

A MORPHOLOGICAL AND PHYSIOLOGICAL  
CLASSIFICATION OF SPIRILLA

by

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## INTRODUCTION

The accumulation of an enormous quantity of information concerning the physiological, biochemical, morphological, and serological characteristics of certain groups of bacteria, has made it possible to establish classification schemes for these bacteria which probably indicate their natural relationships with considerable validity. For examples, one may cite the taxonomic schemes of Edwards and Ewing (1962) for the members of Family Enterobacteriaceae and the scheme of Stanier, Palleroni, and Doudoroff (1966) for the aerobic pseudomonads. In both cases, extensive knowledge of the characteristics of the organisms being classified was available and was drawn upon for purposes of deciding natural relationships and also suitable diagnostic keys. In comparison, current knowledge of the characteristics of the species of genus Spirillum appear to be meager for both diagnostic purposes and establishment of natural relationships. Even the most recent taxonomic treatments of this genus have been based largely on morphological characteristics, although a limited number of physiological characteristics have also been employed.

There are perhaps three reasons for the apparent lack of interest in the spirilla as follows.

(1) The spirilla have been considered to be of little medical or industrial importance; in fact, Spirillum minor has been considered the only pathogenic species of the genus, causing one form of rat-bite fever in man. One may argue that more attention is paid to characterizing bacteria of medical or industrial importance than to harmless saprophytes.

(2) Difficulties in the isolation of spirilla have been experienced, because contaminants present in the source materials usually outnumber and overgrow the spirilla during the isolation procedure. In order to enhance the growth of spirilla, enrichment substrates have been added to the natural samples containing spirilla. This has allowed several kinds of spirilla to predominate or to at least increase in numbers to permit a sufficient chance for isolation by the streak plate method. While this technique has been useful, there are spirilla which have presented even greater difficulties. For example, Spirillum volutans, while responding somewhat to enrichment techniques, was not isolated until 1962 because of the unrecognized factor of obligate microaerophilism (Wells and Krieg, 1965) and also because of the difficulty of culturing the organism on solid media, even with a suitable gaseous

atmosphere. A laborious capillary tube method of isolation must be used even today with this species (Rittenberg and Rittenberg, 1962). One species, Spirillum gracile, was isolated only by passage through a membrane filter into agar (Canale-Parola, Rosenthal, and Kupfer, 1966). A more recent isolation procedure based on the use of 5-fluorouracil has been reported by Cody (1968); this method may prove to be extremely useful for the selective isolation of spirilla in the future.

(3) Difficulty has been experienced in maintaining some of the species in type culture collections by lyophilization. The development of newer preservation techniques, using ultra-low temperature storage, e. g. as in liquid nitrogen, has now made possible the preservation of such organisms.

Several early taxonomic schemes for spirilla, based entirely on morphology, had been devised from 1830 to 1936, but Giesberger (1936) was the first investigator to study certain physiological characteristics of spirilla. He indicated that regardless of the use of either morphological or nutritional criteria, the same four distinct groups developed with the available isolates. Williams and Rittenberg (1957) in a more recent attempt to systematize the genus Spirillum, similarly found that all the cultures of a single morphological group had very similar

nutritional characteristics; however, in contrast to what Giesberger had reported, Williams and Rittenberg found that isolates belonging to distinctly different morphological groups did, in certain cases, demonstrate the same nutritional pattern. Nevertheless, Williams and Rittenberg used only morphological characteristics to separate their isolates into thirteen distinct groups, but indicated that if a larger number of compounds were to be examined, there would likely be differences in the nutritional patterns between cultures in the same morphological group, as well as, similarities in the nutritional patterns of cultures belonging to different morphological groups. Their report contains the comment that " it is not surprising that these differences should occur, when one considers the possibilities of mutation, gene recombination, transduction, and other mechanisms of gene alteration in individuals, but they can hardly have major taxonomic significance... For purposes of classification, therefore, two possibilities exist: one can ignore morphological differences and group on the basis of metabolism or else one can separate metabolically identical types and maintain morphologically homogenous groups. Recognizing that either choice is an arbitrary one, we have taken the latter course."

More recent concepts of bacterial taxonomy are based on the application of a large number of biochemical,

physiological, morphological, and serological characteristics for each organism, to give a "total picture" of the organism. For example, 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 goal of numerical taxonomy, namely, to provide objectivity in bacterial classification. Thus, the old arguments as to whether or not morphology should hold precedence over physiology in classification become irrelevant, if one holds the view that all characteristics of an organism are important in its classification. It follows that the more one knows about an organism, the more satisfactory will be attempts to establish its relationship to other organisms.

In accordance with the above considerations, the present investigation has been made in an attempt to develop further a comprehensive characterization of spirilla as begun by Giesberger and by Williams and Rittenberg, with the hope that a more satisfactory classification of spirilla may result. The type strains of 20 species of the genus Spirillum plus additional strains of S. volutans, S. serpens, S. itersonii, and S. gracile have been subjected to over ninety physiological characterization tests as well as certain morphological

measurements. Furthermore, an attempt has been made to develop standardization in characterization of strains, in order to provide a more satisfactory basis of comparison for spirilla than has been available. In addition, several quite modern characterization procedures have been employed, e. g. determination of the % GC content of DNA, in order to make the characterization as up-to-date as possible.

It must be emphasized that the present investigation of genus Spirillum does not deal with the problems of isolation of spirilla, nor does it attempt to define variation within most of the species since these are largely represented by only a single available strain--the type strain. Nevertheless, it is hoped that the physiological characterizations presented, together with a concurrent study of the nutrition of spirilla from this laboratory, will provide the most comprehensive characterization of genus Spirillum yet available, making it possible for a more satisfactory classification to be devised.

## REVIEW OF THE LITERATURE

The early taxonomic studies of the genus Spirillum were entirely morphological and the early taxonomic history of this genus has been discussed in depth by Williams and Rittenberg (1957) and Williams (1959). Although mention will be made of some of the early work, the present review will be principally concerned with the work of Williams and Rittenberg (1957) and some of the more recent investigations of members of this genus.

The genus was originally published by Ehrenberg (1830) with the diagnosis, "corpore filiforme rigido spirali", and contained two species, namely:

- (1) Spirillum volutans Ehrenberg, originally Vibrio spirillum Müller.
- (2) Spirillum undula (Müller, 1773), originally Vibrio undula.

Stiles (1905) designated S. volutans as the type species. In his treatment he stated: "Judged from the zoological point of view, Vibrio spirillum is the type species by absolute tautonomy, but the zoological name Spirillum, 1830, was a stillborn homonym, having been used by Oken, 1815, for a genus of polychaete worms. It is not the function of the zoological code to determine the names in bacteriology, but it is undoubtedly

not the most wise or farseeing policy for either zoologists or botanists (including bacteriologists) to accept unnecessarily a generic name for organisms so near the border line when that name is a homonym in either zoology or botany; should further investigations again bring bacteriology into zoology, the generic names would come under the zoological code, and in this case Spirillum, 1830, would be rejected. If Spirillum is retained in bacteriology, consistency calls for the rejection of the name Spirillum volutans Ehrenberg in favor of Spirillum spirillum (Müller)."

Vuillemin (1913) in a revision of the genera of bacteria retained the genus Spirillum as a genus conservandum. S. undula was considered the type species by Vuillemin.

The descriptions of the species of Spirillum published prior to 1936 are, on the whole, incomplete and inadequate for determination purposes. According to Williams (1959) Giesberger eliminated the majority of the species as being species dubia. Only those species were retained whose morphological and physiological characteristics were adequately described or whose morphological characteristics were so distinctive that identification was possible on this basis alone, such as S. volutans. Giesberger recognized nine species:

S. undula, S. serpens, S. volutans, S. tenue, S. minus,  
S. kutscheri, S. itersonii, S. virginianum, and S.  
cardiopyrogenes.

Haynes based his description of the species in Bergey's Manual (6th ed.) almost entirely on Giesberger's work. The key to the species of genus Spirillum as found in Bergey's Manual (6th ed) was as follows:

- I. One micron or less in diameter.
  1. Volutin granules present.
    - a. Slow to rapid liquefaction of gelatin.
      - b. Grayish to brown growth on potato.
        1. Spirillum undula.
      - bb. Light yellowish-orange growth on potato.
        2. Spirillum serpens.
    - aa. No liquefaction of gelatin. Of small size (0.5 micron in diameter).
      - b. Colonies on agar white becoming brownish black and slightly wrinkled.
        3. Spirillum itersonii.
      - bb. Colonies on agar white and smooth.
        4. Spirillum tenue.
  2. No volutin granules observed.
    - b. Single flagellum.
      5. Spirillum virginianum.
    - bb. Tuft of flagella.
      6. Spirillum minus.

II. Over one micron in diameter.

1. Grows poorly on peptone agar and potato.

7. Spirillum kutscheri.

2. Not positively known to have been cultivated on artificial media. Very evident volutin granules.

8. Spirillum volutans.

3. Cells more or less deformed by fat drops.

9. Spirillum lipoferum.

The main differences from Giesberger's scheme were that S. cardiopyrogenes was relegated to the appendix because of an incomplete description and S. lipoferum was included. Giesberger had previously isolated several strains of S. lipoferum from soil and concluded that, morphologically, the organism was a vibrio.

Bergey's Manual (7th ed) included a description and key to the genus Spirillum by Breed which was almost identical to that presented in Bergey's Manual (6th ed); however, S. cardiopyrogenes was not presented, and the appendix was completely eliminated from this edition.

Myers (1940) in a study of the isolation procedures and identifications of the spirilla was one of the last investigators to report the isolation of S. undula. Along with morphological and cultured characteristics Myers followed the method of differentiation used by Giesberger, except he used only six of the 29 carbon

sources employed by Giesberger. The nitrogen and carbon sources were examined by adding them to an inorganic medium containing 0.05% MgSO<sub>4</sub>, 0.05% K<sub>2</sub>HPO<sub>4</sub>, and 0.05% CaCl<sub>2</sub>. The pH was adjusted to 7.0 - 7.2 and the media were sterilized by filtration through a Seitz filter.

Using 1.0% calcium lactate as the carbon source, the four nitrogen compounds, NH<sub>4</sub>Cl, KNO<sub>2</sub>, KNO<sub>3</sub>, and asparagine were tested as sources of nitrogen. NH<sub>4</sub>Cl gave at least fair growth with nearly all strains and was therefore the nitrogen source used in the series of various carbon sources. Glucose, fructose, glycerol, ethanol, calcium pyruvate, and calcium lactate were studied as sources of carbon. The last two were used in a concentration of 1.0% and without CaCl<sub>2</sub> in the basal mineral medium; glucose, fructose, glycerol, and ethanol were used in a concentration of 0.5%.

Microscopic observations were made on 12-18 hour broth cultures (30 C) for all strains. On this basis the isolates were divided into four groups: S. serpens, S. itersonii, an un-named group, and S. undula. Myers stated that the "morphological grouping showed good correlation with the data on the utilization of the various sources of carbon." Of special interest today was Myers' Group IV, Spirillum undula (Müller) Ehrenberg, which was given the following description:

Cells widely-coiled, motile by means of biopolar bundles of flagella. Cytoplasm often highly granular. Cell thickness, 0.9-1.2 $\mu$ ; cell length, 8-16 $\mu$ ; width of spiral, 3.0-3.5 $\mu$ ; wavelength, 6-8 $\mu$ .

Gelatin not liquefied.

Agar colonies: circular, smooth, entire, convex, translucent.

Agar slant: beaded-filiform, glistening, gray.

Broth: slight granular growth.

Litmus milk: growth none or very slight; medium unchanged.

Potato: no growth.

Neither nitrites nor gas produced from nitrates.

Indole not produced.

H<sub>2</sub>S not produced.

Aerobic (growth only in upper 15mm of tube shake culture).

Optimum temperature: about 32 C.

Utilized as sources of nitrogen: NH<sub>4</sub>Cl, asparagine; as sources of carbon: lactate, pyruvate.

Isolated from greenhouse pool, stagnant ditch water.

Lewis (1940) in a cytological study of the spirilla also isolated S. undula and reported that it grew well on plain beef-extract peptone agar. He described it as being "similar to S. volutans but on a smaller scale." However, in a photomicrograph, of cells labeled S. undula (Müller) Ehrenberg grown on beef-extract peptone agar for 72 hours, the predominating forms were many short, plump,

slightly curved organisms having 1/2 to 1-1/2 spirals per cell. The same strain when grown by Williams and Rittenberg (1957) grew as a tangled mass of very long, almost straight cells with only a few of the shorter forms present. It was found that these shorter forms were more common in early cultures and even became more prominent in cultures grown in lactate synthetic media. Williams and Rittenberg found that cells with 5 to 8 tightly coiled spiral waves were also prominent in young and even old cultures; therefore, they considered the organism to be a new species and gave it the name Spirillum sinuosum instead of S. undula.

Williams and Rittenberg (1957) recognized nineteen species of spirilla on the basis of a) their new isolates, b) the species included by Giesberger, c) those species found in Bergey's Manual (6th ed), and d) S. manconiense, described by Cayton and Preston (1955) as being catalase negative, thus distinguishing it from the other spirilla. These species were differentiated by the following diagnostic key.

I. Microcysts predominate in old cultures.

A. Marine forms.

1. Microcysts spherical.

a. Salts of lower fatty acids utilized as single sources of carbon.

1. Spirillum linum.

- 2a. Salts of lower fatty acids not utilized as single sources of carbon.
  - b. Good growth in synthetic medium, containing individual single source of carbon.

2. Spirillum curvatum.

- 2b. Growth poor or absent in synthetic medium containing single source of carbon.

3. Spirillum virginianum.

- 2. Microcysts ellipsoidal in early stages of formation.

- a. Brownish-black pigment produced.

4. Spirillum beijerinckii.

- 2a. Brownish-black pigment not produced.

- b. Alcohols utilized as single source of carbon.

5. Spirillum atlanticum.

- 2b. Alcohols not utilized as single source of carbon.

6. Spirillum lunatum.

B. Fresh water forms.

- 1. Anaerobic growth with nitrate as hydrogen acceptor.

- a. Sugars utilized as single sources of carbon.

- b. Nitrates not utilized as sole nitrogen source.

7. Spirillum itersonii  
var itersonii.

2b. Nitrates utilized as sole nitrogen source.

7a. Spirillum itersonii  
var vulgatum.

2a. Sugars not utilized as single sources of carbon.

8. Spirillum polymorphum.

2. No anaerobic growth with nitrate.

9. Spirillum mancunien-  
ense.

II. Vegetative cells predominate in old cultures.

A. Good growth on synthetic medium containing single source of carbon.

1. Cell diameter greater than one micron.

a. Single flagellum at each pole.

10. Spirillum anulus.

2a. Tufts of flagella at each pole.

11. Spirillum giesbergeri.

2. Cell diameter less than one micron.

a. Gelatin liquefied.

b. Alcohols utilized as single sources of carbon.

12. Spirillum undula.

2b. Alcohols not utilized as single sources of carbon (Nitrates not utilized as sole nitrogen source).

13. Spirillum serpens var  
serpens.

2a. Gelatin not liquefied (Nitrates utilized as sole nitrogen source).

- 13a. Spirillum serpens  
var azotum.
- b. Sugars utilized as single carbon source.
  - 14. Spirillum tenue.
- 2b. Sugars not utilized as single carbon source.
  - c. Long, multiwaved cells.
    - 15. Spirillum sinuosum.
  - 2c. Short, S-shaped cells.
    - 16. Spirillum graniferum.
- B. Growth poor or absent in synthetic medium containing single carbon source.
  - 1. Isolated in pure culture: growth on enriched peptone media.
    - 17. Spirillum kutscheri.
  - 2. Not isolated in pure culture.
    - a. Large water forms.
      - 18. Spirillum volutans.
    - 2a. Animal pathogen.
      - 19. Spirillum minus.

With regard to a classification scheme Williams and Rittenberg separated their cultures into thirteen distinct groups based primarily on morphology. For example, distinction was made between S. anulus and S. giesbergeri on the basis that the latter, although similar to S. anulus metabolically, differed morphologically in that typical cells had two or three wide,

regular curves instead of the halfmoon or S-shaped cells of S. anulus; moreover, the diameter was less, the spiral depth was much shallower, and the granules more prominent. S. anulus was also distinguished from S. giesbergeri because it possessed a single polar flagellum. Electron microscopy has since shown that both species have tufts of polar flagella (Williams, 1960). Another example concerns the distinction between S. linum and S. lunatum. The latter had a much larger cell diameter, looser and more irregular spirals, oval rather than spherical microcysts and did not grow on hexoses as a sole source of carbon. Here again, a distinction based on single vs. multiple polar flagella was proven to be incorrect (Williams, 1960).

The organisms were further differentiated on their requirement for sea water or fresh water; the presence or absence of microcysts in old cultures; whether they grew on unsupplemented peptone media or required the addition of yeast extract or casein hydrolysate; physiological tests such as gelatin liquefaction, nitrate reduction and catalase production; and the testing of 22 carbon compounds and 4 nitrogen compounds as sole sources of carbon and nitrogen.

As a result of analysis of their isolates, Williams and Rittenberg recognized 10 new species which they described and named: S. linum, S. curvatum, S. bejerinckii, S. atlanticum, S. lunatum, S. polymorphum,

S. anulus, S. giesbergeri, S. graniferum, and S. sinuosum. In addition to these, they also described two varieties of S. serpens, S. serpens var serpens and S. serpens var azotum, the latter was distinguished from S. serpens var serpens in its inability to liquefy gelatin. Also two varieties of S. itersonii were created, S. itersonii var itersonii and S. itersonii var vulgatum. Distinction was made on the basis of the ability of S. itersonii var vulgatum to utilize nitrates as the sole source of nitrogen.

The species S. linum, S. lunatum, S. curvatum, S. beijerinckii, and S. atlanticum were the only organisms isolated from marine sources. These strains grew in media prepared with either natural or synthetic sea water however larger inocula were required to obtain growth in the synthetic sea water media as compared to natural sea water. Continued subculture in the synthetic sea water medium eventually resulted in failure of the organisms to grow on transfer.

Spherical or oval coccoid bodies which predominated in older cultures of some of the spirilla have been termed "microcysts" by Williams and Rittenberg (1956). They demonstrated that microcyst formation occurred from helical cells of S. lunatum by (1) fusion of two entwined organisms to form one or more microcysts; (2) the production of a protuberance at some point along the cell

into which the entire organism was gradually absorbed; or (3) the gradual shortening and rounding of the organism to form an oval to spherical body. When an old culture of S. lunatum, completely in the microcyst stage, was inoculated into fresh media, the microcysts germinated to form a helical cell. Germination occurred by either unipolar or bipolar emergence of the germ tube.

Williams (1957) demonstrated that fusion of spirilla also occurred in nonmicrocyst-forming species. After the fusion process, the fusion cells enlarged into giant cells, several times the diameter of the ordinary vegetative cell; a possible sexual mechanism was suggested.

Although S. serpens was characterized as not producing microcysts in old cultures (Williams and Rittenberg, 1957), Bisset and Hale-McCaughey (1967) isolated a strain of S. serpens that produced microcysts. When examined by electron microscopy, the cyst wall appeared to be double, and continuous with the double wall of the spirillum. According to Bisset and Hale-McCaughey the development of the cyst represented a true reproductive process, and resembled the process in Azotobacter.

Mitomycin C, and ultraviolet light were found by Clark-Walker (1969) to induce the formation of microcysts in S. itersonii. These spherical cells contained phage

tail parts, rhabdosomes, and a granular substance not seen in normal cells. Spontaneously occurring microcysts rarely contained phage tails or granular substance; however, many of these microcysts contained rhabdosomes. It was suggested that the spherical cells were formed as the result of the induction of a defective phage, with the production of phage lysozyme within the cell leading to the formation of spherical forms as the cells lose their structural mucopeptide layer.

In 1959, Watanabe isolated from marine shell-fish the new species S. japonicum, S. halophilum, S. minutulum, and S. maritimum. In order to isolate the spirilla Watanabe devised two types of media (Table 1), one for enrichment and one for isolation. Descriptions of the four species were based on morphological, cultural, and physiological characteristics. The four species were reported to be catalase negative. These species resembled some of the fresh water species from a morphological point of view; for example, S. halophilum resembled S. itersonii. However, unlike the fresh water species all four of these species required the addition of 2 to 2.5% NaCl to the medium on isolation and repeated subculture.

Isolates since 1957 included S. putridiconchylum, S. metamorphum, and S. crassum isolated from the putrid infusions of fresh water snails by Terasaki (1961).

TABLE 1

WATANABE'S ENRICHMENT AND ISOLATION MEDIA.\*

<u>Enrichment medium</u> g per l		<u>Isolation medium</u> g per l	
NaCl	25	NaCl	25
MgCl <sub>2</sub>	3	Calcium lactate	10
MgSO <sub>4</sub>	1	NH <sub>4</sub> CL	1
CaSO <sub>4</sub>	1	K <sub>2</sub> HPO <sub>4</sub>	0.5
K <sub>2</sub> SO <sub>4</sub>	1	MgSO <sub>4</sub>	0.5
CaCO <sub>3</sub>	0.5	Peptone	2-5
Peptone	2-5		

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\*The pH of the media was adjusted to 7.0-7.2.

Morphological, cultural and physiological characteristics were presented, which indicated differences from known species. For example, S. putridiconchylum, although morphologically similar to many of the species previously described, differed from them in its ability to liquefy gelatin, in its catalase reaction, in the type of nitrogen and carbon compounds that were used for growth in the synthetic media, and in some other minor points. The three species were reported to be catalase negative.

Leifson (1962) isolated and named the species S. delicatum from a specimen of distilled water. Leifson stated that this organism differed so much morphologically from typical spirilla, (individual cells being vibrio shaped and averaging 0.4 by 1-2 $\mu$ ), that classification in the genus Spirillum was not entirely satisfactory, e.g. it was reported to have a single polar flagellum.

While doing a systematic study of the genus Spirillum in oxidation ponds, Pretorius (1963) frequently found the three known species S. itersonii, S. serpens, and S. volutans and one new species which was strictly aerobic, catalase positive, did not grow under anaerobic conditions in the presence of nitrate, formed microcysts (round bodies), and differed in carbon source utilization from previously described species; it was further characterized and named Spirillum peregrinum.

Canale-Parola, Rosenthal, and Kupfer (1966) isolated spirilla measuring 0.25 to 0.30 $\mu$  in diameter from pond and stream water. While previous isolations of spirilla from natural sources were achieved through a number of procedures, generally after primary enrichments, S. gracile, because of its small diameter, was isolated by using cellulose ester filter discs (0.45 $\mu$  pore size) which allowed the thin spirilla to penetrate to the underlying surface of culture plates, but retained larger unwanted organisms. The organisms were reported to exhibit a microaerophilic behavior in that all strains formed colonies beneath the surface of the agar on initial isolation. After prolonged subculturing in liquid media a number of the spirilla lost the ability to diffuse through the agar medium and formed smaller surface colonies, although a part of each colony was still below the surface. If one of these colonies was subcultured in liquid media and then streaked on agar media a few colonies of the subsurface type were observed. None of the strains grew anaerobically.

Freshly isolated strains of S. gracile consistently gave negative catalase tests. However, after prolonged subculturing, a weak positive reaction was detected in all strains.

Although there have been reports to the contrary

(Belozersky, 1941; Bünning and Gossel, 1956), it was not until 1962 that S. volutans was first obtained in pure culture (Rittenberg and Rittenberg, 1962). In order to isolate the organism, it was necessary to exploit one property of S. volutans: its rapid, unidirectional motion. Observations of the organisms under the microscope showed that individual spirilla at the edge of a newly placed drop would swim at a rapid, steady rate in a straight line to the opposite edge of the drop before changing their direction of motion. Using an ingenious capillary tube technique they were able to observe that S. volutans outdistanced the contaminants in the mixed culture and they were able to separate the spirilla from the other organisms on this principle. Pure cultures were achieved by growing the separated spirilla inside of dialysis sacs in contact with mixed cultures on the outside and also by growth in asparagine-mineral salts medium supplemented with an extract of Escherichia coli. No chemical supplements added were able to substitute for this dependence on the other bacteria or the extract of E. coli.

Wells and Krieg (1965), using the capillary tube technique of Rittenberg and Rittenberg, isolated S. volutans and cultured the organism in dialysis sacs in the presence of other organisms. Wet mounts made

of the pure culture growing in the dialysis sacs showed the formation of a narrow band of spirilla some distance in from the cover-slip edge, indicating a microaerophilic response of S. volutans. Using nutrient broth, they found that pure cultures of S. volutans could be propagated indefinitely provided that the atmospheric oxygen concentration was reduced to 3%.

## MATERIALS AND METHODS

Origins of the Strains.--The strains of spirilla used in the present work 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. S. anulus type strain. Obtained from the ATCC, agar slant culture. ATCC 19259.
2. S. atlanticum type strain. Obtained from the ATCC, agar slant culture. ATCC 12753.
3. S. beijerinckii type strain. Obtained from the ATCC, agar slant culture. ATCC 12754.
4. S. delicatum type strain. Obtained from the ATCC, agar slant culture. ATCC 14667.
5. S. giesbergeri type strain. Obtained from the ATCC, agar slant culture. ATCC 11334.
6. S. giesbergeri synonymous with number 5. Obtained from the NCIB, lyophilized. NCIB 9073.
7. S. gracile type strain. Obtained from the ATCC, agar slant culture. ATCC 19624.
8. S. gracile. Obtained from the ATCC, agar slant culture. ATCC 19625.
9. S. gracile. Obtained from the ATCC, agar slant culture. ATCC 19626.
10. S. graniferum type strain. Obtained from the NCIB, lyophilized. NCIB 8230.
11. S. halophilum type strain. Obtained from the ATCC, agar slant culture. ATCC 19192.

12. S. itersonii type strain. Received from Dr. R. M. Smibert, Anaerobic Bacteriology Laboratory, Virginia Polytechnic Institute. ATCC 12639.
13. S. itersonii. Same source as 12. ATCC 11331.
14. S. itersonii. Received from Dr. S. C. Rittenberg, Department of Bacteriology, University of California.
15. S. japonicum type strain. Obtained from the ATCC, agar slant culture. ATCC 19191.
16. S. linum type strain. Obtained from the ATCC, agar slant culture. ATCC 11336.
17. S. lunatum type strain. Obtained from the ATCC, agar slant culture. ATCC 11337.
18. S. metamorphum type strain. Obtained from the ATCC, agar slant culture. ATCC 15280.
19. S. minutulum type strain. Obtained from the ATCC, agar slant culture. ATCC 19193.
20. S. peregrinum type strain. Obtained from the ATCC, agar slant culture. ATCC 15387.
21. S. polymorphum type strain. Obtained from the ATCC, agar slant culture. ATCC 11332.
22. S. polymorphum synonymous with number 21. Obtained from the NCIB, lyophilized. NCIB 9072.
23. S. putridiconchylium type strain. Obtained from the ATCC, agar slant culture. ATCC 15279.
24. S. serpens type strain. Same source as 12. ATCC 12638.
25. S. serpens. Same source as 12. ATCC 11335.
26. S. serpens. Same source as 12. ATCC 15278.
27. S. serpens. Same source as 12. ATCC 11330.
28. S. serpens unnumbered strain. Same source as 14.

29. S. serpens Victoria strain. Same source as 14.
30. S. serpens. Same source as 14. ATCC 12289.
31. S. serpens strain VH. Received from Dr. R. G. E. Murray, Department of Bacteriology and Immunology, The University of Western Ontario.
32. S. serpens strain VHA. Same source as 31.
33. S. serpens strain VHL. Same source as 31.
34. S. serpens strain VHS. Same source as 31.
35. S. serpens St. Rhodes strain. Same source as 31.
36. S. sinuosum type strain. Obtained from the ATCC, agar slant culture. ATCC 9786.
37. S. volutans type strain. Same source as 14. ATCC 19553.
38. S. volutans Wells strain. Isolated from pond water-hay infusion. ATCC 19554.
39. Un-named strain 101 of Dr. H. W. Jannasch, Woods Hole Oceanographic Institution.
40. Un-named strain 102. Same source as 39.
41. Un-named strain 103. Same source as 39.
42. Un-named strain 104. Same source as 39.
43. Un-named strain 204. Same source as 39.
44. Un-named strain 206. Same source as 39.

The Witkin strain of Escherichia coli B used as a standard in determining GC ratios and also the presence of deaminase, was obtained from Dr. G. R. Carta, Department of Biology, Virginia Polytechnic Institute.

Maintenance of Cultures.--All cultures were maintained in semi-solid (0.15% agar) peptone-succinate-salts

(PSS) medium, with serial transfers being made weekly. These transfers served as sources of organisms for preparation of inocula in all experiments except in the determination of the moles % GC of DNA and in the determination of deaminase activity.

The cultures were also preserved in a So-Low Environmental Chamber (So-Low Environmental Equipment Co., Cincinnati, Ohio) set at -80 C in PSS medium + 15% glycerol. On two occasions a number of the cultures stored in the freezer were ruined due to electric failure. After each such incident, replacements were obtained using spirilla maintained by serial transfer. The frozen cultures were used as sources for preparation of inocula for the determination of moles % GC of DNA and deaminase activity; these cultures were also used for confirmation purposes in several experiments which provided data contradictory to that reported by other investigators, particularly with regard to nitrate reduction, catalase production and gelatin hydrolysis.

Media and Chemicals.--MacConkey, Sellar's TSI, and EMB agars; nutrient, MRVP, and urea broths; litmus milk, skim milk, peptone, agar, yeast extract, oxgall bile, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), gelatin, starch (soluble), reagent; melibiose, trehalose, inulin, salicin, aesculin, and dulcitol were obtained

from Difco Laboratories, Detroit, Michigan.

Ammonium sulfate, reagent; calcium chloride, reagent; ferrous sulfate, reagent; ferric chloride, reagent; lead acetate, C.P.; magnesium chloride, reagent; manganese sulfate, reagent; potassium chloride, reagent; sodium chloride, reagent; boric acid, reagent; succinic acid, reagent; and octanol, reagent were products of the J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Glucose, galactose, lactose, mannose, fructose, ribose, arabinose, xylose, rhamnose, raffinose, C. P.; melezitose, sorbose, cellobiose, glycogen, amygdalin (Trihydrate), adonitol (adonite), erythritol, mannitol (mannite), sorbitol (hydrate), dextrin (White, Tech.), glycerol, C. P.; cysteine, tryosine, phenolphthalein diphosphate (pentasodium), and phenolphthalein disulfate (Trihydrate) were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio.

Ethanol, U.S.P. was manufactured by U. S. Industrial Chemical Co., New York.

Magnesium sulfate, reagent; potassium hydroxide, reagent; potassium nitrate, reagent; sodium citrate, reagent; sodium lauryl sulfate, reagent; maltose, reagent; sucrose, certified A.C.S.; selenous acid, certified; and ethylene glycol monoethyl ether, purified were obtained from Fisher Scientific Co., Fairlawn,

New Jersey.

Oxidase test disks were a product of the Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Md.

Inositol, phenylalanine, M. A., and tryptophan, M. A. were supplied by the Mann Research Laboratories, Inc., New York.

Bovine pancreatic ribonuclease (RNAase), A-grade and T<sub>1</sub> RNAase (crystalline) were products of Calbiochem, Los Angeles, California.

Phenol liquefied, A. R. was obtained from the Mallinckrodt Chemical Works, St. Louis, Missouri.

Composition of Peptone-Succinate-Salts Medium.--Peptone-succinate-salts (PSS) broth had the following composition (g per l): peptone, 10.0; succinic acid, 1.0; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.0; FeCl<sub>3</sub>·6H<sub>2</sub>O, 0.002; and MnSO<sub>4</sub>·H<sub>2</sub>O, 0.002. The pH was adjusted to 6.8 with 2N KOH. For solidified media, 1.5% agar was incorporated into the PSS broth; semi-solid media were prepared by adding 0.15% agar to the PSS broth. For the cultivation and characterization of marine spirilla, artificial sea water (Society of American Bacteriologists, 1957, p. 113) was employed in the preparation of all media. S. lunatum grew in the presence or absence of sea water; therefore, this organism was examined under

both conditions with respect to all tests.

Preparation of Inoculum used in Biochemical and Physiological Tests.--All strains of spirilla, except those of S. volutans, were cultured in screw-capped 13 x 125 mm tubes, containing 10 ml of PSS broth, for 24 hrs.; 0.04 ml from the second serial transfer was used as inoculum for all test media unless otherwise indicated. A similar inoculum was employed for S. volutans, with the exception that the organisms were cultivated in 75 ml quantities of PSS broth contained in 250 ml side-arm flasks having a mixture of 30% air and 70% nitrogen (amounting to an oxygen concentration of approximately 6%).

Atmospheric Conditions.--All spirilla, except S. volutans, were grown and characterized under aerobic conditions in all experiments except when determining ability to grow anaerobically in the presence of nitrate; S. volutans, being obligately microaerophilic, was grown either in liquid media stratified with 0.15% agar or on solid media in a mixture of 30% air and 70% nitrogen (amounting to an oxygen concentration of approximately 6%).

Estimation of Growth Responses.--The media employed for the purpose of determining a growth response was examined every day during the incubation period; cultures

were rated by a visual examination with respect to growth in these media.

Temperature of Incubation.--Cultivation and characterization of spirilla were carried out at 30 C, after this value was determined to be optimal for all strains (see below).

Determination of Optimal Temperature.--The optimal temperature was determined by incubating the cultures at temperatures of 10, 15, 20, 25, 30, 37, 42, and 45 C; for the temperatures 10, 20, and 25 C a Fisher Low Temperature Incubator (Fisher Scientific Co., Fairlawn, N. J.) was employed, the 15 C temperature was obtained in a Psychrotherm Incubator Shaker (New Brunswick Scientific Co., Inc., New Brunswick, N. J.), Box Incubators (Precision Scientific Co., Chicago, Ill.) were employed for the temperatures 37, 42, and 45 C, and 30 C was obtained in a Fisher Isotemp Incubator (Fisher Scientific Co., Fairlawn, N. J.). The medium was conditioned to the various temperature for 2 hr. prior to inoculation. After 1 week incubation, a visual estimation of growth was made.

Biochemical and Physiological Methods.

A number of biochemical and physiological tests were employed in the characterization of the strains of spirilla.

Oxidase Test.--A positive purple color reaction was

sought with 48 hr. semi-solid (0.15% agar) PSS cultures by two methods: (a) moistened oxidase test disks (BBL) were smeared with a loopful of the culture, and (b) several drops of paraamino dimethylaniline solution were added directly into the culture.

Phosphatase and Sulfatase Production.--Phenolphthalein-diphosphate or disulfate was added to PSS agar at a final concentration of 0.01%. Inoculation was accomplished by streaking a loopful of the second of two serial 24 hr. cultures across the agar plates. After incubation for 4 days, ammonia fumes were used for the detection of free phenolphthalein.

Deaminase Activity.--The Conway microdiffusion technique was employed (Conway, 1950). The spirilla were cultivated in 300 ml of PSS broth contained in 1 liter flasks for 24 hrs. The cells were harvested, washed twice in sterile saline, and resuspended in 5 ml of sterile saline. The turbidity of this suspension was adjusted so that a 1:10 dilution would give a reading of 200 Klett units. Five-tenths ml of the undiluted suspension was then used to determine the deaminase activity, using the protocol in Table 2.

Catalase Test.--The microscopic production of a froth of bubbles was determined by the addition of 4-5 drops of 2% H<sub>2</sub>O<sub>2</sub> to 24 hr. semisolid PSS cultures, with subsequent incubation up to 30 minutes.

TABLE 2

PROTOCOL AND REAGENTS FOR MICRODIFFUSION  
ASSAY OF DEAMINASE ACTIVITY<sup>1</sup>

Dish No. <sup>2</sup>	Inner Well	Outer Well		
	Boric acid Indicator <sup>3</sup>	M/15 phosphate buffer, pH 7.0	10% Peptone	Cell Suspension
1	1.0 ml	0.5 ml	0.5 ml	0.5 ml
2	1.0 ml	0.5 ml	0.5 ml	0.5 ml
3	1.0 ml	0.5 ml	0.5 ml	0.5 ml

<sup>1</sup>Dishes were incubated for 1 hr. at 30 C, followed by addition of 1.0 ml of saturated K<sub>2</sub>CO<sub>3</sub> to outer wells. After further incubation for 2 hr., the contents of the inner wells were titrated with 0.01 N or 0.001 N HCl.

<sup>2</sup>65 x 15 mm plastic microdiffusion dishes (Falcon Plastics, Los Angeles, California) were used.

<sup>3</sup>Boric acid indicator: 2% boric acid containing 0.002% methyl red and 0.008% brom cresol green to give grayish endpoint color.

Urease Test.--Because of the normal alkaline reaction of cultures grown in PSS medium, this medium was not satisfactory for the urease test; consequently commercial urea broth (Difco) was employed, with incubation of cultures for up to one week. A deep red color indicated urease activity.

H<sub>2</sub>S Production.--For testing hydrogen sulfide production, 0.2% cysteine was incorporated into PSS broth. Dried test strips impregnated with lead acetate were suspended approximately 30 mm above the surface of the medium; cultures were incubated for 7 days before negative results were reported.

Casein Hydrolysis.--Clear zones around a streak of spirilla grown on PSS agar containing single-strength skim milk were used as indication of casein hydrolysis. The milk was combined aseptically with melted cooled double strength medium. Plates were incubated for up to 2 weeks before being reported as negative.

Amylase Activity.--Clear colorless zones around a streak of spirilla grown on PSS agar containing 10% soluble starch were used as indication of starch hydrolysis. Plates were flooded with Lugol's iodine to detect zones of hydrolysis. Plates were tested after both one and two weeks of incubation.

Hippurate Hydrolysis.--Sodium hippurate was incorporated into PSS broth in a final concentration of 1.0%.

The cultures were allowed to grow in the medium for 4 days and the hydrolysis of hippurate was detected by Method 1 of Cowan and Steel (1966).

Indole Production.--Indole production was determined by adding 0.1% tryptophan to the PSS broth. The inoculated medium was incubated for 48 hr. and indole was detected by extraction with ether and addition of Kovac's reagent (Cowan and Steel, 1966).

Aerobic Nitrate and Selenite Reduction.--The reduction of nitrate and selenite was determined by incorporating, respectively, potassium nitrate (0.1%) and sodium selenite (0.3%) into PSS broth; ammonium sulfate was omitted from the PSS broth when potassium nitrate was added. The cultures were incubated for 1 week; the reduction of nitrate was tested for by the method of Cowan and Steel (1966), while the reduction of selenite was checked by observing the cultures for a pink color reaction.

Gelatin Hydrolysis.--The hydrolysis of gelatin was detected by the incorporation of 12% gelatin to the PSS broth; after incubation for one week tubes were chilled to detect liquefaction.

Aesculin Hydrolysis.--Aesculin hydrolysis was determined by adding 0.1% aesculin and 0.05% ferric citrate to the PSS broth; tubes were incubated for one week and observed for blackening.

Pigment Production.--Phenylalanine, tryptophan,

or tyrosine were incorporated into PSS agar in concentrations of 0.2%; the production of water soluble pigments was detected in slants of this media after one week incubation. The production of deaminases specific for these amino acids was determined by first acidifying the slants with 0.2 ml of 0.1 N HCl and then adding 0.2 ml of 10% FeCl<sub>3</sub> aqueous solution.

Nuclease Production.--Spirilla were grown on PSS agar containing either 0.1% DNA or RNA; a clear zone around the streak after flooding the plates with 0.1 N HCl was used as indication of DNAase and RNAase production; plates were incubated for up to one week before reporting negative results.

Bile and Glycine Tolerance.--Growth in the presence of bile or glycine was determined by incorporating bile or glycine in concentrations of 1% to PSS broth; cultures were incubated for 2 weeks.

Sodium Chloride Tolerance.--Growth in PSS broth containing sodium chloride in concentrations of 3%, 4%, 5%, 7%, and 10% was estimated after 2 weeks. With marine organisms, the 2.75% sodium chloride already present in the medium was considered in calculating the final concentration of salt.

Growth on EMB and MacConkey Agar.--Cultures were incubated for one week on EMB and MacConkey agar for

the detection of growth.

Growth on TSI Agar and Seller's Slants.--Cultures were inoculated on TSI and Seller's slants by stabbing the butt and streaking the slant with an inoculating needle. The slants were observed for growth and any subsequent changes in the media.

Reaction in Litmus Milk.--Spirilla were incubated in litmus milk for 72 hrs; the cultures were observed daily for any reactions producing a color change in the litmus milk. The marine strains were not examined for their reactions in litmus milk; the artificial sea water had a precipitating effect on litmus milk even when added aseptically after autoclaving.

Acid Reactions from Carbohydrates.--For demonstration of acid reactions from sugars, PSS broth without succinate and with peptone decreased to 0.2% was used; phenol red indicator (18  $\mu$ g per l) was added. Thirty-two compounds (Table 3) were employed in the determination of acid production; the compounds were added aseptically to the peptone-salts broth at a final concentration of 1%. The cultures were incubated for up to 3 weeks.

Anaerobic Growth in the Presence of Nitrate.--Ability to grow anaerobically in the presence of nitrate was detected by incorporating 0.1% potassium nitrate to freshly-autoclaved PSS semi-solid medium; tubes were

TABLE 3

COMPOUNDS EMPLOYED FOR DETERMINATION  
OF ACID PRODUCTION FROM CARBOHYDRATES.\*

Arabinose	Melizitose
Ribose	Cellulose
Xylose	Dextrin
Glucose	Glycogen
Galactose	Inulin
Mannose	Pectin
Fructose	Erythritol
Sorbose	Mannitol
Rhamnose	Sorbitol
Lactose	Dulcitol
Melibiose	Adonitol
Sucrose	Inositol
Maltose	Amygdalin
Cellobiose	Glycerol
Trehalose	Salicin
Raffinose	Mucin

\*The compounds were prepared in 10 x stock solutions and added aseptically to the PSS broth.

inoculated and immediately sealed with sterile vaspar. Incubation was for 1 week.

Methods used for Morphological Determinations.--

Cell dimensions were obtained from measurement of living cells from 18 hr. PSS broth cultures, using a Leitz Ortholux microscope equipped with a Heine phase contrast condenser and Pv Apo 90x/1.15 objective. In 5 separate fields, a total of about 50 of the longest and the shortest cells were chosen for measurement of the range of cell length; the range of cell diameter, diameter of helix, wavelength, and number of turns were also obtained from these cells. Flagellar tufts were observed with dark field microscopy, using a 150 watt Xenon lamp for illumination and a 95x oil immersion fluorite objective, N.A = 1.10. The presence of coccoid bodies was determined by observing 4 week PSS cultures by phase contrast microscopy. Measurements of colonies growing on PSS agar plates were obtained after 72 hr.

Cultivation of Cells for DNA Isolation.--Spirilla were cultivated in shaken cotton-stoppered liter flasks containing 300 ml of medium having the following composition (g per l): nutrient broth, 6.0; yeast extract, 2.0; and succinic acid, 1.0. The pH was adjusted to 6.8 with 2N KOH. Aeration was accomplished by a 30 C water bath shaker (Rotary Shaker, New Brunswick Scientific

Co., New Brunswick, N.J.). The cells were centrifuged at 5000 x g after 18 to 24 hr. E. coli cells were obtained under similar conditions.

DNA Isolation. --After harvesting, the cells were suspended in 1X saline--EDTA (0.15 M NaCl, 0.01 M EDTA, pH 8.0) and lysed by the addition of 1% sodium lauryl sulfate. Ten ml of liquefied phenol were added, and the mixture was shaken (Wrist-Action Shaker, Burrell Corp., Pittsburgh, Pa. at a setting of 2) for 5 to 10 minutes or until homogenous. The mixture was cooled and centrifuged (5000 x g for 10 min.), and the aqueous phase was extracted again with liquefied phenol. After precipitation from the aqueous phase by addition of 2 volumes of ethanol, the DNA was dissolved in 0.1X SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The SSC concentration was increased to 1X (by addition of sufficient 20X SSC) and the precipitation procedure repeated. After increasing the SSC concentration to 1 X, 0.5 ml of a ribonuclease mixture at a concentration of 500 µg per ml of bovine pancreatic RNAase and 25 units per ml of T<sub>1</sub> RNAase was added and the preparation was incubated at 37 C for 2 hr. The DNA was deproteinized with 5-10 ml of chloroform-octanol (97% chloroform, 3% octanol). The mixture was shaken, cooled and centrifuged at 5000 x g, and the DNA was precipitated from

the aqueous phase with 2 volumes of ethylene glycol monoethyl ether. After a final ethyl alcohol precipitation, the DNA was dissolved in ca. 5 ml of 0.1 X SSC and stored at -18 C.

Melting Point (Tm) Measurements.--The DNA was adjusted to a concentration of 50 µg per ml with 0.5 x SSC and dialyzed against 0.5 X SSC, prior to Tm measurements. Melting temperature (Tm) determinations were obtained by a variation of the method of Marmur and Doty (1962); the starting temperature for determining the Tm profile was 60 C and the temperature was increased linearly 0.5 degree per minute over the entire range. An automatic recording Gilford Model 2400 Spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio) was employed.

## RESULTS

### Cultural and Morphological Tests

The characterization tests included in this category deal with the relation of spirilla to oxygen, temperature, colony formation on PSS agar, size of cells growing in PSS broth, the visibility of flagellar tufts under dark field, and the presence of coccoid bodies in older cultures.

Relationship to Oxygen.--All strains of spirilla except S. volutans grew moderately to abundantly well aerobically in PSS broth in 24 hr. The two strains of S. volutans, being obligate microaerophiles, failed to grow aerobically, but grew well in stratified (0.15% agar) PSS broth or in broth under an atmosphere of 30% air and 70% nitrogen. S. giesbergeri appeared to be a facultative microaerophile, in that although growth did occur aerobically, in stratified broth (0.15% agar) the organism characteristically formed a microaerophilic band of growth 3-4 mm below the surface of the medium.

Ability to Grow Anaerobically in Presence of Nitrate.--Anaerobic growth could occur only in the cases of S. lunatum, and the 3 strains of S. itersonii when 0.1%  $\text{KNO}_3$  was added to the PSS medium.

Temperature Range for Growth.--The optimum temperature was 30 C for all strains of spirilla, although moderate growth did occur at 20, 25, and 37 C. All of the marine strains had scant to moderate growth at 15 C, except S. beijeinckii which failed to grow; none of the fresh water strains grew at 15 C. At 42 C, only S. metamorphum, S. lunatum, S. japonicum, S. putridiconchylium, and certain strains of S. serpens: (Victoria, VH, VHA, VHL, VHS, St. Rhodes, and the unnumbered strain) were able to grow; growth was scanty. No growth occurred at 10 for any of the strains. Only the unnumbered Rittenberg strain of S. serpens was able to grow at 45 C.

Colony Characteristics.--Spirilla grown on PSS agar plates for 72 hr. formed colonies ranging from 0.5 to 1.5 mm in diameter, except the species S. anulus, S. volutans, S. polymorphum, S. gracile, and the un-named strain 101 all of which formed pinpoint colonies. All colonies were white, circular, and convex with smooth edges, except for the strains S. volutans and S. gracile which produced white colonies with fimbriated edges. The three strains of S. gracile formed colonies which were partially below the surface of the agar, indicating a possible microaerophilic response. Colonies from S. volutans were formed best in soft PSS agar (0.7% agar) incubated under reduced oxygen.

Dimensions of Cells.--Ranges for cell diameter, diameter of helix, wavelength, and length of cells are presented in Tables 4 and 5.

Visibility of Flagellar Tufts by Dark Field. All strains of spirilla were actively motile; flagellar tufts were clearly visible under dark field microscopy in many of the organisms. The strains of S. giesbergeri, S. linum, and the un-named strains 101, 102, and 206 all had helical tufts; similar helical structures were seen in at least 50% of the cells of S. delicatum, a species said to possess predominantly only a single polar flagellum. These flagellar tufts are of interest because they permit the investigator to view the action of such structures which, although present on a number of bacteria, are invisible by microscopic observations, and can be seen only after staining; the action of the flagellar tufts can best be described as a series of helical waves moving down the length of the structures. Straight tufts of flagella were seen under dark field on the species S. graniferum, S. anulus, S. putridiconchylum, S. japonicum, S. volutans and the S. serpens strain ATCC 12289; S. volutans flagellar tufts were observed by phase contrast microscopy also. The action of straight flagellar tufts were not readily observed on motile spirilla, except S. volutans, and were seen only on cells

TABLE 4

CELL DIMENSIONS OF FRESH WATER  
STRAINS OF SPIRILLA

Organisms	Range in Microns			
	Cell Diameter	Wave-length	Helix Diameter	Cell Length
<u>S. volutans</u>	1.4-1.7	16.0-18.0	5.0-8.0	14.0-60.0
<u>S. anulus</u>	1.4-1.5	4.9- 7.0	2.8-3.0	7.0-15.0
<u>S. giesbergeri</u>	1.1-1.4	<1	2.0-2.5	4.0-10.0
<u>S. metamorphum</u>	1.1-1.3	<1	2.8-3.0	4.0- 9.0
<u>S. putridicon- chylum</u>	1.0-1.2	6.0- 7.0	1.8-2.0	4.0-22.0
<u>S. graniferum</u>	1.0-1.2	7.0- 8.4	3.5-4.2	7.0-17.0
<u>S. serpens</u> ATCC 12289	0.9-1.0	4.2- 5.0	2.8-4.2	4.0-22.0
<u>S. serpens</u>	0.7-1.0	3.5-10.0	1.4-2.4	6.0-42.0
Un-named strain 103	0.7-0.8	2.0- 3.5	1.4-2.1	2.1- 6.5
<u>S. peregrinum</u>	0.5-0.7	3.5- 4.0	1.4-1.6	5.0-22.0
<u>S. sinuosum</u>	0.5-0.7	8.5-10.5	1.4-3.5	5.0-20.0
Un-named strain 104	0.5-0.7	2.0- 2.5	1.0-1.4	3.0- 8.0
<u>S. serpens</u> ATCC 11330	0.5-0.6	2.0- 2.8	0.8-1.0	2.5- 7.0
<u>S. itersonii</u>	0.4-0.6	3.0- 3.5	1.2-1.6	2.8- 7.0
<u>S. polymorphum</u>	0.3-0.5	<1	0.5-0.8	3.5- 8.4
<u>S. delicatum</u>	0.3-0.4	<1	0.4-0.7	3.0- 5.0
<u>S. gracile</u>	0.2-0.3	2.8- 3.5	0.5-2.0	4.0-14.0

TABLE 5

CELL DIMENSIONS OF MARINE  
STRAINS OF SPIRILLA

Organisms	Range in Microns			
	Cell Diameter	Wave- Length	Helix Diameter	Cell Length
<u>S. lunatum</u>	0.8-1.0	7.8- 3.0	1.0-1.3	2.4- 5.4
<u>S. japonicum</u>	0.7-0.8	8.0-20.0	1.0-3.0	20.0-75.0
Un-named strain 102	0.6-0.7	5.6- 7.0	2.0-2.8	8.5-18.0
Un-named strain 204	0.5-0.7	<1	0.8-1.0	2.1- 3.0
Un-named strain 206	0.5-0.7	6.5- 7.0	2.0-2.8	7.0-21.0
<u>S. beijerinckii</u>	0.5-0.7	6.3- 7.2	1.5-1.6	7.0-14.0
Un-named strain 101	0.5-0.6	3.5- 4.0	1.4-2.0	7.0-40.0
<u>S. atlanticum</u>	0.4-0.6	3.2- 3.5	1.2-1.4	4.0-14.0
<u>S. linum</u>	0.4-0.5	1.8- 2.0	0.8-1.0	7.0-30.0
<u>S. halophilum</u>	0.3-0.6	2.0- 2.8	1.4-1.5	3.5- 8.0
<u>S. minutulum</u>	0.3-0.4	2.0- 2.3	0.6-0.8	3.0- 8.0

which had stopped their motion; in all such cases the flagellar tufts extended out from the cell at an angle of approximately 45 degrees. S. volutans was unique in its flagellar action because its action during rapid movement was readily observed (Krieg, Tomelty, and Wells, 1967).

#### Presence of Coccoid Bodies in Older Cultures.--

The presence of coccoid bodies in 1 to 4 week old cultures was found in all of the marine strains, except the un-named strain 204; in the fresh water forms they were observed in only S. polymorphum, S. itersonii, and S. peregrinum. The diameters of these coccoid bodies ranged from 3.0 to 5.0  $\mu$ .

#### Physiological Tests

Certain physiological tests were found to be uniformly positive or negative for all or nearly all of the strains studied; other kinds of tests resulted in a mixture of positive and negative results among the strains.

Positive Physiological Tests.--Reactions uniformly or nearly uniformly positive are summarized in Table 6. All strains were oxidase positive, deaminase positive (quantitative results are presented in Table 7), and catalase positive, although the catalase reaction was considered very weak with S. volutans, S. japonicum, S.

TABLE 6

POSITIVE PHYSIOLOGICAL REACTIONS OF SPIRILLA

---

Oxidase

Deaminase

Catalase (but weak with S. volutans, S. japonicum, S. beijerinckii, and S. atlanticum.)

Phosphatase (except S. halophilum, S. minutulum, and un-named strains 101, 102, and 206.)

H<sub>2</sub>S from cysteine (except S. atlanticum, S. halophilum, S. minutulum, and S. japonicum.)

Growth with 1% bile (except S. volutans, S. anulus, and S. delicatum.)

TABLE 7

DEAMINASE ACTIVITY OF SPIRILLA AS  
DETERMINED BY MICRODIFFUSION ANALYSIS

Organism	$\mu$ Moles NH <sub>3</sub> Produced per ml of cell suspension	Organism	$\mu$ Moles NH <sub>3</sub> Produced per ml of cell suspension
<u>S. anulus</u>	35.28	<u>S. serpens</u> ATCC 12638	185.76
<u>S. atlanticum</u>	32.40	<u>S. serpens</u> ATCC 11335	103.68
<u>S. beijerinckii</u>	118.80	<u>S. serpens</u> ATCC 15278	47.88
<u>S. delicatum</u>	39.60	<u>S. serpens</u> VH	47.00
<u>S. giesbergeri</u>	21.60	<u>S. serpens</u> VHA	48.68
<u>S. gracile</u> ATCC 19624	17.64	<u>S. serpens</u> VHL	46.80
<u>S. gracile</u> ATCC 19625	16.80	<u>S. serpens</u> VHS	60.84
<u>S. gracile</u> ATCC 19626	17.28	<u>S. serpens</u> St. Rhodes	30.24
<u>S. graniferum</u>	28.40	<u>S. serpens</u> Victoria	47.88
<u>S. halophilum</u>	25.56	<u>S. serpens</u> unnumbered	151.20
<u>S. itersonii</u> unnumbered	108.00	<u>S. serpens</u> ATCC 12289	36.00
<u>S. itersonii</u> ATCC 11331	125.20	<u>S. serpens</u> ATCC 11330	131.04
<u>S. itersonii</u> ATCC 12639	108.00	<u>S. sinuosum</u>	68.76
<u>S. japonicum</u>	33.48	<u>S. volutans</u> ATCC 19553	158.40
<u>S. linum</u>	172.00	<u>S. volutans</u> ATCC 19554	146.40
<u>S. lunatum</u>	52.30	Un-named strain 101	108.00
<u>S. metamorphum</u>	72.00	Un-named strain 102	52.56
<u>S. minutulum</u>	24.60	Un-named strain 103	568.00
<u>S. peregrinum</u>	127.80	Un-named strain 104	428.40
<u>S. polymorphum</u>	41.40	Un-named strain 204	72.00
<u>S. putridicon-</u> <u>chylum</u>	54.00	Un-named strain 206	33.48

beijerinckii and S. atlanticum; in the case of the latter 4 species, a froth of bubbles appeared only after 15 to 30 minutes incubation, with uninoculated control tubes being negative in all cases. All strains were phosphatase positive except S. halophilum, S. minutulum, and the 3 un-named marine strains 101, 102, and 206. As for the production of hydrogen sulfide from cysteine all strains were positive except the marine strains of S. atlanticum, S. halophilum, S. minutulum, and S. japonicum. All strains were able to grow in the presence of 1% bile except S. volutans, S. anulus, and S. delicatum.

Negative Physiological Tests.--Reactions which were uniformly or nearly uniformly negative are summarized in Table 8. Growth in urea broth was scanty except for S. lunatum, S. putridiconchylum, S. peregrinum, S. metamorphum, and the un-named strains 103, 104, and 204; S. peregrinum was the only species to produce a color change, and this occurred within 24 hr. From Table 8, it can be seen that S. metamorphum and all strains of S. serpens (except S. serpens ATCC 12638 and S. serpens ATCC 11335) were the only organisms to hydrolyze gelatin. S. lunatum, S. peregrinum, and S. serpens ATCC 11330 were the only species to produce any reaction in litmus milk; all three strains produced a slight

TABLE 8

NEGATIVE PHYSIOLOGICAL REACTIONS OF SPIRILLA

---

Indole production

Hippurate hydrolysis

Amylase

Sulfatase

Casein hydrolysis

Urease (except S. peregrinum.)

Gelatin hydrolysis (except S. metamorphum and 8 out of  
10 strains of S. serpens.)

Aesculin hydrolysis (except S. polymorphum, S. itersonii,  
and S. peregrinum.)

Reactions in litmus milk (except S. lunatum, S. peregrinum,  
and S. serpens ATCC 11330.)

Acid reaction from sugars (except S. itersonii, S. gracile,  
S. peregrinum, and the un-named strain 204.)

alkaline reaction at the surface of the milk.

Acid Reactions from Carbohydrates.--A reduced peptone concentration (0.2%) was necessary for the detection of acid production from carbohydrates; when 1.% peptone was used, no acid reactions from carbohydrates occurred with any spirilla. Only 9 strains of spirilla were found to be able to produce an acid reaction from carbohydrates (see Table 9). More strains produced acid from fructose than from any other compound, while dextrin appeared to be the least frequently utilized substrate for acid production among the strains. Table 9 indicates that not only was there a limited number of strains capable of producing acid from carbohydrates but the variety of sugars used by each strain was highly restricted.

Physiological Tests Giving Varied Responses Among Spirilla.--Tables 10 and 11 indicate the results of physiological tests in this category. With fresh-water spirilla, selenite was reduced by only 6 out of 16 species. The unnumbered strain of S. itersonii and S. itersonii ATCC 11331 reduced selenite, while S. itersonii ATCC 12639 did not. In regards to nitrate reduction, although several strains could reduce nitrate, a smaller number could further reduce the resulting nitrate to  $N_2$  or  $NH_3$ . Growth on MacConkey agar was positive for

TABLE 9

SPIRILLA PRODUCING ACID FROM CARBOHYDRATES

<u>Organisms</u>	<u>Carbohydrates used for acid production</u>
<u>S. itersonii</u> unnumbered strain	Glycerol, fructose
<u>S. itersonii</u> ATCC 11331	Glycerol, fructose
<u>S. itersonii</u> ATCC 12639	Glycerol, fructose
<u>S. gracile</u> ATCC 19624	Glucose, arabinose, galactose
<u>S. gracile</u> ATCC 19625	Glucose, arabinose, galactose
<u>S. gracile</u> ATCC 19626	Glucose, arabinose, galactose
<u>S. peregrinum</u>	Fructose
<u>S. lunatum</u>	Fructose, mannose, glucose, galactose, dextrin, mannitol
Un-named strain 204	Fructose, mannose, glucose, galactose, mannitol

the following strains of S. serpens: the unnumbered strain, Victoria, VH, VHA, VHL, VHS, and the St. Rhodes strain. Growth in MRVP broth was positive for the following strains: Victoria, VH, VHA, VHL, VHS and St. Rhodes. On Sellaer's slants, VHL, VHS, and St. Rhodes strains of S. serpens gave positive growth reactions. Growth of spirilla on Sellaer's slants resulted in all cases in the production of alkaline slants and greenish colonies. The 2 un-named strains 103 and 104 produced alkaline butts in addition to the alkaline slant reaction. The strains of spirilla which gave a positive growth response on TSI agar did not produce any gas or color changes. Ribonuclease, deoxyribonuclease, or both were formed in several species. Very few of the fresh-water species possessed the ability to grow in the presence of 1% glycine, in contrast to the marine species, where every strain except S. beijerinckii and strain 204 gave a positive response. With regard to marine spirilla, most tests appeared to be almost uniformly negative for the various species except for the reduction of selenite, growth in MRVP broth and growth on TSI slants: no gas or color changes occurred in the latter.

Ability of S. lunatum to Grow in Fresh-Water or Sea Water.--S. lunatum was the only strain of spirilla capable of growing in either fresh-water or sea water

TABLE 10

PHYSIOLOGICAL TESTS YIELDING VARIED RESULTS  
AMONG FRESH-WATER STRAINS OF SPIRILLA STUDIED\*

Organisms	Selenite reduction	$\text{NO}_3^- + \text{NO}_2^-$	$\text{NO}_2^- + \text{N}_2$ or $\text{NH}_3$	EMB agar	MacConkey agar	TSI agar	Seller's slants	MRVP broth	Ribonuclease	Deoxyribonuclease	1% glycine
<i>S. volutans</i>	-	-	-	-	-	-	-	-	-	-	-
<i>S. sinuosum</i>	-	-	-	-	-	-	-	-	+	+	-
<i>S. anulus</i>	-	-	-	-	-	-	-	-	+	+	-
<i>S. giesbergeri</i>	-	-	-	-	-	-	-	+	+	+	-
<i>S. graniferum</i>	-	-	-	-	-	-	-	+	+	+	-
<i>S. metamorphum</i>	-	-	-	+	-	+	-	-	+	-	-
<i>S. putridicon- chylum</i>	-	-	-	+	-	+	-	-	+	-	-
<i>S. peregrinum</i>	+	-	-	+	-	+	+	-	+	-	-
<i>S. serpens</i>	-	-	-	+	d	+	d	d	+	+	-
<i>S. gracile</i>	-	+	-	+	-	+	-	-	+	+	-
<i>S. itersonii</i>	d	+	+	+	+	+	+	+	+	+	-
<i>S. delicatum</i>	-	+	-	-	-	+	-	-	+	-	-
<i>S. polymorphum</i>	+	+	-	+	+	-	-	-	-	-	-
Un-named strains:											
103	+	+	+	+	+	+	+	+	-	-	+
104	+	+	+	+	+	+	+	+	-	-	+

\*+ = positive for all strains used.

- = negative for all strains used.

d = varied results among the strains within a species.

TABLE 11

PHYSIOLOGICAL TESTS YIELDING VARIED RESULTS  
AMONG MARINE STRAINS OF SPIRILLA STUDIED\*

Organisms	1% glycine	Deoxyribonuclease	Ribonuclease	MRVP broth	TSI agar	MacConkey agar	EMB agar	NO <sub>2</sub> <sup>-</sup> → N <sub>2</sub> or NH <sub>3</sub>	NO <sub>3</sub> <sup>-</sup> → NO <sub>2</sub> <sup>-</sup>	Selenite reduction
<u>S. linum</u>	+	-	-	+	+	-	-	-	-	+
<u>S. beijerinckii</u>	-	+	+	-	-	-	-	-	-	-
<u>S. atlanticum</u>	+	-	+	+	+	-	-	-	-	+
<u>S. japonicum</u>	+	-	+	+	+	-	-	-	-	+
<u>S. halophilum</u>	+	-	+	+	+	-	-	-	-	+
<u>S. minutulum</u>	+	-	+	+	+	-	-	-	-	+
<u>S. lunatum</u>	+	-	+	+	+	-	-	-	-	+
Un-named strains:										
101	+	-	-	+	+	-	-	-	-	-
102	+	-	-	+	+	-	-	-	-	-
206	+	-	-	+	+	-	-	-	-	-
204	+	-	-	+	+	-	-	-	-	+

\*+ = positive for all strains used.  
 - = negative for all strains used.  
 d = varied results among the strains within a species.

media; therefore, both types of water were employed in the characterization tests for this organism, with identical results in all cases.

Deamination and Pigment Production with Aromatic Amino Acids.--Deamination of aromatic amino acids was uniformly negative; however a number of the strains could not be examined for the specific color reaction because of the production of distinctive water soluble pigments which would have obscured the results (Table 12). S. peregrinum was unusual in that it was the only species to produce such a pigment in the presence of phenylalanine. The un-named strain 204 was the only strain to form a jet black pigment on tyrosine. This pigment could be decolorized by treatment with sodium hydrosulfite; because of this, it is possible that the pigment was melanin.

Salt Tolerance.--All fresh-water strains were inhibited by the addition of 3% or greater NaCl to the PSS broth; tolerance to lower concentrations was not determined. All of the marine strains were capable of growth in 9.75% NaCl; S. lunatum, S. halophilum, and the un-named strain 204 were able to grow in a concentration of 12.75% NaCl.

Base Composition of DNA.--DNA was isolated from all 42 strains of spirilla and the base compositions, expressed as moles % G + C ( $T_m$ ) are presented in Tables 13 and 14 in ascending order.

TABLE 12

FORMATION OF WATER SOLUBLE PIGMENTS  
IN PRESENCE OF AROMATIC AMINO ACIDS

Organisms	Amino Acids		
	Tyrosine	Phenylalanine	Tryptophan
<u>S. peregrinum</u>	---	yellow	---
<u>S. lunatum</u>	brown	---	brown
<u>S. atlanticum</u>	brown	---	---
<u>S. beijerinckii</u>	brown	---	---
<u>S. halophilum</u>	---	---	brown
<u>S. linum</u>	brown	---	brown
Un-named strains:			
204	black	---	brown
206	brown	---	---

TABLE 13

SPIRILLA POSSESSING DNA BASE COMPOSITION  
VALUES FROM 38 TO 52 MOLES % G + C

Species	Strain	T <sub>m</sub> (C)	% GC
<u>S. volutans</u>	ATCC 19553	84.8	38
<u>S. volutans</u>	ATCC 19554	84.9	38
<u>S. halophilum</u>	ATCC 19192	86.7	42
<u>S. minutulum</u>	ATCC 19193	86.6	42
<u>S. japonicum</u>	ATCC 19191	87.9	45
Un-named	102	87.9	45
Un-named	206	87.8	45
Un-named	101	88.0	46
<u>S. beijerinckii</u>	ATCC 12754	88.6	47
<u>S. atlanticum</u>	ATCC 12753	89.0	48
<u>S. linum</u>	ATCC 11336	88.9	48
<u>S. serpens</u>	ATCC 12638	89.9	50
<u>S. serpens</u>	ATCC 15278	90.0	50
<u>S. serpens</u>	VH	90.0	50
<u>S. serpens</u>	VHA	89.9	50
<u>S. serpens</u>	VHL	90.0	50
<u>S. serpens</u>	VHS	89.8	50
<u>S. serpens</u>	Victoria	89.9	50
<u>S. serpens</u>	Un-numbered	89.7	50
<u>S. serpens</u>	St. Rhodes	90.1	51
<u>S. serpens</u>	ATCC 11335	90.3	51
<u>S. putridiconchylum</u>	ATCC 15279	90.6	52

TABLE 14

SPIRILLA POSSESSING DNA BASE  
COMPOSITION VALUES FROM 57 TO 65 MOLES  
% G ÷ C

Species	Strain	T <sub>m</sub> (C)	% GC
<u>S. sinuosum</u>	ATCC 9786	92.8	57
<u>S. graniferum</u>	NCIB 8230	92.8	57
<u>S. anulus</u>	ATCC 19259	93.1	58
<u>S. giesbergeri</u>	ATCC 11334	93.2	58
<u>S. giesbergeri</u>	NCIB 9073	93.0	58
<u>S. serpens</u>	ATCC 12289	93.0	58
Un-named	204	93.7	60
<u>S. itersonii</u>	Un-numbered	94.7	62
<u>S. itersonii</u>	ATCC 11331	94.8	62
<u>S. itersonii</u>	ATCC 12639	94.6	62
<u>S. polymorphum</u>	ATCC 11332	94.9	62
<u>S. polymorphum</u>	NCIB 9072	94.8	62
<u>S. peregrinum</u>	ATCC 15387	94.6	62
<u>S. lunatum</u>	ATCC 11337	95.3	63
Un-named	103	95.4	63
<u>S. metamorphum</u>	ATCC 15280	95.4	63
<u>S. delicatum</u>	ATCC 14667	95.5	63
<u>S. gracile</u>	ATCC 19624	96.1	65
<u>S. gracile</u>	ATCC 19625	96.2	65
<u>S. gracile</u>	ATCC 19626	96.1	65
<u>S. serpens</u>	ATCC 11330	95.8	65
Un-named	104	96.1	65

## DISCUSSION

Limits of Genus and Species Variation. Unlike the work of Williams and Rittenberg (1957), which was based largely on the examination of their own isolates, and the more recent investigators who have isolated, described, and named several new species of the genus Spirillum, the present study involved a comprehensive examination of all strains presently available from public collections, plus some strains from private collections. It is, however, unfortunate that most of the species are currently represented by only a single strain, the type, except in the cases of S. serpens (12 strains), S. gracile (3 strains), S. itersonii (3 strains), and S. volutans (2 strains). For some species, such as S. virginianum and S. curvatum, a type strain could not be obtained from a collection even though the collection carried the name of the strain in its catalog. One possible reason for this is the difficulty of preservation by lyophilization, although lyophilized cultures of the species S. graniferum, S. polymorphum, and S. giesbergeri were received

from the NCIB; attempts to obtain technical details of the method used were unsuccessful. The ATCC has been unable to maintain strains of spirilla by lyophilization. Examination of several strains belonging to a species is important in determining variation within a species; for example, it would be important for the development of a diagnostic key to be able to know those characteristics that were positive or negative for 95 - 100% of the strains, as some of these might represent good diagnostic characters. Obviously tests yielding mixed reactions among the strains of a species would not be of suitable diagnostic value for the species. With regard to the two strains of S. volutans, the type strain (Pringsheim strain) was first obtained in mixed culture from the cooling vat of a sugar beet refinery in England in 1951; this strain was later obtained in pure culture by Rittenberg and Rittenberg (1962). The Wells strain of S. volutans was obtained in mixed culture and isolated in 1964 from pond-water in Virginia (Wells and Krieg, 1965). It is noteworthy that although the two strains originated from different continents, and although thirteen years separated their discovery, they proved to be identical with respect to all the morphological, physiological,

and biochemical characteristics used in the present investigation. In the cases where species were represented by only one strain, that strain was always the nomenclatural type, that is, the strain to which the name of the species is permanently assigned. It was felt to be highly desirable to determine the characteristics of such name-bearers because these strains serve as permanent examples of what is meant by the various species, although they are not necessarily typical of the species. An important aspect of future work, however, would be the isolation and characterization of new strains of spirilla belonging to species which previously had been characterized solely on the basis of the type strains.

The Usefulness of Standardized Conditions in Characterizing Strains of Spirilla. The present investigation of the genus Spirillum employed standardized conditions in the study of all the strains of spirilla, with the object of providing a valid comparison of the various strains. Lack of uniformity in the methodology among previous investigators has made such a comparison very difficult. For example, previous investigators obtained a number of morphological measurements for many of the spirilla, but in a number of cases the morphological dimensions were

either obtained from stained cells (Williams and Rittenberg, 1957; Watanabe, 1959; Terasaki, 1961) or from living cells (Giesberger, 1936); in the case of the latter, the method of observation (brightfield, phase contrast, or dark field) was not specified. Measurement of stained cells is not as desirable as for living cells, because of the distortions produced by drying and flattening. In the literature, a variety of media and times of incubation have been used as a basis for morphological measurements; e.g. Giesberger (1936) used peptone water medium and examined the cultures after 24 hr. incubation at 30C; Williams and Rittenberg (1957) employed a nutrient broth-yeast autolysate broth (time of incubation was not specified); Watanabe (1959) employed bouillon, peptone-water and other unspecified media, which contained 2.5% (w/v) NaCl, besides the two types of artificial sea water media for purposes of identification, but he did not specify which was employed for morphological measurements, and the time of incubation was not specified; Canale-Parola, Rosenthal, and Kupfer (1966) employed a medium composed of peptone, yeast extract, Tween 80,  $K_2HPO_4$ , and tap water, and the time of incubation was not specified. In the present investigation, a standard medium and time of incubation were employed for

morphological measurements, and all observations were made of living cells by phase contrast. Growth curves were not determined for any of the spirilla, and therefore one cannot say that morphological dimensions were obtained from cells growing in the log phase. It is realized that cells growing in the log phase are normally more uniform in size and shape than cells from other stages of growth; however, since conditions of medium, temperature, age and size of inoculum, time of incubation, and method of observation were clearly defined, the stated morphological characteristics should be reproducible; moreover, since the same conditions were applied to all strains, a valid comparison of the spirilla can be made.

In the literature, a variety of media have been employed for the determination of physiological characteristics, such as nitrate reduction, catalase production, and gelatin hydrolysis. Possibly even more important was a lack of details concerning the methodology employed for the performance of the various tests. For example, in the 7th edition of Bergey's Manual (Breed, Murray, and Smith, 1957) a number of characteristics were presented for each species but many details were not explained; e.g. the nature of the media employed for physiological tests; the age of the cells employed;

the length of time for which certain tests were allowed to proceed (especially important in the catalase test). Giesberger (1936) used peptone water but did not specify testing techniques; Williams and Rittenberg (1957) used nutrient broth-yeast autolysate medium but did not specify methodology for physiological tests; Canale-Parola, Rosenthal, and Kupfer (1966) did not present details of their methods for determining catalase production; Watanabe (1959) did not present details for determination of gelatin liquefaction, indole production, reduction of nitrate, and catalase production (the latter being especially important since cultures were reported as catalase-negative). Pretorius (1963) did specify much of his methodology, in that nitrate broth (Difco) was used for determination of nitrate reduction, nutrient gelatin (Difco) for gelatin liquefaction, and growth on nutrient agar for catalase production; moreover, times of incubation were given.

In the present comprehensive study, the types of media, incubation periods, and methodology for the determination of reactions have been clearly specified for all strains, so that a valid basis of comparison exists. To indicate the importance of specifying conditions, a number of examples may be presented. The time element was critical in determining the production

of water-soluble pigments from aromatic amino acids, since the jet black water soluble pigment produced by the un-named strain 204 appeared only after five days growth. Attempts to reproduce this work would result in erroneous results if the experiment were to be terminated earlier. The catalase test, if examined immediately after the addition of  $H_2O_2$ , gave a negative reaction in several cases, whereas if the test were examined after 30 minutes, a definite froth of bubbles could be seen, as compared with uninoculated controls.

Concerning acid reactions from sugars, the necessity of reducing the peptone concentration in order to demonstrate an indicator change is important due to the alkaline reactions produced by these organisms growing in the presence of peptone. Acid reactions were not detected for any of the spirilla when the peptone concentration was 1.0%. Because certain compounds may have toxic effects, the need for specifying in detail concentration of the medium components employed is necessary; for example, phosphate is toxic to S. volutans at concentrations greater than 0.0029 M (Wells, 1966). Another example is the necessity for explaining whether the nitrate test was carried out under aerobic or anaerobic conditions, since oxygen

is known to inhibit nitrate reduction in some groups of bacteria (Doelle, 1969).

The possibility exists that some of the conditions employed in the present study are not optimal for detection of certain characteristics, as perhaps in the employment of Difco urease broth for the urease tests or in the use of only four days of incubation to determine gelatin liquefaction; nevertheless, because the same conditions were used for all strains, a valid frame of reference for comparison does exist.

Discrepancies Arising from a Comparison of the Present Data With Earlier Results. In most cases, results obtained in the present study coincided with those in the literature, but there are a few cases where discrepancies were found. A contradiction involving the production of catalase concerned five species previously reported as catalase negative: S. halophilum, S. minutulum, and S. japonicum (Watanabe, 1959); S. metamorphum (Teraski, 1961; and S. putridiconchylium (Terasaki, 1961). All of these but S. japonicum have exhibited strong catalase reactions; S. japonicum exhibits a weak reaction. One possible explanation for these apparent contradictions in the catalase reactions may be the same as that suggested by Canale-Parola, Rosenthal, and Kupfer (1966) for the strains of S. gracile; they

suggested that subculturing over a period of time may have selected for cells producing higher levels of catalase, since all their isolates gave negative catalase tests immediately after isolation, while a definite positive reaction was observed in all strains after prolonged subculturing.

Such considerations of the possible changes that may occur upon continued transfer are not only applicable to the catalase reactions, but have a more general implication as well. In the present study, one has no assurance that a strain used is identical in those characteristics to the same strain upon its initial isolation. For example, the type strain of S. lunatum at present can grow in PSS broth in the presence or absence of sea water; yet Williams and Rittenberg (1957), who isolated this strain, reported that this strain could not grow unless at least 50% sea water were present; moreover, only natural rather than synthetic sea water was effective. It is possible that a difference in formulation of synthetic sea water is responsible for the latter effect (no formulation for artificial sea water is given by Williams and Rittenberg) but it is also possible that the strain has changed during the thirteen years between its isolation and the present study. In the cases of discrepancies

concerning these and other tests, it is equally likely, however that differences in methodology may be responsible, and no firm decision can be made.

Although the use of nitrate as an electron acceptor under anaerobic conditions was reported for S. polymorphum by Williams and Rittenberg (1957), in the present study, negative results were obtained for this organism.

S. delicatum was reported by Leifson (1962) to possess predominantly a single polar flagellum; our own darkfield observations of this organism indicate the presence of visible helical structures, suggesting the presence of bundles of flagella. Williams (1960) was able to detect flagellar bundles in spirilla previously reported by her to be monotrichous, and therefore the use of the electron microscope may clarify the situation with S. delicatum. The problem is taxonomically important since monotrichous helical cells would be classified in other genera such as Vibrio and Campylobacter.

Morphological discrepancies arose in regard to S. putridiconchylum, S. japonicum, S. halophilum, and S. minutulum (Watanabe, 1959). From Tables 4 and 5, it can be seen that the cell diameters obtained in the present study differs from those previously

reported. The latter were 0.8 - 1.0  $\mu$ , 1.2 - 1.5  $\mu$ , 0.5 - 0.6  $\mu$ , and 0.2 - 0.3  $\mu$ , respectively. The differences could possibly be attributed to the cultivation media, length of incubation, or methods of observation.

In regard to hydrogen sulfide production, Terasaki (1961) reported that S. metamorphum did not produce hydrogen sulfide; however, his methods were not specified; since we obtained positive results when S. metamorphum was grown in the presence of cysteine, we feel that differences in methodology may be responsible for this discrepancy.

A discrepancy concerning the % GC composition of spirilla was found in the case of S. linum. In a review by Hill (1966), an unnumbered strain of S. linum was reported to have a % GC content of 28-30. Our present investigation of the type strain of S. linum revealed a % GC content of 48. However, since the strain reported by Hill was not clearly identified, the possibility exists that this strain may not have been a strain of S. linum.

Division of Genus Spirillum on the Basis of Single, Highly-Weighted Characters. In evaluating a taxonomic character, the number of genes involved (or at least some approximate estimate, based on knowledge of the

number of enzymes involved in expression of the character) would ideally be determined. The more genes involved, the greater would be the taxonomic weight. Some characters may individually carry great weight - such as sporulation, photosynthetic metabolism, methane fermentation, etc., which involve many enzymes. Do separate characters of sufficient weight exist within genus Spirillum to justify a division of the genus into two or more genera?

A possible division of the genus Spirillum into two genera might be suggested on the basis of a distinction between obligately microaerophilic vs. aerobic spirilla. The only members of the obligately microaerophilic category would be S. volutans, and such a generic group might be further justified by the low % GC value (4% lower than any other species of spirillum). One hesitates to weigh obligate microaerophilism highly, however, because oxygen is used as electron acceptor for both aerobic and microaerophilic organisms; thus the difference may be a superficial quantitative difference rather than a more fundamental qualitative difference. To support this, spirilla intermediate between the two categories appear to exist: the two species S. giesbergeri and S. gracile appear to demonstrate facultative microaerophilic responses in that the former grows in a

microaerophilic band a few millimeters below the surface of PSS medium containing 0.15% agar, while the latter, growing on PSS agar, forms colonies which are partially embedded beneath the surface. This partial submergence of colonies suggested a microaerophilic nature to Canale-Parola, Rosenthal, and Kupfer (1966).

A requirement for sea water might be suggested as sufficient basis to create separate genera for fresh-water and marine spirilla. In regard to requirement for sea water, the number of genes involved in distinguishing these organisms from fresh-water forms is not known; moreover, the distinguishing feature separating marine bacteria from most nonmarine species is generally agreed to reside in the possession of a requirement for  $\text{Na}^+$  by the marine organisms. A requirement for natural sea water, a characteristic weighted heavily by Williams and Rittenberg (1957), may carry taxonomic weight since these authors reported that continued subculture in a synthetic sea water medium did not permit continued growth of the marine spirilla; however, Watanabe (1959) was successful in isolating and subculturing four species of marine spirilla on a synthetic sea water; moreover, in the present study we have successfully subcultured and examined these species, as well as those reported by Williams and Rittenberg, on an artificial sea water

medium. A possible explanation for our success in obtaining growth on synthetic sea water may be that continued subculturing over the years has selected for variants with a reduced dependency for natural sea water; a difference in formulation of artificial sea water represents another possibility. It was also observed in the present investigation that S. lunatum had the ability to grow in media completely devoid of any type of sea water; this may perhaps be attributed, as previously mentioned, to continuous subculturing over the years. In regard to this, MacLeod (1965) states, "Although much remains to be done to elucidate the differences between marine and terrestrial species at the molecular level, enough information is available to suggest that a quite limited number of successive mutations could convert a marine species to a form which would not be dependent on the sea for its survival".

The possibility exists of dividing the genus Spirillum into different genera on a morphological basis since the spirilla are diverse with respect to cell diameter (see Tables 4 and 5). However, we hesitate to divide the genus on this basis because there appears to be an almost continuous spectrum of sizes within the genus; and any grouping would be purely arbitrary.

Another possibility for division of genus Spirillum into two or more genera might be based on DNA base composition. The % GC composition of only a relatively few strains of spirilla have been reported in the literature; these have been reviewed by Hill (1966), where the range of moles % GC was from 28 to 61.5. Our own investigation revealed a % GC range of 38 to 65. This range is a large range for a genus when compared with certain other genera of bacteria; for example, the GC values of the genus Pseudomonas range from 57 to 70 moles % (Mandel, 1966); that of the genus Bacillus from 35 to 51 moles % (Marmur, Falkon, and Mandel, 1963); and that of the genus Escherichia from 50 to 53 moles % (reviewed by Hill, 1966). The genus Vibrio at one time possessed a rather large range of % GC, (29.5 to 65 moles %, reviewed by Hill, 1966); however the genus Vibrio was separated, on the basis of DNA base compositions and other features, into several genera by Sebald and Véron (1963) and such a division represents an example of what possibly could apply to the genus Spirillum. Vibrios possessing a GC range of 29.5 to 35.5 moles % were placed in the genus Campylobacter (also distinguished from Vibrio as being microaerophilic to anaerobic and being non-fermentative); the remaining vibrios have been further separated into

genus Comamonas (% GC = 64-65, aerobic, non-fermentative) and genus Vibrio (% GC = 44-50, aerobic or facultatively anaerobic, fermentative). The two strains V. ichthyodermis (% GC = 61) and V. cunsatus (% GC = 63) were removed from the genus Vibrio and placed in the genus Pseudomonas not only because of their % GC values, but also because of their pigment production, aerobic growth and non-fermentative ability. Therefore, possible divisions within the genus Spirillum may be warranted on the basis of the large range of values of moles % GC within the present genus; however, a number of other correlated characteristics are required before a definite decision can be made (see following section); e.g. nutrition, serology, and possibly most important, DNA homology. Sebald and Véron (1963) divided the genus Spirillum into two subgroups on the basis of GC values: Spirillum subgroup A with a % GC value of 49-54 and Spirillum subgroup B with a % GC value of 57-61. We do not feel that such a division is satisfactory at this level, because of the almost continuous spectrum of values we have found to exist within the genus; any divisions made on the basis of DNA base composition would seem to be arbitrary.

Division of Genus Spirillum on the Basis of Correlated Characters. Although no single factor

appears to carry enough weight according to present knowledge to justify division of genus Spirillum into two or more genera, the total effect of several correlated factors may carry such weight. Do such correlations of characteristics occur within genus Spirillum?

Obligate microaerophilism can be correlated with a large cell diameter (1.4 - 1.7 $\mu$ ) and a very low GC ratio (38 moles %). Yet it appears to the author that these factors, even when considered together, carry insufficient weight to justify division at the generic level. Further development of knowledge concerning the fundamental nature of microaerophilism may necessitate revision of this opinion.

Requirement for sea water can be correlated with the tolerance of the marine strains to salt, their ability to tolerate 1% (w/v) glycine, the presence of coccoid bodies in older cultures of all marine strains except the un-named strain 204, and the % GC values in the range 42-48, except for the two strains, S. lunatum (63 moles %) and the un-named strain 204 (60 moles %). The fact that the marine strains were tolerant to 7 to 10% (w/v) added salt to the medium also distinguishes them from the fresh-water spirilla, which were unable to tolerate even 3% (w/v) NaCl. The assumption could be made that marine bacteria, since they

live in the sea, are naturally salt-tolerant. However, Tyler, Bielling, and Pratt (1960) examined 15 marine strains of bacteria with the following results: all strains grew at 4.7% NaCl, 9 grew at 8.2% NaCl and none grew at 15.2% NaCl. In contrast, a number of terrestrial species, not normally found in a high salt environment, can tolerate much higher concentrations of salt; many aerobic sporeformers can grow at 15 to 20% NaCl while many staphylococci tolerate 25% NaCl. Indeed, some staphylococci can grow well in saturated NaCl. Therefore, the possibility exists that there is some other basis for the salt tolerance of the marine spirilla besides ecological considerations.

The ability of the marine strains of spirilla to tolerate 1% (w/v) glycine is of interest since many gram negative bacteria are readily converted to spheroplasts by the inclusion of glycine into their growth medium. This indicates that the marine strains may differ somewhat in their cell wall structure from the fresh-water strains, or possibly that the higher salt concentrations in the synthetic sea water serve in a protective manner. That there may be differences in cell wall composition is also suggested by the abundance of coccoid bodies found in older cultures of the marine strains.

In regard to the development of coccoid bodies, although Williams and Rittenberg (1957) assigned considerable weight to this characteristic, it would appear that not enough is known about their fundamental nature to permit accurate estimations of the importance of this character; unlike sporulation, for which we know a number of genes are involved, we have no idea if a large or small number of genes is involved in the formation of coccoid bodies. Indeed, the work of Clark-Walker (1969) indicates that they may simply result from release of lysozyme from defective phages which softens the cell wall.

The fact that the moles % GC content for the marine strains was largely in the range 42-48 may be sufficient evidence for separation; however, since there were two marine strains with higher % GC values possibility exists that when more marine spirilla are isolated and examined, % GC values between these two groups may be found.

The above correlation of five characteristics does carry more taxonomic weight than any characteristic considered separately; yet even when these characters are considered together, it would seem premature to divide the genus Spirillum into two genera on this basis. The discovery of additional correlations may at

some future date provide sufficient support for a generic separation.

Description of Genus Spirillum (Ehrenberg 1830, 38) Based on Morphological and Physiological Results. On the basis of the morphological and physiological results, the following description of the genus Spirillum can be offered: Rigid, helical cells, with cell diameters ranging from 0.25 to 1.7  $\mu$ , cell lengths ranging from 3 to 5  $\mu$  (as in S. delicatum) to 14 to 60  $\mu$  (as in S. volutans), and number of turns ranging from less than one to five. Motile with bipolar polytrichous flagella. Gram negative. Chemoorganotrophic, having a strictly respiratory metabolism, with oxygen as the terminal electron acceptor. A few species can grow anaerobically with nitrate as the terminal electron acceptor. Oxidase, deaminase, and catalase positive. Phosphatase usually positive. Hydrogen sulfide usually produced from cysteine. Indol, sulfatase, and amylase negative. Casein and hippurate not hydrolyzed. Gelatin usually not hydrolyzed. Urease usually negative. Acid reaction usually not produced from carbohydrates; where this occurs, the kinds of sugars are extremely limited. Incorporation of natural or artificial sea water into media is required for marine species. Aerobic, facultatively microaerophilic, or obligately microaerophilic. Optimum temperature, 30 to 32 C, with

no growth at 10 or 45 C. Fresh water species do not grow with 3% (w/v) NaCl, while marine species grow in up to 7 to 10% (w/v) NaCl.

Physiological Tests Important for Species Differentiation. Several physiological tests yielded results so nearly uniform for the genus that the few exceptions were clearly evident. Such exceptions as failure to produce hydrogen sulfide from cysteine; urease production; failure to exhibit phosphatase activity; gelatin hydrolysis; aesculin hydrolysis; alkaline reactions in litmus milk; ability to grow in presence of 1% glycine and 1% bile; acid reactions from sugars; and anaerobic growth in the presence of nitrate would appear to have taxonomic value in differentiating the species of spirilla.

In contrast, several physiological tests when applied to spirilla yielded a greater variety of positive and negative reactions; these tests also appear to be useful in differentiating among species, and are listed as follows: selenite reduction; DNAase and RNAase activity; growth on EMB, MacConkey, and Sella's agar; and ability to grow in MRVP broth.

Scheme of Classification of Species of Genus Spirillum. Using the morphological and physiological characters as determined in the present investigation,

the following classification has been devised. It should be emphasized that this scheme is not a diagnostic key; such a key follows the present section.

1. Spirillum volutans (Ehrenberg, 1830).

This fresh-water species is distinguished by a large cell diameter (1.4 - 1.7 microns), by a great cell length (14 - 60 microns), by the easy visibility of its flagellar tufts by darkfield or phase-contrast microscopy, by its obligately microaerophilic nature, and by the low moles % GC content of its DNA (38%). The organism is only weakly catalase positive.

In regard to the type species of the genus Spirillum, we agree with Williams (1959) that S. volutans, rather than S. undula, should be the type species of the genus, but for different reasons. Williams indicated that S. volutans was the only species named by Ehrenberg in his paper to the Berlin Academy (1830), and therefore this was said to be designation of type species by monotypy. An examination of Ehrenberg's paper, however, reveals that two species were listed: S. volutans and S. undula. No descriptions were given, but the organisms were indicated as species previously classified by Müller in 1773 as vibrios: V. spirillum (S. volutans) and

V. undula (S. undula). In 1905, Stiles designated S. volutans as the type species of genus Spirillum, but pointed out that Ehrenberg should have retained Müller's specific epithet, and that therefore the correct name of the organism should be Spirillum spirillum. However, Rule 25 (c) of the International Code of Nomenclature of Bacteria (International Congress of Microbiology, 1966) states that a specific epithet is illegitimate when such epithet exactly repeats the generic name. In view of this, Ehrenberg's name would appear to stand, and should be written: Spirillum volutans Ehrenberg, 1830. In contrast, Ehrenberg's second species should be designated as Spirillum undula (Müller) Ehrenberg, 1830. The designation of S. volutans as the type species by Stiles (1905) appears to be the earliest such designation of the type. Vuillemin (1913) designated S. undula as the type, and also the 7th edition of Bergey's Manual (Breed, Murray, and Smith, 1957) designates S. undula as the type.

We agree with Stiles that S. volutans is the type species of the genus, not only on the basis of priority, but also because of the following practical reasons:

- (a) Strains of S. undula are not presently

available from any collection as far as is known. The last literature references where the use of S. undula is indicated are apparently those of Lewis (1940) and Myers (1940). It would seem more useful to have a type species for which strains are available, so that the strains may be studied by modern methods as they develop; only in this way can the description of the type be continually updated and made relevant to current methodology.

(b) S. volutans can be easily identified on the basis of its large size by the unmistakable morphological description given by Ehrenberg (1838): "Lange der Spirale 1/192 bis 1/48 Linie. Dicke etwa 1/1200 Linie." (A Linie is equivalent to 1/12 of an inch, therefore the length of S. volutans was 11 to 43  $\mu$  and its diameter was about 1.8  $\mu$ ).

In contrast, Ehrenberg's dimensions for S. undula could apply to several current species; such as S. serpens, S. putridiconchylum, S. graniferum, and S. metamorphum. In fact, even the most recent descriptions of S. undula, namely those of Giesberger (1936), Myers (1940), Williams and Rittenberg (1957), and Bergey's Manual (Breed, Murray, and Smith, 1957), do not provide sufficient information to distinguish S. undula from other well-studied species. For example, Giesberger

(1936) gives the following description of S. undula: "In peptone water extremely motile, beautiful, broadly spiral shaped cells; width of cell 0.9  $\mu$ ; length of turn 6  $\mu$ ; width of turn 3  $\mu$ . Motile by means of bipolar flagellar tufts; volutin occurs as reserve material; gram negative; catalase positive, non-spore forming; scanty growth on peptone agar; colonies gray-white, smooth; liquefies gelatin very slowly; gray-brown growth on potato....". Myers (1940) describes S. undula as having a cell diameter of 0.9 - 1.2  $\mu$ , not liquefying gelatin, not growing on potato, not producing either nitrites or gas from nitrates, not producing indole or H<sub>2</sub>S and having an optimum temperature of about 32 C. Williams and Rittenberg (1957) include Giesberger's description in their treatment of this species. Bergey's Manual (Breed, Murray, and Smith 1957) use Giesberger's description of S. undula, although they do include the indole and nitrate reactions as observed by Myers.

We therefore believe that with the greater number of species that are now available, it would be extremely difficult to identify any one organism with these descriptions.

(c) Two strains of S. volutans are presently available: strain #19553 of the American Type Culture

Collection, and the Wells strain, available from the stock culture collection of the Biology Dept. Virginia Polytechnic Institute, Blacksburg, Virginia 24061. We propose that the former strain be designated as the type strain of the species.

2. Spirillum anulus (Williams and Rittenberg, 1957).

This fresh-water species is also distinguished by its large cell diameter (1.4 - 1.5 microns) but it is strictly aerobic and its DNA composition is 58 moles % G + C. It possesses 1 - 3 helical turns, is strongly catalase positive, and produces both DNAase and RNAase activity.

3. Spirillum putridiconchylum (Terasaki, 1961).

This fresh-water species has a moderately-large cell diameter (1.0 - 1.2 microns) and exhibits 2 - 4 helical turns. The G + C content of its DNA is 52 moles %. It forms weakly a yellow-green water-soluble fluorescent pigment (Hylemon, personal communication), produces hydrogen sulfide from cysteine, grows on TSI and EMB agars, but does not grow in MRVP broth and does not produce DNAase activity.

4. Spirillum graniferum (Williams and Rittenberg, 1957).

This fresh-water species is morphologically similar

to S. putridiconchylum, but has a DNA base composition of 57 moles % G + C. Moreover, no fluorescent pigment is produced, hydrogen sulfide is not produced from cysteine, growth fails to occur on TSI or EMB agars but does occur in MRVP broth, and DNAase activity is produced.

5. Spirillum metamorphum (Terasaki, 1961).

This fresh-water species has a cell diameter similar to the two preceding species (1.1 - 1.3 microns); however less than one helical turn is present, and the G + C content of its DNA is 63%. Moreover, an unusual characteristic, gelatin liquefaction, is present. A yellow-green water-soluble fluorescent pigment is formed (Hylemon, personal communication), growth occurs on Sella's slants (alkaline reaction in slant only), and RNAase and DNAase activities are not produced.

6. Spirillum giesbergeri (Williams and Rittenberg, 1957).

With a cell diameter of 1.1 - 1.4 microns, this fresh-water species is similar to the three preceding species; however unlike S. putridiconchylum and S. graniferum (but like S. metamorphum) the cells have less than one helical turn. The G + C content of the DNA of 58 moles % is similar to that of S. graniferum. Two most interesting characteristics, however, sets this species apart from the three preceding ones,

namely: its facultative microaerophilic nature and its slow rate of growth. When 0.15% agar is incorporated into PSS broth, a microaerophilic band of growth consistently forms a few mm below the surface of the medium; as growth increases, this band eventually moves to the surface. In non-stratified broth, moderate growth occurs with bottom sediment and clear supernatant. Recent results of Hylemon (personal communication, 1970) indicate that in a defined medium containing succinate as carbon source, no growth occurs unless 0.15% agar is added; in the latter case, a microaerophilic band forms 3 - 6 mm below the surface of the stratified medium, but even after continued growth, this band fails to move to the surface. The slow rate of growth of S. giesbergeri in PSS broth is unusual, and 3 to 4 days of incubation are required to achieve a crop of cells comparable to what most other spirilla produce in 24 hr.

7. Spirillum peregrinum (Pretorius, 1963).

This fresh-water species has a cell diameter of 0.5 - 0.7 microns and exhibits 4 - 5 helical turns. Coccoid bodies predominate in older cultures, and it is strictly aerobic, with no anaerobic growth in the presence of nitrate. The G + C content of its DNA is 62 moles %. It forms weakly a yellow-green water-soluble fluorescent pigment (Hylemon, personal communication), produces urease, reduces selenite, and produces

a slight alkaline reaction in litmus milk, but nitrate is not reduced, no growth occurs on MacConkey agar and RNAase and DNAase activities are not produced. A yellow water-soluble pigment is produced in the presence of 0.2% phenylalanine, and an acid reaction is produced from fructose in PSS broth with reduced peptone.

8. Spirillum itersonii (Giesberger, 1936).

This fresh-water species is similar to S. peregrinum, morphologically, with a cell diameter of 0.4 - 0.6 microns; moreover, coccoid bodies predominate in older cultures, the G + C content of its DNA is 62 moles %, and it produces a yellow-green water-soluble fluorescent pigment (Hylemon, personal communication). However, it exhibits from less than one to two helical turns, and is strictly aerobic, but can grow anaerobically in the presence of nitrate. It reduces nitrate to ammonia or nitrogen, reduces selenite in only two out of three strains, grows on MacConkey agar and produces RNAase and DNAase activities, but it does not produce urease and no reactions are produced in litmus milk. A yellow water-soluble pigment is produced in the presence of 0.2% tryptophan, a brown water-soluble pigment is produced in the presence of 0.2% tyrosine, and acid reactions are produced from glycerol, in addition to fructose, in PSS broth with reduced peptone.

Williams and Rittenberg (1957) considered two varieties of S. itersonii to exist, based on their nitrogen metabolism; S. itersonii var. itersonii did not reduce nitrate, while S. itersonii var. vulgatum strongly reduced nitrate. The three strains examined in the present investigation were all of the latter variety.

9. Spirillum serpens (Müller, Winter 1884).

Strains belonging to this fresh-water species have cell diameters ranging from 0.6 to 1.0 microns, and possess from less than one to two helical turns. The G + C contents of their DNA ranges from 50 - 51 moles %. Some strains produce a yellow-green water-soluble fluorescent pigment (Hylemon, personal communication). Most strains produce RNAase activity, grow on TSI and EMB agar and tolerate 1% glycine. Some strains grow on MacConkey agar, MRVP broth and on Sellar's slants (when positive, slant only is alkaline). One strain reduced nitrate weakly. Most strains hydrolyze gelatin.

Williams and Rittenberg (1957) separated S. serpens into two varieties based on their nitrogen metabolism and gelatin hydrolysis; S. serpens var. azotum reduced nitrate weakly and did not hydrolyze gelatin, while S. serpens var. serpens did not reduce nitrate but did hydrolyze gelatin. In the present investigation two out of ten strains failed to hydrolyze

gelatin, and only one of these very weakly reduced nitrate; consequently, it would appear that the distinction between var. azotum and var. serpens is of questionable value.

Two strains, S. serpens ATCC 12289 and S. serpens ATCC 11330, were found to differ from the other strains to such a degree that they were not considered in the above classification of this species. S. serpens ATCC 12289, is morphologically similar to the other strains of S. serpens; however, the G + C content of its DNA is 58 moles % and it is extremely fastidious in its nutritional requirements (Hylemon, 1970). S. serpens ATCC 11330, morphologically, is much smaller than the other S. serpens strains; moreover, nitrate is strongly reduced to ammonia or nitrogen, gelatin is not liquefied, and the G + C content of the DNA is 65 moles %. Therefore, we feel that these two strains should not be placed in S. serpens, and that their classification should await the further results of nutritional and serological studies currently in progress.

10. Spirillum sinuosum (Williams and Rittenberg, 1957).

Morphologically, this strain is similar to S. serpens, but it has one to two helical turns and a DNA base composition of 57 moles %. In regard to its

DNA base composition, it is similar to S. anulus, S. graniferum, and S. giesbergeri, however, with a cell diameter of 0.5 - 0.7 microns, it is smaller than these species. DNAase activity is produced, but not RNAase activity (similar to S. graniferum); however, hydrogen sulfide is produced from cysteine and growth does not occur in MRVP broth, unlike S. graniferum. Although the production of hydrogen sulfide and growth in MRVP broth are not major differences when considered alone, the additional character of morphological differences does appear to be sufficient for keeping these species separated.

11. Spirillum gracile (Canale-Parola, Rosenthal, and Kupfer, 1966).

This fresh-water species is distinguished by a small cell diameter (0.25 to 0.30 microns) and exhibits 3 - 4 helical turns. Coccoid bodies are not formed in older cultures. The G + C content of its DNA is 65 moles %. It does not produce a yellow-green water-soluble fluorescent pigment. It produces RNAase activity, reduces nitrate to nitrite, produces acid reactions from glucose, galactose, and arabinose, but does not grow on MacConkey or TSI agar or in MRVP broth; moreover, it does not hydrolyze aesculin or reduce selenite.

It is aerobic and does not form microaerophilic bands in semi-solid PSS agar; although Canale-Parola,

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 distances 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, since we did not observe these discs, and we have not observed subsurface colonies on agar plates, although colonies which form partially below the surface are a common occurrence.

Canale-Parola, Rosenthal, and Kupfer also reported that on initial isolation, S. gracile was catalase negative by a microscopic test, but that after several transfers the organisms became catalase positive. In the present study, all three strains were found to be catalase positive, and many transfers have intervened since their initial isolation. An initial negative catalase test would seem to be taxonomically important for this species, but the possibility exists that if a

30 minute incubation period for the catalase test, as used in the present study, had been used after initial isolation of S. gracile, a weakly positive result might have been seen. Therefore it is difficult at this time to assess properly the catalase test in the classification of this species.

12. Spirillum polymorphum (Williams and Rittenberg, 1957).

This fresh-water species is also distinguished by its small diameter (0.3-0.5 microns) but it exhibits less than one helical turn and coccoid bodies predominate in older cultures. The DNA base composition is 62 moles % G + C, similar to S. gracile, but it does produce a yellow-green water-soluble fluorescent pigment (Hylemon, personal communication), hydrolyzes aesculin, reduces selenite (weakly) and grows on MacConkey agar, but acid reactions are not produced with any sugar in complex media and it does not grow on TSI agar or in MRVP broth.

13. Spirillum delicatum (Leifson, 1962).

With a cell diameter of 0.3 - 0.4 microns, this fresh-water species is very similar to the two preceding species. It exhibits less than one helical turn, similar to S. polymorphum, and with a DNA base composition of 63 moles % G + C, it is similar to both S. gracile

and S. polymorphum; however coccoid bodies are not formed in older cultures and it does not form a yellow-green water-soluble fluorescent pigment, unlike S. polymorphum; moreover, it does not hydrolyze aesculin, reduce selenite, nor grow on MacConkey agar, but it does grow on TSI agar and in MRVP broth (unlike either S. gracile or S. polymorphum). In addition, acid reactions are not produced with any sugar in complex media (unlike S. gracile).

Leifson (1962) placed this species provisionally with the spirilla, but because of his observation that the organisms possessed predominately single polar flagella rather than flagellar bundles (presumably by flagella staining), he suggested that the species might more appropriately be included with the non-fermentative vibrios. In the present study, darkfield examination clearly indicated the presence of helical flagella; indeed, helical waves moving along the filaments could easily be seen. Such visibility under darkfield suggests that flagellar bundles rather than single flagella may predominate in this species; electron microscopy is needed to resolve the problem. It is noteworthy that certain species of spirilla initially reported by Williams and Rittenberg (1957) as possessing single flagella, on the basis of stains, were later reported

as possessing flagellar bundles on the basis of electron microscopy (Williams, 1960).

14. Spirillum lunatum (Williams and Rittenberg, 1957).

This marine species has a cell diameter of 0.8 - 1.0 microns and exhibits less than one helical turn. It possesses a G + C content of 63 moles % and produces a yellow-green water-soluble fluorescent pigment (Hylemon, personal communication). It reduces selenite and nitrate, forms acid reactions from glucose, fructose, galactose, mannose, mannitol, and dextrin in complex media (reduced peptone), produces dark brown water-soluble pigment in presence of 0.2% tryptophan and a light brown water-soluble pigment in presence of 0.2% tyrosine. Anaerobic growth occurs in the presence of nitrate.

15. Spirillum linum (Williams and Rittenberg, 1957).

This marine species possesses a cell diameter of 0.4 - 0.5 microns and exhibits 5-6 helical turns. The base composition of its DNA is 48 moles %. It reduces selenite weakly but not nitrate, and it does not form acid reactions with sugars. It produces a deep brown water-soluble pigment in the presence of 0.2% tryptophan or tyrosine. RNAase and DNAase activities are not produced. Growth occurs on TSI agar and in MRVP broth.

16. Spirillum beijerinckii (Williams and Rittenberg, 1957).

This marine species possesses a cell diameter (0.5-0.7 microns) similar to the preceding species, but exhibits only 1-2 helical turns. The G + C content of its DNA is 47 moles %, similar to the preceding species; however, it does not reduce selenite, does not produce a deep brown water-soluble pigment in the presence of tryptophan, but it does produce RNAase and DNAase activities. It does not grow on TSI agar or in MRVP broth; hydrogen sulfide is produced from cysteine.

17. Spirillum atlanticum (Williams and Rittenberg, 1957).

With a cell diameter of 0.4 - 0.6 microns, this marine species is similar to the two preceding species; moreover, it possesses a DNA base composition of 48 moles % G + C. It produces RNAase activity (unlike S. linum), but does not produce DNAase activity (unlike S. beijerinckii). It does not produce water-soluble pigments in the presence of aromatic amino acids (unlike S. linum and S. beijerinckii), but it does reduce selenite and does grow on TSI agar and in MRVP broth (unlike S. beijerinckii). It does not produce hydrogen sulfide from cysteine (unlike S. linum and S. beijerinckii).

18. Spirillum japonicum (Watanabe, 1959).

With a cell diameter of 0.7 - 0.8 microns this

marine species is similar to S. lunatum; however it is distinguished by its great length (20 - 75 microns), exhibits less than one to one full helical turn, and has a G + C content of 45 moles %, (similar to S. linum, S. beijerinckii, and S. atlanticum); however it does not produce hydrogen sulfide from cysteine (unlike S. linum and S. beijerinckii, but like S. atlanticum). It does not produce RNAase and DNAase activities and water-soluble pigments are not produced from aromatic amino acids; moreover, growth does not occur on either TSI agar or in MRVP broth (unlike S. atlanticum).

19. Spirillum halophilum (Watanabe, 1959)

This marine species is smaller than the preceding marine species with a diameter of 0.35 - 0.6 microns; it exhibits 1-2 helical turns. The base composition of its DNA is 42 moles % G + C; moreover, it reduces selenite and nitrate, produces a dark brown water-soluble pigment in the presence of 0.2% tryptophan, and grows on TSI agar, but fails to produce phosphatase activity or hydrogen sulfide from cysteine.

20. Spirillum minutulum (Watanabe, 1959).

This marine species is very similar to S. halophilum, with a cell diameter of 0.3 - 0.4 microns and 1-2 helical turns. The DNA base composition and all physiological reactions were identical, except that this species did

not produce a pigment in the presence of 0.2% tryptophan. Therefore, unless future tests indicate that S. halophilum and S. minutulum are different, we feel they should be placed in the same species; since they were both first named in the same publication, either name would be suitable for the resulting species. A future test which would be beneficial in determining their relationship is a DNA homology determination. In our diagnostic scheme we will treat S. halophilum and S. minutulum as synonyms, with the feeling that pigment production with tryptophan does not justify separation of the two species.

Un-named Strains of Spirilla. Un-named strains 101, 102, and 206 appear to be similar to the type strain of S. linum, but certain differences are evident. All three strains fail to reduce selenite, and the moles % G + C contents of their DNA differ slightly from the value of 48% for the type strain. Both kinds of differences appear to be minor and these strains should be classified as S. linum. The results of future nutritional and serological studies may necessitate a revision of this opinion.

Un-named strain 204 is similar to the type strain of S. lunatum in several respects, particularly in

regard to its production of an acid reaction from certain sugars. The base composition of its DNA differs slightly (60 moles % G + C as compared to 63% for the type strain), and its cell diameter is somewhat smaller (0.5 - 0.7  $\mu$  as compared to 0.8 - 1.0  $\mu$  for the type strain). Unlike the type strain, 204 produces a jet black pigment on tyrosine, and fails to produce DNAase and RNAase activity. The cumulative effect of these differences makes it difficult to include this strain in the species S. lunatum, and it would be desirable to have additional nutritional and serological data before making a final decision.

Un-named strains 103 and 104 are closely similar to each other but do not appear to be related to any of the established species. These fresh-water forms have a cell diameter of 0.5 - 0.8  $\mu$  and tufts of flagella are not evident under dark field microscopy. Coccoid bodies are not evident in older cultures. They are distinguished from all other fresh-water forms by their ability to grow in the presence of 1% glycine. Their DNA base compositions are similar (63% for strain 103 and 65% for strain 104). Selenite, nitrate, and nitrite were reduced; growth occurred in MRVP broth, MacConkey agar, EMB agar, TSI agar, and Seller's slants (with alkaline reaction in slant and butt); DNAase and

RNAase activities were negative. In other respects the general description for the genus was found to apply. From the present study, it seems likely that strains 103 and 104 should be placed in a de novo species. However, a complete description of such a species must await further data concerning nutrition and serology, and would be premature at this time.

Diagnostic Key to the Species of Genus Spirillum.

In order to develop a good diagnostic key, several strains belonging to a particular species need to be examined, with regard to a large number of characters. With data of this type, one may select characters which are good diagnostic characters for that particular species. Such characteristics would be those which were positive or negative for 95 to 100% of all the strains of a species examined. Since strains other than the type strains were not available for most species of spirilla, it is difficult to present a diagnostic key that will stand the test of time. However, we consider the following to be the best diagnostic scheme at present.

Key to the Species of Genus Spirillum.

- I. Growth only in the absence of sea water.
  - A. Cell diameter, 1.4 to 1.7 microns; cell length, 14 to 60 microns; obligately microaerophilic.

1. Spirillum volutans
- B. Cell diameter, 1.4 to 1.5 microns; cell length, 7 to 15 microns; aerobic.
2. Spirillum anulus
- C. Average cell diameter between 1.0 and 1.4 microns; aerobic or facultatively microaerophilic.
  1. Two to four helical turns.
    - a. H<sub>2</sub>S produced from cysteine; no DNAase activity; no growth in MRVP broth.
  3. Spirillum putridiconchylium
    - aa. H<sub>2</sub>S not produced; DNAase activity; growth in MRVP broth.
  4. Spirillum graniferum
2. Less than one helical turn.
  - a. Gelatin liquefied; strictly aerobic.
5. Spirillum metamorphum
  - aa. Gelatin not liquefied; facultatively microaerophilic.
6. Spirillum giesbergeri
- D. Average cell diameter between 0.5 and 0.9 microns.
  1. Urease positive.
7. Spirillum peregrinum.

2. Urease negative.

a. Anaerobic growth in the presence of nitrate.

8. Spirillum itersonii.

aa. No growth anaerobically with nitrate.

b. DNA base composition of 50-51 moles % G + C.

9. Spirillum serpens.

bb. DNA base composition of 57 moles % G + C.

10. Spirillum sinuosum.

E. Average cell diameter between 0.25 and 0.4 microns.

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

11. Spirillum gracile.

2. No acid reaction from sugars.

a. Aesculin hydrolyzed; growth on TSI agar and in MRVP broth.

12. Spirillum polymorphum.

aa. Aesculin not hydrolyzed; growth occurs on TSI agar and in MRVP broth.

13. Spirillum delicatum.

II. Sea water required.

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

14. Spirillum lunatum.

- B. No acid reactions from sugars.

1. Phosphatase positive.

- a. H<sub>2</sub>S produced from cysteine.

- b. Grows on TSI agar and in MRVP broth; does not produce RNAase and DNAase activities.

15. Spirillum linum.

- bb. Does not grow on TSI agar and in MRVP broth; produces RNAase and DNAase activities.

16. Spirillum

beijerinckii

- aa. H<sub>2</sub>S not produced from cysteine.

- b. Grows on TSI agar and in MRVP broth; produces RNAase activity.

17. Spirillum atlanticum.

- bb. Does not grow on TSI agar and in MRVP broth; does not produce RNAase activity.

18. Spirillum japonicum.

2. Phosphatase negative.

a. Cell diameter 0.35 to 0.6 microns.

19. Spirillum halophilum.

20. Spirillum minutulum.

## SUMMARY

In order to develop further a comprehensive characterization of spirilla and to devise a more satisfactory classification of the species, the type strains of 20 marine and fresh-water species of the genus Spirillum, plus additional strains of S. gracile, S. itersonii, S. serpens, and S. volutans, and 6 un-named strains of spirilla, were subjected to over 90 physiological tests and also various morphological tests using standardized inocula, media, incubation times, and testing methods. Determination of DNA base compositions was also performed.

The morphological, physiological, and DNA base composition data were combined to present a modified description of the genus Spirillum (Ehrenberg, 1830).

A scheme for the classification of species of the genus Spirillum was presented, using those characters which best distinguished each species. The two species S. halophilum and S. minutulum were combined as a result of their morphological, physiological, and DNA base composition similarities.

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A MORPHOLOGICAL AND PHYSIOLOGICAL  
CLASSIFICATION OF SPIRILLA

by

Jerry Scott Wells, Jr.

ABSTRACT

In order to develop further a comprehensive characterization of spirilla and to devise a more satisfactory classification of the species, the type strains of 20 marine and fresh-water species of the genus Spirillum, plus additional strains of S. gracile, S. itersonii, S. serpens, and S. volutans, and 6 un-named strains of spirilla, were subjected to over 90 physiological tests and also various morphological tests using standardized inocula, media, incubation times, and testing methods. Determination of DNA base compositions was also performed.

All strains of spirilla grew aerobically except S. yolutans which was an obligate microaerophile; S. giesbergeri and S. gracile appeared to be possible

facultative microaerophiles. S. lunatum and S. iter-sonii were the only species to grow anaerobically in the presence of nitrate. The optimum temperature was 30 C for all strains examined. The cell diameters for all strains formed an almost continuous spectrum, ranging from 0.25 to 1.7 microns. All spirilla were actively motile and a number of the strains possessed flagellar tufts visible with living cells by dark field microscopy and in the case of S. volutans also by phase contrast. Coccoid bodies were present in 4 week cultures in the case of most marine strains; only 3 fresh-water species exhibited these forms. Several physiological characteristics were found to be uniformly or nearly uniformly positive (oxidase, deaminase activity with peptone, catalase, phosphatase, H<sub>2</sub>S from cysteine, growth in 1% bile) or negative (indol; amylase; sulfatase; hydrolysis of gelatin, casein, aesculin, and hippurate; urease; alkaline reaction in litmus milk; acid reaction from carbohydrates) for all or nearly all of the strains. In the few cases where acid was produced from carbohydrates, a reduced peptone concentration was necessary. Several physiological characteristics yielded varied reactions among the spirilla (reduction of selenite and nitrate; growth on EMB, MacConkey, TSI, and Sellar's agars and in MRVP broth; RNAase and DNAase activity; and

tolerance to 1% glycine). Water-soluble pigments were produced by a few strains growing on aromatic amino acids. Three percent NaCl inhibited all fresh-water strains, while the marine strains could all grow in the presence of 9.75% (w/v) NaCl. The DNA base compositions of the spirilla, determined from their  $T_m$  values, gave a broad range of values from 38 to 65 moles % G + C.

The limits of genus and species variation, the usefulness of standardized conditions in characterizing strains of spirilla, discrepancies arising from a comparison of the present data with earlier results, the division of the genus Spirillum on the basis of single, highly-weighted characters, and the division of genus Spirillum on the basis of correlated characters, were discussed. A modified description of the genus Spirillum (Ehrenberg, 1830) based on the present study was presented. Various physiological tests important for species differentiation were indicated. A modified classification scheme for the species of genus Spirillum was devised, and a temporary diagnostic key was also presented.