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A TAXONOMIC STUDY OF THE GENUS *SPIRILLUM* EHRENBERG

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INTRODUCTION

Spirillum Ehrenberg, 1830 (22) is one of the oldest of the bacterial generic names, of those still in use only Vibrio, Polyangium and Serratia are older. As a specific epithet spirillum was used by Müller a half century earlier in the species name Vibrio spirillum, which became Spirillum volutans Ehrenberg, 1830. Spirillum volutans, for example, can be recognized with certainty from the description and drawings given by Ehrenberg in 1838 (23). Despite the antiquity of its descriptions, the genus Spirillum has been one of the least studied of all the genera of the Eubacteriineae. The lack of attention undoubtedly relates, in part, to the fact that none of the spirilla, excepting Spirillum minus whose taxonomic position is uncertain and a few incompletely described isolates, are pathogens and none has had direct significance in the economic affairs of man.

The most important and detailed study of this genus was published by Giesberger (26) in 1936. He developed methods of enrichment culture and isolation and studied the gross morphology and physiology of his isolates, but paid little attention to the detailed cytology of the group. On the basis of his own data and a thorough review of the literature, Giesberger prepared the first adequate characterization of the genus and differentiated nine species, the diagnosis of some being admittedly incomplete. The description of the species in the sixth edition of Bergey's Manual (7) is based almost entirely on Giesberger's work, differing mainly in

the retention of Spirillum lipoferum as a valid species and in the exclusion of Spirillum cardiopyrogenes.

From a systematic viewpoint, one of Giesberger's most interesting contributions was the demonstration that each species distinguished morphologically was also metabolically distinct in that each was capable of utilizing a different group of compounds as sole carbon and energy sources. Thus the venerable controversy as to the relative weight to be assigned to morphology and physiology in bacterial taxonomy did not enter into his separation of the species.

Unlike the situation with respect to physiology and nutrition, for which Giesberger's thesis is still essentially the sole source of information, a considerable literature has developed on the cytology of the spirilla. In fact, because of the large size of certain spirilla, this group was a favorite subject for cytological investigation during the last decade of the nineteenth century and the first decade of the present century. Zettnow (68) studied vital staining as well as other cytological features using Kutscher's (36) pure cultures. Grimme (29) also used Kutscher's cultures in his extensive investigation of volutin which Arthur Meyer (43) had named because of its occurrence in S. volutans. Ellis (24) studied motility, flagellation, cell division, and the growth cycle of the same organisms. Swellengrebel (59), in a comparative study of the spirilla and spirochetes, described a spiral nucleus in Spirillum giganteum which he stated could be differentiated from volutin and other cell inclusions by its staining reaction. More recently, the spirilla were used by Lewis (39) in his investigation of the chromidial theory of the nucleus in bacteria. Lewis found no evidence of chromidia in the spirilla and reached the conclusion that the structures which earlier investigators had interpreted as nuclei were either volutin or fat granules.

The occurrence of flagella or fascicles of flagella in bacteria (spirilla) was first described by Ehrenberg in 1838 (23) and later by Cohn in 1872 (14). Much of the early work on flagella, thoroughly reviewed by Meyer (43), was done with the larger spirilla, and modern cytologists in re-investigating the problems of flagellation and motility have used the same bacteria (33, 48, 49, 50, 61, 62).

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Our interest in the genus Spirillum was aroused by the chance observation of these forms in enrichment cultures set up for other purposes. The elegance of form of the organisms exerted a fascination not stimulated by the usual bacteria to which one is so accustomed. The initial desultory and easily successful attempts to isolate these organisms led to more detailed investigations.

ENRICHMENT AND PURE CULTURE METHODS

Source materials. The organisms studied were isolated from fresh water sources, mainly small lakes and fish ponds, and from sea water samples collected from the intertidal zone.

Enrichment procedures. Enrichment materials included decomposing algae, peptone, yeast autolysate, and simple carbon compounds.

Isolations direct from algal infusions were readily accomplished. Stagnant water samples from small ponds were collected in wide mouth jars along with a considerable amount of algae from the same source. The samples were allowed to stand at room temperature for ten days to two weeks by which time microscopic examination showed the spiral organisms to be abundant. They showed marked positive aerotaxy as evidenced by their occurrence almost exclusively at the surface of the culture where they predominated. Pure cultures were obtained by diluting the surface liquid of the infusion 1:100 with sterile tap water and streaking on solid media. Only a single morphological type was obtained from the algal infusions despite the testing of numerous source waters. Similar attempts to isolate marine forms using infusions of fresh and dried seaweed in nonsterile sea water were unsuccessful. Harold and Stanier (31), however, report the successful use of particles of marine algae in sea water for spirilla enrichments.

The results obtained using peptone and yeast autolysate were similar. One gram of nutrient was added to 100 ml of source water and the mixture incubated at room temperature. Spiral organisms were usually observed within three days but other morphological types were always in great excess. The spirilla reached their maximum development in seven days

although remaining a minority form. As in the algal infusions, the spirilla were most abundant at the surface of the fresh water cultures and just below the pellicle that always formed in the marine enrichment cultures. Isolations were not successful from the initial enrichment cultures because of the large excess of other bacteria. Serial transfers through sterile sea or pond water containing 1 per cent peptone or yeast autolysate did not increase the proportion of spirilla.

Good enrichment of spirilla over the other forms was obtained by the following procedure. Part of the initial enrichment culture was added to an equal quantity of the source water and the mixture was sterilized by autoclaving. The resulting solution, without the addition of other nutrients, was inoculated from the unsterilized portion of the initial culture. The spirilla grew well, apparently thriving on the diluted nutrients from the initial culture, and overgrew the other bacteria. The spirilla predominated after one to three transfers through successively exhausted medium and were isolated by streaking on solid media. The peptone and yeast autolysate enrichments were superior to the algal enrichments in that spirilla of more varied morphology were observed and their development was more rapid.

Carbon compounds in 0.5 to 1.0 per cent concentration were also used individually for enrichment. The best results were obtained with calcium malate and lactate, as was observed by Giesberger (26). Not all of the compounds that support growth of the pure cultures are suitable for enrichment. For example, spirilla were never observed in water samples enriched with pyruvate even though this compound proved a good carbon source for all isolates.

Calcium malate or lactate (one per cent) solutions in fresh water from various sources were incubated. Spirilla were usually abundant in these cultures after one week at room temperature. Serial transfers were then made into sterile source water containing one per cent of the carbon compound. After three to four transfers the spirilla predominated and could be isolated. If the transfer medium was supplemented with other mineral nutrients, in particular, with ammonium chloride, as was done by Giesberger, other bacteria grew more abundantly than the spirilla and isolation

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of the latter was difficult or impossible, even after repeated serial transfers.

For the marine forms, the sea water being examined was mixed with equal quantities of Giesberger's base medium (NH_4Cl , 0.1 per cent; K_2HPO_4 , 0.05 per cent; MgSO_4 , 0.05 per cent) plus one per cent of the carbon compound. For subcultures, the NH_4Cl was omitted from the formula and the modified salts solution was mixed with equal quantities of sterilized used initial culture medium. One to three subcultures in this transfer medium were sufficient to establish the spirilla as the predominating type. For the marine forms, successful enrichment cultures were obtained only with calcium lactate as the carbon source. Calcium malate, an excellent substrate for the fresh water forms, was not successful for the marine spirilla although these organisms all utilized malate when grown in pure culture.

Not all of the observed spirilla were successfully isolated from the enrichment cultures noted above. One very large form was repeatedly observed in malate enrichments from fresh water ponds, but its colonies were never observed after streaking these enrichment cultures on either malate or nutrient agars. Since the organism resembled S. volutans morphologically, attempts were made to grow it in the soil infusion medium developed by Pringsheim* (52) for S. volutans. Transfers were made from the malate enrichment cultures containing the large spirillum to Pringsheim's medium and, although the desired form grew it was greatly outnumbered by the other forms and could not be isolated. By chance, a culture in this medium that had been standing at room temperature for four months was examined before discarding and the large spiral forms were abundant whereas other organisms were not evident. Colonies of the desired organism were obtained by streaking from this culture and others, deliberately aged, on malate agar.

*Pringsheim's soil infusion medium: Place one grain of wheat in a large test tube and cover with 3 to 4 cm of garden soil. Fill the tube almost to the top with tap water. Pringsheim sterilized his media by intermittent sterilization at 100 C for three successive days but it was found that the medium could be used if autoclaved. Better growth of S. volutans is obtained by allowing the tubes to stand at room temperature for 24 to 48 hours before autoclaving.

The difficulty of isolating spirilla from enrichment cultures has been commented on by nearly everyone who has attempted to isolate these organisms from natural sources. Beijerinck (2, 3) was apparently the first to obtain a pure culture of a Spirillum species, isolating Spirillum tenue from malate enrichment cultures. Also notable was the early work of Kutscher (36) who isolated four species from liquid manure, evidently with ease, as there is no comment in his article of any difficulty in isolation. The typical enrichment media for spirilla from the time of Ehrenberg has been hay infusion. Hay infusions were used without success in the present investigation. Giesberger (26), although reporting some success with hay infusions, preferred individual carbon compounds for enrichment purposes. Recently, Cayton and Preston (12) have reported the isolation of a new species, Spirillum mancunienne, from an infusion of grass cuttings from a compost heap.

Since most of the primary enrichment media employed for growing spirilla are relatively nonselective nutritionally, additional procedures by which the spirilla can become the predominating form have usually been required. Giesberger (26) accomplished this by incubating his cultures at 40 C, which prevented the overgrowth of the spirilla by the contaminating forms. More recently, Giesberger (27) used an atomizer to spray a diluted enrichment culture onto the solid medium for the isolation of a photosynthetic spirillum. This method was also used by Pease (47) for the isolation of two species of spirilla. In this investigation, apart from the algal infusions, from which a single type was easily obtained from the initial culture, isolation of the spirilla was achieved by limiting the available nutrients, in particular the nitrogen source, either by re-use of a partially spent medium or by relying on the nitrogen present in the source waters. These methods had the effect of reducing the growth of the undesired types rather than stimulating the development of the spirilla.

Pure culture isolation. Samples from positive cultures were diluted 1:100 to 1:100,000 with sterile tap or sea water, depending on the relative abundance of contaminating forms. The dilution bottles were shaken vigorously and allowed to stand at room temperature for 20 minutes, since it was observed that the spirilla tended to gather at the

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surface of the diluent while the other forms remained dispersed throughout the water. A loopful of the surface water was streaked either on nutrient agar containing 0.3 per cent yeast autolysate (stock agar) or on Giesberger's base medium containing one per cent calcium malate or lactate. Colonies of the spirilla were never obtained by the pour plate method even when pure cultures were used as the inoculum. The plates were incubated at 30 C. After 24 hours incubation, the colonies that developed were examined under the 4 mm lens and with the hand lens. Colonies of spirilla have a distinctive granular, ground-glass appearance when examined with the hand lens by transmitted light (Fig. 1). The colonies of the larger forms appear umbonate (Fig. 2); those of the smaller forms may be pulvinate. Under the 4 mm objective, the colonies have a typical wavy, interlaced texture with fimbriated edges. Distinct spirilla, some of which show an active motility, can be seen at the edges of the colonies of the larger forms. After some familiarity is gained, a large number of plates can be rapidly and accurately scanned with the hand lens even though the colonies of some of the larger rod bacteria have a similar appearance.

The plates were re-incubated for another 24 hours and wet mounts prepared from typical colonies to confirm the presence of spirilla. Samples from positive colonies were restreaked serially until all colonies developing on a plate were uniform in appearance and microscopic examination showed a uniform spirillar morphology.

One hundred eighty-seven cultures were isolated in this manner. These differed considerably in appearance. It was possible to assign each isolate to one of ten groups with fairly distinctive morphologies. The sources and average dimensions of the ten groups are given in Table 1. The measurements were made using carefully standardized conditions. A species name is given to each group for ease of reference throughout the succeeding portion of the paper; justification for recognition of the several species is presented later.

It must be emphasized that the average dimensions shown in Table 1 did not serve as the basis for assigning a particular isolate to a particular group. Actually the converse was true, that is, measurements were made on a culture

TABLE 1. Numbers, Source, and Dimensions of Cultures of Spirillum¹ Isolated

Morpho- logical Group	Species Name	Number of Isolates	Source and type of Enrichment ¹	Microcysts Formed	Diameter	Spiral Amplitude	Spiral Depth	Length of Cell
I	<u>S. serpens</u> var. <u>azotum</u>	18	FW-a	Unknown	0.8-0.9	5.0-5.6	1.5-2.0	10-16
II	<u>S. linum</u>	26	SW-p	Yes	0.3-0.35	1.5-2.0	0.9-1.0	2.8-4.2
III	<u>S. lunatum</u>	20	SW-p, l	Yes	1.0-1.2	6.0	3.0-3.5	7-21
IV	<u>S. curvatum</u>	23	SW-p, l	Yes	0.4-0.6	3.5	2.5	7-15
V	<u>S. polymorphum</u>	15	FW-m	Yes	0.2	0.5	0.3	2-7
VI	<u>S. undula</u>	33	FW-m, Pr	Unknown	1.5-1.75	7.5-9.6	4.5-6.0	11-33
VII	<u>S. itersonii</u> var. <u>vulgatum</u>	5	FW-p	Yes	0.5	2.0-3.0	1.5-2.0	3.5-6
VIII	<u>S. giesbergeri</u>	6	FW-p	Unknown	0.9-1.2	6.0-7.0	2.5-3.5	11-25
IX	<u>S. beijerinckii</u>	26	SW-ya	Yes	0.9-1.0	4.3	2.0	16-30
X	<u>S. atlanticum</u>	15	SW-ya	Yes	0.4-0.6	2.4	2.0	10.5-16

¹FW = fresh water lakes and ponds; SW = sea water; a = algal infusions; l = lactate infusions; p = peptone enrichments; m = malate enrichments; ya = yeast autolysate enrichments; Pr = Pringsheim's soil infusion.

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arbitrarily chosen from the group after its establishment on the basis of overall morphological resemblances. This procedure implies that the average cell dimensions do not give a picture of a species of Spirillum that can be relied on exclusively for identification. That this is the case can be seen from an examination of Figures 3, 4, and 5 that show photographs of heat-fixed and stained preparations of Spirillum lunatum, S. serpens var. azotum, and Spirillum giesbergeri and of Figures 6 and 7 that show photographs of living cells of S. lunatum and S. serpens var. azotum taken by dark phase contrast. The morphological uniqueness of each type is apparent yet the average dimensions of the cells (Table 1) show so much overlap that these values alone could lead one to the conclusion that separation is not warranted.

Maintenance of stock cultures. The spirilla are best maintained in liquid media and growth can be obtained from broth cultures up to 3 or 4 months of age, using stock broth or stock broth supplemented with calcium lactate. There is an increasing tendency of the spirilla to "straighten out" with increasing length of storage, and to maintain typical morphology stocks should be transferred monthly. Stocks are stored at room temperature; storage in the refrigerator, deep freeze, or under mineral oil results in rapid loss of viability. Attempts at storage by lyophilization have been unsuccessful; a similar experience was reported by Cayton and Preston (12) for S. mancuriense.

PHYSIOLOGY AND METABOLISM

Methods

Cultures. In addition to our own isolates, we studied two cultures obtained from the American Type Culture Collection, No. 9785 labelled Spirillum serpens and No. 9786 labelled S. undula; also a mixed culture, containing S. volutans, was secured from Professor E. G. Pringsheim. Efforts to obtain cultures of Spirillum from other type culture collections and from individual investigators were uniformly unsuccessful, showing the scarcity of available strains. The cultures on which Giesberger conducted his extensive studies (26) had died out during the war and were not available for comparative purposes. Recently Giesberger

was kind enough to isolate two cultures for us that corresponded in properties to those used for his description of S. serpens and S. itersonii and a few tests were made with these strains.

Culture procedures. Cultures were maintained on slants of "stock" agar of the following composition: Peptone, 5.0 g; beef extract, 3.0 g; yeast autolysate (Basamin, Anheuser-Busch, Inc., St. Louis, Missouri), 3.0 g; agar, 16.5 g; distilled or sea water, 1000 ml. Routine broth cultures were made in the same medium minus the agar. Giesberger's mineral salts solution was used as the base for all synthetic media. When substrates other than calcium salts were used, 0.05 per cent CaCl_2 was added to the basal medium. The pH of the base solution was adjusted to 7.0 with KOH and the solution autoclaved at 15 lbs for 15 minutes. The clear supernatant, formed on cooling of the solution, was decanted off the voluminous precipitate that forms during sterilization and sufficient carbon source was added to give a final concentration of 0.05 per cent (0.02 per cent for the saturated fatty acids). When organic acids were used as carbon sources they were first neutralized with KOH.

Available nitrogen sources were checked by substituting nitrate, urea, or asparagine for ammonia in the base solution, in 0.1 per cent concentration. Potassium pyruvate and calcium lactate were used as carbon sources when testing the nitrogen compounds.

The pH limits for growth were tested in synthetic media with pyruvate as the carbon source. The pH was adjusted with HCl or KOH to give a range between 5.5 and 9.0 in 0.5 units.

Salt tolerance of the marine strains was determined in stock broth and pyruvate synthetic medium prepared with distilled water and appropriate amounts of NaCl. Tests were also made using media with varying proportions of fresh and natural or synthetic sea water (69).

All aerobic growth experiments were done in 15 x 125 mm test tubes containing 10 ml of medium. The inoculum consisted of 0.1 ml of a 48 hour broth culture washed twice in basal salts solution and resuspended in the original volume. Tubes were incubated at 32 C and observed over a period of

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one week. Growth was determined by turbidity measurements using a Klett photoelectric colorimeter. Anaerobic cultures were set up in glass stoppered bottles completely filled with medium.

Experimental Results

Growth on complex media. With the exception of the organisms of groups I and VII, the fresh water strains grew poorly and the marine strains failed to grow on ordinary nutrient agar and broth. Those organisms that did grow tended to straighten out and lose their typical spiral morphology when maintained on these media. All strains grew luxuriantly on nutrient agar supplemented with yeast autolysate or casein hydrolysate. The growth response was roughly proportional to the concentration of yeast autolysate or casein hydrolysate added up to the highest level tested, 0.5 per cent. Similar growth stimulation was obtained using single pure compounds such as pyruvate or lactate as supplements in place of yeast autolysate. It can be concluded, therefore, that the deficiencies of the nutrient agar relate to a lack of suitable carbon (or energy) sources rather than to a lack of specific growth factors.

Utilization of single compounds of carbon. Altogether 22 compounds were tested for their ability to support growth of spirilla. In general, organisms grouped together on the basis of morphology gave similar growth patterns on most of the compounds tested. Results on a few compounds, in particular the higher fatty acids and disaccharides, were quite variable between strains of a single morphological group. In a few instances, strains of essentially identical morphologies showed important differences in the carbon compounds utilized.

Table 2 presents the growth results with 14 compounds that gave fairly consistent group patterns. All of the isolates grew on at least five of the compounds; pyruvate, succinate, malate, fumarate, and lactate. The same compounds were also used by all the isolates studied by Giesberger (26). Group differences existed with respect to the utilization of the hexoses, the lower fatty acids, the alcohols and citric and malonic acids. Some groups appeared very restricted in their metabolic potentialities (VI, VIII, ATCC 9785, and

TABLE 2. Utilization of Various Compounds Used Singly as Carbon Sources by Species of *Spirillum*

Morpho- logical Group	Species Name	Compound													
		Alcohols		Sugars		Acids									
		Ethanol	Glycerol	Glucose	Fructose	Acetic	Propionic	Butyric	Pyruvic	Succinic	Fumaric	Malic	Lactic	Citric	Malonic
I	<u>S. serpens</u> var. <u>azotum</u>	-	-	-	-	(+)	(+)	(+)	+	+	+	+	+	-	-
II	<u>S. linum</u>	(+)	-	-	-	+	+	+	+	+	+	+	+	+	(+)
III	<u>S. lunatum</u>	-	-	+	+	-	-	-	+	+	+	+	+	-	(+)
IV	<u>S. curvatum</u>	-	-	(+)	(+)	-	-	-	+	+	+	+	+	-	-
V	<u>S. polymorphum</u>	+	+	-	-	(+)	-	-	+	+	+	+	+	+	+
VI	<u>S. undula</u>	-	-	-	-	-	-	-	(+)	(+)	(+)	+	(+)	-	-
VII	<u>S. itersonii</u> var. <u>vulgatum</u>	+	+	+	(+)	+	(+)	+	+	+	+	+	+	(+)	
VIII	<u>S. giesbergeri</u>	-	-	-	-	-	-	-	+	(+)	(+)	+	+	-	(+)
IX	<u>S. beijerinckii</u>	-	(+)	(+)	-	(+)	-	+	+	+	+	+	+	(+)	(+)
X	<u>S. atlanticum</u>	(+)	(+)	(+)	-	(+)	-	-	+	+	+	(+)	+	+	(+)
ATCC 9785	<u>S. graniferum</u>	-	-	-	-	-	-	-	+	(+)	(+)	+	+	-	(+)
ATCC 9786	<u>S. sinuosum</u>	-	-	-	-	-	-	-	+	(+)	(+)	+	+	-	-

+ = Good growth

(+) = Poor growth

- = No growth

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ATCC 9786) while others grew on all compounds tested (VII).

Group VI and ATCC 9786, which are morphologically quite distinct, utilize the same group of compounds; this is also true for groups VIII and ATCC 9785. If growth on malonate is omitted from consideration, then four distinct morphological groups VI, VIII, ATCC 9786, and ATCC 9785, cannot be distinguished on the basis of carbon sources utilized. The same holds true for groups III and IV.

Nitrogen sources. All groups were capable of using ammonia, nitrate, and asparagine as the sole nitrogen source in a synthetic medium with pyruvate or lactate as the carbon source. All but groups IX and X were capable of using urea as well. With nitrate as the nitrogen source, nitrite accumulated in cultures of groups I, II, V, and VII; ammonia was formed in cultures of the other groups. The organisms of groups V and VII grew anaerobically in the presence of nitrate, the others lacked this ability.

These results are not in agreement with those of Giesberger (26) who failed to obtain growth of any of his isolates with nitrate or urea as the sole nitrogen source. When Giesberger's reisolated strains of *S. serpens* and *S. itersonii* became available to us the experiments on nitrogen sources were repeated using both his isolates and ours. The results with our isolates did not change, his cultures showed very sparse growth with nitrate that could only be detected by microscopic examination of the cultures. It could be that trace amounts of ammonia present as impurities in the media components were responsible for the growth, but in any case, the data show distinct strain differences in this property.

pH. The pH ranges over which growth was initiated in a pyruvate synthetic medium were as follows: group VII, less than 5.5 to 9.0; group I, 5.5 to 9.0; groups II, IV, VI, VIII, IX and X, 6.0 to 9.0; groups III, ATCC 9785, and ATCC 9786, 7.0 to 9.0; group V, 7.0 to 8.5. All groups grew best on the alkaline side and most were quite sensitive to even slightly acid conditions. Essentially the same pH relationships held for Giesberger's (26) isolates. It is interesting that the group VII organisms, which were the most acid tolerant, were the only ones which could use both sugars and alcohols for growth.

The pH in all tubes in which growth occurred shifted to higher levels during the growth period. The general tendency for the medium to become more alkaline during growth relates to the use of a salt, potassium pyruvate, as the carbon source and suggests that the pyruvate is completely oxidized.

The organisms of groups III, IV, and VII, which grew on glucose and fructose, were tested for acid production from these substrates and other sugars and alcohols in synthetic media. No significant reduction in pH occurred with cultures of groups III and IV, suggesting complete oxidation of the substrate, but considerable acid was formed by those of group VII from glucose, fructose, ethyl alcohol and glycerol.

Temperature. Except for those of group I, all strains grew at 35 C but not at 40 C, having 30-32 C as the optimum. Group I strains grew at 40 C but died out rapidly at this temperature. The maximum growth temperature of Giesberger's isolates was apparently higher since he incubated his enrichment cultures at 40 C to aid in the isolation of the spirilla.

Oxygen. All isolates were aerobic. None grew anaerobically in either complex or synthetic media with the exception of the group V and VII organisms which used nitrate for anaerobic growth. All the spirilla grew only in the upper portion of shake tubes and all were catalase positive. With the exception of S. volutans, which frequently showed a distinct growth zone (mixed culture) below the surface of liquid cultures, none exhibited the "microaerophilic" character of growth described by Beijerinck (2) for his spirillum (S. tenue). Cayton and Preston (12) report microaerophilic growth for S. manconiense.

Salt and marine strains. The marine strains grew in media prepared with either natural or synthetic sea water, but growth was very poor if 3 per cent NaCl was used. Larger inocula were required to obtain growth in the synthetic sea water media as compared to natural sea water and initiation of growth in the former was approximately 6-12 hours slower than in the latter. Continued subculture in a synthetic sea water medium eventually resulted in failure of growth on transfer.

Attempts were made to adapt the marine spirilla to decreasing concentrations of sea water. All of the strains grew when the sea water content was reduced to 50 per cent

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but only the organisms of group II grew when the sea water concentration was lowered still further. These organisms were finally adapted to 30 per cent sea water but growth was poor and the cells were fragile and morphologically abnormal. When such cells were transferred to the regular sea water medium, they regained their normal morphological characteristics. Thus the marine spirilla not only require a minimum osmotic pressure in their growth environment but appear adapted to the particular balance of salts found in sea water.

CYTOLOGY

Methods

Cytological procedures. Smears were made from broth or impression smears were made by the agar block method, either on slides or number 0 coverslips, and for most procedures, fixed in Carnoy's acetic acid-alcohol (1:3) (1 part glacial acetic acid, 3 parts absolute alcohol) for 10 minutes. They were transferred through a graded series of alcohol and distilled water to distilled water before being stained with the dye being used. Smears for cell wall stains and fat stains were fixed in neutral formalin (10 per cent) for 10 minutes and washed twice with distilled water before staining. Heat fixed preparations were used in routine and exploratory procedures but were not satisfactory for critical work due to the distortion and shrinkage of the cells.

Smears for acid hydrolysis were either fixed in Carnoy or exposed to the fumes of 2 per cent osmic acid for 5 minutes prior to hydrolysis. Hydrolysis was carried out with 1 N HCl at 60 C, usually for 5 to 8 minutes, or with 10 per cent perchloric acid at 4 C overnight (46). After hydrolysis, the preparations were washed in distilled water before being stained or subjected to the Feulgen reaction. Smears were also digested with ribonuclease (26 micrograms/ml) in veronal buffer, pH 6.0, for 24 hours at 37 C. The resulting preparations were washed well in distilled water and phosphate buffer before staining.

Smears were stained with various aniline dyes and also by Gram's method, using standard procedures. Cell wall stains were made using the methods of Gutstein (30), Knaysi (34), and Dyar (20). Gray's (28) and Leifson's (38) techniques were used for flagellar stains.

Fat stains were made on both wet and fixed preparations using Sudan IV or Sudan black B in ethylene glycol or 70 per cent alcohol (10), or by the isopropyl alcohol technique of Lillie (40). Coverslip preparations were placed in 2 per cent osmic acid solution for periods of 36, 48, 72, and 96 hours and examined unstained and stained with safranin for 20 to 30 seconds in order to detect unsaturated fats.

Giemsa stains (64) were made on unhydrolyzed, acid hydrolyzed and ribonuclease digested preparations, according to the method for the malaria parasites. Attempts to identify volutin were based on the properties of this material as cited by Grimme (29) and Meyer (43).

Direct observation of living cells. Direct observation of cell division, motility, and microcyst formation and germination were made by dark phase contrast on wet mount preparations, sealed with immersion oil.

Photography. Photographs were made with both the light and dark phase contrast microscopes (Leitz Ortholux) on Panatomic X sheet film. Interference filters, ranging from 500 to 580 mμ, were used for photographing stained preparations.

EXPERIMENTAL RESULTS

Living cells. Observations of wet mounts showed the typical wave form of all the isolates. Motility was rapid and involved not only forward progression but also spinning of the cell around the long axis. While in rapid motion the cells frequently appeared as straight rods. This was assumed to be an optical illusion due to the rapid spinning of the cells. Pijper and coworkers (50), however, have recently presented cinematographic records of S. serpens in rapid motion which show a change from the typical curved form to that of a straight rod-like form in $1\frac{1}{2}$ seconds, with a concomitant lengthening and thinning of the cell. Because of the rapidity with which the spirilla move, it is impossible to determine, by direct observation alone, how much of the apparent straightening of the moving cell is due to an optical artifact and how much is due to an actual change in cell shape.

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Refractile granules were observed in most of the species and were especially prominent in the larger forms. These granules have been interpreted as either fat or volutin by the majority of investigators of the spirilla species. When dilute methylene blue (1:10,000) was used as a vital stain, the majority, but not all, of the granules stained blue. Red-violet staining of the granules of the living cells was never observed. As observed by Zettnow (68) successful sub-culture could be made from cultures so stained with methylene blue.

Cell division was followed in rapidly dividing cultures in both wet mount and sealed preparations by dark phase contrast. The cell about to divide darkens slightly at the point where separation will occur. Then the two portions of the cell bend together at the darkened point and twist about, one part clockwise and the other counterclockwise. Ellis (24) also observed this bending and twisting during division of the spirilla and a similar process has been described for the spirochetes (18). As the twisting continues, the twisted portion becomes elongated and pulls out into a fine thread. This can be observed in the larger forms by dark phase contrast but cannot be photographed by still photography in the living condition because of the rapid movement of the two dividing cells. Figure 8 shows the elongation between two undivided but separated cells of Spirillum undula stained with Giemsa. Dimitroff's (19) camera lucida drawings (his Plate 1) also show this filament.

In the large spirilla of group VI (S. undula), whose flagella can be observed by dark phase contrast, a flagellum is immediately observable after cell division on the newly separated ends of the cells. From the phase microscopic observations of this species it appears as if the flagellum arises from the elongated thread formed during cell division.

Gram's method. All of the cultures were Gram negative. The cells usually did not stain uniformly with the Gram procedure or with single aniline dyes such as crystal violet or safranin. The granules remained unstained or lightly stained, using normal procedures, but were much darker than the remainder of the cell when overstained. On occasions, the granules retained the crystal violet whereas the cytoplasm was Gram negative.

Cell wall stains. The cell wall of the species of Spirillum did not stain by the procedures of Gutstein (30), Knaysi (34), or Dyar (20). The outline of the cell could be seen and if the cells were in a state of division, the septum could be observed; the cell wall, however, could not be distinguished from the cytoplasm by these methods. The outline of the cell and the septum, in dividing cells, could be observed much better in flagellar preparations (Gray's method) than in cell wall stains (Fig. 9). In some flagellar preparations, in which cells of S. volutans or S. undula were suspended in distilled water, the cytoplasm has retracted from the cell wall (Figs. 11 through 13) which can be seen distinctly. The difficulties experienced in attempting to demonstrate cell walls by standard staining techniques are in line with the results of other investigators that show the cell walls of the spirilla are chemically and structurally unique (55, 56, 13, 32).

Flagella. The flagella of living cells of the spirilla species we have investigated can be observed by dark phase contrast in only two species, the group VI organisms (S. undula) and the Pringsheim strain of S. volutans. In both cases, only a single flagellum can be observed when the organisms are in rapid motion but when motility ceases due to the drying out of the wet mount preparation, as many as five flagella may be observed in a few individuals of both species, at both ends of the cell. These flagella show independent motion and appear to have their own point of contact (or insertion) with the cell. Even among these motionless cells, tufts of flagella are a rarity.

Two types of flagellation were observed when Gray's (28) method of staining was used. The spirilla of groups I, II, V, VII, and VIII, and the two cultures obtained from the American Type Culture Collection, 9785 and 9786, showed bipolar tufts of flagella (Fig. 10). The others showed only a single flagellum at each pole in the majority of preparations (Fig. 11). However, using Leifson's (38) method, all the organisms but those of groups V and VI showed bipolar tufts of flagella. (Dr. Leifson prepared the slides, using our strains of Spirillum and kindly sent them to us for examination.) The only explanation which can be given for these differences in results by the two methods lies in the preparation of the specimen. In Gray's method the cells are

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placed on the slide and stained without fixation. In Leifson's method the cells are killed with neutral formalin before the organisms are placed on the slides and the possibility exists that the latter procedure allows a fascicle of flagella to be separated into its component parts. The tufts of flagella in S. volutans, arising from what Pijper (48) calls the "stem" (Figs. 12 and 13) might have formed in this fashion.

The mode of flagellation in the spirilla is still an unresolved problem. All individuals who have studied the flagella of living cells of the spirilla from the time of Cohn (15) have maintained that they observe only a single whip-like flagellum at each pole (25, 48, 49, 54). On the other hand, all investigators using stained preparations have stated that the spirilla have tufts of flagella (38, 39, 43). Meyer (43), who reviewed the early literature, took the position that the single flagellum, reported by various investigators from examination of living material, is a fascicle (Geisselbuscheln) or tuft (Geisselzopfes); a view that is confirmed for, at least one spirillum species, by a recent electron microscope picture (32). Since staining procedures give variable results, depending on the technique used, and since flagella can be seen in the living state only on the larger spirilla (with dark phase contrast), a comparative study using electron microscope techniques will probably be required before a decision can be reached as to whether one or more modes of flagellation occur in this group.

Fat. The refractile granules observable in the living cells of the spirilla that do not stain with aniline dyes have been assumed to be fat globules by the majority of investigators of this genus. Grimme (29) shows drawings in which the granules have been stained by a fat dye and Lewis (39) shows photographs of fat stained cells, but the majority of investigators have assumed the unstained granules to be fat. Fat stains were tried on cells taken from all stages of growth, ranging from five hours to week-old cells, without positive results. Even when glycerol was added to the growth medium the results were essentially negative. When the Sudan dyes were applied to washed cell suspensions for up to 30 minutes, fat staining of the granules was not observed. With longer staining periods, however, the entire cell was stained black if Sudan black B was used. Lewis' procedure (Löffler's methylene blue, Lugol's iodine,

and vesuvin) was repeated and it was found that positive results were obtained (black staining granules) only if the preparations were not washed between each staining period but merely blotted. When the preparations were washed between stains, however, the granules showed staining with methylene blue and the cytoplasm was a pale brownish-yellow from the vesuvin. Since the black staining characterizes both volutin and fat (39), it is difficult to see how Lewis could differentiate between them by this method.

In the larger spirilla, in preparations fixed with neutral formalin, some of the refractile granules, in a small percentage of the cells, were outlined by the fat stain (Sudan black B) if the preparations were examined in high focus (Fig. 14). Essentially the same results were found by Lillie's method.

Smears exposed to osmic acid were dark brown, macroscopically, but microscopically no darkening of the granules was observed. Since osmic acid is supposed to blacken only unsaturated fats (42) the results imply that there is no great concentration of such in the cells.

It can be concluded that in the strains investigated few, if any, of the granules observed could be composed exclusively of fat. The results suggest, however, that the cell wall and the periphery of some of the granules contain considerable lipid material.

Volutin. The investigations of Grimme (29) and Meyer (43) form the basis for the statements by later investigators of the species of Spirillum that the granules, prominent in the cells, are composed of volutin. Grimme (29) characterizes volutin as staining a dark blue or reddish-blue with dilute methylene blue (1:10); he states that the granules swell when exposed to methylene blue and that the center of the granules appears a bright, peculiar shade of red, often giving the impression of being composed of a different material from the periphery. He states that the granules (when stained with dilute methylene blue) are not decolorized with 1 per cent sulfuric acid; dissolve in 5 minutes in hot water at 80 C; dissolve in 5 minutes in 5 per cent acid or alkali at room temperature. Grimme's procedures were followed exactly in studying the granules in our isolates.

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The granules of the spirilla species stained dark blue with the dilute methylene blue (Fig. 15) and were not decolorized by 1 per cent sulfuric acid (Fig. 16). Reddish staining of the granules was seldom observed but the swelling of the granules (mentioned by Grimme) was observed and the impression was gained that the center of the granules differed from the periphery. This impression is also obtained when the granules are examined by dark phase contrast microscopy. Heating a wet mount preparation to steaming resulted only in the disappearance of the center of the granule, rather than the entire granule, as was observed by Grimme. The outer portion of the granule always remained stained a dark blue.

Suspensions of cells were prepared in 5 per cent acetic, hydrochloric, and sulfuric acids and sodium carbonate solution; allowed to remain at room temperature for a two hour period. A portion of the cells was removed at 10 minute intervals, smeared on a slide, air dried, heat fixed, washed with tap water, and stained with dilute methylene blue. Suspensions of cells were also placed in hot water at 80 C for a similar period of time and treated as above. At the end of the treatment, the granules in acid and hot water treated cells appeared intact; those exposed to alkali showed some disintegration at the edges of the granules.

Similar treatments were given to unfixed preparations from 5 and 10 hour cultures. When the unfixed, hot water treated smears were stained with methylene blue, some of the granules showed empty spaces, as described by Grimme, but others stained blue (Fig. 17). Smears treated with 5 per cent acid, washed well with tap and distilled water, and stained with methylene blue gave the same picture (Fig. 18) but those treated with alkali showed mostly empty vacuoles, which had a pinkish tinge, where the granules had been. As observed by Grimme, formalin fixed cells resisted the hot water and acid treatments; formalin fixed cells treated with alkali showed some disintegration of the periphery of the granules, which were stained a reddish-blue. Thus the granules of the spirilla species gave some but not all of the reactions described as characteristic of volutin by Grimme (29) and Meyer (43).

Nuclear staining. Both unhydrolyzed and acid hydrolyzed smears of the spirilla showed staining reactions similar to

those of the malaria parasites when Giemsa stained. The unhydrolyzed cells showed a blue stained cytoplasm containing red stained granules (Fig. 19); the hydrolyzed cells showed essentially the same picture with less intense staining of the cytoplasm, that is, a pale blue or colorless cytoplasm with red stained granules (Fig. 20). Preparations digested with ribonuclease and stained with Giemsa showed staining of the same granules that were stained by the other methods (Fig. 21). When the Feulgen reaction was used, the same granules were Feulgen positive.

From the results of the various staining techniques employed it can be concluded that the refractile granules observed in the living spirilla fulfill all the histochemical criteria considered critical for the demonstration of chromatinic bodies in bacteria. Since the granules are apparent in the unstained spirilla and can be demonstrated in stained cells without recourse to special pre-treatments like acid hydrolysis or enzyme digestion, it is obvious that there are important differences in the cytology of the spirilla as compared to "typical" Eubacteriineae like Escherichia coli and Bacillus subtilis, which were used as controls on all staining techniques used for the spirilla. It is commonly assumed that the uniform staining reaction obtained with bacterial cells is due to the large amount of ribonucleic acid in the cytoplasm during the period of rapid growth (35) and most experiments designed to reveal internal structure use some technique to remove opaque ribonucleic acid from the cells. Since the chromatinic bodies of the spirilla are easily observed in both living and unhydrolyzed stained cells, these forms must have either a lower ribonucleic acid content than the usual Eubacteriineae or else this material must be distributed differently in the cells.

The granules that react cytochemically like nuclei also give some of the reactions by which volutin is identified and show peripheral staining with fat stains. If volutin is a type of ribonucleic acid as is claimed by some (35, 43), one could postulate that the granules are a composite of ribo- and desoxypentose-nucleoproteins within a lipid-containing boundary layer. Alternatively, the staining reactions could be explained by the presence of a unique type of nucleic acid. The latter would be consistent with the findings of Belozersky (4) who isolated a new type of nucleic acid from

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the nucleoprotein of S. volutans which contained all the purines and pyrimidines, including thymine, but which, in addition to desoxyribose, contained another pentose. Clarification of the exact structure and composition of the refractile granules of the spirilla species must await chemical analysis of these elements.

DISSOCIATION, LIFE CYCLES AND PLEOMORPHISM

Dissociation. The species of *Spirillum* exhibit dissociation of two distinct types. One is the mucoid type, in which the colonies have a slimy, mucoid appearance and the cells are difficult to stain by ordinary methods. A thick, slimy material is produced and some preliminary experiments showed this substance has some of the characteristics of a dextran. The other type of dissociation is what is called "extreme rough" in other bacterial species, in which the colonies have a piled up formation, are very hard and come off the agar as whole colonies which require some pressure with a coverslip to disperse. In both cases, the individual cells tend towards straight rods, losing their spiral characteristics. In broth, the dry, rough organisms grow as matted pellicles at the surface of the tubes and it is difficult to disperse the growth for examination.

Cultivation of those organisms which will grow on nutrient agar or broth, without the addition of supplements, yields both types of dissociants upon plating. Cultivation in potassium pyruvate broth gives an extreme mucoid type of growth but the cells remain spiral, rather than becoming straight. In those species producing microcysts, growth in synthetic pyruvate broth or in nutrient broth to which potassium pyruvate has been added, is characterized by extreme turbidity and sliminess of the medium. After about three days' growth the medium clears suddenly and microscopic examination shows the presence of microcysts almost exclusively. In species in which microcysts are not found abundantly, the sliminess of the liquid medium increases throughout the growth period. Attempts to show capsules on cells from slimy growths have been unsuccessful. The dry rough type of growth is found upon transfer of old stock cultures whether they are maintained on supplemented media or plain nutrient agar or broth. The two ATCC cultures are especially prone to the rough dry type of dissociation.

The normal type can be recovered from dissociated cultures by growing the organisms in yeast autolysate broth, streaking out on yeast autolysate agar, and picking the typical granular, ground-glass colonies.

Life cycles. A cyclic alternation between morphologically distinct vegetative and resting forms occurs in many, if not all, of the spirilla investigated. The species can be divided into two distinct groups, those that exist predominately or exclusively in the microcyst form in old cultures (groups II, III, IV, V, VII, IX, and X and Giesberger's S. itersonii) (Fig. 22) and those that even after long periods of storage, at room temperature, remain primarily as typical vegetative cells (groups I, VI, VIII, ATCC 9785 and 9786). The pattern of the life cycles of the former group is similar: namely, all germinate from microcysts by both unipolar and bipolar germination; grow as typical vegetative cells for a restricted period of time; and again form microcysts. The details of the life cycle for the group III organisms (S. lunatum) have been presented elsewhere (65) and will not be repeated here.

Among the morphological groups whose cells remain largely in the vegetative stage in old cultures there are observed, although infrequently, forms which resemble the microcysts of the other groups. In some instances these have been observed to form in much the same fashion as in the groups where microcysts are abundant, i.e., by the formation of a protuberance. However, some are formed in an entirely different manner. For example, in group VI (S. undula) a cell will bend, as if about to divide, but no division occurs. Instead, a ring is formed by the central portion of the cell with the two ends crossing each other. Eventually, these two end portions are absorbed or disappear and the ring gradually closes with the formation of a spherical body resembling a microcyst. This sequence is reconstructed from stained preparations in Figure 23 but has not been studied by following sequential changes in living cells as was done with S. lunatum (65). A similar phenomenon was noted by Dimitroff for Spirillum virginianum and described in detail, with camera lucida drawings, in his section on coccoid bodies (19).

Pleomorphism. All of the species of Spirillum studied show definite morphogenesis which differs, in degree, among

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the various species. Branched forms have been observed in all species but they are more commonly observed in the larger forms. Although branched forms are common in old cultures, and thus could be taken for degenerate forms, they are more commonly found in young cultures, 5 to 10 hours after inoculation (Fig. 24). Entwined forms were noted by Cohn (15) and can be found in all species examined. These forms occur only in young cultures and are found in both broth and agar growths. In certain instances, two individual cells twist about each other but in others, one end of a particular cell twists about the other end (Fig. 25). It has been shown that the entwined cells fuse (65) and that fusion and rearrangement of the chromatinic material then occurs (66). Also prominent in cultures of all strains are extremely large and delicate cells which have been termed "giant" cells (Fig. 26). All the above mentioned forms occur in the normal media used for culturing the spirilla. Although some resemble the large bodies reported for other bacterial species (17) no special inducing agent is needed to promote their occurrence. Pleomorphism of the spirilla has been noted by many investigators of this genus (68, 59, 19, 24). With the exception of Dimitroff (19) who compared the "coccoid" bodies of the spirilla to similar bodies reported for the spirochetes (1), the other investigators regarded these as involution or degenerative forms. Recently, Cayton and Preston (12) reported the production of spherical bodies, undoubtedly microcysts, by a new species of Spirillum but failed to attach any significance to them.

The demonstration that the spherical bodies represent a morphologically distinct resting stage of the spirilla clarifies at least part of the literature on pleomorphism. In addition, the finding that microcysts are produced with different frequencies by different species and that they may arise in different fashions, i.e., by contraction of the entire cell, by protuberance formation, after fusion of entwined forms (65) and by ring formation, may lead, with further study, to a firm basis for distinguishing species within the genus.

Whether other pleomorphic forms noted by us and others are phases in other aspects of the life cycle of the spirilla cannot yet be stated with certainty, despite suggestive evidence for a sexual cycle in which they participate (65,66).

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Diagnosis of the genus. Giesberger (26) proposed the following diagnosis for the genus Spirillum Ehrenberg based on his own and previous work: "Spiral shaped, rigid cells, which are motile by means of bi-polar flagella tufts. Do not form endospores. Gram negative. Catalase positive. Chemoheterotrophic; oxidize various organic substances, preferentially salts of organic acids. Volutin generally occurs as reserve material." Essentially the same diagnosis is used in the 6th edition of Bergey's Manual (7) although the phraseology is somewhat changed and the nutritional characteristics of the group are qualified in order to permit the inclusion of two species of more complex requirements, S. volutans and S. minus.

On the basis of the work reported we feel that the diagnosis should be modified in several respects. Both Giesberger's and Bergey's definition would exclude S. volutans and the organisms of group III, IV, and VI, since these forms show, predominately or exclusively, only a single flagellum at each pole, when Gray's flagellar staining method is used. As has already been pointed out, the true nature of the lophotrichous state is still a matter of controversy, and for the present, at least, the distinct spiral form should be considered more important than the mode of flagellation in assigning an organism to the genus.

The statement regarding volutin could well be eliminated from the diagnosis on two bases. First, not all species of the genus show "volutin" granules and many species of other genera do. Thus the characteristic has little diagnostic value. Secondly, the exact chemical and functional nature of the prominent granules in members of this genus is uncertain; our data show that they have the cytochemical characteristics of nuclei and only a few of the characteristics of volutin.

Finally, the formation of microcysts by at least certain species is of great taxonomic significance and should be mentioned.

The following diagnosis is therefore proposed. "Spiral shaped cells, which are motile by bi-polar flagella occurring

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singly or in tufts. Microcysts formed by some species, endospores not formed. Gram negative. Aerobic, catalase positive (one exception). Chemotrophic heterotrophs; most species oxidative and non-exacting, growing well on salts of organic acids. Primarily water forms. A few species have not been cultivated in pure culture."

Relationships of the genus to other groups of bacteria.

In practically all early classifications (44), the curved organisms, vibrios, spirilla, and spirochetes, were closely grouped. Separation of the spirochetes and the spirilla, when made, was at the genus level on the basis of one or more of the following differences; flexibility of the cells, motility without flagella, and coiled structure. With time, as bacterial classifications grew more pretentious and efforts were made to indicate natural relationships, two major changes in the groupings of the curved bacteria have gained widespread acceptance. The first is the recognition that a close relation exists between the spirilla, the vibrios, and the Gram negative, polarly flagellated pseudomonads. The results of Giesberger (26) and those reported here, showing that most of the spirilla have the same type of oxidative metabolism characteristic of the pseudomonads, are in accord with this grouping and nothing further will be said on this point.

The second is the wide separation of the spirilla and the spirochetes in the belief that the latter group has little phylogenetic relation to the Eubacteriales. In the American literature, this viewpoint was crystalized by the creation of the order Spirochaetales by Buchanan (8) and the subsequent acceptance of this order by the Bergey Committee and by almost every textbook author since. In Europe, Lehmann and Neumann (37) followed suit, although not so drastically, by the acceptance of the family Spirochaetaceae, first proposed by Swellengrebel (59). Likewise in more recent systems the spirilla and spirochetes are widely separated at the order or even class level (51, 58).

This point of view probably resulted from the controversy that developed after the discovery of the syphilis organism as to whether the spirochetes should be considered protozoa or bacteria. Buchanan's original diagnosis of the order Spirochaetales included the vague general statement "protozoan-like in many characteristics" and mentions only two specific

properties that separate the spirochetes from the spirilla; being "flexuous" and multiplying by "longitudinal" fission. It was soon learned that the belief in longitudinal fission of the spirochetes was based on an artifact and the order, as now defined, includes only two characteristics which have differential significance; "flexuous" as opposed to "rigid" for the Eubacteriales and motility without flagella.

The term flexuous (or its Latin or German equivalent) has been applied to the spirochetes at least since the time of Ehrenberg (23) by numerous investigators, but has never been exactly defined. It has been used to express the ability to bend or flex (and was used in this meaning by Ehrenberg (23)) and thus accounts for the secondary waves that are often imposed on the primary coil of the pathogenic spirochetes. It also has been applied to describe cells that can stretch or contract. In either sense the term flexuous can also apply to the spirilla, especially the longer forms. Not only do the spirilla bend or flex, but they also straighten during motion and increase in length as was shown by Pijper (50). In addition, during microcyst formation, the entire cell contracts to form an oval or spherical body showing marked "flexuousness" during this transitional period of the life cycle. It is difficult, therefore, to justify separation of the two groups on the basis of this property since the groups differ here, if at all, only quantitatively.

Flexuousness has also been interpreted as meaning the absence of a cell wall and this property is sometimes mentioned in the diagnosis of the spirochetes. Dyar (21), however, using her cell wall stain, demonstrated a heavily stained surface layer in a free living spirochete which she termed the "cell membrane". It is not clear from her picture or technique why the same structure could not have been called a cell wall, as indeed, Knaysi (35) did when referring to her work. Bradfield and Cater (6), speak of a....."sheath (representing the cell membrane)....." around the spirochete cell proper and show this structure in an electron micrograph of Treponema duttoni, but again, just what distinguished this structure from a cell wall is not made clear. Our staining results with the spirilla suggest that differences exist in their cell walls as compared to other Eubacteriales and Salton (55) has demonstrated marked differences in the chemical composition of different groups of eubacteria. The possibility

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remains, therefore, that the spirilla and the spirochetes differ only quantitatively on this point.

The mechanism of motion in the spirochetes and whether flagella are involved in their motility are still unsolved problems. Although flagella-like structures have been observed by some (41, 45), evidence for their functionality is lacking. On the basis of electron microscope studies, Bradfield and Cater (6) recently proposed a unique structure for the spirochete cell in which one or more bundles of fibrils (probably contractile) are wound spirally around the main core of protoplasm from end to end; the fibrils lying inside or outside a cell membrane, depending on the species involved. They found no evidence for the occurrence of a similar structure in spirilla. Although they did not discuss the question of motility, the inference to be drawn from their proposed structure is that motion in the spirochetes arises from the contractility of the fibrils. There is a striking similarity in the postulated nature of the fibril bundles and the structure of the bacterial flagella and it is not difficult to envision the development of the former from the latter. Thus, assuming that the unique structure proposed by Bradfield and Cater for the spirochetes is confirmed, there is still no compelling reason to believe in an origin of this group independent of the Eubacteriales.

To sum up, there is very little evidence to justify a wide separation of the spirilla and spirochetes and much in the way of morphological and structural similarities to suggest their close relation. Although we do not propose to offer an alternate classification at this time, we wish to emphasize the artificial nature of the order Spirochaetales.

The belief in the lack of a close relation between the myxobacteria and the eubacteria (58) has been justified, as with the spirochetes, by the absence of a rigid cell wall and creeping motility without flagella. Although this belief may be valid, it is based on an imperfect knowledge of the mechanism of motion and on, essentially, no knowledge of the structure and chemistry of the limiting cell boundary of the myxobacters. It is apparent that investigations of these two problems are required and can contribute greatly to the field of bacterial taxonomy. However, the formation of microcysts, by at least certain species of spirilla, raises

the question of their relation to the myxobacters. The mode of formation and germination of these bodies in the marine spirilla appears to be almost identical to the process described for the Sporocytophaga (57). The occurrence of a common type of resting stage in both the eubacteria and the myxobacters suggests that the origin of these two groups may not be as divergent as most authorities believe today.

Accepted species. The descriptions of the species of Spirillum published prior to 1936 are, on the whole, incomplete and so inadequate that they cannot be used for determinative purposes. Giesberger (26) made a systematic survey of the spirilla described up to that time, and eliminated the majority of the species as being species dubia. He retained in the genus Spirillum only those organisms whose morphological and physiological characteristics were adequately described or whose morphological characteristics were so distinctive that identification was possible on this basis alone. Nine species were recognized: S. undula, S. serpens, S. volutans, S. tenue, S. minus, S. kutscheri, S. virginianum, S. cardiopyrogenes, and S. itersonii.

Bergey's Manual (7) follows Giesberger in all but two particulars. S. cardiopyrogenes is relegated to an appendix as being incompletely described, and S. lipoferum is included. Since Giesberger also comments on the inadequate description of S. cardiopyrogenes its relegation to an appendix is proper. The status of S. lipoferum is uncertain. Giesberger (26) isolated several strains of this organism from soil, following Beijerinck's procedures and concluded that, morphologically, the organism is a vibrio. Although we have had no experience with this organism we accept Giesberger's judgement since it is based on direct observation. Recently, Cayton and Preston (12) described a new species, S. mancunienne, which they describe as catalase negative, a characteristic which would distinguish it from the other well known spirilla. Unfortunately the metabolism of this organism was not adequately studied, but if other catalase negative spirilla species are isolated, this characteristic will have to be included in the diagnosis of the genus.

Criteria for species separation. Giesberger (26) found that the application of either morphological or metabolic criteria resulted in the same grouping of his isolates.

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In other words, all isolates of a given morphological type had the same metabolic pattern which differed from the metabolic patterns of other distinct morphological types. Among our isolates all the cultures of a single morphological type proved to have identical or very similar metabolic patterns in that they would all use the same carbon and nitrogen compounds as growth substrates. It is true, that as the number of carbon compounds individually tested increased, differences in utilization patterns between cultures of the same morphology appeared. It is not surprising that these differences should occur, when one considers the possibilities of mutation, gene recombination, transduction, and other mechanisms of genetic alteration in individuals, but they can hardly have major taxonomic significance.

Contrary to what Giesberger found, isolates of distinctly different morphologies did, in certain instances, show the same metabolic pattern. 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 homogeneous groups.

Recognizing that either choice is an arbitrary one, we have taken the latter course. Using gross morphology alone, 13 distinct groups could be recognized among our 190 cultures. Although there were variations in the morphology of isolates placed in a single group, the mode of each group is fairly distinct and placement of the isolates into the proper category presented little difficulty.

The taxonomic rank to be assigned to these 13 groups is a more difficult problem to deal with. Unfortunately, as has been repeatedly pointed out, the species concept in bacteriology has never been clearly defined and, at least borrowed from other branches of biology, may not be applicable to bacteria at all (53, 60, 63). As stated by Benecke (5), a bacterial species is "...that which the investigator who proposes the species wishes to include in accordance with his scientific tact...", and the subjectivity of the species concept is apparent upon reading even the most recent taxonomic contributions (11, 16).

To resolve the problems inherent in this situation, van Niel (63) has suggested that "....Discontinuation of the terms species and genus for bacteria, along with the introduction of multiple keys, would eliminate some of the difficulties now encountered, because it would insure a far greater autonomy to specialists in dealing with their problems, unencumbered by the exigencies of different groups." (Italics inserted by the authors.) The difficulty here is that such keys, to be useful, must separate and name, in one fashion or another, determinable groups. If the groups are determinable, differences exist, and these differences must have taxonomic significance at some level. Although the problem of what taxonomic level is involved can be ignored, it would appear to be the duty of the systematist to assess the taxonomic significances of the differences even though, at the moment, this can only be done intuitively. From such efforts, subjective as they may be, a satisfactory species concept may ultimately develop in our science. It is our belief that further studies on the life cycles of the spirilla will give a more solid basis for defining the species and that, at some future time, it will be possible to relegate most of the species to some lower rank. It is from this point of view that we feel justified in describing and naming ten new species in the genus Spirillum.

Description of the species. The measurements of the cells given were made on heat fixed preparations. A "type" picture is given for each species. Type cultures have been deposited in the American Type Culture Collection.

GROUP I

FRESH WATER SPIRILLA WHICH GROW READILY IN PEPTONE MEDIA

Morphologically and physiologically, these organisms are very similar to S. serpens as described by Giesberger (26), but differ in the nitrogen compounds that can be used for growth in synthetic media and in several other less important respects. They are considered a variety of S. serpens and are named S. serpens var. azotum (Figs. 4 and 7) because of their nitrogen metabolism.

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(Type culture No. 11335 American Type Culture Collection)

Spiral cells containing granules, having 2 to 3 wide, shallow, spiral curves; cell diameter 0.8 to 0.9 μ ; spiral amplitude 5.0 to 5.6 μ ; spiral depth 1.5 to 2.0 μ ; cell length 10.5 to 16.5 μ ; bipolar tufts of flagella. Gram negative.

Nutrient agar: Small, creamy-white, opaque, umbonate colonies, 1.5 mm in diameter, which have a typical granular, ground-glass appearance when viewed with the hand lens with transmitted light.

Nutrient broth: Moderate turbidity in young cultures; finely granular sediment and clear supernatant in old cultures.

Gelatin: Not liquefied (thus distinguished from S. serpens var. serpens).

Potato: Brownish-orange growth.

Nitrogen compounds: Grows well on peptone media and can utilize ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates weakly reduced to nitrites. (Utilization of nitrate distinguishes this from S. serpens var. serpens.)

Carbon compounds: Grows well on the salts of pyruvic, lactic, and malic acids in synthetic media. Utilizes the salts of acetic, propionic, butyric, succinic, and fumaric acids less readily. Does not utilize sugars, glycerol, ethyl alcohol, citrate or malonate.

Aerobic. Catalase positive. Optimum temperature 32 C, but can grow at 40 C. Growth can be initiated between pH's 6.5 and 9.0 in synthetic media.

Source: Isolated from fresh water.

*Spirillum serpens Giesberger liquefies gelatin and does not utilize nitrate as a sole source of nitrogen. With the description of the new variety (S. serpens azotum) it is recognized that another new variety to include the species type is automatically created (under the rules of the International Bacteriological Code of Nomenclature) with the name Spirillum serpens serpens. Inasmuch as no type culture collection has a type culture of this species (and variety), a culture of a suitable strain has been deposited with the American Type Culture Collection as its No. 11130 with the recommendation that this be recognized as the "neotype" or "standard" culture of Spirillum serpens Giesberger and of its variety Spirillum serpens serpens.

GROUP II

MARINE SPIRILLA WHICH DO NOT GROW IN
UNSUPPLEMENTED PEPTONE MEDIA

The organisms exist in both a vegetative and microcyst stage. They could not be identified with any previously described organism and are considered a new species. The name Spirillum linum is applied from the thread-like form of the vegetative cells (Latin linum flax, thread) (Fig. 27).

Spirillum linum sp. nov.

(Type culture No. 11336 American Type Culture Collection)

Small thread-like spiral cells, usually with 2 to 3 shallow spiral curves, although organisms in rapidly transferred cultures may have 5 to 10 spirals; cell diameter 0.3 to 0.4 μ ; spiral amplitude 1.5 to 2.0 μ ; spiral depth 0.9 to 1.0 μ ; cell length 2.8 to 4.2 μ ; bipolar tufts of flagella. Gram negative.

Microcysts spherical, 0.8 to 1.5 μ in diameter.

Sea water yeast autolysate nutrient agar: Yellowish-white growth, spreading along line of streak, seldom forming discrete colonies. When formed, colonies are flat with an irregular edge. Produces a characteristic pungent odor.

Sea water yeast autolysate broth: Heavy turbidity and pellicle formation in young cultures; the pellicle breaks up into large flakes which do not disperse; viscous sediment in old cultures.

Sea water gelatin: Not liquefied.

Potato: Orange-brown growth on potato slices soaked overnight in sea water.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates weakly reduced to nitrites.

Carbon compounds: Grows well on salts of acetic, propionic, butyric, pyruvic, succinic, fumaric, malic, lactic, citric, and malonic acids. Slight growth on ethyl alcohol. No growth on sugars or glycerol.

Aerobic. Catalase positive.

Optimum temperature 32 C, no growth at 40 C. Growth initiated between pH's 6.0 and 9.0 in synthetic medium. Growth on 30 per cent sea water but not lower.

Source: Isolated from coastal sea water.

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GROUP III

MARINE SPIRILLA WHICH DO NOT GROW
IN UNSUPPLEMENTED SEA WATER PEPTONE MEDIA

These organisms differ from S. linum in the following major respects: a much larger cell diameter, looser and more irregular spirals, oval rather than spherical microcysts, a single flagellum at each pole, and growth on hexoses. The name S. lunatum (Latin adjective lunatus half-moon shaped) is applied from the crescent-shaped cells that are formed during microcyst formation (Figs. 3 and 6).

Spirillum lunatum sp. nov.

(Type culture No. 11337 American Type Culture Collection)

Large spiral cells with prominent granules having 2 to 3 wide, loose, irregular curves. The cells are slightly wider at the center than at the ends which are tapered. Cell diameter is 1.0 μ ; spiral amplitude 6.0 μ ; spiral depth 3.0 to 3.5 μ ; cell length 7.0 to 21.0 μ ; single flagellum at each pole. Gram negative.

Microcysts oval to ellipsoidal in early stages of formation, becoming spherical on aging; 2.0 to 3.0 by 2.5 to 5.0 μ .

Sea water yeast autolysate nutrient agar: Cream-colored, opaque, oval, smooth, umbonate colonies with an entire edge, 2 mm in diameter. Cultures have a pungent odor.

Sea water yeast autolysate broth: Heavy turbidity with pellicle formation in young cultures; the pellicle breaks up into large flakes which do not disperse; viscous sediment in old cultures.

Sea water gelatin: Not liquefied.

Potato: Very slight, grayish-white growth on potato slices soaked overnight in sea water.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates not reduced to nitrites.

Carbon compounds: Grows well on the salts of pyruvic, succinic, fumaric, malic, and lactic acids in synthetic media. Slight growth on malonate. Glucose and fructose used without acid production. Glycerol, ethyl alcohol, and fatty acids not used.

Aerobic. Catalase positive.

Optimum temperature 32 C, no growth at 40 C.

Growth initiated between pH's 7.0 and 9.0 in synthetic media.

Growth on 50 per cent sea water but not lower.

Source: Isolated from coastal sea water.

GROUP IV

MARINE SPIRILLA WHICH DO NOT GROW
IN UNSUPPLEMENTED PEPTONE MEDIA

These organisms are similar, metabolically, to S. lunatum. Morphologically, they are more like S. linum but larger and monotrichous. They are considered a new species and named Spirillum curvatum (latin adjective curvatus curved, bowed) from the bow-like curves of the spirals (Fig. 28).

Spirillum curvatum sp. nov.

(Type culture No. 11338 American Type Culture Collection)

Small, regularly curved spiral cells, having 3 to 5 spiral curves; cell diameter 0.4 to 0.6 μ ; spiral amplitude 3.5 μ ; spiral depth 2.5 μ ; cell length 7.0 to 16.0 μ ; single flagellum at each pole. Gram negative.

Microcysts spherical, 1.5 to 3.0 μ in diameter.

Sea water yeast autolysate nutrient agar: Irregular, mucoid, umbonate yellow-brown colonies, 3 mm in diameter. The cultures have a pungent odor.

Sea water yeast autolysate nutrient broth: Heavy turbidity with pellicle formation in young cultures. Old cultures have clear supernatant and heavy sediment which is composed of large flakes from the pellicle plus viscous material.

Sea water gelatin: Not liquefied.

Potato: Dark brownish-orange growth on potato slices soaked overnight in sea water.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates not reduced to nitrites.

Carbon compounds: Grows well on the salts of pyruvic, succinic, fumaric, and lactic acids. Grows less well on glucose and fructose. Malonate, citrate, fatty acids, ethyl alcohol and glycerol not used.

Aerobic. Catalase positive.

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Optimum temperature 32 C, no growth at 40 C.

Growth initiated between pH's 6.0 and 9.0 in synthetic media.

Growth on 50 per cent sea water but not lower.

Source: Isolated from coastal sea water.

GROUP V

FRESH WATER SPIRILLA EXHIBITING CONSIDERABLE
MORPHOLOGICAL VARIATION, DEPENDING ON THE GROWTH MEDIUM

These organisms are similar to S. itersonii var. vulgatum in their ability to grow anaerobically in the presence of nitrate and in their use of ethyl alcohol and glycerol as growth substrates, but differ, morphologically, and also in their inability to grow on sugars and the lower fatty acids. These spirilla form microcysts similar to those of the marine spirilla, but because of the small size of the cells, microcyst formation and germination have not been studied in this species. They are considered a new species and named Spirillum polymorphum (Greek adjective polymorphus multiform) from the variety of morphological forms found in the same and in different media (Fig. 29).

Spirillum polymorphum sp. nov.

(Type culture No. 11332 American Type Culture Collection)

Very slender cells, having tightly coiled spiral curves in malate media and wide, deeper spiral curves in yeast autolysate media; cell diameter 0.2 μ . In malate media, the spiral amplitude is 0.2 μ and spiral depth 0.3 μ ; in yeast autolysate media, the spiral amplitude is 3.5 to 4.0 μ and the spiral depth is 2.0 μ . In malate medium the typical cell has 5 to 6 curves; in yeast autolysate medium, 1½ curves per cell is usual, although longer forms occur. Bipolar tufts of flagella. Gram negative.

Microcysts formed.

Yeast autolysate nutrient agar: Small, white, translucent, umbonate colonies with an entire edge, 0.8 to 1.5 mm in diameter.

Yeast autolysate nutrient broth: Slight turbidity in young cultures; finely granular sediment and clear supernatant in old cultures.

Gelatin: Not liquefied.

Potato: Grayish-white growth.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates strongly reduced to nitrites. Grows anaerobically in the presence of nitrate.

Carbon compounds: Utilizes salts of pyruvic, succinic, fumaric, malic, lactic, citric, and malonic acids, ethyl alcohol and glycerol as sole carbon sources. Sugars and lower fatty acids not used except for scant growth on acetate.

Aerobic, except in the presence of potassium nitrate. Catalase positive.

Optimum temperature 32 C, no growth at 40 C.

Growth initiated between pH's 7.0 and 8.5 in synthetic media.

Source: Isolated from pond water.

GROUP VI

FRESH WATER SPIRILLA WHICH DO NOT GROW IN UNSUPPLEMENTED PEPTONE MEDIA

Morphologically, these organisms resemble the illustrations of the spirillum designated S. undula by Cohn (15), having the same half-moon type of cell and a single flagellum. They are, however, very unlike the S. undula of Giesberger (26). Since the description of the morphology of Cohn's organism is not distinctive enough for exact recognition and since its physiological characteristics are unrecorded, our group VI isolates are considered representative of a new species Spirillum anulus (Fig. 30).

Spirillum anulus sp. nov.

(Type culture No. 11879 American Type Culture Collection)

Large spirilla, containing many granules, having wide, deep, regular curves with S-shaped or half-moon cells predominating; cell diameter 1.5 to 1.8 μ ; spiral amplitude 7.5 to 9.6 μ ; spiral depth 4.5 to 6.0 μ ; cell length 7.0 to 30.0 μ ; single flagellum at each pole. Gram negative.

Yeast autolysate nutrient agar: Large, creamy-white, granular, umbonate colonies with a fimbriated edge, 3 to 4 mm in diameter.

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Yeast autolysate nutrient broth: Slight turbidity in young cultures; granular sediment and clear supernatant in old cultures.

Nutrient gelatin: No growth.

Potato: No growth.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates not reduced to nitrites.

Carbon compounds: Grows well only on malate in synthetic medium, grows poorly on salts of pyruvic, succinic, fumaric, and lactic acids. Sugars, ethyl alcohol, glycerol, malonic, citric and monocarboxylic acids not used.

Aerobic. Catalase positive.

Optimum temperature 32 C, no growth at 40 C.

Growth initiated between pH's 6.0 and 9.0 in synthetic media.

Source: Isolated from pond water.

GROUP VII

VERY COMMON FRESH WATER FORMS THAT GROW
READILY IN PEPTONE MEDIA

These spirilla are very similar, in most properties, to S. itersonii Giesberger, but differ in their nitrogen metabolism. They are considered to be a variety named S. itersonii var. vulgatum (Latin adjective vulgatus usual, common) because of their common occurrence (Fig. 31).

Spirillum itersonii var. vulgatum var. nov.

(Type culture No. 11331 American Type Culture Collection)

Small, slightly curved spiral cells, having 2 to 3 spiral curves: cell diameter 0.5 μ ; spiral amplitude 2.0 to 3.0 μ ; spiral depth 1.5 to 2.0 μ ; cell length 3.5 to 6.0 μ ; bipolar tufts of flagella. Gram negative.

Microcysts formed.

Nutrient agar: Small, white, translucent, circular, flat colonies, 1 mm in diameter.

Nutrient broth: Uniform turbidity in young cultures; finely granular sediment and clear supernatant in old cultures.

Gelatin: Not liquefied.

Potato: Brownish-black growth.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates strongly reduced to nitrites; grows anaerobically in the presence of nitrate. In this character it is differentiated from S. itersonii itersonii.

Carbon compounds: Grows well on the salts of acetic, pyruvic, succinic, fumaric, malic, and lactic acids. The salts of propionic, butyric, citric, malonic acids are less suitable for growth. Good growth on glucose, fructose, sucrose, lactose, maltose, arabinose, glycerol, ethyl alcohol, n-propyl, and n-butyl alcohols and mannitol. Produces acid only from glucose, fructose, ethyl alcohol and glycerol, both in synthetic and peptone base media.

Aerobic, except in the presence of nitrate. Catalase positive.

Optimum temperature 32 C; no growth at 40 C.

Growth can be initiated between pH's 5.5 and 9.0 in synthetic media.

Source: Isolated from pond water.

GROUP VIII

FRESH WATER SPIRILLA THAT ARE SIMILAR TO S. ANULUS METABOLICALLY BUT DIFFER MARKEDLY IN MORPHOLOGY

The typical cell has 2 to 3 wide, regular curves instead of the half-moon or S-shaped cells of S. anulus. In addition, the diameter is less, the spiral depth is much shallower, the granules are more prominent, and the flagella occur in tufts. The cells have a tendency to break up into slightly curved, almost straight, short cells and appear to be composed of two or more units. These isolates are considered a new species named Spirillum giesbergeri in recognition of the outstanding contributions of Dr. Giesberger to our knowledge of this genus (Fig. 5).

Spirillum giesbergeri sp. nov.

(Type culture No. 11334 American Type Culture Collection)

Large spiral cells, containing very prominent granules, having 2 to 5 wide, regular spiral curves; cell diameter 1.2 to 1.5 μ ; spiral amplitude 6.0 to 7.5 μ ; spiral depth 2.5 to

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3.0 μ ; cell length 11.0 to 25.0 μ ; bipolar tufts of flagella. The cells have a tendency to break up into short, slightly curved cells, giving the impression that the long cells are composed of two or more units. Gram negative.

Yeast autolysate nutrient agar: White, dry, granular, radiately ridged colonies with lobate edges, 2 to 3 mm in diameter.

Yeast autolysate nutrient broth: Light, uniform turbidity in young cultures; finely granular sediment and clear supernatant in old cultures.

Gelatin: Not liquefied.

Potato: Very slight, white growth.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates not reduced to nitrites.

Carbon compounds: Grows well only on the salts of pyruvic, malic, and lactic acids. Poorer growth on succinic, fumaric, and malonic acids. Monocarboxylic acids, sugars and alcohols not utilized.

Aerobic. Catalase positive.

Optimum temperature 32 C, no growth at 40 C.

Growth initiated between pH's 6.0 to 9.0 in synthetic media.

Source: Isolated from pond water.

GROUP IX

MARINE SPIRILLA WHICH PRODUCE A BROWNISH BLACK PIGMENT
BOTH IN PEPTONE AND SYNTHETIC MEDIA

This pigment occurs only on the aging of the cultures and the color of the colonies is yellowish to light orange in young cultures. The pigment appears to be water soluble as it diffuses into the medium. Pigment is more pronounced on solid media but it is also produced in broth. We consider these organisms to be a new species which is named Spirillum beijerinckii in honor of Professor Beijerinck and his contributions, not only to the knowledge of the spirilla, but to the entire field of bacteriology (Fig. 32).

Spirillum beijerinckii sp. nov.

(Type culture No. 12754 American Type Culture Collection)

Large, irregularly curved spiral cells, having $1\frac{1}{2}$ to 2 spiral curves; many half-curved cells are found and filaments of 5 to 7 curves are observed, which are obviously composed of a number of half-curved cells. The majority of the cells are slightly curved, flattened S-shaped cells with tapering ends. The diameter of the cells is definitely larger at the center than at the ends, being 0.9 to 1.0 μ at the center of the cell. Spiral amplitude 4.5 μ ; spiral depth 2.0 μ ; cell length 16.0 to 30.0 μ ; bipolar tufts of flagella but single flagella are also observed. Gram negative.

Microcysts spherical, 2.8 to 4.0 μ in diameter.

Sea water yeast autolysate agar: Small, yellowish-orange, flat colonies with an irregular edge. As the cells age, the surrounding medium becomes a brownish-black and the colonies are also often black. Cultures have a very pungent odor.

Sea water yeast autolysate nutrient broth: Heavy turbidity with pellicle formation in young cultures; the pellicle breaks up into large flakes which do not disperse, forming a coarsely granular sediment in old cultures, which are often black or brownish-black.

Sea water gelatin: Not liquefied.

Potato: Yellowish-orange growth, turning black on prolonged incubation, on potato slices soaked overnight in sea water.

Nitrogen compounds: Utilizes ammonium salts, nitrate, and asparagine as sole nitrogen sources in synthetic media; urea not used. Nitrates not reduced to nitrites.

Carbon compounds: Grows well on the salts of butyric, pyruvic, succinic, fumaric, malic, and lactic acids. The salts of acetic, citric, and malonic acids are used less readily. Glucose and glycerol are used without acid production; ethyl alcohol and fructose are not used.

Aerobic. Catalase positive.

Optimum temperature 32 C; no growth at 40 C.

Growth initiated between pH's 6.0 and 9.0 in synthetic media.

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

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GROUP X

MARINE SPIRILLA WHICH DO NOT GROW IN UNSUPPLEMENTED
PEPTONE MEDIA OR PRODUCE ANY PIGMENT

They are very delicate, thread-like organisms and differ from the organisms of group IX both in morphology and in the lack of pigment production. They are considered a new species and are named Spirillum atlanticum from the source (Fig. 33).

Spirillum atlanticum sp. nov.

(Type culture No. 12753 American Type Culture Collection)

Small, delicate, thread-like cells, deeply spiralled with 2½ to 5 curves. There is no evidence that the longer cells are composed of more than a single cell. The spiral waves are deep, regular, and the cells appear to have a very small, much more delicate filament at either end, which is turned at a 45° angle to the main body of the cell. The diameter of the cells is 0.4 to 0.6 μ; spiral amplitude 2.5 μ; spiral depth 2.0 μ. Single flagellum at both poles as well as tufts of flagella.

Microcysts formed: Spherical in shape and in the early forms, 12.0 μ in diameter; late forms 4.0 μ in diameter.

Sea water yeast autolysate nutrient agar: Small, creamy-white, pinpoint colonies, seldom found except along the line of the streak, where the heaviest inoculum was placed. Discrete colonies seldom observed. Pigment is not formed.

Sea water yeast autolysate nutrient broth: Very slight growth until after 48 hours. A pellicle is not formed but a heavy, creamy-white film forms on the surface of the broth after 48 hours incubation, which is easily dispersed up to 4 days after inoculation. Finely granular sediment and clear supernatant in old cultures.

Sea water gelatin: No growth.

Potato: No growth.

Nitrogen compounds: Utilizes ammonium salts, nitrate, and asparagine as sole nitrogen source in synthetic media. Urea not used. Nitrates not reduced to nitrites.

Carbon compounds: Grows well on the salts of pyruvic, succinic, fumaric, lactic, and citric acids; the salts of acetic, malic, and malonic acids are less suitable for growth. Slight growth on glucose, ethyl alcohol and glycerol.

Aerobic. Catalase positive.

Optimum temperature 32 C, no growth at 40 C.

Growth initiated between pH's 6.0 and 9.0 in synthetic media.

Source: Coastal sea water of Long Island Sound.

GROUP XI

CULTURE 9785, AMERICAN TYPE CULTURE COLLECTION

This was initially isolated by Lewis (39) and identified by him as S. serpens, apparently on the basis of morphology alone. This organism still resembles the original illustration published by Lewis (39) but differs from S. serpens, as described by Giesberger (26) in being much shorter in overall length and in spiral amplitude. Since the cell diameter and spiral depth are about the same as for S. serpens, the general appearance of the ATCC organism is that of a much more deeply curved organism. When Giesberger re-isolated an organism which he considered to be identical with his S. serpens (Fig. 34), comparison of both morphological and physiological characteristics definitely established that the ATCC organism 9785 could not be identical with Giesberger's nor with our isolates. The ATCC culture does not grow on the lower monocarboxylic acids as does S. serpens, and there are other differences in physiology and metabolism. For these reasons we consider this culture to be a new species Spirillum graniferum (Latin adjective granifer grain-bearing) from the prominent granules in the cells (Fig. 35).

Spirillum graniferum sp. nov.

(Type culture No. 9785 American Type Culture Collection)

Very granular, predominately S-shaped cells, although cells having 2 to 3 spiral curves are common; cell diameter 0.9 μ ; spiral amplitude 3.5 to 4.5 μ ; spiral depth 1.5 to 2.0 μ ; cell length 4.5 to 8.5 μ ; bipolar tufts of flagella. Gram negative.

Yeast autolysate nutrient agar: Small, creamy-white, convex colonies with an entire edge, 1.5 mm in diameter.

Yeast autolysate nutrient broth: Slight uniform turbidity with surface film in young cultures; granular sediment and clear supernatant in old cultures.

Gelatin: Not liquefied.

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Potato: Slight, creamy-white growth.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates not reduced to nitrites.

Carbon compounds: Grows well only on the salts of pyruvic, malic, and lactic acids; salts of succinic, fumaric, and malonic acids are utilized less readily. Ethyl alcohol, glycerol, hexoses, monocarboxylic acids and citrate not utilized.

Aerobic. Catalase positive.

Optimum temperature 32 C, no growth at 40 C.

Growth initiated between pH's 7.0 and 9.0 in synthetic medium.

Source: Isolated from fresh water by Lewis (39).

GROUP XII

CULTURE 9786, AMERICAN TYPE CULTURE COLLECTION

This was isolated by Lewis (39) and identified by him as S. undula. This organism grows as a tangled mass of very long, almost straight cells with some of the longer forms measuring over 500 microns in length. Many of the longer forms show lateral tufts of flagella which we believe originate from the ends of single cells which are joined in chains, suggesting some defect in the cell division mechanism. The organism has absolutely no resemblance, morphologically, to S. undula as described by Giesberger (26) nor, curiously, does it resemble the predominating form in Lewis' photograph. His picture shows, in addition to the many short, plump, slightly curved organisms having $\frac{1}{2}$ to $1\frac{1}{2}$ spirals per cell, a single, very lightly stained long spirillum, similar in appearance to the existing culture. The short $1\frac{1}{2}$ curved organisms are often found among the longer forms and can be observed in the upper left hand corner of Figure 25. These shorter forms are common in early cultures and are especially prominent in early cultures grown in lactate synthetic media. As the cultures age, however, the predominating form is the long, almost straight cells. Also prominent in young cultures and in old cultures are cells with 5 to 8 spiral waves, which are tightly coiled. These can be observed only in the living organism, as any type of fixation appears to cause a loosening of the spirals. Figure 36 shows a darkfield photograph of

such a cell, kindly provided by Professor A. Pijper of Pretoria, South Africa. We consider the organism to be a new species and have applied the name Spirillum sinuosum (Latin adjective sinuosus full of curves) from the beautiful winding curves found in some of the living cells (Fig. 36).

Spirillum sinuosum sp. nov.

(Type culture No. 9786 American Type Culture Collection)

Long spiral cells which contain many granules. The majority of these organisms grow as tangled masses of very long, almost straight, cells but shorter, more spiralled forms are always present. Also present in all cultures are tightly coiled, sinuous organisms with 5 to 8 spiral waves. Cell diameter 0.9 μ ; spiral amplitude 6.0 μ ; spiral depth 3.0 μ ; cell length (shorter forms) 8 to 18 μ ; bipolar tufts of flagella. Gram negative.

Yeast autolysate nutrient agar: Small, circular, convex, creamy-white colonies with an entire edge, 0.5 to 0.8 mm in diameter. Also present are small pinpoint colonies which consist entirely of the shorter forms. When the small colonies are transferred, both types are found on plating out. This is also true of the larger colonies.

Yeast autolysate nutrient broth: Slight uniform turbidity in young cultures; flaky sediment and clear supernatant in old cultures.

Gelatin: Not liquefied.

Potato: No growth.

Nitrogen compounds: Utilizes ammonium salts, nitrate, urea, and asparagine as sole nitrogen sources in synthetic media. Nitrates not reduced to nitrites.

Carbon compounds: Grows well only on the salts of pyruvic, malic, and lactic acids; the salts of succinic and fumaric acids are less readily utilized. Ethyl alcohol, glycerol, hexoses, monocarboxylic acids, citrate and malonate not used.

Aerobic. Catalase positive.

Optimum temperature 32 C, no growth at 40 C.

Growth can be initiated between pH's 7.0 and 9.0 in synthetic medium.

Source: Isolated from fresh water by Lewis (39).

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GROUP XIII

THE LARGE SPIRILLUM IN THE MIXED CULTURE
OBTAINED FROM PROFESSOR PRINGSHEIM

This closely resembles the excellent drawings of S. volutans originally made by Ehrenberg (23) and corresponds to the description of this organism as given by Cohn (15). Cohn commented on the flashing motility (which differs from that of other spirilla) and on the single, whip-like flagellum, both properties being characteristic of the Pringsheim culture.

Dimitroff (19) made a comparison of the descriptions of S. volutans in the literature and concluded that the majority of the authors were describing other organisms. Migula (44) emphasized that the isolate named S. volutans by Kutscher (36) was not identical with Cohn's mixed culture, and stated that S. volutans had not been obtained in pure culture. Giesberger (26) and Lewis (39) also comment on their failure to obtain S. volutans in pure culture. Since there has been a tendency to call any spirillum measuring more than 1.0 micron in diameter S. volutans, and since the description in Bergey's Manual (7) is incorrect in one important respect, we are including a morphological description of the Pringsheim strain of S. volutans (Figs. 9, 11, 12, 13).

This organism could not be isolated in pure culture by any method tried. It did not grow on any of the synthetic or complex media that support an abundant growth of the other species of Spirillum. The addition of single carbon compounds, like glucose or pyruvate, to Pringsheim's medium suppresses rather than enhances growth. When growing in Pringsheim's medium, S. volutans tends to concentrate just below the surface of the culture. In some tubes this effect is so marked that a very distinct gray layer composed mainly of S. volutans cells forms 3 to 5 mm below the liquid surface. A similar effect can be observed if a drop of culture about 5 mm in diameter is allowed to stand on a glass slide for 5 to 10 minutes. At the end of this time the cells, initially uniformly distributed, have concentrated into an almost perfect ring about two-thirds the distance out from the center (Fig. 37). These phenomena are very reminiscent of what Winogradsky (67) observed in his microcultures of the sulfur bacteria and suggested that S. volutans might be growing as a chemotrophic autotroph on hydrogen sulfide liberated by the sulfate reducing bacteria in the mixed

culture. Attempts to grow the organism in a mineral medium with sulfide as the energy source were unsuccessful, and nothing definite can be said about the metabolism of this organism.

Spirillum volutans Ehrenberg (Pringsheim strain)

Large spiral cells, containing many small granules. The majority of the cells are S-shaped, although cells having 3 or more spiral curves are not uncommon. In young cultures, the cells are predominately half-curves. The diameter of the cells is 1.5 to 2.0 μ ; spiral amplitude 11.0 to 15.0 μ ; spiral depth 6.0 to 9.0 μ ; cell length 17.0 to 33.0 μ ; single, large whip-like flagellum at each pole. Gram negative. Has not been grown in pure culture.

KEY TO THE SPECIES
OF THE GENUS SPIRILLUM EHRENBURG

The following key includes our own isolates, the species included by Giesberger, those in Bergey's Manual 6th Edition (excluding S. cardiopyrogenes and S. lipoferum for reasons already mentioned) and the new species described by Cayton and Preston (12).

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

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- 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 manconiense
- 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

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- 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

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PLATE 1

- Fig. 1. Typical granular colony of a species of Spirillum, 25 X.
- Fig. 2. Typical umbonate colony of a larger species of Spirillum, 25 X.
- Figs. 3, 4, 5. Heat-fixed cells, stained with methylene blue, of S. lunatum, S. serpens, and S. giesbergeri, respectively. (The micron scale shown on Fig. 3 holds for the remaining figures.)
- Figs. 6 and 7. Dark phase contrast photographs of living cells of S. lunatum and S. serpens.

PLATE 2

- Fig. 8. S. undula, showing elongated thread-like filament connecting the two daughter cells. Giemsa stain.
- Fig. 9. S. volutans, showing outline of cell wall and septum between the two daughter cells. Flagella stain (Gray's method). (The micron scale shown on this figure holds for Figs. 8-13.)
- Fig. 10. S. serpens, showing typical flagellar tufts. Flagellar stain (Gray's method).
- Fig. 11. S. volutans, showing single flagellum at both poles. Flagellar stain (Gray's method).
- Fig. 12. S. volutans, showing cell, plasmolyzed by distilled water, in which the cytoplasm has broken loose from the cell wall, which shows attached flagellum. Flagellar stain (Gray's method).
- Fig. 13. S. volutans, showing retraction of cytoplasm from cell wall and flagellum with cytoplasmic connection to flagellum, which is seen as a tuft. Flagellar stain (Gray's method).
- Fig. 14. S. sinuosum, heat-fixed cell, stained with Sudan black B, showing the granules outlined by the fat dye. Taken at high focus. (The micron scale shown on this figure applies to the remaining figures of Plate 2.)
- Fig. 15. S. serpens, Carnoy fixed cell, stained with methylene blue (1:10), showing volutin granules.
- Fig. 16. S. serpens, Carnoy fixed cells, stained with methylene blue, destained with 1 per cent H_2SO_4 , and lightly counterstained with aqueous safranin, showing the retention of staining by the granules. Classical volutin stain.

PLATE 3

All cells fixed in Carnoy, as outlined in text, unless specified otherwise.

- Fig. 17. S. serpens, hot water treated cells (80 C) stained with dilute methylene blue, showing the retention of the stain by the granules despite the hot water treatment. (The micron scale shown on this figure holds for the succeeding figures.)
- Fig. 18. S. serpens, cells placed in 5 per cent HCl, washed and stained with dilute methylene blue, showing retention of stain by the granules despite the acid treatment.
- Fig. 19. S. serpens, unhydrolyzed cells, stained with Giemsa.
- Fig. 20. S. serpens, hydrolyzed cells, stained with Giemsa, showing that the chromatin material is retained within the granules.
- Fig. 21. S. serpens, ribonuclease digested cells, stained with Giemsa, showing staining of granules after being digested.
- Fig. 22. S. lunatum, 4 week-old culture, showing the pre-dominating form of the cells, "microcysts". Phase contrast photograph.
- Fig. 23. S. undula, showing "ring" formation of bodies similar to the microcysts of the marine organisms. Giemsa stain.
- Fig. 24. S. serpens, showing branched cell from 5-hour culture. Giemsa stain.
- Fig. 25. S. sinuosum, showing entwined cells. Giemsa stain.

PLATE 4

No micron scale is shown, as the figures were printed at different magnifications so that entire cells could be shown.

- Fig. 26. S. giesbergeri, showing "giant" cell. Hydrolyzed and Giemsa stained. 1000 X.
- Fig. 27. Spirillum linum, heat-fixed cells from 48-hour broth culture, stained with methylene blue. 1000 X.
- Fig. 28. S. curvatum, heat-fixed cells from 48-hour broth culture, stained with methylene blue. 1000 X.
- Fig. 29. S. polymorphum, heat-fixed cells, stained with methylene blue. 48-hour broth culture. 1250 X.
- Fig. 30. S. undula, dark phase contrast photograph of living cells from 18-hour culture, showing division of cells. 1000 X.
- Fig. 31. S. itersonii, flagella stain (Gray's method) taken from 48-hour agar slant. 1000 X.
- Fig. 32. S. beijerinckii, flagellar stain (Leifson's method) showing single flagellum at upper pole and two flagella at lower pole. This organism also shows tufts of flagella in the same preparation with Leifson's method. 1250 X.
- Fig. 33. S. atlanticum, heat-fixed cells from 60-hour culture, stained with methylene blue, showing fine filament at ends of cells. 1000 X.
- Fig. 34. S. serpens (Giesberger's strain), flagellar stain (Leifson's method) showing tufts of flagella. 750 X.
- Fig. 35. S. graniferum, heat-fixed cells from 48-hour broth culture, stained with methylene blue, showing large granules. 1000 X.
- Fig. 36. S. sinuosum, darkfield photograph of living cell, kindly supplied by Professor Pijper. 600 X.
- Fig. 37. S. volutans, showing concentric ring of organisms after having remained on slide, at room temperature, for 5 minutes. Methylene blue stain of heat-fixed cells. 75 X.