complement of enzymes, which converts the compound into one or more intermediary metabolites. However, unless information on the biochemistry of the pathway is known, it is quite difficult to assess the number of gene characters the pathway should be assigned. For example, in the genus Pseudomonas, the ability of the fluorescent pseudomonads to grow on L-tryptophan represents a twelve gene or twelve enzyme character; whereas, the ability to grow on L-tartrate is only a two gene character (Stanier, Palleroni, and Doudoroff, 1966). To a numerical taxonomist, these two characters would be assigned unit weight; however, as is apparent, these two characteristics are far from equivalent. As another example, the ability of the acidovarans and fluorescent groups of pseudomonads to use p-hydroxybenzoate as a sole carbon and energy source makes them phenotypically identical; however, after the first step-reaction the pathways follow different routes of degradation. Knowledge concerning the degradation of p-hydroxybenzoate in these two groups of pseudomonads shows one group synthesizes four specific enzymes not synthesized by the other group (Stanier, Palleroni, and Doudoroff, 1966). I feel that in view of the paucity of information regarding the intermediary metabolism of spirilla, a numerical taxonomic analysis would be of limited value and this is why I have not fed all the data into a computer.

Comparison of the Genera: Spirillum, Aquaspirillum,
Oceanospirillum, and Pseudospirillum with genera
Pseudomonas, Vibrio, and Campylobacter

Members of the genera Spirillum, Campylobacter, Oceanospirillum, Vibrio, Aquaspirillum, Pseudospirillum, and Pseudomonas are all gram negative, polarly flagellated rods. Some of the more important taxonomic characteristics of these seven genera are summarized in Table XVII.

In regard to the taxonomy of these bacteria, the genera Aquaspirillum and Pseudomonas appear to be similar for the following reasons: (1) the guanosine plus cytosine content of the DNA of most Aquaspirillum species overlaps that of the genus Pseudomonas; (2) the morphology of Aquaspirillum species and Pseudomonas species are similar in that both are gram negative, polarly flagellated rods or helical rods; (3) both genera produce fluorescent pigments and synthesize poly-beta-hydroxybutyrate; (4) neither Aquaspirillum nor Pseudomonas species can ferment carbohydrates. On the other hand, probably most species of Aquaspirillum can be distinguished from pseudomonads by morphology alone, one exception being that some members of Aquaspirillum are typically only slightly curved rods e.g. delicatum, polymorphum, and metamorphum and may therefore be difficult to distinguish from pseudomonads by only morphology. The nutritional and physiological tests should be helpful in resolving this problem. An illustration of this is that most species of Pseudomonas can use a large variety of sugars, and/or aromatic compounds such as p-hydroxybenzoate, benzoate, phenol, and anthranilate as sole carbon and energy sources.

This is in contrast to all species of spirilla. And though the guanosine plus cytosine content of the DNA of some species of Aquaspirillum does overlap that of the genus Pseudomonas (58-69%), in the cases of other species they are clearly distinct e.g. A. serpens and A. putridiconchylum (G + C % of 50-51 and 52 respectively).

The genus Pseudomonas is very similar to genus Pseudospirillum because of the cell morphology (both are straight or slightly curved rods), nutritional and physiological characteristics e.g. both genera can use a large spectrum of carbohydrates, amino acids (including glycine), and alcohols as a sole carbon and energy source, reduce nitrate to NH_3 or N_2 , produce fluorescent pigments, and have a similar guanosine plus cytosine content of their DNA (Table XVII). On the other hand, genus Pseudospirillum is different from any described species of Pseudomonas e.g. the negative growth response on p-hydroxybenzoate, benzoate, phenol, 2-keto-gluconate, pentoses, inability to liquify gelatin, growth at 42 C, serological difference from pseudomonads (McElroy, 1970), and formation of coccoid bodies in older cultures distinguishes this genus from genus Pseudomonas.

A description of genus Campylobacter (Sebald and Veron, 1963) is not included in the 7th. ed. of Bergey's Manual of Determinative Bacteriology because this genus was not proposed until after its publication. This genus includes what was formally Vibrio bubulus and Vibrio fetus. The major taxonomic characteristics of this genus are summarized in Table XVII. Genus Spirillum is probably slightly related to genus Campylobacter because both are obligately microaerophilic, do not ferment carbohydrates, and have a similar guanosine

Table XVII. Comparison of genera: Spirillum, Vibrio, Aquaspirillum, Oceanospirillum, Pseudospirillum and Pseudomonas

Genus	guanine plus cytosine content of the DNA (per cent)	fermentative metabolism of carbohydrates	morphology	sea water required for growth	acid reaction from sugars in complex media	p-hydroxy-benzoate and 2-keto-gluconate can usually be used as sole carbon source	flagellation	nitrate reduction
<u>Spirillum</u>	38	-	large spiral rods	-	-	-	large tuft of polar flagella	-
<u>Campylobacter</u>	30-34	-	slender curved rods	-	-	-	mono or multi-trichous	+
<u>Vibrio</u>	40-48	+	short curved rods, but never spiral	d	+	-	single polar flagellum	d
<u>Oceanospirillum</u>	42-48	-	spiral rods	+	-	-	tuft of polar flagella	d
<u>Aquaspirillum</u>	50-65	-	spiral rods	-	d	-	tuft of polar flagella	d
<u>Pseudospirillum</u>	63	-	spiral rods	+	+	-	tuft of polar flagella	+
<u>Pseudomonas</u>	58-69	-	short rods sometimes curved but not spiral	d	+	+	one or more polar flagella	+

+ = All species positive. - = All species negative.

d = Some species are positive and others negative.

plus cytosine content of their DNA; however, these two genera are different in regard to cell morphology and nitrate reduction (Table XVII).

The distinction of the genera Spirillum, Campylobacter, Oceanospirillum, Aquaspirillum, Pseudospirillum and Pseudomonas from the genus Vibrio should not be a major problem since only genus Vibrio can ferment carbohydrates (Sebald and Véron, 1963).

Carbohydrate Catabolism of Genera Aquaspirillum
and Pseudospirillum

The experimental approaches employed to determine a biochemical pathway in bacteria have historically progressed through the following stages: (1) secretion of a product in nature by a mixed culture of bacteria; (2) isolation and culturing the organism secreting the product in pure culture; (3) determination of the nutritional requirements and metabolic end products; (4) measurement of enzyme activity using a resting cell suspension; (5) measurement of enzyme activity in a crude or partially purified cell free extract; (6) measurement of radioactive products as an indication of enzyme activity (bacterial mutants have been helpful in this regard); (7) isolation, purification, and determination of the properties of enzymes involved in the formation of the products; (8) study of the control mechanisms involved in formation of the product including transcription, translation, and control of enzyme activity.

In reference to the pathways of carbohydrate catabolism in genera Aquaspirillum and Pseudospirillum, progress has reached the point of measurement of enzyme activities and, to some degree, description of some of the properties of the enzymes. Lagging behind other studies is

progress in identification of the acidic products which accumulate when these organisms are cultured in the presence of various sugars.

Carbohydrate Catabolism in the Genus Pseudospirillum

Pseudospirillum lunatum ATCC 11337 produces acidic products in complex media when various sugars are added (Wells, 1970). It has been reported for the genus Pseudomonas (Cho and Eagon, 1967) that the accumulation of acidic products from sugar catabolism under aerobic cultivation conditions is probably due to (1) a sluggish tricarboxylic acid cycle which is rate-limited by the supply of NADP^+ and (2) sugar repressing key enzyme synthesis of the tricarboxylic acid cycle.

Pseudospirillum lunatum ATCC 11337 can utilize several sugars as sole carbon and energy sources (Table I).

Since very few spirilla can attack carbohydrates, it is of interest to determine how these sugars are degraded.

The present investigation of the pathway(s) of carbohydrate catabolism in P. lunatum employed the method of enzyme assay analysis only. The results presented in Table VII indicates high levels of hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrase, and 2-keto-3-deoxy-6-phosphogluconate aldolase and very low levels of 6-phosphogluconate dehydrogenase and fructose 1,6-diphosphate aldolase when P. lunatum is cultured on D-glucose/casamino acids or gluconate/casamino acids. Table VII indicates very low levels of activity of all enzymes assayed when P. lunatum is cultured on succinate/casamino acids. The evidence presented in Table VII clearly indicates that, by enzyme assay analysis, the Entner-Doudoroff Pathway is the major carbohydrate catabolism pathway in this bacterium, and

the pathway is apparently inducible. However, before drawing any firm conclusions regarding the pathway of carbohydrate catabolism in P. lunatum, it would be helpful to run radiorespirometry and growth rate experiments to provide good correlative evidence for the enzyme assay analysis.

When P. lunatum is cultured on D-mannitol/casamino acids, a NAD^+ specific mannitol dehydrogenase is induced; it is non-reversible with fructose-1-phosphate or fructose-6-phosphate (Table VII). This enzyme has been reported in other bacterial genera (Tanaka, Lerner, and Lin, 1967). Regarding mannitol catabolism, it has been reported that the initial steps in D-mannitol catabolism can occur in at least two different ways: (1) D-mannitol is first phosphorylated by the PEP-phosphotransferase system and a NAD^+ specific mannitol-1-phosphate dehydrogenase converts mannitol-1-phosphate to fructose-6-phosphate which can be further metabolized; (2) D-mannitol can be initially oxidized to D-fructose by a NAD^+ specific mannitol dehydrogenase and then phosphorylated by fructokinase to fructose-6-phosphate which can be further metabolized. Some investigators (Phibbs and Eagon, 1970) have inferred indirectly that the presence of mannitol dehydrogenase is highly correlated with the absence of the PEP-phosphotransferase system of carbohydrate phosphorylation, as was first reported by Kundig, Ghosh, and Roseman (1964). The PEP-phosphotransferase system of Escherichia coli apparently consists of three components, called enzyme I, enzyme II, and HPr, which catalyze the following reactions:

phosphoenolpyruvate + HPr

phospho-HPr + pyruvate

phospho-HPr + sugar

sugar phosphate + HPr

HPr is a small protein (m.w. 10,000) and contains a histidine residue which functions as a phosphate carrier during the transferase reaction (Anderson and Wood, 1969). Enzyme II constitutes a class of enzymes, most of which are inducible and differ in their specificities for sugars (Anderson and Wood, 1969). Of the various genera of bacteria which have been reported to have the PEP-phosphotransferase system e.g. Aerobacter, Escherichia, Salmonella, Bacillus, Staphylococcus, Lactobacillus, and Clostridium (Patni and Alexander; 1971, Tanaka, Lerner, and Lin; 1967, Berbonitz; 1971, and Romano et al, 1970), all initiate mannitol catabolism by an initial phosphorylation and subsequent oxidation. However, bacterial genera which initiate mannitol catabolism by a mannitol dehydrogenase, e.g. Acetobacter, Azotobacter, Cellvibrio, and Pseudomonas, apparently lack the PEP-phosphotransferase system (Romano et al; 1970, and Phibbs and Eagon, 1970). The only exception in these observations was the report of a mutant of Aerobacter aerogenes (usually phosphorylating mannitol via a PEP-phosphotransferase system), which may also initiate mannitol catabolism fortuitously by a NAD^+ specific dehydrogenase, normally induced by xylitol but not mannitol (Tanaka, Lerner, and Lin, 1967). However, until further evidence is accumulated, it is difficult to assess the value of this type of reasoning.

Table VIII presents evidence which indicates glucose-6-phosphate dehydrogenase from P. lunatum is specifically inhibited by ATP. The inhibition of glucose-6-phosphate dehydrogenase by ATP has been reported in several other systems including mammalian (Passaneau,

Schulz, and Lowry, 1966), in Pseudomonas aeruginosa (Lessie and Neidhardt, 1967), in Hydrogenomonas H-16 (Blackkolb and Schlegel, 1968), and in Thiobacillus intermedius (Matin and Rittenberg, 1970). One might argue that the inhibition of glucose-6-phosphate dehydrogenase by ATP in bacteria such as Pseudospirillum, Pseudomonas, and Thiobacillus, which obtain their energy by oxidative phosphorylation, is to prevent the wasteful degradation of sugars which could be used for synthetic purposes.

Glucose-6-phosphate dehydrogenase from P. lunatum is apparently non-specific for NAD^+ or NADP^+ , with each serving approximately equally well (Table VIII). However, the possibility of isoenzymes of glucose-6-phosphate dehydrogenase cannot be eliminated without further investigation although, to my knowledge, there are no reports in the literature concerning isoenzymes of this type. Apparently three categories of glucose-6-phosphate dehydrogenases can be distinguished on the basis of their nucleotide specificity. The first type reacts exclusively with NADP^+ and is found in brewer's yeast, Candida utilis, and Escherichia coli. The second type is characterized by a strong reaction with NADP^+ , by displaying weak activity with NAD^+ , and by being found primarily in animal systems. The third type of glucose-6-phosphate dehydrogenase reacts approximately equally well with NAD^+ or NADP^+ . This type of enzyme would apparently include the type found in P. lunatum, and has also been reported in Leuconostoc mesenteroides, Pseudomonas aeruginosa, Hydrogenomonas H-16, and Thiobacillus ferrooxidans (Olive, Geroch, and Levy, 1971). Moreover, a recent report by Olive, Geroch, and Levy (1971), indicated that by using crystalline

pure glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides, NADP^+ , NADPH, and NADH bind to a different enzyme isomer (i.e. possessing the same amino acid sequence but a different conformation) than does NAD^+ . Furthermore, Kemp and Rose (1964) demonstrated that in Leuconostoc mesenteroides the NADPH generated from glucose-6-phosphate dehydrogenase is used primarily for fatty acid synthesis, whereas, NADH formed is used in ATP yielding reactions. Thus, glucose-6-phosphate dehydrogenase in Leuconostoc mesenteroides catalyzed the formation of two different products which possessed distinctly different metabolic functions. The investigation of transhydrogenases in P. lunatum may possibly offer a physiological explanation for the non-specificity of glucose-6-phosphate dehydrogenase for NAD^+ or NADP^+ . One might argue that since the tricarboxylic acid cycle enzymes are apparently repressed in certain aerobic organisms growing on sugars (Cho and Eagon, 1967) the production of NADPH would possibly be limiting (e.g. isocitrate dehydrogenase), thereby limit the synthesis of fatty acids and other essential metabolites.

P. lunatum is a bacterium which is ideal for physiological studies in that it grows rapidly on a large spectrum of compounds as sole carbon and energy sources (Table I) and appears to be closely related to members of the genus Pseudomonas, which may be of interest to study from a comparative physiology view-point. It may also be of interest to study the possibility of cytochrome linked glucose dehydrogenases in P. lunatum since most species of Pseudomonas are reported to possess this enzyme (Doelle, 1969). Furthermore, P. lunatum would be an ideal bacterium for investigating mannitol dehydrogenase to ascertain if this

enzyme requires 3'-5'-cyclic AMP for induction. Another problem which may be interesting to investigate is the unusual ability of this bacterium to grow on urea as a sole nitrogen source, yet is apparently urease negative.

Carbohydrate Catabolism in the Genus *Aquaspirillum*

Three different species of genus *Aquaspirillum* have the ability to produce acid from sugars in complex media (Wells, 1970); these species include: *A. itersonii*, *A. peregrinum*, and *A. gracile*. Furthermore, *A. peregrinum* and *A. itersonii* produce acid from D-fructose, but no other sugars, and can use this compound as a sole carbon and energy source (Tables I and II). *A. gracile* produces an acid reaction from D-glucose, D-galactose, and D-arabinose but no other sugars. This bacterium cannot, however, use these sugars as sole carbon and energy sources under standard conditions.

In the present investigation, I was concerned primarily with the carbohydrate catabolism of *A. itersonii* and *A. peregrinum*. In reference to the carbohydrate catabolism in *A. itersonii*, the results presented in Table IX indicate activities of hexokinase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrase, 2-keto-3-deoxy-6-phosphogluconate aldolase, and fructose 1,6-diphosphate aldolase, but very little 6-phosphogluconate dehydrogenase. This array of enzymes strongly suggests the existence of the Entner-Doudoroff Pathway (ED) and the Embden-Meyerhof-Parnas Pathway (EMP) being present when *A. itersonii* is cultured on D-fructose/casamine acids. For example, 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase are the key enzymes in the Entner-Doudoroff Pathway and

fructose 1,6-diphosphate aldolase is the key enzyme in the Embden-Meyerhof-Parnas Pathway. The hexose monophosphate pentose pathway is apparently absent in this organism because the key enzyme, 6-phosphogluconate dehydrogenase, is not detected under my assay conditions ($< 1 \text{ EU} \cdot 10^{-4}$). Moreover, since the key enzymes of the ED and EMP Pathways are both present it is impossible, without further investigation, to access the per cent contribution of each pathway. The use of specifically labeled fructose (fructose $^{14}\text{C}_3$ and $^{14}\text{C}_4$ as well as fructose $^{14}\text{C}_1$ and $^{14}\text{C}_4$) and the use of radiorespirometry techniques may be useful in resolving this problem. The possibility of the hexose phosphoketolase pathway being functional cannot be discounted, especially since this pathway uses fructose-6-phosphate as a substrate. However, the pathway is rare (Devries and Stouthamer, 1967) and bacteria which have been reported to use this pathway are missing glucose-6-phosphate dehydrogenase as well as fructose 1,6-diphosphate aldolase activities. Table IX indicates that only basal levels of activity of the enzymes of carbohydrate catabolism are detectable when A. itersonii is cultured on succinate/casamino acids.

With regard to the carbohydrate catabolism in A. peregrinum, Table X indicates activities of hexokinase and fructose 1,6-diphosphate aldolase; but no activities of enzymes characteristic of other pathways were detected under my assay conditions when this organism is cultured on D-fructose/casamino acids. The specific activities are much lower when this organism is cultured on succinate/casamino (Table X). The evidence indicates the EMP Pathway as the major pathway of carbohydrate catabolism in this organism. The

apparent absence of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and the key enzymes of the DE Pathway indicates the absence of the hexose monophosphate shunt and the DE Pathway as the major pathway of carbohydrate degradation. Exhaustive efforts were made in an attempt to demonstrate glucose-6-phosphate dehydrogenase activity in this bacterium, including: incubation in the presence of glucose-6-phosphate, addition of divalent metal ions to the reaction mixture, and usage of a commercial preparation of glucose-6-phosphate dehydrogenase as a control when added to cell free extracts. However, all attempts were unsuccessful in detecting glucose-6-phosphate dehydrogenase activity in this organism.

It is of interest that both A. itersonii and A. peregrinum produce an acid reaction from D-fructose in complex media, and could also use this compound as a sole carbon and energy source; whereas, D-glucose or other sugars will not yield these results. There exists several possibilities which might explain the latter: (1) glucose may not be phosphorylated upon entering the cell; (2) D-glucose may not be permeable; or (3) most of the carbon of carbohydrate catabolism may be metabolized via the EMP Pathway; but, if glucose-6-phosphate isomerase activity is low, this could prevent the rapid degradation of sugars such as glucose. However, the catabolism of D-fructose would not be affected, the latter being directly phosphorylated by hexokinase or fructokinase. The first possibility is the most unlikely, in that hexokinase activity was detected in cell free extracts of this bacterium; however, the enzyme may not be active in vivo. The isolation and identification of phosphorylated sugars would be helpful in eliminating

this possibility. To eliminate the second possibility (impermeability of D-glucose), studies should be made involving the uptake of labeled D-fructose and D-glucose. In regard to the third possibility, glucose-6-phosphate isomerase activity has not yet been determined, and enzyme assays should be performed to resolve this problem.

Not yet attempted, an interesting future investigation would be that of the carbohydrate catabolism in A. gracile especially since it is the only spirillum that can produce an acid reaction in complex media from a pentose (L-arabinose) (Wells, 1970). The catabolism of the pentose indicates the pathway of carbohydrate catabolism may be somewhat different in this bacterium as compared to other aquaspirilla.

SUMMARY

A collection of 44 strains (including 20 type strains) of spirilla has been subjected to detailed study with particular emphasis on nutritional, biochemical, and physiological characters. A total of 67 different organic compounds were tested for their ability to serve as sources of carbon and energy. In addition, 30 compounds were tested for their ability to serve as sole nitrogen sources. Other characters that were studied included: effects of calcium ions on growth of Spirillum strains; NaCl tolerance of fresh-water Spirillum strains; fluorescent pigment production; urease production; enzyme assays of key enzymes of carbohydrate catabolism in P. lunatum, A. itersonii, and A. peregrinum; and effects of various inhibitors on glucose-6-phosphate dehydrogenase from P. lunatum.

The resultant data of this study plus that of McElroy (1970) and Wells (1970) has been used to divide the genus Spirillum into four genera of bacteria. Each of the four genera (Spirillum, Aquaspirillum, Oceanospirillum, and Pseudospirillum) has its own unique characteristics and species. A diagnostic key was presented for the four genera and keys to the species of each genera. Each of the four genera were compared with other related genera of bacteria.

The species Oceanospirillum minutulum (formally Spirillum minutulum) and Oceanospirillum halophilum (formally Spirillum halophilum) were combined as a result of their morphological, physiological, nutritional, serological, and DNA base composition similarities. The two species Oceanospirillum linum (formally Spirillum linum) and Oceanospirillum atlanticum (formally Spirillum atlanticum) were also

combined because of their similarities. The unnamed strains 101, 102, and 206 were proposed as a new species, Oceanospirillum maris. With regard to the genus Aquaspirillum, one new species was proposed Aquaspirillum mobile, (formally unnamed strains 103 and 104).

Characteristic enzymes of the Entner-Doudoroff Pathway were found in P. lunatum and A. itersonii. Key enzymes of the Embden-Meyerhof-Parnas Pathway were detected in A. itersonii and A. peregrinum. The characteristic enzymes of the hexose monophosphate pentose pathway were not detected in any of the above species. Glucose-6-phosphate dehydrogenase from P. lunatum was found to be inhibited by ATP and non-specific for NAD^+ or NADP^+ .

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APPENDIX

Hexokinase

Reagents:

- (1) Glucose, 0.15 M.
- (2) MgCl_2 , 0.2 M.
- (3) Tris-HCl, 0.2 M, pH 7.6.
- (4) NADP^+ , 0.0013 M.
- (5) EDTA, 0.0001 M.
- (6) ATP (sodium salt), 0.3 M, pH 7.6.
- (7) Glucose-6-phosphate dehydrogenase (excess).

Procedure:

Mix 0.3 ml each of glucose, MgCl_2 , Tris-HCl, EDTA, NADP^+ , and glucose-6-phosphate dehydrogenase and distilled water to bring the volume to 2.8 ml, add 0.1 ml of centrifuged crude extract and then 0.1 ml of ATP. The rate is measured from the second to the tenth minute.

Controls:

The controls contained no ATP in one case and no glucokinase in the other.

Glucose-6-phosphate Dehydrogenase

Reagents:

- (1) Glucose-6-phosphate, 0.2 M.
- (2) Tris-HCl, 0.1 M, pH 7.8.
- (3) NAD^+ or NADP^+ , 0.0027 M.
- (4) MgCl_2 , 0.1 M.

Procedure:

To a 3 ml cuvette with a 1-cm light path 1.5 ml of Tris-HCl

buffer, 0.2 ml of NAD^+ or NADP^+ , 0.1 ml of MgCl_2 , 0.1 ml of centrifuged crude extract, and 1.0 ml of distilled water. The reaction is started by adding 0.1 ml of glucose-6-phosphate.

Controls:

The O.D. is adjusted to zero before glucose-6-phosphate is added. The following compounds were added in place of glucose-6-phosphate with no change in O.D.: fructose; glucose; mannose; glucose-1-phosphate; galactose-1-phosphate; and fructose-6-phosphate.

6-phosphogluconate Dehydrogenase

Reagents:

- (1) Glycylglycine buffer, 0.05 M, pH 7.6.
- (2) NADP^+ , 0.01 M.
- (3) MgCl_2 , 0.1 M.
- (4) 6-phosphogluconate (sodium salt), 0.15 M.

Procedure:

Add to a spectrophotometric cuvette with a 1-cm light path 0.26 ml of distilled water, 0.5 ml of reagent 1, 0.2 ml of reagent 3, 30 μl of reagent 2, 10 μl of reagent 4, and a quantity of enzyme to produce an optical density change of 0.01 to 0.025 per minute at 340 nm.

Controls:

A commercial preparation of 6-phosphogluconate dehydrogenase was obtained from Calbiochem, Los Angeles, Calif., and used to test the assay system.

2-keto-3-deoxy-6-phosphogluconate Aldolase

The assay simply involves a lactic dehydrogenase-NADH coupling system.

Solution (I)

- (1) 0.10 ml of 0.01 M NADH.
- (2) 0.20 ml of 0.5 M imidazole buffer, pH 8.0.
- (3) 0.70 ml of distilled water.
- (4) 0.20 ml of lactic dehydrogenase (Calbiochem).

Procedure:

The assay is run in microcuvettes, capacity 0.25 ml, by adding 0.01 ml of 0.05 M KDPG, 0.08 ml of solution (I), and enzyme plus water up to 0.15 ml. The disappearance of color is followed at 340 nm in a Gilford spectrophotometer fitted with a pinhole beam mask.

The KDPG is prepared from the barium salt by dissolving the 10 mg barium salt in 0.25 ml of water with an excess of Dowex 50, hydrogen form. The mixture is then filtered through a sintered glass filter. The filtrate should give a positive congo red; if not, add more resin and repeat. The filtrate is brought to 0.5 ml after which it is useable for the assay.

Controls:

The controls contain no enzyme in one case, and no KDPG (2-keto-3-deoxy-6-phosphogluconate) in the other.

Coupled: 6-phosphogluconate dehydrase and
2-keto-3-deoxy-6-phosphogluconate

Reaction mixture:

Tris-HCl buffer, pH 7.65. 200 μ moles
Gluthione sodium salt (reduced) 3 μ moles
Ferrous sulfate 6 μ moles
6-phosphogluconate 7 μ moles

NADH 0.008 Molar.

Lactic dehydrogenase (excess)

Procedure: (Note: order of addition is important).

- | | |
|------------------------------|---------|
| (1) Tris-HCl buffer, pH 7.65 | 0.35 ml |
| (2) Enzyme + water | 0.25 ml |
| (3) Glutathione | 0.10 ml |

Add within 3 minutes.

- | | |
|--|---------|
| (4) Ferrous sulfate | 0.10 ml |
| (5) Lactic dehydrogenase
plus water | 0.10 ml |

Incubate 10 min. at 37C.

- | | |
|----------------------------|---------|
| (6) Add NADH | 0.05 ml |
| (7) Add 6-phosphogluconate | 0.05 ml |

Controls:

The experiment is run without any 6-phosphogluconate and the non-specific decrease in optical density is subtracted from the experiment.

Fructose 1,6-diphosphate Aldolase

Reagents:

(1) Buffer K-SH solution- glycylglycine, 0.1 M, pH 7.5 (neutralized with 2 N KOH), potassium acetate, 0.2 M, and beta-mercaptoethanol, 0.05 M (solution unstable).

(2) Fructose 1,6-diphosphate (sodium salt), 0.02 M, pH 7.5.

(3) NADH (sodium salt), 0.002 M in 0.001 M NaOH at 0-4 C.

(4) Alpha-glycerophosphate dehydrogenase/triose phosphate isomerase 10 mg/ml, diluted 1:5 in distilled water at 0 C.

Procedure:

0.5 ml of buffer, 0.1 ml of fructose 1,6-diphosphate, 0.1 ml of NADH, and 10 ul of alpha-glycerophosphate dehydrogenase/triose phosphate

isomerase diluted to 1.0 ml with distilled water in a 1.0 ml cuvette with a 1.0 cm light path. The reaction is started by addition of 10 ul of enzyme.

Controls:

A strain of Streptococcus lactis with known fructose 1,6-diphosphate aldolase activity was used to test the assay system.

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A TAXONOMIC STUDY OF THE GENUS SPIRILLUM EHRENBERG,
WITH SPECIAL REFERENCE TO NUTRITION AND CARBOHYDRATE CATABOLISM

by

Phillip Brooks Hylemon

(ABSTRACT)

Forty-four strains (including 20 type strains) of spirilla, have been subjected to a comprehensive nutritional characterization. A total of 67 different organic compounds were tested for their ability to serve as sources of carbon and energy. A total of 30 different compounds were tested for their ability to serve as sole nitrogen sources. Other characters that were studied included: urease production; NaCl tolerance of fresh-water Spirillum strains; effects of calcium ions on growth of Spirillum strains; production of fluorescent pigments; enzyme assays of key enzymes of carbohydrate catabolism in P. lunatum, A. itersonii, and A. peregrinum; and the effects of various inhibitors on glucose-6-phosphate dehydrogenase activity from P. lunatum.

The resultant data, plus that of Wells (1970) and McElroy (1970), have been used to divide genus Spirillum into four genera (Aquaspirillum, Spirillum, Oceanospirillum, and Pseudospirillum), each possessing its own unique characteristics. Diagnostic keys are presented for the four genera and also the species of each genus. Each of the four genera were compared with other related genera of bacteria.

The species O. minutulum (formally Spirillum minutulum) and O. halophilum (formally Spirillum halophilum) were combined into a single species (O. minutulum) on the basis of their morphological,

physiological, nutritional, serological, and DNA base composition similarities. The two species O. linum (formally Spirillum linum) and O. atlanticum (formally Spirillum atlanticum), were also combined into a single species (O. atlanticum) because of their similarities. Previously unnamed strains 101, 102, and 206 were proposed as a new species, O. maris. With regard to the genus Aquaspirillum, one new species was proposed, A. mobile (formally unnamed strains 103 and 104).

Characteristic enzymes of the Entner-Doudoroff Pathway were found in P. lunatum and A. itersonii. Key enzymes of the Embden-Meyerhof-Parnas Pathway were detected in A. itersonii and A. peregrinum. The characteristic enzymes of the hexose monophosphate pentose pathway were not detected in any of the above species. Glucose-6-phosphate dehydrogenase from P. lunatum was found to be inhibited by ATP and also to be non-specific for NAD^+ or NADP^+ .