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Biology of the Chemoheterotrophic Spirilla

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INTRODUCTION 56
DIVISION OF THE *SPIRILLUM* 57
THE REDEFINED GENUS *SPIRILLUM* 58
GENUS *AQUASPIRILLUM* 58
Typical Characteristics 59
Exceptions to Typical Characteristics 59
Other Characteristics 59
OTHER ORGANISMS POSSIBLY RELATED TO *AQUASPIRILLUM* 63
Pathogenic Spirilla 63
Nitrogen-Fixing Spirilla 66
Spirilla in Insects 67
Bdellovibrio-Like Spirilla 68
Phototrophic Spirilla 69
Oxidative Vibrios 69
GENUS *OCEANOSPIRILLUM* 69
Typical Characteristics 70
Other Characteristics 71
OTHER SPIRILLUM-LIKE ORGANISMS 71
Sporeforming Spirilla 71
Gastric Spirilla 72
ECOLOGY OF SPIRILLA 72
Habitat and Numbers in Nature 72
Ecological Studies of Spirilla by Steady-State Cultivation 74
ISOLATION OF SPIRILLA 75
CULTIVATION AND NUTRITION OF SPIRILLA 76
Complex Media 76
Defined Media 77
Defined Medium for *A. gracile* 78
RESPIRATION OF SPIRILLA 78
Cytochromes of Spirilla 79
Nitrate Reduction 80
Catalase Activity 80
Microaerophilism of *Spirillum volutans* 81
SUGAR CATABOLISM AND TRANSPORT BY SPIRILLA 83
MOTILITY AND FLAGELLA OF SPIRILLA 84
Number, Configuration, and Behavior of Flagella 84
Coordination and Uncoordination in *S. volutans* 86
Practical Applications of Flagellar Uncoordination 88
Source of Energy for Motility of Spirilla 89
Structure and Chemistry of Flagella and Related Organelles 89
TACTIC RESPONSES OF SPIRILLA 91
Aerotaxis 91
Geotaxis 92
Thermotaxis 92
Chemotaxis 93
CELL WALL PERIPLASMIC SPACE OF SPIRILLA 94
Profile of the Cell Wall in Thin Sections 94
Mucopeptide and the Shape of Spirilla 95
Outermost Layers of Cell Walls 97
Growth of Cell Walls 101
Lipopolysaccharide Layers 102
Periplasmic Space 102
DNA OF SPIRILLA 102
DNA Base Composition 102
BACTERIOPHAGES FOR SPIRILLA 104
COCCOID BODIES AND LIFE CYCLES 104
Budlike Bodies 109
LITERATURE CITED 109

INTRODUCTION

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.

M. A. Williams and S. C. Rittenberg (198)

Members of the genus *Spirillum* were probably first described in the 1670s by van Leeuwenhoek (54) and a century later by Müller (132, 133). The genus *Spirillum* was created in 1832 by Ehrenberg (58), and from his descriptions of members of the genus in 1838 (59) it is clear that rigid helical cells were being described rather than flexible cells such as spirochetes. Phototrophic spirilla were distinguished by their placement in a different genus, *Ophidomonas (Thiospirillum)*. The subsequent development of the taxonomy of the chemoheterotrophic spirilla, reflecting an increasing knowledge of their characteristics, was thoroughly reviewed by Williams (193, 194) and by Giesberger (Ph.D. thesis, Delft Univ., Delft, The Netherlands, 1936).

Giesberger was the first investigator to provide a comprehensive study of a large number of isolates. He noted the great difficulty experienced by previous investigators in isolating strains of spirilla for study, as well as a lack of available strains from collections. Consequently, using new enrichment and isolation methods, he isolated 64 strains from surface waters or from liquid manure. It is unfortunate that these strains were lost during World War II (198). Giesberger's report provided a firm basis for the study and classification of a genus that previously had been poorly described and difficult to isolate, but which nevertheless seemed to be distributed widely in nature. He found *Spirillum* species to be gram negative and catalase positive and to possess bipolar tufts of flagella. The organisms were aerobic chemoheterotrophs, which oxidized rather than fermented various organic substances (perferentially salts of organic acids), were able to grow in simple defined media, and contained "volutin" granules. It is interesting that the name "volutin" is based on a term used in 1902 to describe the granules of *S. volutans* (117); however, Martinez (117) in 1963 was the first to show that, despite their ability to be stained with metachromatic stains such as toluidine blue, the granules of *S. serpens, S. itersonii,* and *S. anulus* were, in fact, composed of poly-β-hydroxybutyrate (PHB) rather than polyphosphate (which "volutin" has since come to mean). Indeed, no trichloroacetic acid-extractable, barium-precipitable polyphosphates could be isolated from these spirilla.

An interesting observation arising from Giesberger's use of defined media for nutritional studies was the apparent preference of spirilla for the calcium salts of organic acids rather than the sodium salts. Although he considered that calcium was required for proper ionic balance of the media, more recent studies (83) indicate that freshwater spirilla have a low tolerance to sodium ions rather than a requirement for calcium ions. However, it should also be noted that certain strains of *S. serpens* do require calcium ions to prevent massive lysis of cultures when the stationary phase of growth is reached (20).

An even more comprehensive study of the genus *Spirillum* was made by Williams and Rittenberg in 1957 (198). In general, their concept of *Spirillum* was in accord with Giesberger's, but a considerable amount of new information appeared; e.g., several species of *Spirillum* seemed to possess only a single flagellum at each pole when flagella stains were examined. However, subsequent electron microscopy revealed that tufts of flagella were, in fact, present in these species. The individual flagella had a tendency to aggregate into a single fascicle that appeared as a "single flagellum" by ordinary staining (195, 196). With regard to catalase, Williams and Rittenberg found all of their strains to be catalase positive, but they noted that in 1955 Cayton and Preston (30) had found a negative reaction for an organism called *Spirillum mancuniense*. Since this time, several other species of *Spirillum* have been found to give only a negative or a very weak catalase reaction, not demonstrable by ordinary methods (82, 83, 180). Williams and Rittenberg also isolated and studied marine spirilla for the first time and found that they required a seawater-based medium. (Although in 1926 Dimitroff [52] isolated *S. virginianum* from mud adhering to an oyster shell, this may not have been a marine spirillum, as it apparently did not require seawater.) Another point of interest was that all of the marine spirilla, and a few of the freshwater spirilla, formed large numbers of round forms (termed "microcysts" by Williams and Rittenberg) in old cultures. Such forms had been previously described by Dimitroff (52) for *S. virginianum* and by Cayton and Preston (30) for *S. mancuniense*. Williams and Rittenberg believed that such forms represented a resting stage and that they were part of a life cycle occurring in the spirilla; the validity of this interpretation is not yet clear, however (see Coccoid Bodies and Life

Cycles, below). As noted by Hylemon et al. (83), it is preferable at present to use the term "coccoid body" rather than "microcyst" to avoid implying a relationship to the microcysts formed by myxobacteria.

One of the major contributions by Williams and Rittenberg that greatly helped to stimulate studies of spirilla was their deposition of representative strains in national collections. Even when maintained in collections, however, spirilla were prone to die out, and they were notoriously difficult to lyophilize (198). Therefore, the development of suitable methods for preservation of spirilla, such as drying culture-impregnated filter paper strips in a vacuum (L-drying) (104) and the use of liquid nitrogen (83), has been important. One of the most difficult of all spirilla to preserve, *S. volutans*, can now be easily preserved by the use of dimethyl sulfoxide and liquid nitrogen (142).

DIVISION OF THE GENUS *SPIRILLUM*

In an analysis of the deoxyribonucleic acid (DNA) base compositions of a large number of *Spirillum* species, Hylemon et al. (83) concluded that the range of moles percent guanine plus cytosine (G+C) values was too great to justify inclusion of all percent strains in a single genus. They noted that all of the freshwater spirilla, except *S. volutans*, were aerobic, could not tolerate 3% NaCl, and possessed a G+C range of 50 to 65%. *S. volutans* was distinguished from these forms by its great size, its microaerophilic nature, and a G+C content of 38%. The marine spirilla were aerobic, required seawater for growth, and possessed a G+C range of 42 to 48%. Consequently, Hylemon et al. proposed that the genus *Spirillum* be divided into three genera representing the above-mentioned groups (*Aquaspirillum, Spirillum,* and *Oceanospirillum,* respectively). The name *Spirillum* was reserved for that genus containing the type species *S. volutans* (82, 83, 96).

The general characteristics of the three genera are presented in Table 1, and a list of the species and type strains presently included in each genus is indicated in Table 2. Tables 3 and 4 list the characteristics of the species of *Aquaspirillum* and *Oceanospirillum,* respectively.

TABLE 1. *Characteristics of the genera Spirillum, Aquaspirillum, and Oceanospirillum*[a]

Characteristic	*Spirillum*	*Aquaspirillum*	*Oceanospirillum*
Helical shape	+	Usually +, one exception	+
Gram negative	+	+	+
Chemoorganotrophic	+	+	+
Strictly respiratory type of metabolism	+	+	+
Bipolar tufts of flagella	+	Usually +[b]	Usually +, one exception
G+C content (mol %) of DNA	38	50 to 65	42 to 48
Relation to oxygen	Obligately microaerophilic	Aerobic	Aerobic
Require seawater for growth	−	−	+
Tolerant to 3% NaCl	−	−	+
Oxidase	+	+	+
Catalase	W	Usually +; one species W or −	Differs among species
Indole	−	−	−
Hydrolysis of casein, starch, hippurate	−	−	−
Carbohydrates oxidized	−	Usually −[b]	−
Carbohydrates fermented	−	−	−
Cell diameter (μm)	1.7	0.2 to 1.5	0.3 to 1.2
Anaerobic growth with nitrate	−	Usually −[b]	−
Coccoid bodies predominant in cold cultures	−	Usually −[b]	Usually +, one exception
Intracellular PHB present	+	Usually +, one exception	+
Habitat	Freshwater	Freshwater	Marine

[a] Symbols: +, all species tested are positive; −, all species tested are negative; W, very weak reaction, not detectable by ordinary tests.

[b] See Table 3.

TABLE 2. *Species presently included in the genera Spirillum, Aquaspirillum, and Oceanospirillum*

Genus	Species[a]	Type strain[b]	References for characteristics
Spirillum	*S. volutans*[c]	ATCC 19554	58, 59, 82
Aquaspirillum	*A. serpens*[c]	ATCC 12638	29, 83, 180, 198, and Giesberger[d]
	A. gracile	ATCC 19624	25, 29, 83
	A. delicatum	ATCC 14667	29, 83, 110, 180
	A. polymorphum	NCIB 9072	29, 83, 180, 198
	A. itersonii	ATCC 12639	29, 83, 180, 198, and Giesberger[d]
	A. peregrinum	ATCC 15387	29, 83, 146, 180, 181
	A. dispar	ATCC 27510	83
	A. aquaticum	ATCC 11330	83, 180
	A. sinuosum	ATCC 9786	29, 83, 180, 198
	A. psychrophilum	IFOJ 13611	181
	A. putridiconchylium	ATCC 15279	29, 83, 175, 180
	A. bengal	ATCC 27641	102
	A. metamorphum	ATCC 15280	29, 83, 176, 180
	A. giesbergeri	ATCC 11334	29, 83, 180, 198
	A. anulus	NCIB 9072	29, 83, 180, 198
	A. fasciculus	ATCC 27740	173
Oceanospirillum	*O. linum*[c]	ATCC 11336	29, 83, 180, 198
	O. minutulum	ATCC 19193	29, 83, 180, 186
	O. pusillum	IFOJ 13613	181
	O. multiglobuliferum	IFOJ 13614	181
	O. hiroshimense	IFOJ 13616	181
	O. pelagicum	IFOJ 13612	181
	O. beijerinckii	ATCC 12754	29, 83, 180, 198
	O. maris	ATCC 27509	83
	O. japonicum	ATCC 19191	29, 83, 180, 186

[a] The following species were originally assigned to genus *Spirillum*, but their taxonomic position is uncertain because of lack of sufficient data and/or apparent lack of available type or reference strains: *S. undula* (58, 132); *S. minus* (28); *S. virginianum* (52); *S. tenue* (59); *S. kutscheri* (130); *S. curvatum* (198); *S. mancuniense* (30); *S. maritimum* (186); and *S. lipoferum* (9). *S. lunatum* (198) was not included because the characteristics of the type strain (ATCC 11337) do not match the original description (29, 83, 180).

[b] ATCC, American Type Culture Collection, Rockville, Md.; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; IFOJ, Institute for Fermentation, Osaka, Japan.

[c] Type species of the genus.

[d] G. Giesberger, Ph.D. thesis, Delft Univ., Delft, The Netherlands, 1936.

THE REDEFINED GENUS *SPIRILLUM*

The redefined genus *Spirillum* contains only a single species, *S. volutans* (Table 2). This species has been described in detail by Hylemon et al. (82). It contains the largest of the chemotrophic spirilla (members of the phototrophic genus *Thiospirillum* are larger), being 1.4 to 1.7 μm in diameter and up to 60 μm in length, with from less than one to a maximun of five helical turns (see Fig. 1). Prominent granules of PHB occur intracellularly. One of the most characteristic features of the organisms is their possession of prominent crescent-shaped bipolar fascicles of flagella, which rotate at high speed, forming oriented cones of revolution that reverse their configuration during reversal of swimming direction (101, 129, see also Fig. 5 and 6). The organisms possess a strictly respiratory type of metabolism but are microaerophilic, requiring an atmosphere of 1 to 9% oxygen for growth (191). Positive reactions are obtained for the oxidase and phosphatase tests, but catalase activity is very weak (41, 82). Nitrates are not reduced, and growth does not occur anaerobically with nitrate. Carbohydrates are neither oxidized nor fermented. The indole test is negative, and there is no hydrolysis of casein, gelatin, starch, or esculin. An unusual feature of the species is inhibition of growth in peptone-succinate-salts (PSS) broth by low concentrations of phosphate (82). No growth occurs in the presence of 3% NaCl.

GENUS *AQUASPIRILLUM*

The aerobic freshwater chemoheterotrophic spirilla having a G+C content of from 50 to 65% were placed in the genus *Aquaspirillum* by Hylemon et al. (83). The species in this genus (Table 1) have been thoroughly characterized in several taxonomic studies (29, 83, 102, 126, 173,

180, 181). A list of the characteristics of the various species is presented in Table 3, and photomicrographs of many of the species are presented in Fig. 1. All species possess polar flagella, have a strictly respiratory type of metabolism, are oxidase positive, and fail to grow in the presence of 3% NaCl. Cell diameters range from 0.2 to 1.5 μm.

Typical Characteristics

A number of characteristics apply to most, but not all, of the species of *Aquaspirillum*. These characteristics are as follows: (i) helical shape, (ii) bipolar tufts of flagella, (iii) intracellular PHB granules, (iv) no coccoid bodies formed in older cultures, (v) catalase positive, (vi) carbohydrates neither oxidized nor fermented, (vii) no growth anaerobically in the presence of nitrate, (viii) phosphatase positive, (ix) no growth in the presence of 1% glycine, (x) optimal growth temperature is ca. 30 C, and (xi) occurrence of growth in simple defined media, with tricarboxylic acid cycle intermediates being used preferentially as sole carbon sources. Ammonium salts can usually serve as nitrogen sources.

Exceptions to Typical Characteristics

(i) *A. fasciculus* is a straight rod which, on the basis of many other characteristics, appears to warrant inclusion in the genus (173). These characteristics include: large bipolar fascicles of flagella; a strictly respiratory metabolism; inability to attack carbohydrates; intracellular PHB granules; positive reactions for catalase, oxidase, and phosphatase; lack of tolerance to 3% NaCl or glycine; and a G+C content of 62 to 65%. Furthermore, the occurrence of a "polar membrane" was demonstrated in thin sections; this structure has been found mainly in *Aquaspirillum* and other helical bacteria (75, 76, 97, 135, 149, 150). However, it has also been observed in the rod-shaped organism *Chromatium* (135), and a structure similar in certain respects has also been demonstrated in *Selenomonas* (31). With regard to the rod shape of *A. fasciculus*, there is a tendency of certain species of *Aquaspirillum* to become nearly straight rods on prolonged serial transfer or by selection of mutants (83, 180, 198). Moreover, Strength et al. (173) observed that one of their isolates, initially identical morphologically to *A. fasciculus*, eventually exhibited many cells that were curved or even S-shaped. In view of the many similarities of *A. fasciculus* to the genus *Aquaspirillum*, it does not appear reasonable to exclude the organisms from the genus merely because of their shape, a single and probably mutable characteristic. (ii) *A. delicatum* possesses unipolar rather than bipolar flagellation. Generally, one or two flagella occur at one pole, but tufts with up to six flagella have been seen (83). *A. polymorphum* has predominantly bipolar, single flagella, but tufts of up to three flagella occur occasionally (83). With *A. psychrophilum*, one or more flagella occur usually at only one pole, but bipolar, single flagella or tufts occur occasionally (181). (iii) *A. gracile* does not possess intracellular PHB granules (83). *A. psychrophilum* has not yet been tested for PHB but seems to lack visible granules in stained preparations (181). (iv) Four species form coccoid bodies in old cultures: *A. itersonii*, *A. peregrinum*, *A. fasciculus*, and *A. polymorphum*. (v) *A. putridiconchylium* exhibits a very weak (83) or negative (180) catalase reaction. (vi) In contrast to the majority of species in the genus, three species are capable of oxidizing carbohydrates: *A. itersonii*, *A. peregrinum*, and *A. gracile* (83, 180). The variety of carbohydrates attacked is extremely limited. Although acidification of carbohydrate-containing media sealed with oil or wax may occur under certain conditions, no visible growth response occurs (83). (vii) Three species can grow anaerobically in the presence of nitrate (nitrate respiration): *A. itersonii*, *A. fasciculus*, and *A. psychrophilum* (83, 173, 180, 181). (viii) *A. polymorphum* and *A. psychrophilim* do not exhibit phosphatase activity (83, 181). (ix) Only *A. dispar* and *A. aquaticum* appear to be able to tolerate 1% glycine (83), although *A. psychrophilum* has not yet been tested. (x) The optimal growth temperature of *A. bengal* is 41 C (102). *A. psychrophilum* is unable to grow at temperatures as high at 30 C; it grows best at ca. 20 C and can grow at temperatures as low as 2 C (181). (xi) *A. gracile*, *A. aquaticum*, and an unclassified strain (ATCC 12289) appear to have more complex nutritional requirements than do other aquaspirilla (83).

Other Characteristics

Several species produce a yellow-green water-soluble fluorescent pigment when cultured on PSS agar and examined under ultraviolet light (254 nm) (83). This suggests a possible relationship to the genus *Pseudomonas* (44), but whether pigment is the same as that formed by fluorescent pseudomonads is not known. *A. serpens* is one species in which most strains form a fluorescent pigment, but the DNA base composition of this species (50 to 51% G+C) is well below that for pseudomonads (58 to 70% G+C) (56). Several *Aquaspirillum* species exhibit urease activity (83). A number of

TABLE 3. *Characteristics of species of the genus Aquaspirillum*[a]

Test	*A. gracile*	*A. delicatum*	*A. polymorphum*	*A. itersonii*	*A. peregrinum*	*A. dispar*	*A. aqaticum*	*A. sinuosum*	*A. psychrophilum*	*A. fasciculus*	*A. serpens*	*A. putridiconchylium*	*A. bengal*	*A. metamorphum*	*A. giesbergeri*	*A. anulus*
Cell diameter (μm)	0.2–0.3	0.3–0.4	0.3–0.5	0.4–0.6	0.5–0.7	0.5–0.7	0.5–0.6	0.6–0.9	0.7–0.9	0.7–0.9	0.6–1.1	0.9–1.1	0.9–1.2	1.1–1.3	1.0–1.4	1.1–1.5
G+C content (mol%) of DNA[b]	64–65	63	61–62	57–64	60–62	63–65	64–65	57–59		62–65	49–51	52	51	63	57–58	58–59
Helical shape	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
Type of flagellation (usual)[c]	BT	U	BS	BT	BT	BT	BT	BT	U	BT	BT	BT	BT	BT	BT	BT
Acidic reaction from sugars[d]	+	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+	+	W	+	+	+	+
Phosphatase	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+
Deoxyribonuclease	+	−	−	+	−	−	+	+			d	−	v	−	+	−
Ribonuclease	+	+	−	+	−	−	+	−			d	+	−	+	+	d
Nitrate reduced to nitrite	+	+	+	−	−	−	+	−	−	+	d	−	−	−	−	−
Nitrate reduced beyond nitrite state	−	−	−	+	−	+	−	−	+	−	−	−	−	−	−	−
Anaerobic growth with nitrate	−	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−
Esculin hydrolysis	−	−	+	+	+	−	−	−		−	−	−	−	−	−	−
Growth in presence of:																
1% Bile (oxgall)	+	−	+	+	+	+	+	+		+	+	+	+	+	+	−
1% Glycine	−	−	−	−	−	+	+	−		−	−	−	−	−	−	−
Growth on:																
Eosin methylene blue agar	+	−	+	+	+	+	+	−			+	+	−	+	−	−
MacConkey agar	−	−	+	+	−	+	+	−			d	−	−	−	−	−
Triple-Sugar iron agar	−	+	−	+	+	+	+	−			d	+	+	+	−	−
Seller agar	−	−	−	+	+	+	+	−			d	−	+	+	−	−
Methyl red-Voges-Proskauer broth	−	+	−	+	−	+	+	−			d	−		−	+	−
Water-soluble brown or yellow pigment from:																
Tyrosine	−	−	−	+	−	−	−	−			−	−	+	−	−	−
Phenylalanine	−	−	−	−	+	−	−	−			−	−	−	−	−	−
Tryptophan	−	−	−	+	−	−	−	−			−	−	+	−	−	−
Growth at <2 C; no growth at 30 C	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−

Optimal growth at 41 C	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
Coccoid bodies predominant in old cultures	−	−	+	+	+	−	−	−	−	+	−	−	−	−	−	−
Versatile with respect to tricarboxylic acid cycle intermediates and related compounds as sole carbon sources[e]	−	+	−	+	+	+	−	−		+	−	+	+	+	−	−
Versatile with respect to sole nitrogen sources[e]	−	+	+	+	+	+	−	−		+	+	+	i	i	−	−
Nitrogenase activity present					+					+						
Water-soluble fluorescent pigment	d	−	+	+	+	+	+	−		+	d	−	−	+	−	−
Intracellular PHB	−	+	+	+	+	+	+	+		+	+	+	+	+	+	+
Litmus milk, alkaline reaction	−	−	−	−	+	−	+	−			−	−	−	−	−	−
Type of spiral (clockwise [C] or counterclockwise [CC])[f]			CC	CC	CC		C	C	C	−	C	C		C	C	C
Urease	+	−	−	−	+	−	−	+		+	d	−	−	−	+	−

[a] Symbols: +, all strains positive; −, all strains negative; d, difference among strains; W, very weak; demonstrable only by special test; i, intermediate range; blank space, not determined; BT, bipolar tufts; BS, bipolar single; U, unipolar; v, variable in same strain.

[b] DNA base compositions are based on the following references: 29, 39, 43, 82, 83, 148, 158. See also footnote *b*, Table 6.

[c] *A. delicatum* usually possesses one or two flagella at one pole; *A. psychrophilum* usually possesses one to several flagella at one pole.

[d] *A. itersonii* produces an acidic reaction from D-fructose and glycerol; *A. gracile* produces an acidic reaction from D-glucose, L-arabinose, and D-galactose.

[e] As determined by the methods of Hylemon et al. (83).

[f] As defined by Terasaki (180), the type of spiral is determined by focusing on the bottom of the cell. The pattern / / / / indicates a clockwise spiral; the pattern \ \ \ \ indicates a counterclockwise spiral.

TABLE 4. *Characteristics of species of the genus Oceanospirillum*[a]

Test	*O. minutulum*	*O. pusillum*	*O. linum*	*O. multiglobuliferum*	*O. hiroshimense*	*O. pelagicum*	*O. beijerinckii*	*O. maris*	*O. japonicum*
Cell diameter (μm)	0.3–0.4	0.3–0.5	0.4–0.6	0.6–0.8	0.7–1.0	0.7–1.1	0.7–1.0	0.7–1.0	1.0–1.2
G+C content (mol%) of DNA[b]	42–44		48–50				46–47	45–46	45–46
Type of flagellation (usual)	BT	BS	BT	BT	BT	BT	BT	BT	BT
Catalase	+	−	+/W	+	−	+	W	+	−/W
Phosphatase	−	−	+	+	+	+	+	−	+
Deoxyribonuclease	−		−				+	−	−
Ribonuclease	−		d				+	−	−
Nitrate reduced to nitrite	+	+	−	−	−	−	−	−	−
Nitrate reduced beyond nitrite stage	−	−	−	−	−	−	−	−	−
Anaerobic growth with nitrate	−	−	−	−	−	−	−	−	−
Acidic reaction from sugars	−	−	−	−	−	−	−	−	−
Esculin hydrolysis	−	−	−	−	−	−	−	−	−
Growth in presence of:									
1% Bile (oxgall)	+		+				+	+	+
1% Glycine	+		+				−	+	−
Growth on:									
Eosin-methylene blue agar	−		−				−	d	−
MacConkey agar	−		−				−	−	−
Triple-sugar iron agar	+		+				−	+	−
Seller agar	−		−				−	−	−
Methyl red–Vogues-Proskauer broth	−		+				−	+	−
Water-soluble brown pigment from:									
Tyrosine	−		+				+	d	−
Phenylalanine	−		−				−	−	−
Tryptophan	d		d				−	d	−
Growth at 3-4 C; no growth at 35 C	−	−	−	−	+	−	−	−	−
Coccoid bodies predominant in old cultures	+	+	+	+	+	+	+	+	−
Versatile with respect to tricarboxylic acid cycle intermediates and related compounds as sole carbon sources[c]	+		−				−	−	i
Versatile with respect to sole nitrogen sources	+		−				−	+	+
Water-soluble fluorescent pigment	−		+				−	d	−
Intracellular PHB	+		+				+	+	+
Growth in PSS-seawater broth containing 9.75% total NaCl	+		+				+	+	+
Type of spiral (clockwise [C] or counterclockwise [CC])[d]	C	CC	C	C	C	C	C		C
Urease	−		−				−	−	−

[a] Symbols: +, all strains positive; −, all strains negative; d, difference among strains; W, very weak, demonstrable only by special test; i, intermediate range; blank space, not determined; BT, bipolar tufts; BS, bipolar single.
[b] DNA base compositions are based on references 29 and 83.
[c] As determined by the methods of Hylemon et al. (83).
[d] See footnote *f*, Table 3.

species can reduce nitrate to nitrite, but only three *(A. itersonii, A. dispar,* and *A. psychrophilum)* can reduce nitrate beyond the nitrite stage (83, 180, 181), presumably to ammonia (68). Only *A. psychrophilum* forms gas (181); the gas has not yet been identified. Most species can grow in the presence of 1% oxgall bile (83). Three species form yellow or brown water-soluble pigments when cultured with certain aromatic amino acids: *A. itersonii, A. bengal,* and *A. peregrinum* (83). *A. psychrophilum* has not yet been tested. A number of species of *Aquaspirillum* can liquefy gelatin (180), but only *A. metamorphum* and certain strains of *A. serpens* appear to be able to give a positive reaction within 4 days (83). Terasaki (180) noted that aquaspirilla may exhibit smooth (S) or rough (R) colonies. In general, colonies of aquaspirilla tend to be small, ranging from pinpoint in size to 1 or 2 mm in diameter.

Terasaki (180) also observed that the type of cell helix is characteristic for a given species: When the bottom of a helical cell is brought into focus under a microscope, the pattern / / / / is indicative of a clockwise helix, whereas \ \ \ \ is indicative of a counterclockwise helix.

The nitrogenase activity exhibited by *A. peregrinum* and *A. fasciculus* is discussed below (see Nitrogen-Fixing Spirilla).

OTHER ORGANISMS POSSIBLY RELATED TO *AQUASPIRILLUM*

Pathogenic Spirilla

A spirillum-like organism causing one of the two forms of rat-bite fever in humans was described by Carter in 1888 (28). (Although Carter named the organism *Spirillum minor,* Robertson [152] has pointed out that the specific epithet was not grammatically correct and must be changed to *minus.*) Although a human pathogen, its primary reservoir of infection appears to be wild rats. The disease in humans has been described in detail by Beeson (6) and by Watkins (188). The spirillum has a rigid shape (77, 125, 152) and has been said to have planar waves (125). The cell diameter is ca. 0.2 μm and the length is ca. 3 to 5 μm (152). At both poles are flagella, usually single but sometimes multiple (152). The cells are highly motile and move forward and backward, spinning about the long axis as do spirilla or vibrios (28, 152). Except for these morphological characteristics, little else of a fundamental nature is known about *S. minus* because of the great difficulties encountered in growing the organism in vitro.

Although *S. minus* has been claimed to have been cultivated in vitro on several occasions, such reports have not been subsequently confirmed. In the hope that the literature on this point may hold important clues for satisfactory, reproducible cultivation, the following summary is offered. Futaki et al. (65) claimed successful primary growth from blood in Shimamine medium, but subsequent transfers were not successful. A possibly significant observation was that in the primary cultures the organisms "diffused in the medium to within 1 cm of the surface." This suggests that possibly the organism is an anaerobe or a microaerophile. Robertson (152) attempted to culture *S. minus* in Shimamine medium without success; he suggested that since Futaki's cultures had exhibited many forms of much greater cell length than was characteristic of *S. minus* in vivo, perhaps a spirochete had been cultured rather than the spirillum. Futaki et al., however, had indicated the occurrence of short as well as long forms in cultures. In 1925 Joekes (95) claimed to have cultured short forms of *S. minus* from laboratory guinea pigs (infected previously using the blood from infected wild rats) aerobically for many transfers, using inspissated serum slants covered with Vervoort medium. After initial isolation, the use of 1% glucose broth was found to be as satisfactory as the original medium. Joekes noted that, despite the aseptic conditions used for cultivation, "a motile coliform bacillus" was invariably present along with the spirilla. Furthermore, mice could not be infected with the cultures. Also, cultures could be obtained only from guinea pigs, not from mice previously infected with rat blood. McDermott (125) was unable to repeat isolation of *S. minus* by using the methods of Joekes. Inocula of citrated guinea pig blood containing many spirilla per field were made into a variety of media, including those suitable for growth of spirochetes, trypanosomes, vibrios, and entamoebae, in addition to more common media, all without success. In citrated blood or in broth, the organism survived for 24 to 48 h at room temperature or at 37 C. Hitzig and Liebesman (77) claimed to have cultured *S. minus* six times from a case of subacute endocarditis. The procedure involved the use of 2% veal broth or 10% tomato extract-veal infusion broth, inoculated with blood from the patient. When first isolated, the spirillum grew only in an atmosphere of 3.5% carbon dioxide, but after 5 months of serial transfer it grew aerobically. The addition of citrated human or rabbit blood to the media was required for successful subculture. The organisms were said to have been carried for 80 subcultures over a period of 11 months before they finally died. Organisms from cultures appeared to be able to infect mice. Although the results obtained by Hitzig and Liebesman appear to be valid, in view of other claims for successful cultivation of *S. minus* that subsequently were not confirmed, substantiation by other investigators would be desirable.

A case of spirillum hepatitis was recently described in a patient having hypogammaglobulinemia (189). On three occasions a spirillum-like organism was isolated from blood cultures from the patient. The organism grew rather slowly both anaerobically and aerobically on horse blood agar. Good growth was also obtained in nutrient broth containing added horse serum. The cells were 0.5 μm in diameter and possessed a single flagellum at each pole when observed by electron microscopy. Although the patient had no history of contact with rats, it is possible that the organism was *S. minus.*

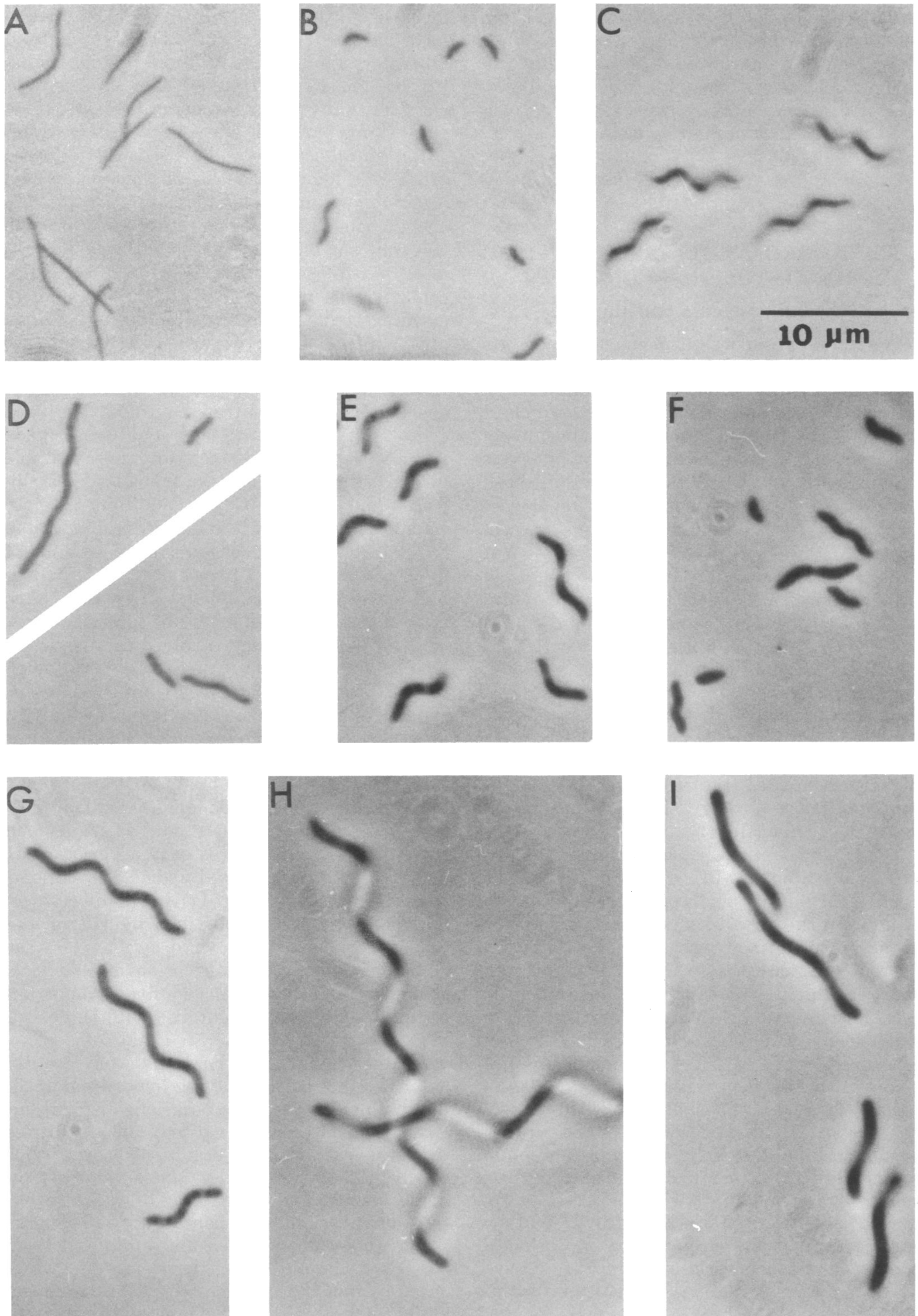
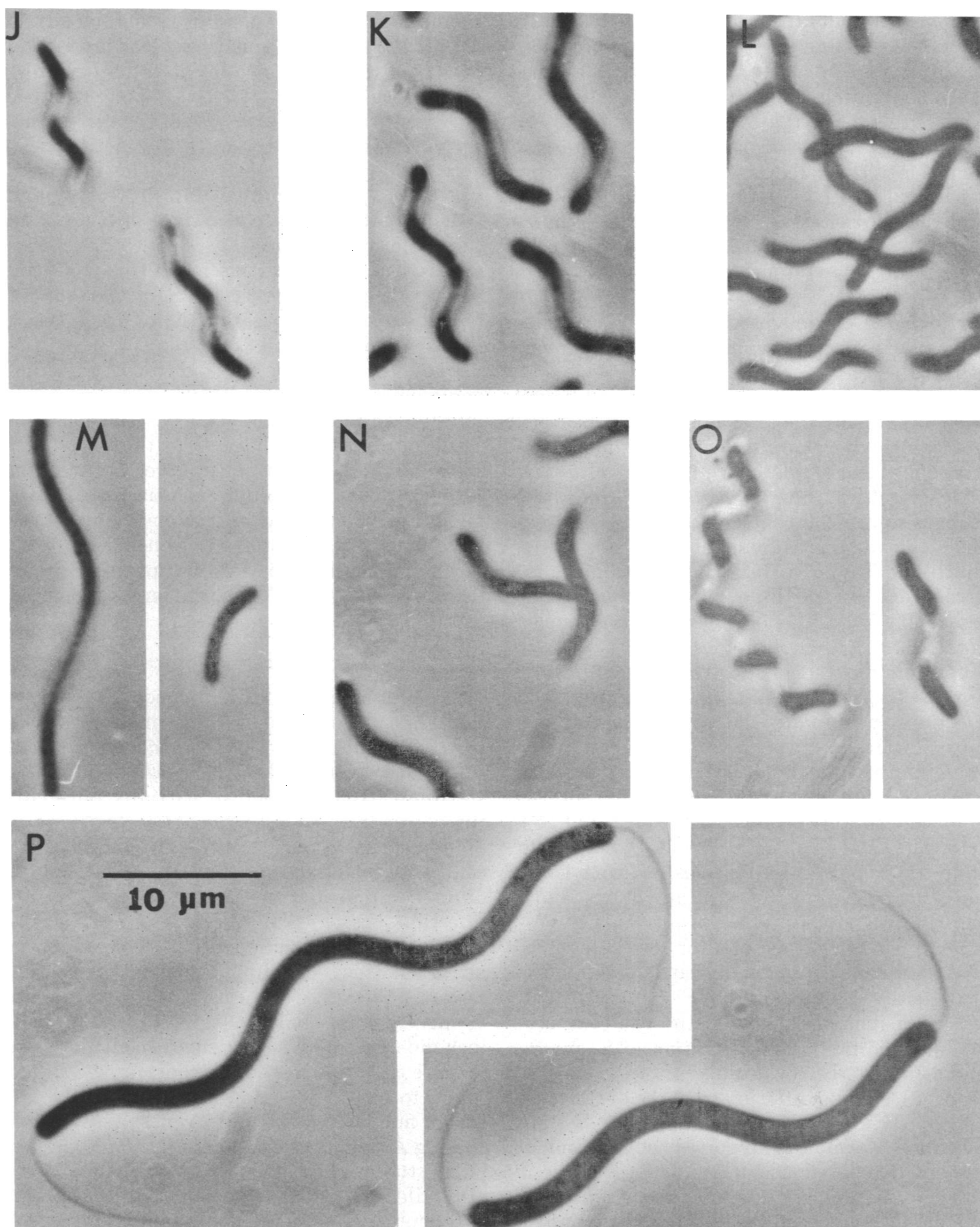


FIG. 1. *Phase-contrast photomicrographs of several species of the genus Aquaspirillum; Spirillum volutans is also depicted. The spirilla were cultured in MPSS broth (26) at 30 C for 24 to 48 h; however, A. bengal*



was incubated at 37 C, and A. delicatum was cultured in nutrient broth since its morphology and motility appeared to be more characteristic in this medium. All photomicrographs were taken at the same magnification. (A) A. gracile ATCC 19624. (B) A. delicatum ATCC 14667. (C) A. polymorphum NCIB 9072. (D) A. aquaticum ATCC 11330. (E) A. itersonii ATCC 12639. (F) A. dispar ATCC 27510. (G). A. peregrinum ATCC 15387. (H) A. sinuosum ATCC 9786. (I) A. putridiconchylium ATCC 15279. (J) A. serpens VH. (K) A. serpens ATCC 12638. (L) A. bengal ATCC 27641. (M) A. metamorphum ATCC 15280. (N) A. anulus NCIB 9012. (O) A. giesbergeri (previously A. graniferum) NCIB 8230. (P) S. volutans ATCC 19554.

A case of endocarditis was reported recently in which spirilla resembling *S. minus* were cultured from blood samples in 0.3% glucose broth containing thioglycolate (128). The organisms grew when the broth was incubated anaerobically; no growth occurred under aerobic conditions. The cells were 0.4 by 4.0 μm with a spiral width of 1.3 μm and a wavelength of 2.0 μm; they possessed a single polar flagellum as observed by electron microscopy. Pinpoint colonies formed slowly on blood agar plates incubated under hydrogen containing 10% carbon dioxide. The organisms gave positive catalase and oxidase reactions and failed to ferment carbohydrates.

Assuming that *S. minus* is a rigid, flagellated organism, the possibility must be seriously considered that the species may belong to, or may be closely related to, the genus *Campylobacter* rather than to *Aquaspirillum*. It is perhaps significant that, in describing the organism causing contagious abortion in cattle, Smith and Taylor in 1919 (168) continually referred to the "spirillum," even though they named it "*Vibrio fetus*" (now *Campylobacter fetus* [166]). Although *C. fetus* subsp. *fetus* is not a human pathogen, *C. fetus* subsp. *intestinalis* and *C. fetus* subsp. *jejuni* can cause blood infections in humans (166, 173). It is also perhaps significant that *C. fetus* is obligately microaerophilic and cannot be easily grown under aerobic or anaerobic conditions since it requires an oxygen concentration of between 3 and 15% (166). The dimensions of cells of *C. fetus* (0.2 to 0.5 by 1.5 to 5.0 μm) are consistent with those that have been given for *S. minus* (0.2 by 3 to 5 μm). Both *C. fetus* and *S. minus* usually possess a single flagellum at one or both poles (152, 166). Sufficient points of similarity seem to exist to justify a comparison of *S. minus* with *C. fetus*, and it is possible that the use of microaerobic conditions for cultivation of *S. minus* might prove to be rewarding.

Certain spirilla differing from the description of *S. minus* have been isolated from human infections. Kowal (100) reported the isolation of a polar-flagellated *Spirillum* from a patient with chronic aplastic anemia, recurrent fever and malaise, and systolic murmurs. The *Spirillum* was isolated from blood samples on two occasions 1 year apart, growing readily on blood agar, chocolate agar, and mannite agar aerobically at 37 C. The ease of cultivation and also the cell diameter (0.5 to 1.0 μm) would not seem to be compatible with descriptions of either *S. minus* or *C. fetus*. Edwards and Kraus (57) reported the isolation of a spirillum from the spinal fluid and blood samples of a patient with meningitis. The organism grew aerobically in Trypticase soy broth and horse blood broth after 24 h at 37 C, and it also formed small colonies on horse blood agar in 4 days. The organism was identified as *S. serpens*, but the cell diameter was not stated, nor were the motility and type of flagellation described. The organism was 5 to 10 μm in length, catalase positive, and indol negative, did not reduce nitrate, and could neither oxidize nor ferment carbohydrates. The ease of cultivation of this organism again suggests that it was not *S. minus*, but the paucity of characterization data precludes a precise identification.

In addition to *S. minus* and the other pathogenic spirilla described above, another spirillum-like organism has been described as the cause of diphtheritic lesions in the mouths of chickens (124). The organisms were rigid cells, 1 by 5 to 12 μm (although measurements were made under darkfield microscopy, which may have falsely augmented the dimensions) with bipolar single flagella. All attempts to cultivate the organism failed. The organism was named *Spirillum pulli*.

Nitrogen-Fixing Spirilla

An organism initially named *Azotobacter spirillum* was observed by Beijerinck (8) in 1922 in enrichment cultures of *Azotobacter chroococcum* and was isolated in 1925 (9). Although the spirillum fixed nitrogen in partially pure cultures (free from *Azotobacter* or *Clostridium*), it did not do so in pure cultures. In general, the cells were rod-shaped or slightly curved, but on dilute bouillon agar they were definitely spiral. The cells possessed lipid droplets (PHB granules?), and the specific epithet *lipoferum* was based on this. The lipid droplets sometimes distorted the shape of the cells. Calcium malate or lactate supported good growth, but Beijerinck did not state whether sugars could be used by pure cultures. Beijerinck considered *S. lipoferum* to be a connecting link between *Azotobacter* and *Spirillum* and even suggested including *Azotobacter* in the family *Spirillaceae*. Later studies of *S. lipoferum* by Schröder (156) also failed to demonstrate nitrogen fixation in pure cultures. Peptone or ammonium salts were necessary for growth, and a variety of sugars and organic acids could be used as carbon sources. The organisms were generally long thick rods, but a few cells having a half-turn were observed. A few cultures of *S. lipoferum* were isolated by Giesberger in 1936 (Ph.D. thesis), who found that the organisms were rod-shaped or faintly curved. Cells with a few turns were seen rarely. The motility of the

organisms was characteristic of vibrios or spirilla, however. Because only a single polar flagellum was present, Giesberger considered the organism to belong to the genus *Vibrio* rather than *Spirillum*. Several aerobic spirilla that apparently could fix nitrogen were isolated by Russian investigators in 1956 and 1965 (cited by Dalton [46]), but confirmation by the use of $^{15}N_2$ was not done. *S. lipoferum* was forgotten until 1963, when Becking (5) isolated a vibrio or spirillum that assimilated $^{15}N_2$. The organism could not grow in a completely nitrogen-free medium, however, and required 0.01 to 0.005% yeast extract. In broth cultures, the organisms became localized below, rather than at, the surface of the medium. In general, the cells were only slightly curved, but in peptone media they were spirals. Many intracellular PHB granules were present, and the cells possessed a single polar flagellum.

J. Döbereiner and J. M. Day (*In: International Symposium on N_2-Fixation*, sponsored by the Charles F. KeHering Research Laboratory and the National Science Foundation, 3 to 7 June 1974, Washington State Univ., Pullman, Wash.; see also reference 121) reported that *S. lipoferum* could be isolated from the root cortex cell of the tropical grass *Digitaria decumbens* by the use of a semisolid nitrogen-free malate enrichment medium. After enrichment, colonies were obtained on solid nitrogen-free medium containing a small amount of yeast extract. Enrichment in semisolid rather than liquid nitrogen-free medium was required since the nitrogenase activity (determined by acetylene reduction techniques) was found to be severely inhibited by oxygen. In the semisolid medium, the organisms formed a pellicle several millimeters below the surface, apparently at a location where there was sufficient oxygen for respiration and energy production but not enough to inhibit nitrogenase activity; i.e., in nitrogen-free media, the organisms were obligately microaerophilic but, when provided with a source of fixed nitrogen (yeast extract, peptone, or ammonium salts), they were capable of aerobic growth. The failure of Beijerinck (9) and Schröder (156) to demonstrate nitrogen fixation in pure cultures using liquid media can probably be explained by these results. In Beijerinck's partially pure cultures, in which nitrogen fixation could be demonstrated, it is likely that the contaminants may have decreased the level of dissolved oxygen to a point suitable for the spirilla. Furthermore, Döbereiner (personal communication) has found that certain strains of *S. lipoferum* cannot initiate growth in a completely nitrogen-free medium unless a low concentration of yeast extract is present. Beijerinck's strain may have been of this type, and it is perhaps significant that Döbereiner has found that impure cultures could, in some cases, fix nitrogen without addition of the yeast extract.

Whether *S. lipoferum* is closely related to *Aquaspirillum*, to *Vibrio*, or to members of *Azotobacteraceae* (i.e., *Azotobacter, Azomonas, Beijerinckia*, and *Derxia*) is not yet clear, and thorough characterization of Döbereiner and Day's strains is needed. A photograph of the proposed type strain (Sp 7) is presented in Fig. 2. Although the type of flagellation has not yet been determined, the type of motility exhibited by the strains is indicative of polar flagellation. The isolate described in 1963 by Becking (5) exhibited a single polar flagellum. Although this type of flagellation is more typical of vibrios than spirilla, it should be noted that *A. delicatum* generally possesses one or two flagella at only one pole. Furthermore, Döbereiner and Day have indicated that their strains are catalase positive, indole negative, and unable to acidify sugar media; these characteristics are typical in the genus *Aquaspirillum*. The possession of nitrogenase activity in *S. lipoferum* does not appear to preclude inclusion in the genus *Aquaspirillum* since *A. peregrinum* can reduce acetylene when cultured by the methods of Döbereiner and Day (173). The possibility that *S. lipoferum* might be closely related to polar-flagellated members of *Azotobacteraceae*, as for example *Azomonas insignis* and *Azomonas macrocytogenes* (3), should also be considered.

Nitrogenase activity, demonstrated by the acetylene reduction method, occurs in *A. peregrinum* and *A. fasciculus* when cultured aerobically in the semisolid nitrogen-free medium of Von Bülow and Döbereiner (183), provided 0.005 to 0.05% yeast extract is added. The function of the yeast extract is not yet understood. Under these conditions the organisms, like *S. lipoferum*, are microaerophilic, and no nitrogenase activity occurs aerobically in liquid medium. It is possible that other aquaspirilla may exhibit such microaerophilic nitrogenase activity; no comprehensive survey has yet been made.

Spirilla in Insects

In a survey of North Mississippi mosquitoes performed in 1972 to 1973 by Fulton et al. (64), larvae that appeared to be diseased were dissected and examined for microsporidia, fungi, viruses, and bacteria. In certain larvae of *Culex erraticus*, organisms identified as *Spirillum*

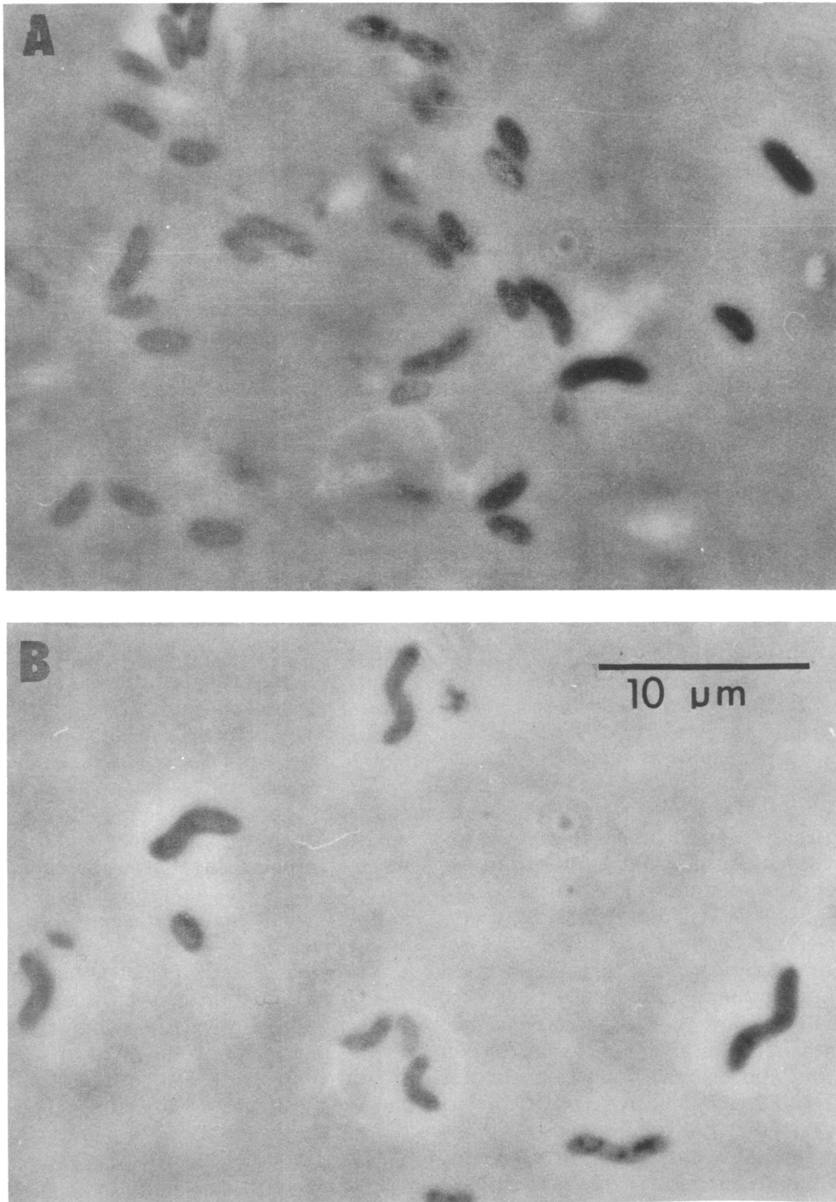


FIG. 2. *Cells of Spirillum lipoferum Sp 7 grown in the semisolid, nitrogen-free malate medium of Döbereiner and Day with 0.005% yeast extract added: (A) 48-h-old culture; (B) 25-day-old culture. Phase-contrast microscopy.*

were found, although it is not clear what criteria were used for identification. The relationship of these organisms to the genus *Aquaspirillum* remains to be determined. It is possible that the spirilla were pathogenic for the insects.

Bdellovibrio-Like Spirilla

Schnepf et al. (155) recently described a *Spirillum*-like organism that could infect the green alga *Scenedesmus*. The organism invaded the algal cells by perforating the walls, generally at one of the two cell tips. Within the cell, the bacterium digested the cytoplasm and eventually even the inner layer of the cell wall. The organisms had a cell diameter of 0.2 to 0.5 μm and seemed to be remarkably similar to members of *Bdellovibrio*, a genus that consists of small, curved bacteria capable of entering and multiplying within other bacteria (170).

Members of *Bdellovibrio* normally are vibrioid in shape and usually possess a single, sheathed polar flagellum, but spiral cells and cells with bipolar flagella or flagellar tufts do occur (159, 172). Parasitic strains as well as host-independent strains derived from parasitic strains elongate into helical forms during growth. These observations suggest a possible relationship to the genus *Aquaspirillum*. Also supportive of such a relationship is the aerobic nature of the host-independent strains, their possession of a strictly respiratory metabolism, their failure to catabolize carbohydrates, and a positive oxidase reaction (170). However, members of *Aquaspirillum* are not known to prey on other bacteria and, although host-independent strains of *Bdellovibrio* occur, many cells of which are apparently incapable of attacking host bacteria (159), all such strains that have been described so far have been derived from parasitic strains rather than from nature, and all produce an exocellular protease capable of lysing heat-killed bacteria (159). Burnham and Robinson (23) noted that it might be difficult to recognize host-independent strains isolated from nature as belonging to *Bdellovibrio*. However, in the multiplication of *Bdellovibrio*, the initial small, vibrioid cells develop into large, nonmotile forms, followed by formation of constrictions and subsequent segmentation into a number of flagellated vibrioid cells (170). This mode of replication does not occur in *Aquaspirillum*. Also, in contrast to most members of *Aquaspirillum*, the flagella of *Bdellovibrio* are sheathed. Furthermore, a "polar membrane" such as that described for *A. serpens* (135) and *C. fetus* (97, 150) does not appear to be present in *Bdellovibrio*. The DNA base composition of *Bdellovibrio* is 42 to 43% G+C for some strains and ca. 50% G+C for others (23). Although the latter value is within the range for *Aquaspirillum* (50 to 65% G+C), all of the small aquaspirilla that bdellovibrios might resemble (such as *A. delicatum*, *A. gracile*, and *A. polymorphum*) have a DNA base composition greater than 62% G+C.

Phototrophic Spirilla

Although phototrophic spirilla (*Rhodospirillum* and *Thiospirillum*) require anaerobic conditions for photosynthesis, the ability of some to grow aerobically or microaerophilically in the dark with greatly decreased pigmentation (144) suggests a possible relationship to *Spirillum* or *Aquaspirillum*. It is possible that the chemoheterotrophic spirilla may represent colorless derivatives of the phototrophic genera or that the phototrophic spirilla may have evolved from the colorless chemoheterotrophic genera. This possibility is reinforced by the finding of a "polar membrane" in *Rhodospirillum rubrum* (76) and *R. molischianum* (75); such a structure was originally described by Murray (135) in *A. serpens*. Additional similarities between *Rhodospirillum* and *Aquaspirillum* occur with respect to DNA base composition and the presence of intracellular PHB. It is likely that only DNA/DNA homology studies will resolve the question. The large cell diameters characteristic of *Thiospirillum* (144), as well as the occurrence of large, easily visible, polar fascicles of flagella (153), suggest a possible relationship of this genus to *S. volutans*. However, the DNA base composition of *Thiospirillum jenense*, the only *Thiospirillum* so far obtained in pure culture, is 45% G+C (144), compared to 38% for *S. volutans*; moreover, *T. jenense* is obligately phototrophic and anaerobic (144).

Oxidative Vibrios

The genus *Vibrio* (161) contains organisms that are capable of fermenting carbohydrates and thus differs from *Aquaspirillum* (in which only a few species can catabolize carbohydrates and this is by oxidation rather than fermentation) and *Oceanospirillum* (in which carbohydrates are not catabolized by any species). Moreover, the DNA base composition of the genus *Vibrio* is 40 to 50% G+C (161), whereas that for *Aquaspirillum* is 50 to 65% G+C (83). However, there exists a number of vibrioid organisms such as *V. percolans* (72, 131) that have a strictly respiratory metabolism, a DNA base composition of 64% G+C, and one flagellum or a tuft of flagella at one or both poles. Such organisms, initially placed in the genus *Vibrio* because of their shape, were subsequently placed in the genus *Comamonas* (47, 158), but they appear to have many characteristics in common with the genus *Aquaspirillum*, especially *A. delicatum*. They also are similar to certain members of the genus *Pseudomonas*, especially *P. testosteroni* (79).

GENUS *OCEANOSPIRILLUM*

The aerobic chemoheterotrophic marine spirilla having a G+C content of 42 to 48% were placed in the genus *Oceanospirillum* by Hylemon et al. (83). The species in this genus (Table 2) have been thoroughly characterized in several taxonomic studies (29, 83, 126, 180, 181). A list of the characteristics of the species is presented in Table 4, and photomicrographs of many of the species are presented in Fig. 3. All species are helical, possess bipolar flagella, require seawater for growth, have a strictly respiratory type of metabolism, fail to oxidize or ferment carbohydrates, fail to grow anaerobi-

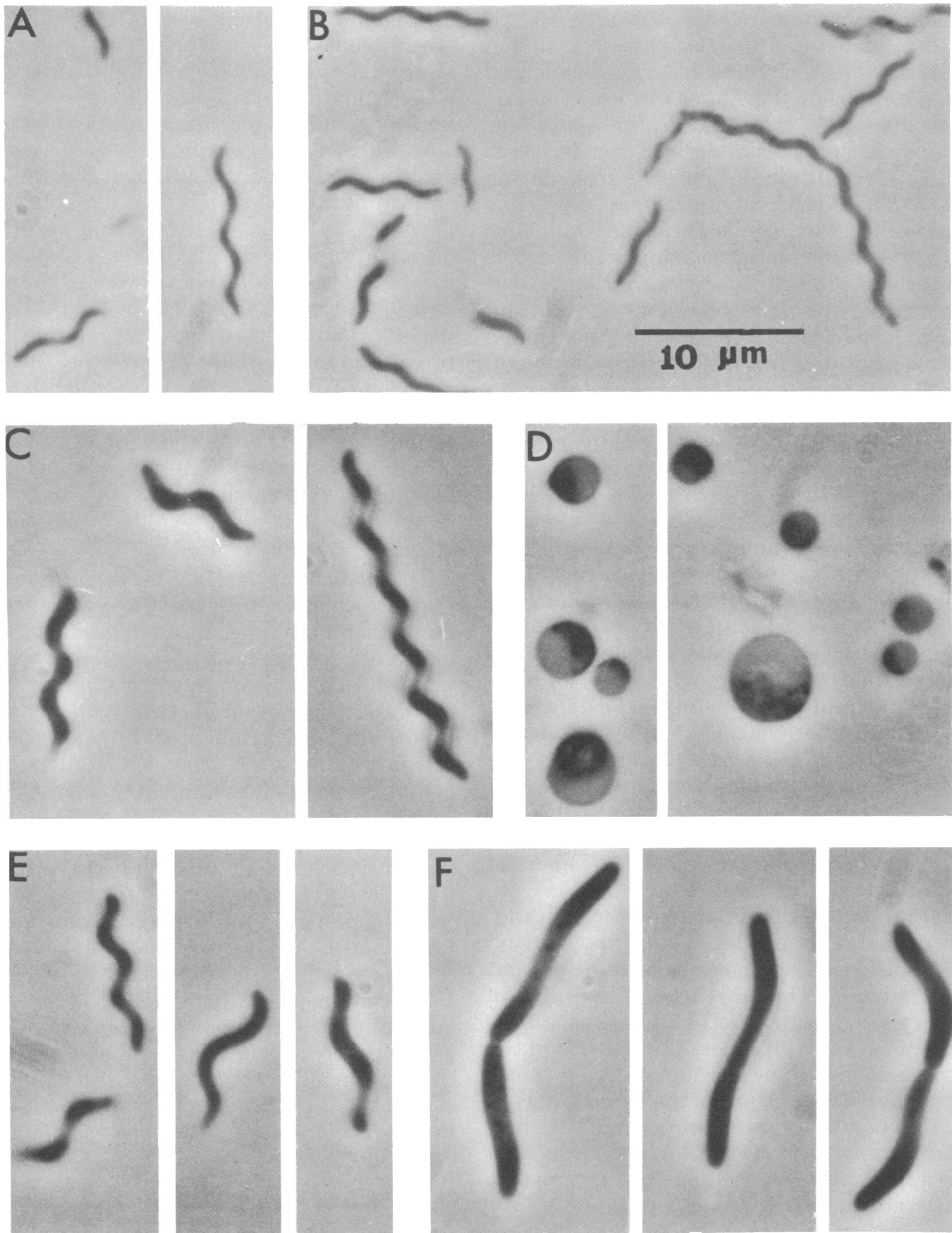


FIG. 3. *Phase-contrast photomicrographs of several species of the genus Oceanospirillum. The spirilla were cultured in seawater-MPSS broth (26) at 30 C for 24 to 48 h. All photomicrographs were taken at the same magnification. (A) O. minutulum ATCC 19193. (B) O. linum ATCC 11336. (C) O. maris ATCC 27509. (D) Coccoid bodies of O. maris formed at 7 days of incubation. (E) O. beijerinckii ATCC 12754. (F) O. japonicum ATCC 19191.*

cally in the presence of nitrate, fail to reduce nitrate beyond the nitrite stage, and are oxidase positive. Cell diameters range for 0.3 to 1.2 μm.

Typical Characteristics

A number of characteristics apply to most, but not all, species of *Oceanospirillum*. (i) Bipo-

lar tufts of flagella are present. However, *O. pusillum* possesses predominantly bipolar, single flagella (181). (ii) Intracellular PHB granules are apparent. The presence of PHB has not yet been tested in *O. pusillum, O. multiglobuliferum, O. hiroshimense,* and *O. pelagicum.* (iii) Coccoid bodies are predominant in old cultures. Only *O. japonicum* appears to be an exception (83, 181). (iv) Nitrates are not reduced to nitrites. However, *O. minutulum* and *O. pusillum* are exceptions. (v) The optimal growth temperature is ca. 30 C, but *O. hiroshimense* grows best at ca. 25 C and can even grow at 3 to 4 C (181).

Other Characteristics

Catalase reactions range from positive to very weak or negative (83, 180). Some species form water-soluble brown pigments from certain aromatic amino acids (83). All strains so far tested can grow in the presence of 1% oxgall bile, but the ability to tolerate 1% glycine varies (83). Some species form a water-soluble, yellow-green fluorescent pigment when cultured on PSS agar containing artificial seawater and examined under ultraviolet light (254 nm) (83). Nutrition varies from versatile to extremely limited with respect to sole carbon or nitrogen sources (83). Some strains exhibit phosphatase activity.

OTHER SPIRILLUM-LIKE ORGANISMS

Many habitats contain bacteria that have a spirillum morphology, and many of these probably cannot be isolated or cultivated by current techniques. Sometimes an organism of great morphological distinction is found regularly in a particular habitat and so can be described with some confidence even though it cannot yet be cultivated. These deserve the close attention of those bacteriologists who have a particular facility for growing difficult organisms. The most extreme forms that would certainly be likely to fall into independent genera are to be found in the intestinal tract of animals.

Sporeforming Spirilla.

Collin (42) and Delaporte (48, 49) have described the occurrence of very large, rigid helical bacteria in the intestinal contents of tadpoles. Endospore-like structures occurred within the cells. The organisms could not be cultivated in vitro, but the intestinal contents of tadpoles containing the organisms, or even the bottom sediment from their basins, could be used to infect tadpoles that had previously been free of the organisms. The spirilla did not appear to be pathogenic for the tadpoles. Because the spirilla could not be cultivated on artificial media, the thermal resistance of the endospore-like structures was not tested, but it might be possible to do this by determining whether infectivity for tadpoles remained after heating intestinal material. Delaporte (49) suggested that the spirilla might possibly be anaerobic. Although Collin (42) had observed no motility for the spirilla, Delaporte (49) found that the organisms were able to move slowly in a corkscrew-like manner with frequent stopping followed by reversal of direction. Although no flagella could be discerned, it is possible that staining methods or electron microscopy might reveal their existence. A remarkable attribute of the spores was their ability to move within the cytoplasm of the cells, and a series of photomicrographs taken by Delaporte (48) clearly demonstrates the rotation of a curved endospore within a cell. Because of the enormous size of the spirilla and the occurrence of endospores, Delaporte (49) placed the organisms in a new genus, *Sporospirillum,* and distinguished three species on the basis of morphology. The first was *S. praeclarum* (cell diameter, 3.0 to 4.0 μm; length, 50 to 100 μm; diameter of helix, 5 to 10 μm; wavelength, 17 to 23 μm) (a single endospore, 3 to 4 by 9 to 12 μm was present). The second was *S. gyrini* (cell diameter, 1.8 to 2.6 μm; length, 40 to 100 μm; diameter of helix, 3 to 6 μm; wavelength, 13 to 20 μm) (a single spore, 2 by 5 to 7 μm, was present). The third was *S. bisporum* (cell diameter, 3.5 to 4.8 μm; length, 50 to 90 μm; diameter of helix, 3 to 6 μm; wavelength, 27 to 35 μm) (a cylindrical spore, 2 to 4 by 10 to 14 μm was present at each pole). These species are illustrated in Fig. 4. Spores initially developed near the cell poles but eventually moved toward the center of the cells. Prior to rupture of the cell, the cytoplasm disappeared and the cells became distorted and flaccid.

Although these large spirilla would not seem to be closely related to *Spirillum* or *Aquaspirillum,* they do seem to resemble certain very large straight rods that also occur in the intestines of tadpoles and that also have endospores capable of migration within the cytoplasm (42, 48, 50).

The possibility of the existence of smaller sporeforming spirilla is indicated by the observation by Dobell (53) in 1908 of spirilla 1.5 by 6 to 8 μm occurring in the intestines of frogs and tadpoles. These cells contained an endospore-like structure at one pole and were motile by means of bipolar, single flagella or flagellar tufts. Like the large spirilla described by Dela-

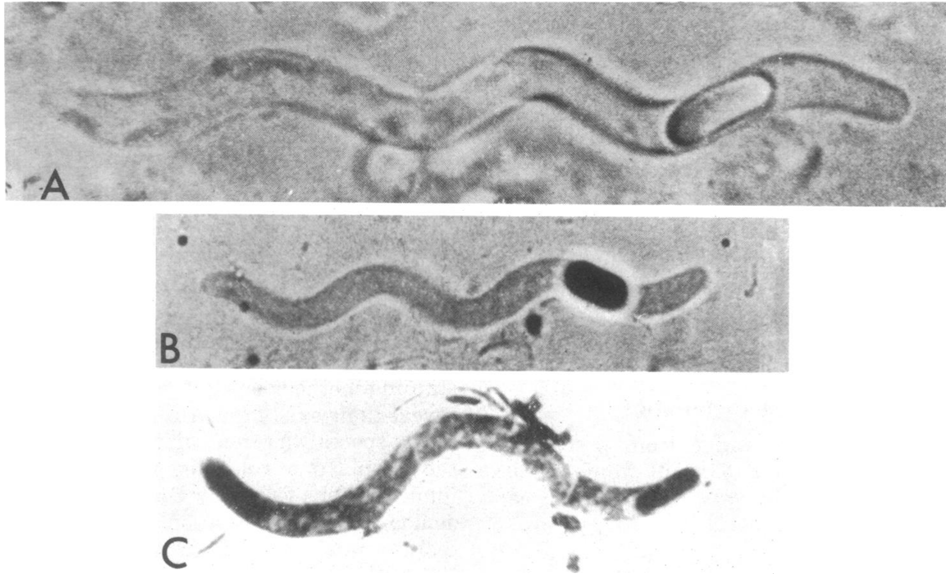


FIG. 4. *Species of Sporospirillum. (A) S. praeclarum in vivo, with a kidney-shaped spore that is just slightly refringent. The cell poles are slightly attenuated. Cell diameter, 4.6 μm; cell length, 69 μm; diameter of helix, 8 μm; wavelength, 20 μm. The spore is 3.3 by 9.3 μm. (B) S. gyrini in vivo with a very refringent mature spore. Cell diameter, 2.6 μm; cell length, 40 μm; diameter of helix, 5.9 μm; wavelength, 14 μm. The spore is 2.6 by 5.3 μm. (C) S. bisporum, fixed with OsO_4 and stained by the method of Piéchaud. The cell contains two almost mature spores, one at each cell pole. Cell diameter, 3.3 to 4.2 μm; cell length, 50 μm; diameter of helix, 11.7 μm; wavelength, 30.3 μm. Spores are 2.1 by 7.1 μm and 2.0 by 8.3 μm. (Reproduced by permission of the Secretariat des Annales from: B. Delaporte, Ann. Inst. Pasteur Paris 107:246–262, 1974)*

porte, these organisms could not be cultivated in vitro. No further reports of them have appeared.

Gastric Spirilla

Ito (85) reported the occurrence of a motile spirillum in the gastric gland of cats, an environment that normally has a pH of 1 and is rich in hydrolytic enzymes. The organism was not cultured but, judging from an electron micrograph of a thin section, it appears to have a cell diameter of ca. 0.6 to 0.7 μm, a helix diameter of ca. 0.85 μm, a wavelength of ca. 1.0 μm, and a length of ca. 7 μm, with six coils being evident. The organism possesses bipolar tufts of sheathed flagella. It is possible that the spirillum is related to the anaerobic genus *Succinivibrio* (18), although the latter has been described as having a smaller cell diameter, fewer coils, and monotrichous flagellation.

ECOLOGY OF SPIRILLA

Habitat and Numbers in Nature

Members of the genus *Aquaspirillum* have been isolated from a variety of natural sources including ditch water and canal water (Giesberger, Ph.D. thesis); creeks, ponds, and small lakes (25, 102, 112, 198); stored distilled water (110); sewage (138); and primary oxidation ponds (146). Aquaspirilla have also been isolated from sources other than water, such as decaying grass cuttings (30). Giesberger isolated many strains from horse and pig manure, finding that success depended on the use of manure that had been in contact for a time with the stable floor, rather than fresh manure. From this he concluded that the spirilla probably had not originated in the manure itself. Terasaki (175–178, 180) isolated many strains of aquaspirilla from freshwater shellfish but concluded that such spirilla were probably not present as normal or parasitic inhabitants of the mussels but were instead present in the adherent mud (178).

S. volutans has been cultivated from the water of the cooling tower of a sugar-beet refinery in England (151) and from the water of a polluted pond (191). The species is probably widely distributed in stagnant water. In hay infusions prepared with pond water, the spirilla can be observed easily when a small portion of the surface scum is pushed aside and a drop of water is taken from immediately below the surface (J. S. Wells, Jr., M.S. thesis, Virginia Polytechnic Institute and State Univ., Blacksburg, 1966). The location of the spirilla just

below the surface scum may possibly reflect the microaerophilic nature of the species.

The occurrence of the nitrogen-fixer *S. lipoferum* in tropical soils and in association with the roots of tropical grasses has been mentioned previously. The high optimal temperature for N_2-dependent growth appears to be related to the tropical habitat of the organisms (122). Tropical soils in general, and also the soils from which *S. lipoferum* has been isolated, seldom show a pH above 5.0. Because N_2-dependent growth of *S. lipoferum* occurs best at a pH of 6.8 to 7.8, this has been suggested as one reason why *S. lipoferum* does not fix nitrogen when free in such soils, but only at root surfaces or within root cells (J. M. Day and J. Döbereiner, Soil Biol. Biochem., in press.) Döbereiner (personal communication) has found two distinct groups of *S. lipoferum*. Organisms of the first group are easy to isolate, grow well in nitrogen-free semisolid media, and have the characteristic actively motile curved forms. This group has been isolated from the roots of *Digitaria, Panicum* maize, sorghum, and several other grasses from the tropical regions of Brazil; members have also been isolated from the Rio Grande de Sul in the extreme south of Brazil (temperate in climate). This group contains the proposed type strain (strain Sp 7). Organisms of the second group are difficult to isolate and require the addition of low concentrations of yeast extract for growth and nitrogen fixation; the reason for the yeast extract requirement is not yet clear. The cells possess a larger diameter than those of the first group and are more pleomorphic. Strains of the second group have been isolated from grasses collected in the state of Washington and from the roots of wheat in Brazil.

When fixing nitrogen in association with plant roots, nodules are not formed as with *Rhizobium*. The spirilla are located within cells of the inner root cortex, and acetylene-reducing activity is localized at discrete sites on the roots. The association is a truly symbiotic one – not the effect of nitrogen fixation in the rhizosphere (183). In a field experiment in Florida, R. L. Smith, M. H. Gaskins, S. C. Schank, and D. H. Hubbell (Abstr. Annu. Meet. South. Branch Agron. South. Assoc. Agric. Sci., 1975, p. 12) inoculated a variety of tropical grass ecotypes with *S. lipoferum*. An increase of 50 to 60% in dry weight yields and 60 to 80% in protein yields of certain grasses compared with uninoculated controls suggested that the nitrogen-fixing grass-bacteria association might be adapted to cultivation. Von Bülow and Döbereiner (183) have detected nitrogenase activity associated with the roots of maize plants, with *S. lipoferum* being isolated from all active root pieces when they were surface-sterilized. Such findings indicate the potential application of *S. lipoferum* in agriculture.

A nitrogen-fixing straight rod considered for many reasons to belong to the genus *Aquaspirillum* (173) was isolated from pond water from Iowa and Virginia and also has been observed in *Sphaerotilus* beds in streams. Morphologically similar organisms have been observed in Germany (93) and Holland (78). The slime matrix for cell flocs of this organism may have possible significance not only for attachment to surfaces in natural environments, but also for decreasing the oxygenation of cells within the flocs, resulting in protection of the nitrogenase.

With regard to *Oceanospirillum* species, Williams and Rittenberg (198) isolated many strains from seawater samples collected from the intertidal zone along the coast of Long Island Sound. Whether *Oceanospirillum* occurs in the open sea appears to be unknown. Jannasch (86) isolated a marine spirillum from decaying seaweed. *S. virginianum* was isolated by Dimitroff (52) from mud adhering to the outside of an oyster shell; whether this organism was a marine spirillum is not clear, however, since seawater was apparently not required for its growth. Watanabe (186) isolated marine spirilla from the viscera of marine shellfish. Although Terasaki (177, 178) sometimes observed spirilla in the alimentary tracts of marine shellfish, his studies suggested that this occurrence was probably fortuitous and that mussel mud was more likely to be the usual source of the organisms.

Few studies have been made of the number and relative proportions of spirilla occurring in natural habitats. Oppenheimer and Jannasch (141) made direct microscope counts of the bacteria present in clear and turbid seawaters near Port Aransas, Tex. Counts ranged from 900,000/ml outside the Gulf surf, where the water was clear, to 28,800,000/ml in turbid water along the shore of Redfish Bay. From 90.0 to 99.5% of the total populations were cocci. Rod-shaped organisms comprised 0.9 to 4.8%, vibrios comprised 0.3 to 3.2%, and spirilla comprised 0.1 to 2.5%. Scully and Dondero (157) enumerated spirilla in samples of algae *(Cladophora)*, pond mud, pond water, stream "Aufwuchs," stream water, trickling filter effluent, cow manure (moist manure from an outdoor, concrete-floored cattle pen), field drainage, cultivated field soil, woods soil, and hay. The enumeration was by means of the most-probable-number (MPN) method, using six different enrichment media. Any helical form moving in a

helical path was considered to be a spirillum, and such organisms were divided into three groups on the basis of cell size. Although these groups may have included organisms other than *Spirillum* or *Aquaspirillum* (e.g., *Vibrio, Desulfovibrio,* or anaerobic vibrios), it was clear that helical bacteria represented only a small proportion of the total bacterial population in all samples. They were about 4% in the *Cladophora* sample; about 0.1 to 0.6% in mud, slime, trickling filter effluent, and cow manure; and less than 0.01% in all of the remaining samples except hay, in which they were not found.

Ecological Studies of Spirilla by Steady-State Cultivation

As noted by Jannasch (86), little was known prior to 1960 about the growth of bacteria in extremely dilute media like seawater. It is of great significance for ecological studies on marine microorganisms that growth may be measured in a chemostat at extremely low concentrations of a limiting nutrient, and these results may be quantitatively comparable to bacterial activities in the sea. Moreover, spirilla are especially suitable for such studies because they do not easily grow on the walls of culture vessels. In a chemostat, the self-adjusting steady state is controlled by two factors: the concentration of the growth-limiting substrate in the inflowing medium, which determines the population density, and the dilution rate, which determines the specific growth rate. Using a marine spirillum isolated from decaying seaweed and a medium composed of 3% commercial seasalt, phosphate buffer, lactate as the carbon source, and asparagine as the nitrogen-limiting nutrient, Jannasch found that at a dilution rate of 0.185/h (= $1/2$ of the maximal growth rate), slow washout of the spirillum occurred at 0.16 mg of asparagine-N per liter. A steady-state population of ca. 4×10^5 cells/ml could be established at 0.28 mg of asparagine-N per liter. Experiments with unsupplemented natural seawater and higher dilution rates (to increase the growth rate) indicated that the spirillum was unable to multiply at a rate of 1 generation in 24 h, even in a sample of extremely polluted water from Naples Harbor, although the addition of lactate and asparagine at the concentrations used previously easily stimulated growth. Jannasch suggested that the growth of the spirillum might therefore be restricted to environments of higher nutrient concentrations than found in ordinary seawater, such as in zones surrounding decaying particulate plant matter. In support of this, he found that all enrichments from seawater were unsuccessful unless pieces of decaying seaweed were added.

For the freshwater spirillum *A. serpens,* as well as for two marine species, the threshold concentration of lactate (as a growth-limiting carbon and energy source) in the reservoir of the chemostat was determined (87, 89). When a dilution rate equivalent to half the maximal growth rate was used, the threshold concentration was 15 mg/liter. Below this, a steady-state culture could not be maintained in the chemostat, and washout occurred. On a theoretical basis, Jannasch had expected lower threshold values for lactate and, consequently, lower threshold population densities. In experiments in which various dilution rates were used to alter growth rates, it was found that the threshold concentration of lactate was a function of the growth rate: the higher the growth rate, the lower became the threshold concentration. This suggested that at low population densities certain growth-stimulating metabolic activities (such as excretion of growth "activators" or neutralization of inhibitory factors) might have an influence that would not be apparent at high population densities. Jannasch noted that high population densities in the chemostat maintained an E_h of −230 mV, whereas when the population decreased to the threshold level the medium became less reducing. The addition of ascorbic acid to restore reducing conditions permitted lower threshold levels of lactate to be used, with resulting lower threshold population densities. On the basis of these results, Jannasch considered the spirilla to be microaerophilic at low population densities.

In dilute environments, spirilla may be able to compete successfully with other bacteria for the nutrients present. In chemostat experiments using a mixture of a marine spirillum *(O. maris)* and a marine pseudomonad, with 50 mg of lactate per liter as the growth-limiting carbon source, Jannasch (90) found that the spirillum predominated by 7:1 after five retention times with a dilution rate of 0.4/h. At a dilution rate of 0.7/h, however, the pseudomonad outnumbered the spirillum by 9:1 after 12 retention times. In batch cultures in which the lactate concentration was varied from 4 to 20 mg/liter, the growth rate of the spirillum exceeded that of the pseudomonad at lactate concentrations below 10 mg/liter. In similar experiments using mixtures of the spirillum with *Escherichia coli,* Jannasch (91) showed that the growth rate of the spirillum exceeded that of *E. coli* when lactate was supplied at 5 mg or less per liter.

ISOLATION OF SPIRILLA

As indicated by Giesberger (Ph.D. thesis), Williams and Rittenberg (198), Jannasch (88), Terasaki (177, 180), and many others, the isolation of spirilla has been relatively difficult compared with that of many other bacteria. The difficulties attendant in the isolation of spirilla are twofold. (i) They are not dominant organisms in natural environments (see previous section). (ii) They are easily overgrown by other bacteria, such as pseudomonads and vibrios, on isolation media containing peptone or yeast extract (88). The reasons for these difficulties are not entirely clear, but an important clue was provided by the investigations by Jannasch (90), who found that *O. maris* could grow faster in continuous culture than a *Pseudomonas* species in dilute media (with lactate as the limiting nutrient); the spirillum was outgrown in more concentrated media. This may explain the success of certain empirical methods of isolating spirilla, such as that employed by Williams and Rittenberg (198). Here, it was found that 1% calcium malate or lactate added to freshwater from various sources usually allowed abundant multiplication of spirilla after 1 week at room temperature. Serial transfers were then made into sterile source water containing the carbon source; after three or four such transfers, the spirilla predominated and could be isolated on solid media. If the transfer medium was supplemented with ammonium chloride, other bacteria grew more abundantly than the spirilla, making isolation difficult or impossible. Jannasch (88) described a method similar to that used by Williams and Rittenberg but employing phosphate-buffered media. The choice of carbon source for enrichment was important; e.g., although Williams and Rittenberg found that all of their isolates could use pyruvate, spirilla were never observed in water samples enriched with this carbon source. For enrichment of marine spirilla, Williams and Rittenberg (198) found that calcium lactate, but not calcium malate, was satisfactory, even though all isolates obtained could use malate. Seawater samples were mixed initially with equal quantities of phosphate-buffered medium containing 1% calcium lactate and 0.1% ammonium chloride. Subsequent serial transfers were made in media lacking the nitrogen source, and spirilla usually predominated after one to three transfers.

In some cases enrichment cultures that allowed spirilla to reach numbers sufficiently high to warrant plating on solid media have employed enrichment media that were not dilute. Such media are exemplified by putrid infusions of freshwater or marine shellfish (175–177, 186), infusion of grass cuttings (30), algal infusions (198), or defined media (88, 198). As noted by Williams and Rittenberg (198) and Jannasch (88), spirilla appear to be enriched at the surface of freshwater cultures or just beneath the surface scum that forms in marine cultures. This accumulation near the surface appears to be the result of an aerotactic response. Isolation of spirilla by plating a loopful of this surface layer, or dilutions made from it, on solid media has often been successful. Jannasch (88) observed that colonies of spirilla show strongly inhibited growth in the immediate presence of colonies of other bacteria and begin to develop usually when metabolic products of the other colonies have diffused away from the region; consequently, a dilute inoculum for plating is often advantageous.

Cody (38) found that growth of *A. serpens* was not inhibited by concentrations of 5-fluorouracil as high as 300 μg/ml, a concentration that inhibited many other bacterial species. This suggested that 5-fluorouracil might be successfully used as a selective agent for some spirilla, and inoculation of pond water samples into media containing 300 μg/ml yielded no organisms other than spirilla. Scully and Dondero (157) found, however, that pure cultures of *A. itersonii* and *A. polymorphum* grew poorly with 5-fluorouracil and that *A. serpens* did not grow at all. For the estimation of the MPNs of spirilla in various natural sources, 5-fluorouracil was generally not effective and suppressed the growth of spirilla having moderate-to-large cell diameters. However, in cow manure, trickling filter effluent, stream "Aufwuchs," and streamwater, it suppressed the MPN of the total bacteria greatly, giving higher relative proportions of small spirilla (but lower MPN values, except in the case of cow manure). In general, the best enrichment medium for recovery of spirilla was a vermiculite-egg albumin medium (expanded mica: hydrated magnesium-aluminum-iron silicate, 1.0 g; egg albumin [scales or powder], 3 g; distilled water, 1 liter).

For isolation of the nitrogen-fixing spirillum *S. lipoferum* from maize, Von Bülow and Döbereiner (183) achieved enrichment by placing surface-sterilized root pieces into a nitrogen-free semisolid sodium malate medium. After 2 days at 30 to 35 C, a white pellicle of organisms formed 2 mm below the surface of the medium, and the spirilla predominated greatly after two serial transfers in the malate medium. Isolation was then achieved by streaking on solid malate agar containing a

small amount of yeast extract (the fixed nitrogen in the latter allowing aerobic growth).

Perhaps the most difficult spirillum of all to isolate has been *S. volutans*. Although described by Ehrenberg in 1832, this organism was not isolated in pure culture until 1962 (151). The reasons for the difficulty are twofold. (i) No satisfactory enrichment medium has ever been developed which allows the organisms to predominate in mixed cultures. In Pringsheim soil medium (151), probably the best medium ever devised for maintenance of mixed cultures of *S. volutans*, the organism is outnumbered by ca. 1,000:1. (ii) *S. volutans* is so far incapable of forming colonies on solid media, even under conditions that are optimal for growth in liquid media (82, 151). In 1962 Rittenberg and Rittenberg (151) nonetheless achieved isolation of the organism by an ingenious method. They noted that the swimming velocity of *S. volutans* was higher than that of the contaminants present, with speeds as high as 200 μm/s being reported. This suggested that a suitable isolation method might be to allow the organism to outswim the contaminants. Capillary tubes (0.1 to 0.3 mm by 15 to 30 cm) were filled with sterile medium and inoculated at one end with the mixed cultures; the tubes were observed at ×100 until the spirilla had passed into the sterile medium well beyond the limits of the mixed population. The tubes were then broken at the observation point, and the separated spirilla were inoculated into sterile media. Despite the success of the mechanical separation, however, the spirilla failed to multiply in any of a large variety of media tested, even when rich supplements were added. Even the use of autoclaved or filtered media in which the spirilla had previously grown well in mixed culture failed to support growth of pure cultures. Pure culture growth was finally achieved by inoculating sterile media contained within a dialysis sac. The sac was suspended in a vessel containing a culture of *E. coli* or other bacteria. Cell extracts of *E. coli*, prepared by sonic oscillation and sterilized by filtration, could also support growth of *S. volutans*. It was believed that, because filtrates of mixed cultures of *S. volutans* or of pure cultures of *E. coli* failed to support growth of pure cultures of *S. volutans*, some kind of extremely labile nutritional factor was being formed continuously by bacteria outside of the dialysis sac. The nature of this factor was an enigma: although it apparently was not present in a wide variety of vitamin mixtures and rich supplements, it seemed to be a common metabolite since a great variety of bacteria were effective in promoting the growth of dialysis sac cultures. The answer to the mystery came about as the result of observations of wet mounts by Wells and Krieg (191). When wet mounts were allowed to stand for ca. 10 min, the regular formation of a ring-shaped band of spirilla occurred near (but not at) the edges of the cover slips. This band formation suggested that the spirilla were being repelled by too much or too little oxygen, and with this tactic response as a guide, Wells and Krieg were able to cultivate *S. volutans* in pure culture without the use of dialysis sacs. Here, the oxygen concentration of the atmosphere within culture vessels was decreased from 21% to 1-9%. Under these conditions, populations of ca. 4×10^5 cells per ml were obtained in ordinary nutrient broth; much higher populations can be obtained by the use of modified PSS (MPSS) broth (26). Therefore, it seems likely that in the experiments of Rittenberg and Rittenberg the function of the bacteria outside of dialysis sac cultures was not to provide an essential nutritional factor, but instead was to decrease the level of dissolved oxygen to a point suitable for growth of the spirilla.

Another method for mechanical separation of spirilla from contaminants was devised by Canale-Parola et al. (25) for the isolation of *A. gracile* from pond and stream water. In this method, sterile cellulose ester filter disks having a pore size of 0.45 μm were placed on plates of media solidified with 1.0% agar. Samples (0.05 ml) of pond or stream water were then deposited in the center of the filter disks. The plates were incubated for 1.5 to 5 h, after which the filter disks were removed, and incubation was continued. After 3 days or longer, some of the plates exhibited spreading, semitransparent areas of growth which developed entirely within the agar medium; this represented the multiplication of small spirilla which, unlike the larger contaminants, had been able to migrate through the pores of the filter into the underlying agar. The diffuse growth on the plates was subsequently streaked on fresh media for isolation of colonies.

CULTIVATION AND NUTRITION OF SPIRILLA

Complex Media

Members of the genera *Aquaspirillum* and *Oceanospirillum* have been cultured in a variety of complex media, but no unusual supplements or agents such as blood, heme, serum, starch, or carbon dioxide are required. One commonly used formulation for stock cultures is that of Williams and Rittenberg (198), as

follows (percentage): peptone, 0.5; beef extract, 0.3; yeast autolysate, 0.3; agar, 1.65; pH = 7.0. Ordinary nutrient agar was used successfully by Terasaki (180). Hylemon et al. (83) used a semisolid PSS medium of the following composition (percentage): peptone, 1.0; succinic acid (free acid), 0.1; $(NH_4)_2SO_4$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.0002; $MnSO_4 \cdot H_2O$, 0.0002; and agar, 0.15. The pH was adjusted to 6.8 with KOH rather than NaOH to avoid possible inhibitory levels of Na^+. For members of the genus *Oceanospirillum*, media similar to those described above have been used, but seawater must be substituted for distilled water (83, 180, 198). Williams and Rittenberg (198) were unable to adapt any marine strain to growth with less than 30% seawater, and even at this level the cells were fragile and morphologically abnormal. The superiority of natural seawater to artificial seawater was also indicated, as continued subculture in artificial seawater led to eventual loss of viability. Hylemon et al. (83), however, found that excellent growth occurred in semisolid PSS medium containing the following artificial seawater formulation (169) NaCl, 2.75%; $MgCl_2$, 0.5%; $MgSO_4$, 0.2%; $CaCl_2$, 0.05%; KCl, 0.1%; and $FeSO_4 \cdot 7H_2O$, 1 to 2 mg/100 ml. Whether this formulation would be suitable for primary isolation of marine spirilla is not known.

The pH range for growth of spirilla has been thoroughly investigated by Terasaki (180). All species, marine and freshwater, grew between pH 6.5 and 8.5, but the best growth occurred in neutral or slightly alkaline media. No spirillum could grow at pH 5.0, but a number of strains could grow at pH 5.5 or 9.0. The tolerance of many spirilla to high pH is reminiscent of the tolerance of the genus *Vibrio* (161).

The optimal temperature for growth of spirilla is usually 30 to 32 C (83, 198). However, *A. bengal* grows best at 41 C (102), whereas *O. hiroshimense* and *A. psychrophilum* have optima of 25 and 20 C, respectively (181).

S. volutans is an obligate microaerophile, as described in the previous section. With an atmosphere of 6% oxygen-94% nitrogen, excellent growth can be obtained in a modified PSS broth (26). After initial turbidity has appeared (ca. 18 h), access of air to the vessel will no longer inhibit growth but will enhance it. Decreased oxygen levels are not necessary if MPSS broth is freshly boiled and cooled just before use, provided a heavy inoculum is used (41). The use of semisolid (0.15% agar) MPSS medium also eliminates the necessity for decreased oxygen levels by stratifying the medium (82).

An important consideration for the cultivation of *S. volutans* is the unusual toxicity of phosphate for the organism. Hylemon et al. (82) indicated that growth of the Wells' strain in PSS broth was inhibited completely by as little as 0.003 M added phosphate.

Defined Media

The synthetic basal mineral salts medium devised by Giesberger (Ph.D. thesis), supplemented with various sole carbon and nitrogen sources, has long served as the basis for determination of the nutritional requirements of spirilla (180, 198). A disadvantage of the formulation is the voluminous precipitate, which forms during autoclaving and which must be removed by filtration. Generally, several autoclavings and filtrations must be performed before a neutral, clear solution can be obtained (180, 198). Such treatment must affect the composition of the medium in undefined and non-reproducible ways. A different formulation devised by Hylemon et al. (83) has eliminated the problem of precipitates in freshwater defined media, but seawater defined media require sterilization by filtration. Giesberger reported that spirilla preferred the calcium salts of organic acids rather than the sodium salts; he considered that calcium ions were required for proper ionic balance in the media. Calcium salts of organic acids were also employed by Williams and Rittenberg (198); when other substrates were used, 0.05% $CaCl_2$ was added to the basal medium. Terasaki (180) used mainly the potassium salts of organic acids, but the basal medium included 0.05% $CaCl_2$. Hylemon et al. (83) found that greater growth responses of spirilla occurred in the absence of 0.05% $CaCl_2$, and it seems likely that Giesberger's original observations may be explained not by a requirement for calcium ions, but instead by the toxicity of sodium ions that has been demonstrated for a number of freshwater spirilla (41, 83, 164). However, it should be noted that certain strains of *A. serpens* have been shown to exhibit massive lysis when the stationary phase of growth is reached in calcium-deficient media (20); here, the calcium requirement for cell wall integrity could be replaced only by strontium and not by any other of a variety of divalent cations.

In comparing the sole carbon and nitrogen sources for spirilla as determined by various investigators, considerable variation is apparent from one investigation to another, even where the same strains were studied. Some of the variation is undoubtedly attributable to the different methodologies used. Furthermore, precise definitions of the size of the inocula used or of what constitutes a "positive growth re-

sponse" have usually been lacking. Growth responses with a given carbon or nitrogen source have usually not been confirmed by a second transfer. In the nutritional studies by Hylemon et al. (83) and by Kumar et al. (102), inocula and growth responses were defined turbidimetrically, and growth was measured after two transfers.

As useful as determinations of the sole carbon and nitrogen sources for spirilla have been, they contain certain limitations that should be considered in future investigations. In particular, the determination of sole carbon or nitrogen sources may well fail to indicate the full potential of the organisms to use various substrates; e.g., besides the lack of appropriate enzymes, inability of a strain to use a given substrate might be attributable instead to impermeability of the cells to the substrate. Also, as noted by Peck (143), the mode of regulation of a metabolic pathway might be such as to allow utilization of a compound for anabolism or catabolism without allowing it to serve as the sole carbon or nitrogen source. Hylemon et al. (83) suggested that a useful approach would be to test for the utilization of substrates by means of a medium containing enough peptone or yeast extract to permit a low basal level of growth. Stimulation of growth could reflect the ability to use a substrate even though the compound might not be able to support growth in the absence of the supplements.

The fact that most *Aquaspirillum* and *Oceanospirillum* species are able to grow with at least some sole carbon sources, with ammonium salts serving as the nitrogen source, indicates that the nutrition of spirilla is usually simple (83, 180). Most spirilla can use various tricarboxylic acid cycle intermediates, organic acids, or amino acids as carbon sources. Hylemon et al. (83) found that only *A. itersonii* and *A. peregrinum* can use carbohydrates as sole carbon sources, and the variety used is extremely limited. *A. itersonii* uses only fructose and glycerol (83); although the organism has all of the catabolic enzymes necessary for utilization of glucose, Hylemon et al. (81) have established that the cells are not permeable to this sugar. Hylemon et al. (83) and Terasaki (180) found that only a few species of spirilla could use nitrate as a sole nitrogen source. With regard to tricarboxylic acid cycle intermediates, organic acids, and amino acids, Terasaki (180) and Hylemon et al. (83) found that some species were quite versatile whereas others could grow with only a very limited range of compounds. Hylemon et al. (83) found that some spirilla failed to grow under their experimental conditions with any sole carbon or nitrogen source provided (*A. aquaticum*, *A. gracile*, and an unclassified strain [ATCC 12289]). In the case of *A. gracile*, the reason appears to be that the spirilla have a vitamin requirement (see below). Other possible reasons for failure to grow are a possible preference for microaerophilic conditions with the use of too small an inoculum (87, 89).

Defined Medium for *A. gracile*

Canale-Parola et al. (25) reported that *A. gracile* could grow in relatively simple, chemically defined media lacking an exogenous supply of vitamins. However, Hylemon et al. (83) reported that the same strains failed to grow in defined media used for determination of sole carbon or nitrogen sources of other aquaspirilla. A satisfactory defined (but not necessarily minimal) medium was subsequently devised for the type strain of *A. gracile* in our laboratory (B. E. Laughon, M.S. thesis, Virginia Polytechnic Institute and State University, Blacksburg, 1973); its formulation is presented in Table 5. With the omission of biotin from the medium, growth of washed inocula could occur initially but not upon subsequent serial transfer.

RESPIRATION OF SPIRILLA

All members of the genera *Aquaspirillum* and *Oceanospirillum* are capable of growth under aerobic conditions in complex liquid media or when forming colonies on complex

TABLE 5. *Composition of a defined growth medium for Aquaspirillum gracile ATCC 19624*[a]

Compound	Concn (g/liter)
Succinic acid (free acid)	0.1
L-Glutamine	0.1
L-Leucine	0.1
L-Phenylalanine	0.05
L-Aspartic acid	0.01
$(NH_4)_2SO_4$	0.1
$MgSO_4 \cdot 7H_2O$	0.1
K_2HPO_4	0.005
$FeCl_3 \cdot 6H_2O$	0.00047
$MnSO_4 \cdot H_2O$	0.00025
$CaCO_3$	0.0001
$ZnSO_4 \cdot 7H_2O$	0.000072
$CuSO_4 \cdot 5H_2O$	0.0000125
$CoSO_4 \cdot 7H_2O$	0.000014
H_3BO_3	0.0000031
$Na_2MoO_4 \cdot 2H_2O$	0.0000245
Biotin	0.0000010

[a] From B. E. Laughon (M. S. thesis, Virginia Polytechnic Institute and State Univ., Blacksburg, 1975). The pH is adjusted to 7.0 with KOH. The medium is sterilized by filtration.

solid media (83, 180, 198), but whether they all prefer aerobic conditions is another question. For example, Cayton and Preston (30) found the maximum growth of *S. mancuniense* (probably a member of *Aquaspirillum*) to occur ca. 5 mm below the surface in gelatin stabs. Although colonies grew aerobically in peptone agar, better growth was obtained at a pressure of 60 mm of Hg. Canale-Parola et al. (25) reported that *A. gracile* developed a few millimeters below the surface of semisolid peptone-yeast extract media and suggested that perhaps the spirilla preferred microaerophilic conditions. However, Laughon (M.S. thesis) recently tested the type strain of *A. gracile* in MPSS broth and also in a defined medium (Table 5) at oxygen concentrations of 6 to 21%. The highest growth responses, measured turbidimetrically, occurred at 21% oxygen (air atmosphere). Nevertheless, with small inocula or very low population densities, it is possible that microaerophilic conditions may be beneficial for the growth of at least some *Aquaspirillum* or *Oceanospirillum* species (87, 89).

Cytochromes of Spirilla

Cytochromes *b* and *c*, as well as terminal oxidases of the *o* type, have been demonstrated in *A. itersonii, A. serpens,* and *S. volutans* (37, 41). Cytochrome *a* could not be detected in these organisms.

The regulation of cytochrome synthesis in *A. itersonii* has been investigated by Clark-Walker et al. (37). Under conditions of low aeration, the levels of cytochromes *b* and *c* were considerably higher than for cells cultured under high aeration. A disproportionate increase in cytochrome *c* suggested that the two pigments were not under strict coordinate control. Despite the higher levels of cytochromes, the respiration rates with succinate or glutamate were not significantly different from those found for cells cultured under high aeration, and there did not seem to be any apparent advantage for the spirilla. Even under low aeration, both cytochromes were fully reducible by succinate and, therefore, apparently integrated with the electron transport chain. Iron deficiency severely inhibited cytochrome synthesis by limiting heme synthesis, but no increase in the apocytochrome occurred under these conditions. Further evidence for failure to form an apocytochrome was obtained by use of a hemin-requiring mutant (107). With regard to synthesis of the prosthetic group of the cytochromes, neither heme, porphobilinogen, nor porphyrins accumulated in the presence of iron when protein synthesis was prevented by chloramphenicol. These findings indicated that the syntheses of the heme group and apocytochrome were closely linked. The synthesis of the first enzyme of the tetrapyrrole biosynthetic pathway, δ-amino-levulinate synthase, was repressed by high aeration, and its activity was inhibited in cell extracts by hemin. These findings suggested a central role for the heme prosthetic group in the regulation of cytochrome synthesis.

Clark-Walker et al. (37) noted that *A. itersonii* synthesized higher levels of cytochromes *b* and *c* when nitrate was present in the medium. In the absence of nitrate, 15 to 25% of the cytochrome *c* was soluble (found in the supernatant fraction of cell extracts and occurring in the periplasmic space of whole cells [66]), whereas up to 45% was soluble when cells were cultured with nitrate (68). When *A. itersonii* was cultured anaerobically with nitrate, 45 to 60% of the cytochrome *c* was soluble. Nitrite was even more effective than nitrate in stimulating synthesis of the soluble pigment. Although the specific activity of nitrate reductase was also stimulated by addition of nitrate or nitrite, synthesis of active enzyme was not necessary for synthesis of soluble cytochrome *c*, because an *A. itersonii* mutant strain that lacked nitrate reductase activity synthesized increased amounts of soluble cytochrome *c* in response to nitrate or nitrite. In the absence of nitrate or nitrite, the mutant formed larger amounts of soluble cytochrome *c* than did the wild type. The function of the soluble pigment is not yet known. Although it may possibly serve as a physiological electron donor for nitrate reductase, as it does in certain other bacteria, attempts to demonstrate this with cell extracts of *A. itersonii* were unsuccessful. Because nitrite was more effective than nitrate in stimulating synthesis of soluble cytochrome *c*, even with the nitrate reductase-negative mutant, Gauthier et al. (68) suggested that the pigment might be concerned in the reduction of nitrite to ammonia.

A detailed study of the synthesis, assembly, and localization of the soluble cytochrome *c* has been made by Garrard (67). From labeled amino acid pulse-chase experiments with whole cells, he concluded that localization of the cytochrome in the periplasmic space occurred in less than 15 s after completion of the polypeptide chain. No soluble cytoplasmic or membrane-bound pool of the cytochrome existed to act as a source of the periplasmic form. It is possible that membrane-bound ribosomes are involved in the synthesis of such periplasmic proteins. Chloramphenicol prevented incorpo-

ration of iron into periplasmic cytochrome *c* but did not block heme synthesis. Strong evidence for the occurrence of a large pool of iron-tetrapyrrole precursor was obtained. The chemical nature of this precursor is not yet known. With regard to the relationship between synthesis of the apocytochrome and synthesis of the prosthetic group, the effect of inhibiting the synthesis of one component upon production of its counterpart was examined. Prosthetic group synthesis was not inhibited when protein synthesis was blocked. On the other hand, inhibition of heme synthesis by levulinic acid appeared to prevent synthesis of the apocytochrome. Although chloramphenicol did not inhibit heme synthesis, it did block iron incorporation into the cytochrome, indicating that assembly of the holocytochrome was tightly coupled to protein synthesis. These results were consistent with assembly and localization of the holocytochrome immediately after synthesis of the polypeptide, with the prosthetic group being derived from an iron-tetrapyrrole precursor pool. The results also suggested that the size of this pool may regulate the amount of apocytochrome synthesized.

The enzyme ferrochelatase (EC 4.99.1.1), which inserts Fe^{2+} into the protoporphyrin nucleus during heme biosynthesis, was shown by Dailey and Lasceles (45) to have high activity in *A. itersonii*. The enzyme was predominantly membrane bound, with only 10 to 15% of the activity occurring in the cytoplasm. The cytoplasmic activity is probably not physiologically significant, since a similar level of cytoplasmic activity occurred in a hemin-requiring mutant that lacked the membrane-bound enzyme. Protoporphyrin had less activity than mesoporphyrin or deuteroporphyrin when used as a substrate for membrane-bound enzyme. Fe^{2+} was the most effective metal for incorporation, although Zn^{2+} and Co^{2+} could also be incorporated. Solubilization of the enzyme from membranes was achieved by an unusual procedure involving the sequential use of sodium chloride, sodium perchlorate, and sodium thiocyanate. Although solubilization could also be achieved by use of Triton X-100, this treatment also solubilized ca. 50% of the total membrane proteins and did not greatly increase specific activity. In contrast, the use of the chaotropic reagents solubilized more than 80% of the enzyme, with release of less than 20% of the total membrane proteins. The solubilized enzyme had properties similar to those of the membrane-bound enzyme, except that succinate could no longer substitute for dithioerythritol for maintenance of iron in the ferrous state.

Nitrate Reduction

Although possessing a strictly respiratory type of metabolism, some members of *Aquaspirillum* can grow anaerobically in the presence of nitrate (nitrate respiration): *A. itersonii, A. psychrophilum,* and *A. fasciculus*. All of these except the last can reduce nitrate beyond the nitrite stage, but only *A. psychrophilum* forms visible gas. The nature of the gas is not known; where no visible gas is formed, it has been presumed that ammonia is the product of the nitrate dissimilation (68).

The nitrate reductase of *A. itersonii* has been investigated by Gauthier et al. (68). Growth curves of *A. itersonii* incubated with nitrate under low aeration were of the diauxic type, the second phase occurring shortly after the appearance and subsequent disappearance of nitrite, when oxygen had become limiting. Synthesis of nitrate reductase appeared to be repressed by high aeration, even in the presence of nitrate. Under anaerobic conditions or low aeration, the enzyme was formed in the presence or absence of nitrate, but the presence of nitrate stimulated its synthesis. Nitrite was more effective as an inducer during the early stages of incubation of cell suspensions. Although dissimilatory nitrate reductases in bacteria are usually membrane associated, 85 to 100% of the nitrate reductase activity of *A. itersonii* was found in the supernatant portion of cell extracts after centrifugation at 105,000 × *g* for 2 h, conditions under which release of membrane-bound reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase activity did not occur. Further evidence for the soluble nature of the reductase was its occurrence as a homogeneous peak in sucrose gradient profiles of the soluble portion of cell extracts. The in vivo electron donor for the reductase is not known, although, as noted previously, soluble cytochrome *c* might possibly fulfill this function.

Catalase Activity

Although most spirilla clearly exhibit catalase activity, *A. putridiconchylium, S. volutans,* and certain members of *Oceanospirillum* exhibit negative or extremely weak reactions (82, 83, 180). Terasaki (180) tested catalase activity by adding 2 to 3 ml of 3% hydrogen peroxide to nutrient agar or seawater-nutrient agar slant cultures. By this method, several spirilla gave no indication of forming oxygen bubbles. Hylemon et al. (83) added 4 to 5 drops of peroxide to cultures grown in semisolid (0.15% agar) PSS medium. In several cases, the development

of a small froth of bubbles occurred only after 15 to 30 min of incubation. Uninoculated controls exhibited no such bubbles. Hylemon et al. suggested that the use of a semisolid medium might have increased the sensitivity of the test by aiding the entrapment and accumulation of oxygen bubbles. Despite the use of uninoculated controls, however, such weak reactions may possibly have been artifacts. Alkalies are known to cause the decomposition of hydrogen peroxide, and, because of the oxidation of succinate by growing cultures, the pH of the culture medium may increase markedly. Cole and Rittenberg (41) reported final pH values of 8.1 to 8.4 in cultures grown in PSS broth. Although no oxygen bubbles are formed within 30 min upon addition of peroxide to sterile, semisolid PSS medium adjusted to pH 8.1, the present author has noted the occurrence of a few bubbles at pH 8.5, with stronger reactions occurring at higher pH values. Consequently, the extremely weak catalase reactions reported for some spirilla should be regarded with caution. In one case, however, such a weak reaction has been confirmed by the use of washed bacterial suspensions and a polarographic electrode: Cole and Rittenberg (41) found *S. volutans* to have a catalase activity of 2 μmol/g of protein per h. This should be compared with activities of 16 to 75 μmol/g of protein per h found for *A. serpens* and *A. itersonii*, respectively.

Microaerophilism of *S. volutans*

Cole and Rittenberg (41) compared the respiration of the microaerophile *S. volutans* with that of two aerobic spirilla, *A. serpens* and *A. itersonii*. The liquid medium used for cultivation of *S. volutans* in an air atmosphere was boiled and cooled before use to expel excess dissolved oxygen. Without this treatment, an inoculum size of 4×10^3 cells per ml failed to grow. Cell suspensions prepared in 0.05 M phosphate buffer were tested for rates of oxygen consumption with various substrates by using a polarographic electrode. With succinate, one of the best oxidizable substrates for *S. volutans* (27), the rate of oxygen consumption (nanomoles of oxygen per milligram of protein per minute) was 30, compared with higher rates for *A. serpens* and *A. itersonii* (127 and 60, respectively). Cole and Rittenberg (41) also found that the levels of activity for various tricarboxylic acid cycle enzymes were considerably lower for *S. volutans* than for the other spirilla. Fumarase (EC 4.2.1.2) and malate dehydrogenase (EC 1.1.1.37) activities were especially low. The levels of activity of catalase, $NADH_2$ oxidase, and cytochrome oxidase for *S. volutans* were also relatively low, although the cytochrome oxidase activity was still higher than the respiration rate and was unlikely to have limited the rate at which oxygen could be reduced. Although all three spirilla synthesized appreciable quantities of cytochromes *b* and *c* (but not *a*-type cytochromes), the level of cytochrome *c* in both soluble and cell wall membrane fractions of *S. volutans* was lower than for the other spirilla.

Cole and Rittenberg (41) suggested that the slower growth and respiration rates of *S. volutans* might be explained on the basis of the low activities of fumarase and malate dehydrogenase in this organism. With succinate as the major energy source, these deficiencies would leave succinic dehydrogenase (EC 1.5.99.1) as the primary supplier of electrons to the electron transport chain. The low activity of this enzyme could then result in a low yield of adenosine 5′-triphosphate (ATP) and low growth and respiration rates. As suggested by Cole and Rittenberg, the low respiration rate might allow a relatively high intracellular concentration of oxygen; thus, a cellular component—equally sensitive to oxygen in all three spirilla—would be damaged in *S. volutans* but protected in the other two spirilla.

Cole (33) incubated cultures microaerobically for 12 h and then aerated them for 8 or 12 h. Such cultures were compared with cultures incubated continuously under microaerobic conditions. The growth yield after 12 h of aeration was threefold greater than that from the microaerobic cultures. The respiration rate per milliliter after 8 h of aeration was twice that of the microaerobic cultures, but by 12 h the rate had fallen to half of that for the microaerobic cultures. However, addition of succinate to the medium after 8 h of aeration increased the growth yield and the respiratory rate, and the spent culture medium supported growth only if more succinate was added. These results suggested that succinate was exhausted after 8 to 12 h of aeration. Aeration for 8 h increased the specific activities of fumarase, malate dehydrogenase, aconitase (EC 4.2.1.3), and isocitrate dehydrogenase (EC 1.1.1.42). Succinic dehydrogenase activity was decreased slightly. After 12 h of aeration, however, fumarase and malate dehydrogenase activities were undetectable, while the other enzyme activities had not changed appreciably from the 8 h level.

Cole proposed the following hypothesis to account for the microaerophilism of *S. volutans*. During active growth with succinate as an oxidizable substrate, the level of intracellular dis-

solved oxygen may be low because of reduction of oxygen by the electron transport chain at the cell surface. The rate of oxygen reduction decreases when succinate becomes exhausted, so that oxygen or toxic oxidation products (such as peroxides) could accumulate within the bacteria. The oxygen or oxidation products might inhibit the synthesis of key catabolic enzymes (such as fumarase or malate dehydrogenase), resulting in insufficient ATP synthesis for maintenance of viability. Loss of viability could be prevented by transferring cells to fresh but oxygen-depleted (microaerobic) medium, or could be accelerated by increased exposure to oxygen, particularly in the absence of an oxidizable substrate.

The very low levels of catalase present in *S. volutans* (41, 82) suggest that perhaps toxic levels of hydrogen peroxide might be allowed to accumulate under aerobic conditions. This seems to be an unlikely explanation, however, since other spirilla with catalase activity so weak as to be nondetectable by ordinary tests (e.g., *A. putridiconchylium* and *O. japonicum*) are capable of aerobic growth. Moreover, Hylemon et al. (83) reported that the addition of exogenous catalase to cultures of *S. volutans* failed to allow aerobic growth. The possibility still exists, however, that low catalase activity might be a contributory factor to the oxygen toxicity. Superoxide dismutase levels have not yet been determined in *S. volutans,* but this enzyme might well play an important role in microaerophilism because of its ability to destroy highly reactive superoxide radicals (O_2^-) commonly formed as intermediates of oxygen reduction (63).

Bowdre et al. (16) recently described a defined medium for *S. volutans* that permitted growth to occur in an air atmosphere under static incubation. In their initial studies, Bowdre et al. found that addition of tyrosine from a stock solution prepared in 2 N KOH and sterilized by autoclaving was responsible for the enhancement of aerotolerance. When the tyrosine was sterilized by filtration, it was ineffective. In 1956 Gorini and Lord (70) noted that autoclaved alkaline tyrosine was stimulatory for the growth of a *Sarcina* and that the tyrosine could be replaced by a low concentration (5×10^{-5} to 5×10^{-7} M) of various dihydroxyphenyl compounds such as norepinephrine. Bowdre et al. (16) found that such compounds (norepinephrine, epinephrine, and 3,4-dihydroxyphenylalanine) were effective at 10^{-5} to 10^{-6} M in enhancing the aerotolerance of *S. volutans* and could substitute for the autoclaved alkaline tyrosine. This suggested that when tyrosine is autoclaved in alkaline solution a small portion of the tyrosine may be oxidized to a dihydroxyphenyl form. With an inoculum of 2.1×10^4 cells of *S. volutans* per ml, growth failed to occur in air unless dihydroxyphenyl compounds were supplied, whereas growth could occur under microaerobic conditions (6% oxygen) in the absence of the compounds. Dihydroxyphenyl compounds were also effective when used at 2×10^{-4} M in permitting growth of an obligately microaerophilic strain of *Campylobacter fetus* (subsp. *jejuni*) to occur at 17 or 21% oxygen on streaked plates of brucella agar.

Hutner (80) noted that the stimulatory effects of dihydroxyphenyl compounds on bacterial growth are probably due to their ferric iron-binding properties. Lankford (103) has summarized many studies indicating that little soluble ferric iron occurs in aerobic environments and that aerobic and facultative bacteria synthesize various ferric iron-binding compounds, including dihydroxyphenyl compounds, to solubilize and transport ferric iron. Bowdre et al. (16) suggested the possibility that a lack of available iron might be responsible for the microaerophilism of *S. volutans* and *C. fetus*. This was supported by the finding that an iron-binding agent which was not stimulatory for the aerotolerance of *C. fetus,* Desferal (methane sulfonate derivative of deferri-ferrioxamine B), antagonized the stimulatory effect of norepinephrine. Moreover, *C. fetus* exhibited aerotolerant growth on streaked plates of brucella agar when high concentrations of iron salts were used in place of low concentrations of dihydroxyphenyl compounds. Bowdre et al. (16) proposed that microaerophiles might be unable to synthesize iron transport compounds at rates sufficient to permit a suitable supply of available iron for aerobic growth. They suggested that the addition of low levels of dihydroxyphenyl compounds or high levels of iron salts might relieve this deficiency. The increased supply of available iron might possibly be used for synthesis of iron-containing enzymes such as succinic dehydrogenase or cytochromes. Higher levels of these enzymes might lead to greater reduction of oxygen at the cell surface, keeping oxygen from reaching vital cell components in the cell interior (41). The levels of other iron-containing enzymes, which protect cells from the toxic products of oxygen metabolism, e.g., catalase or ferrosuperoxide dismutase (71), might also be increased. It is perhaps significant that hematin has been reported to be stimulatory for the growth of certain strains of *C. fetus* (14) and that exogenous catalase has been used to reduce the minimum inoculum size of *C. fetus* (98).

SUGAR CATABOLISM AND TRANSPORT BY SPIRILLA

Members of *Oceanospirillum* and *Spirillum* do not catabolize sugars, but a few species of *Aquaspirillum* (*A. itersonii, A. peregrinum,* and *A. gracile*) can catabolize a very limited variety of sugars. Such spirilla are incapable of growing anaerobically with sugars (although acidification of sealed tubes of sugar media has been reported [83, 180]), and their sugar catabolism is essentially oxidative rather than fermentative.

D-Fructose is the only hexose known to be catabolized by *A. itersonii* (83). Hylemon et al. (81) found that fructose induced high activities of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), glucokinase (EC 2.7.1.2), 6-phosphogluconate dehydratase (EC 4.2.1.12), and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.1.14). The latter two enzymes are characteristic of the Entner-Doudoroff pathway. The activities of fructose-1,6-diphosphate aldolase (EC 4.1.2.13), characteristic of the Embden-Meyerhof-Parnas pathway, remained at an essentially constant and relatively low level, irrespective of the carbon source for growth. 6-Phosphogluconate dehydrogenase (EC 1.1.1.44), characteristic of the hexose monophosphate pathway, could not be detected despite exhaustive efforts. Although succinate was a more effective carbon source for growth of *A. itersonii* than fructose, it did not act as a catabolite repressor of the catabolic enzymes induced by fructose, in contrast to the strong and immediate repression of inducible hexose catabolic enzymes by succinate in another aerobic oxidative organism, *Pseudomonas aeruginosa* (P. B. Hylemon and P. V. Phibbs, Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, P154, p. 161).

An interesting anomaly in the carbohydrate catabolism of *A. itersonii* was that, although the complete enzymatic complement necessary for glucose catabolism was present (including glucokinase), glucose could not serve as a carbon source for the organism (81, 83). This was explained by an investigation of the substrate saturation kinetics for D-fructose and D-glucose uptake by whole cells, which indicated the presence of a carrier-mediated transport system for D-fructose but not for D-glucose. Thus, the inability to catabolize glucose appears to be due to the impermeability of the cells for this substrate. A further anomaly, not yet resolved, was that, although D-fructose could be transported into the cells and could serve as an energy source for growth, an initial ATP-dependent phosphorylation of fructose to fructose-6-phosphate could not be demonstrated. The inducible glucokinase was not able to use fructose as a substrate. Hylemon et al. (81) suggested that a phosphoenolpyruvate:hexose phosphotransferase system might possibly be present, which could simultaneously transport and phosphorylate fructose.

A. gracile has been shown to produce acidification of the medium only when grown with D-glucose, D-galactose, or L-arabinose (83). Laughon and Krieg (108) demonstrated the occurrence of enzymatic activities characteristic of the Entner-Doudoroff and Embden-Meyerhof-Parnas pathways in cells cultured with glucose, but activity of the representative enzyme of the hexose monophosphate pathway, 6-phosphogluconate dehydrogenase, could not be detected. As with *A. itersonii* (81), the activity of fructose diphosphate aldolase was relatively low, suggesting that the Embden-Meyerhof-Parnas pathway may not be of great importance for the two species. The activities of 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase, as well as glucose-6-phosphate dehydrogenase, were 3.5- to 10-fold higher for cells cultured with glucose than for cells cultured with succinate, suggesting a possible induction by glucose or derepression by the absence of succinate. The latter possibility seemed less likely than the former in view of the low activities of most of the Entner-Doudoroff enzymes in cells cultured with arabinose or galactose in the absence of succinate.

The existence of an additional mechanism for glucose catabolism in *A. gracile* was indicated by the occurrence of activities for a soluble NAD-linked glucose dehydrogenase (EC 1.1.1.47) and for gluconokinase (EC 2.7.1.12) (108). The 6-phosphogluconate so formed may be further catabolized by 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase via the Entner-Doudoroff pathway. It was unlikely that gluconic acid (formed as a result of glucose dehydrogenase activity) could be oxidized to 2-keto- or 5-ketogluconic acid, because of the lack of detectable 2-keto- or 5-ketogluconate reductase (EC 1.1.1 group, EC 1.1.1.69) activity.

The nature of the products of sugar catabolism responsible for acidification of sugar media by spirilla has been investigated only for *A. gracile* (108). Paper chromatographic analysis of the spent culture supernatant media from glucose-cultured cells indicated an accumulation of gluconic acid, and this was confirmed by the use of gluconokinase. 2-Keto- and 5-ketogluconic acids were not detectable. Cells cultured with either D-galactose or L-arabinose possessed high specific activities for both soluble NAD-linked galactose dehydrogenase (EC

1.1.1.48) and soluble NAD-linked arabinose dehydrogenase (EC 1.1.1.46), and the results of analysis of spent culture supernatant media by paper chromatography and by use of gluconokinase were consistent with the formation of galactonic acid from galactose and of arabonic acid from arabinose.

MOTILITY AND FLAGELLA OF SPIRILLA

Number, Configuration, and Behavior of Flagella

Spirilla typically swim rapidly back and forth in straight lines and exhibit a characteristic vibratory appearance while swimming. These characteristics are also shared by vibrios. Members of *Spirillum, Aquaspirillum,* and *Oceanospirillum* typically possess bipolar tufts of flagella, but some exceptions occur. *A. polymorphum* and *A. psychrophilum* possess mainly bipolar, single flagella, and *A. delicatum* possesses mainly one or two flagella at only one pole (83, 181).

Certain species of spirilla that were initially thought to possess bipolar, single flagella, as observed by flagella staining, were later found to possess bipolar tufts when electron microscopy was employed (195). The error was attributable to the tendency of the flagella in a tuft to aggregate, forming a coherent fascicle. Caraway and Krieg (26) found that when cells of *S. volutans,* which has large bipolar flagellar fascicles that can be seen easily by darkfield microscopy (101, 129), were washed in distilled water the flagellar fascicles were no longer visible; however, the addition of as little as 0.001 M NaCl or other salts rendered the fascicles visible again. This observation suggests that neutralization of electrical charges is probably responsible for the aggregation of the individual flagella.

As noted by Leifson (109), the typical flagella of spirilla have a very long wavelength (over 3 μm) and usually less than one complete wave. This seems generally true of the large spirilla (e.g., *A. serpens, A. bengal, A. anulus,* etc.), but at least one large spirillum (*O. maris*) and spirilla having a medium or small diameter may exhibit flagella with several waves and a short wavelength (83).

The bipolar fascicles of *S. volutans* are extremely large (Fig. 5) and consist of ca. 75 individual flagella (196). The fascicles do not appear to be corkscrew shaped or helically curved but rather seem to be crescent shaped in a single plane. As first described by Metzner (129) in 1919, the front fascicle appears to rotate in the form of a wide bell opened toward the back, while the rear fascicle seems to rotate in the form of a wide goblet opened away from the end of the cell (Fig. 6a and b). According to Metzner, the mechanical effect of the flagella is to cause the cell body to rotate in a direction opposite to that of the flagella. Since the cell body is helical, its rotation causes the cell to move forward. As described by Chwang et al. (34), this mechanism is analogous to the movement of a corkscrew, the fluid reaction to the rotation of the polar flagella acting in a manner analogous to a "screwdriver." This mechanism for motility is to be contrasted with the more familiar one for most flagellated bacteria, in which the rotation of a corkscrew-shaped flagellum provides the propulsion for a straight rod. A study of the hydrodynamics of *S. volutans* locomotion according to this model has been made by Chwang et al. (34).

An important point must be noted with respect to the fascicles of *S. volutans*. To an observer looking at *S. volutans* from the side (as in Fig. 6a or b), both polar fascicles would appear to be rotating in the same direction. However, if an observer were to be located theoretically inside the cell, looking first toward one pole and then toward the other, the direction of apparent rotation of one polar fascicle would be opposite to that of the other fascicle. Another important point to be made is that electron micrographs of the flagellar fascicles of *S. volutans* clearly indicate that each individual flagellum in a fascicle has a separate insertion point in the cell wall (92, 196). Consequently, although the flagellar fascicles appear to rotate about the cell, it cannot be that they are rotating as a unit. However, it is possible that each individual flagellum within a fascicle might rotate. It is also possible that each individual flagellum might merely wave about in a circular manner, by means of circumferential periodic contraction, without actually turning. Using an organism identified as *S. volutans* (but which possessed a long, helical fascicle rather than the shorter, crescent-shaped fascicles characteristic of this species), Mussill and Jarosch (137) obtained evidence that seemed to support the concept of rotation rather than contraction. When squeezed between two slides to immobilize the flagellar fascicle (the latter having a helix diameter larger than that of the cell body), the cell body was seen to rotate. The situation seemed to be analogous to the turning of the field coils of a freely suspended electric motor when the armature is prevented from turning. However, Harris (74) has noted that even if the rotation of a flagellum were to be apparent rather than actual, prevention of the apparent rotation would still cause the cell body

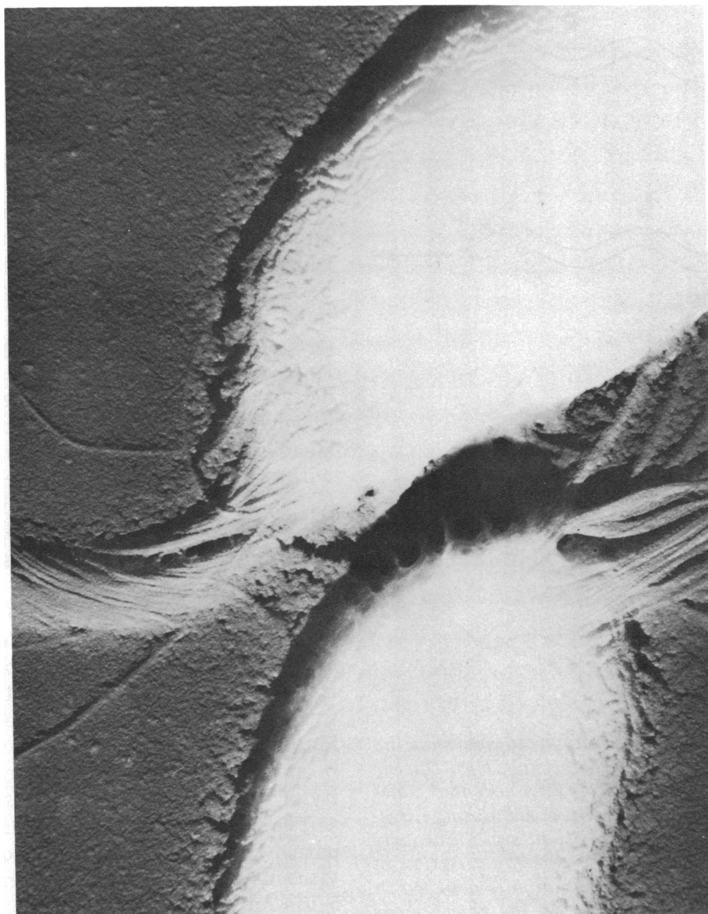


FIG. 5. *Ends of two cells of Spirillum volutans showing the large, polar flagellar fascicles. Shadowed with tungsten oxide. Ca. ×40,000.*

to rotate. Therefore, the possibility that the individual flagella of *S. volutans* are capable of actual rotation is not yet established. However, strong evidence has been obtained that the flagella of *E. coli* are capable of actual rotation (163) and also that the direction of rotation can be reversed (106), and in view of this it would seem unlikely that the flagella of *S. volutans* would operate in a completely different manner.

Although many other spirilla exhibit flagellar behavior similar to that of *S. volutans* (i.e., fascicles describing cones of revolution), some spirilla exhibit the more conventional type of behavior—wave propagation along a helical fascicle. Linn and Krieg (unpublished data) observed that *O. maris* (ATCC 27509) possessed helical flagellar fascicles having two to four helical waves, as seen by darkfield microscopy. Both front and rear fascicles were always extended out from their respective cell poles regardless of the direction of swimming or reversal of swimming direction. During swimming, the front fascicle exhibited wave propagation from tip to base, while the rear fascicle exhibited it from base to tip. When a cell reversed its direction of swimming, both fascicles appeared to reverse their direction of rotation, judging from the reversal of the direction of wave propagation along the fascicles. It is possible, however, that the front fascicle may not be active during swimming but instead may merely rotate with the cell body; in this case, the rear fascicle would provide all of the propulsion. This possibility is suggested by observations of cells that were adherent to the slide by the cell body and one fascicle, with only the other fascicle free to rotate. Wave propagation along the latter was invariably from base to tip, never the reverse. This situation may not necessarily be comparable to that for a free-swimming cell, however.

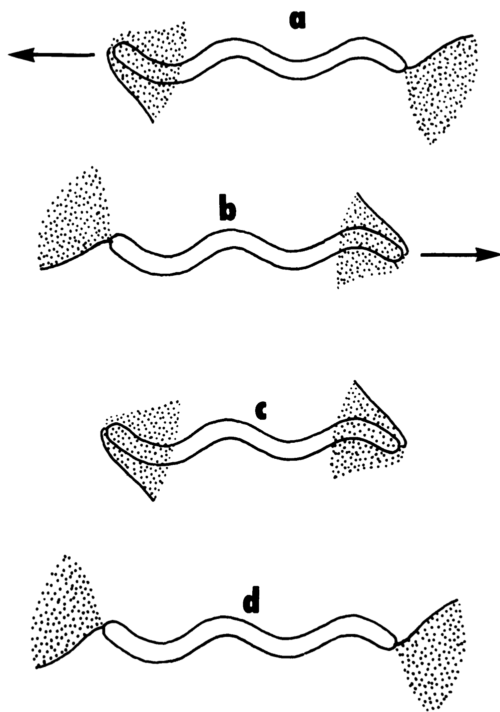


FIG. 6. *Diagram of Spirillum volutans showing orientation of the bipolar flagellar fascicles. During normal swimming, the fascicles form oriented cones of revolution (a). During reversal of swimming direction, both flagellar fascicles reorient simultaneously (b). Arrows in (a) and (b) indicate the direction of swimming. Dual-head uncoordination is depicted in (c): both fascicles have assumed the forward or "head" configuration. Although the fascicles actively rotate, the cell is motionless because of the opposing action of the flagella. Dual-tail uncoordination is depicted in (d): both fascicles have assumed the rear or "tail" configuration. Again, despite the activity of the flagella, the cells are unable to swim. (Reproduced by permission of the Virginia Water Resources Research Center from: J. H. Bowdre and N. R. Krieg, Va. Polytech. Inst. State Univ. Water Resour. Res. Cent. Bull. no. 69, 1974)*

In 1953 Houwink (78) described a "rod-like" *Spirillum* that was generally unable to swim in a normal fashion. Instead, the organism was described as having a "floundering" motility. Large bipolar flagellar fascicles were present, and in nonmotile cells these were seen to assume the form of a coiled spring. A few cells could swim in a straight line for ca. 100 μm, and in these the front flagellar fascicle appeared to be wound around the cell body while the rear fascicle was extended behind the cell. When the cells reversed direction, the flagellar fascicles changed their orientation. In 1969, Jarosch (93) described a similar rod-shaped organism found in mud. Again, this organism could not swim freely, but it could move about on surfaces. When moving in this manner, the rear flagellar fascicle was extended and exhibited propagation of helical waves, while the front fascicle was wound backward around the cell. Organisms morphologically similar to those described by Houwink and by Jarosch were isolated by Strength and Krieg (174) and on the basis of many characteristics have been considered, despite their rod shape, to be members of genus *Aquaspirillum* (173). One of the most interesting aspects of the flagellar fascicles was their ability to coil up frequently like springs (Fig. 7). Despite the intense flagellar activity, the cells were unable to swim freely; however, under certain conditions of growth, steady straight-line motility was observed within the viscous flocs characteristically formed by the organisms on initial isolation. In viscous suspensions of cells prepared by homogenization of such flocs in a small quantity of water, steady straight-line free swimming could be observed; here, the "tail" fascicle was extended behind the cell, while the "head" fascicle was either coiled into a polar loop or was coiled around the cell. Non-floc-forming cultures, occurring after prolonged transfer, were found to exhibit poor motility when suspended in media of low viscosity, but when the viscosity of the medium was increased nearly every cell could swim freely and steadily in straight paths. One strain was found to swim best at a viscosity of 200 cP, but its velocity was slow (ca. 5 μm/min) compared with most other types of flagellated bacteria. The results suggested that the organisms, although apparently defective in their motility by ordinary standards, may be highly adapted to life within viscous flocs. The high viscosities that are optimal for the motility of *A. fasciculus* are inhibitory for the motility of other flagellated bacteria (154).

Coordination and Uncoordination in *S. volutans*

While swimming, *S. volutans* frequently reverses direction, with the cell body reversing its corkscrew-like rotation. This appears to be caused by the simultaneous reversal of the rotation and also of the orientation of both flagellar fascicles (Fig. 6a and b). Cinephotomicrography has shown that both fascicles change their orientation within 0.06 s (101), even though the leading end of the cell must receive the stimulus for such change (e.g., an unfavorable oxygen concentration [191]) before the other and a large distance (up to 60 μm) exists between the two cell poles. This observation strongly indicates that some kind of coordination mechanism exists between the cell poles (101).

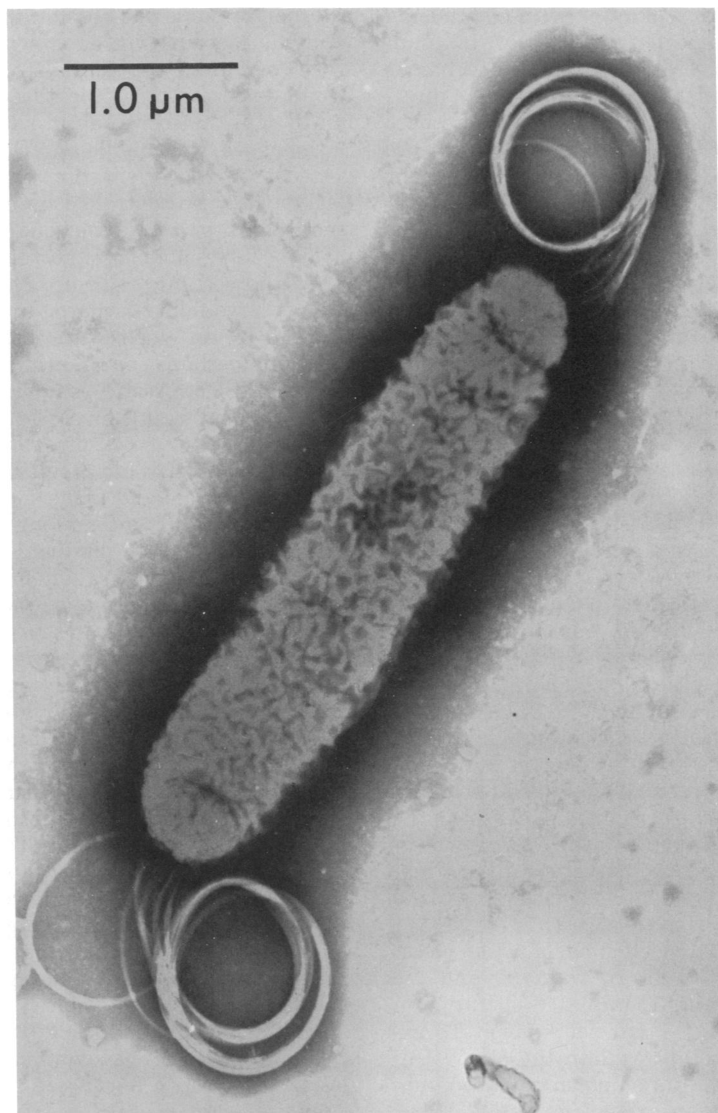


FIG. 7. *Formalin-fixed cell of Aquaspirillum fasciculus showing the bipolar fascicles of flagella coiled into loops. When the flagellar fascicles are extended, they have a helical configuration with several waves. Despite the rod shape of the organism, many other characteristics indicate that the organisms are members of the genus Aquaspirillum. (Reproduced by permission of the National Research Council of Canada from: W. J. Strength and N. R. Krieg, Can. J. Microbiol. 17:1133–1137, 1971)*

Metzner (129) was the first to find that certain chemical compounds could cause uncoordination of the polar fascicles of *S. volutans*. With some compounds, such as chloroform or ether, both fascicles would assume the front or "head" type of orientation (dual-head uncoordination) as depicted in Fig. 6c. With other compounds, such as halogen salts, both fascicles would assume the rear or "tail" orientation (dual-tail uncoordination) as depicted in Fig. 6d. In both cases, the cell body was motionless despite the active rotation of the flagellar fascicles. As indicated by Metzner, both fascicles would be trying to turn the cell body but, because they were rotating in opposite directions (from the standpoint of an observer looking at the cells from the side), the cell body could not respond. Krieg et al. (101) expanded the spectrum of effective uncoordinating compounds for *S. volutans* and determined their optimal concentrations, using pure cultures. They also found that the simultaneous application of an agent caus-

ing dual-head uncoordination with one causing dual-tail uncoordination would result in a null point at which coordinated flagellar behavior and swimming (with frequent reversal of direction) could occur; here, both flagellar fascicles were intermediate in orientation between the head and tail type, appearing to be at right angles to the cell axis. Caraway and Krieg (26) described a different type of dual-tail uncoordination caused by application of $HgCl_2$ or polyethylene glycol, in which the cones of revolution for each fascicle were unusually narrow (dual-straight-tail uncoordination). The effect of polyethylene glycol was interesting since this compound is nonmetabolizable and relatively inactive chemically. Caraway and Krieg also noted that certain uncoordinating agents could cause more than one type of uncoordination; e.g., ethanol caused cells to exhibit either dual-head, dual-tail, or dual-straight-tail uncoordination, with any given cell being committed to one or another of these types. High or low pH values were also effective in causing uncoordination. A pH of 4.4 caused cells to exhibit dual-tail uncoordination, whereas a pH of 9.9 caused dual-head uncoordination. Uncoordination caused by metal salts was found to be reversed by application of metal-chelating agents. Uncoordination caused by agents such as phenol, chloral hydrate, polyethylene glycol, or phenethyl alcohol could be reversed by washing the cells free from the agent. In the case of uncoordination by high or low pH values, recoordination could be achieved by readjusting the pH to 6.8.

The fundamental mechanism of action of uncoordinating agents for *S. volutans* and the nature of the normal coordinated operation of the flagellar fascicles are not known. To explain one would be to explain the other. Uncoordinating agents are known to cause substantial decreases in oxygen uptake (26), but this is not related to uncoordination: normal coordinated states have been maintained for prolonged periods in the absence of oxidizable substrate even in the presence of cyanide—where no detectable oxygen uptake was occurring. Although it might seem that coordination is passively produced by the forward motion of the spirilla through the medium, sweeping the flagella backwards, the occurrence of dual-tail or dual-head orientations in the absence of cell motility clearly indicate that flagellar orientation is not dependent on movement of the cell through a medium. A number of other possibilities have been suggested for coordination, most of which concern the cell membrane. The generation of an action potential along a polarized cell membrane is one possibility but, although many uncoordinating agents might interfere with membrane potentials, certain other agents expected to act on the cell membrane fail to cause uncoordination (26). Doetsch and Seymour (55) and Biddle and Doetsch (12) suggested that the orientation of head and tail flagella may be controlled by electrostatic attraction or repulsion, based on the presence of a mobile double-ionic layer surrounding the cell. When the cell moves in a dielectric medium, this layer would be displaced toward the posterior end by an electrostatic drag, inducing a lower and perhaps opposite charge at the anterior end. Electrostatic repulsion of the rear flagella would result in a "tail" orientation, whereas less repulsion, or even attraction, of the front flagella would result in a "head" configuration. Biddle and Doetsch (12) noted that, if the charge differences along the bacterium are not continuously dissipated into the medium at rates equal to their formation, there will ultimately occur an attraction between the differential head and tail charges sufficiently strong to induce flagellar reorientation and reversal of direction of swimming. Presumably, uncoordinating agents would interfere with the dielectric constant of the medium. Certain experimental evidence supports the concept that flagellar orientation is dependent on the bioelectric characteristics of bacterial surface layers. Harris and Kline (73) found that alignment of motile cells of *A. serpens* occurred in an electric field. Although motile cells of *S. volutans* did not show such alignment, Caraway and Krieg (26) found that flagellar reorientation and reversal of swimming direction occurred when the field polarity was suddenly reversed or even when the circuit was simply broken or closed. Cells swimming at right angles to the electric field were generally unaffected.

Practical Applications of Flagellar Uncoordination

Using a defined motility medium, Bowdre and Krieg (15) developed a simple method whereby uncoordination of *S. volutans* could be used potentially for the biological in-plant monitoring of the levels of toxicants in industrial effluents. The spirilla were found to be incapable of motility in the defined medium in the presence of zinc, nickel, copper, mercury, or lead ions at concentrations of 2 or 3 μg/ml. The sensitivity to zinc ions was comparable to that of monitoring systems using fish, and the results could be obtained in a matter of minutes rather than hours. Biological monitoring has

received considerable attention as a useful supplement to (but not a substitute for) monitoring by chemical or physical methods (24). Although such monitoring will not identify the pollutants present in a complex effluent, it could indicate the occurrence of an adverse biological effect that might be difficult to predict by analyzing physical and chemical parameters alone.

Source of Energy for Motility of Spirilla

The possibility exists that the energy source for the motility of flagellated bacteria might be ATP, although recent evidence by Larsen et al. (105) indicates that, at least in *E. coli*, a chemomechanical coupling to the respiratory chain is involved instead. In an attempt to determine whether adenosine 5'-triphosphatase activity might be present in high levels at or near the basal structures of bacterial flagella, Vaituzis (182) employed a cytochemical method in which the formation of insoluble lead phosphate indicated the location of the enzyme activity. In thin sections of *A. serpens*, no adenosine 5'-triphosphatase activity appeared to be preferentially associated with the regions in which the polar flagella had their origin; instead, activity seemed to be located uniformly around the cell in the periplasmic space. This was in contrast to *Vibrio metchnikovii*, where large concentrations of lead phosphate were found in the cytoplasm at the poles and none was found in the periplasmic space around the cells. *Bacillus lichenformis*, a peritrichously flagellated organism, showed deposits of lead phosphate in the cytoplasm near the base of the flagella.

Although *S. volutans* possesses a strictly respiratory metabolism for growth, with oxygen as the electron acceptor, Caraway and Krieg (27) found that coordinated reversing motility could occur for long periods in a non-nutritive defined medium containing no oxidizable substrate. Motility continued even upon addition of KCN. Even in the absence of this respiratory inhibitor, no oxygen uptake by the cells could be detected (by manometric methods). The addition of diethyl-*p*-nitrophenyl phosphate, an esterase inhibitor that has been shown to markedly inhibit PHB depolymerase in *Micrococcus halodenitrificans* (162), quickly abolished motility of cells suspended in defined motility medium in the absence of an oxidizable substrate but had no effect when an oxidizable substrate was supplied unless KCN or 2,4-dinitrophenol was also added. In capillary tube experiments where an aerotactic band of spirilla migrated in response to a self-created oxygen gradient formed by oxidation of succinate, cells were motile in the anaerobic region of the capillary behind the migrating band unless diethyl-*p*-nitrophenyl phosphate was present. These results suggested that the energy for motility of *S. volutans* is generated by either of two alternative means: (i) by respiration with an oxidizable substrate or, if this is prevented, by (ii) another means not dependent on respiration. Although the specificity of diethyl-*p*-nitrophenyl phosphate is not known, its reported effect on PHB depolymerase suggests that PHB might serve as the source of energy for (ii) above.

Structure and Chemistry of Flagella and Related Organelles

Structural details of the proximal ends of the polar flagella of *A. serpens* and their mode of attachment in the cell envelope have been studied by electron microscopy, using cells damaged by *Bdellovibrio bacteriovorus* (D. Abram, Bacteriol. Proc., 1969, p. 29). In cells whose flagella had been removed prior to exposure to the parasite, protoplasts retracted uniformly from the cell wall, and the flagellar insertion sites could be visualized as pits, 12 nm in diameter, the ends of the cells being reminiscent of the top of a saltcellar. In flagellated cells that had lost most of their cytoplasm, each flagellum was seen to originate within the protoplast in a individual spherical vesicle, 38 to 43 nm in diameter, having a delicate envelope and enclosing dense material. The mode of attachment of such polar flagella appeared to be similar to that found in gram-negative bacteria having peritrichous flagella.

In thin sections of the polar region of *A. serpens*, Murray and Birch-Andersen (135) found that the flagella passed through the cell wall and the plasma membrane, terminating just below the latter in the form of a small button or knob (possibly corresponding to the bottom rings associated with the flagellar base of other gram-negative bacteria such as *E. coli* [51]). On either side of this area, ca. 20 nm below the cell membrane, occurred a thin membranous component connected to the cell membrane by short barlike links (Fig. 8). Serial sections indicated that this structure, termed "polar membrane," occurred in the form of a hemispherical cap, with the hole in the center located directly beneath the region where the flagella entered. This structure has since been observed in the other helical bacteria: *Rhodospirillum rubrum* (76), *R. molischianum* (75), *Ectothiorhodospira mobilis* (149), and *Campylobacter fetus* (97, 150). It has also been observed in *Aquaspirillum fasciculus*, a straight rod considered to be essentially an *Aquaspiril-*

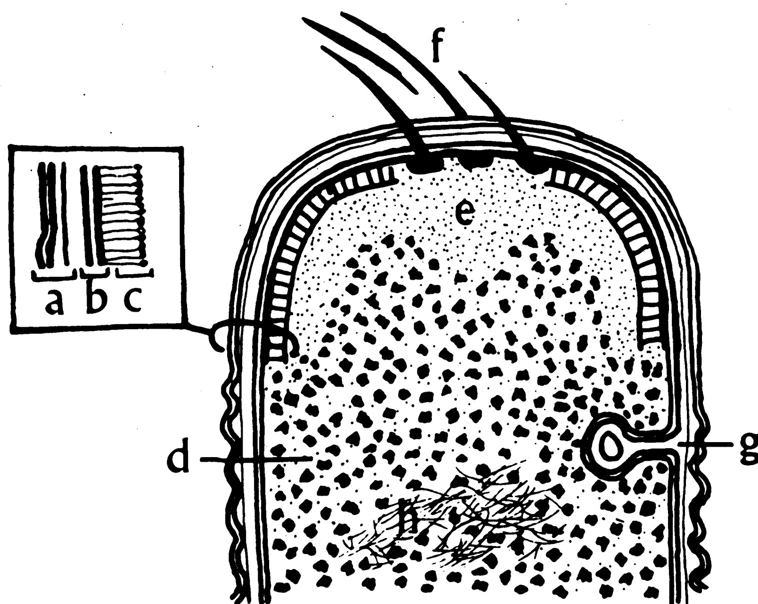


FIG. 8. *Diagrammatic representation of a thin section of the polar region of Aquaspirillum serpens. (a) Cell wall (outer, trilaminar, lipopolysaccharide wall membrane plus inner, thin, mucopeptide layer). (b) Cytoplasmic membrane. (c) Polar membrane with linkers to cytoplasmic membrane. (d) Ribosome-packed cytoplasm. (e) Ribosome-free polar matrix. (f) Flagella; the flagellar hooks and basal disk assemblies are not represented. (g) Cytoplasmic membrane intrusion (mesosome). (h) Nucleoplasm. (Reproduced by permission of the National Research Council of Canada from: R. G. E. Murray and A. Birch-Andersen, Can. J. Microbiol. 9:393-401, 1963)*

lum (173), and also in the rod-shaped organism *Chromatium* (135). In some cases, segments of polar membrane have been seen not only at the poles but also at other locations along the cell (135, 150), possibly at points where cell division would later occur. A platelike structure resembling in certain respects the polar membrane of spirilla has also been observed in the curved organism *Selenomonas ruminantium* (31). This appeared to be part of an organelle composed of membrane-bounded, differentiated cytoplasm lying just below the flagellar tuft in the middle of the concave side of the organism. The external flat side of the organelle appeared to be in contact with the flagellar bases, while the internal side bore the platelike structure. Although no central hole was evident in the plate, short bars (such as are associated with the polar membrane of *A. serpens*) projected from it.

The function of the polar membrane in spirilla is unknown, but one possibility is that it might be involved in energy production for motility. In *A. serpens*, the cytoplasm immediately beneath the polar membrane and flagellar insertion sites appears to be relatively free from ribosomes (135). Another unusual feature is that the outer portion of the cell wall (lipopolysaccharide membrane) did not exhibit its characteristic waviness at the polar region.

Purified suspensions of flagella from *A. serpens* have been prepared by adsorption of crude suspensions on diethylaminoethyl-cellulose columns in phosphate buffer, followed by elution with NaCl (115). Only one kind of flagella appeared to be made by the spirilla. Besides consisting of protein, the purified flagella contained 1.5% carbohydrate (116). Even when the flagella were dissociated to the monomeric form by acid or heat, with subsequent chromatography on Sephadex G-50, no separation of the carbohydrate from the protein occurred. Although this suggests a glycoprotein nature for the flagellin, Smith and Koffler (167) have noted that proof would depend on demonstration of a covalent linkage between the carbohydrate and the protein. The flagellin monomers of *A. serpens* appear to be of a single kind by acrylamide gel electrophoresis and have a molecular weight of ca. 40,000 (118). Amino acid analysis indicated lack of cysteine or tryptophan, and only a few residues of histidine, proline, and tyrosine (118). Alanine, aspartic acid, and glutamic acid were present at high levels. These results are similar to those for other

flagellated bacteria (167). The flagellin of *A. serpens* also contains a few residues of an unusual amino acid, ϵ-*N*-methylysine (69); this amino acid has also been reported for the flagellins of certain salmonellae (69, 167).

The flagellar filaments of *A. serpens* appear to possess antigenic determinants that are not apparent in flagellin monomers. Monomers have been found to yield a single precipitin line by double diffusion on agar gel using antiflagella serum but, when the same antiserum was tested against whole purified flagella, two lines were seen – one showing identity with flagellin (118). Furthermore, absorption of antiflagella serum with the homologous flagellin eliminated the flagellin precipitation line without affecting the flagella precipitation line. A similar situation occurred with other types of flagella, and Smith and Koffler (167) have suggested that exposure of new antigenic sites on flagellin might occur due to change in shape of the monomers during assembly into filaments.

The flagella of *A. serpens* can be dissociated by agents effective for the flagella of other organisms: acid, alkali, guanidine, or urea (118). Temperature has also been shown to be effective (116, 120). The conversion of ordered flagella of *A. serpens* into their monomers at a constant rate of heating has been followed turbidimetrically at 250 nm, with a sharp decrease in absorbance indicating dissociation. The midpoint of this transition was taken as the dissociation temperature (T_d). The T_d was found to increase with increasing molarities of phosphate buffer, but calcium chloride or magnesium chloride reduced the T_d, and sodium and potassium chloride had little effect. Smith and Koffler (167) noted that, if hydrophobic bonds are involved in intermolecular structure, salts should stabilize flagellar filaments; therefore, they suggested that ionic as well as hydrophobic bonds are involved.

The regeneration of flagella on deflagellated cells of *A. serpens* occurs rapidly. Murray and Birch-Andersen (135) indicated that half the normal length of a flagellum could be synthesized within ca. 10 min (0.5 μm of flagellum per min). Martinez and Gordee (119) reported that deflagellated *A. serpens* regained motility and flagella within one doubling time (70 min at 30 C). Regeneration occurred even in the presence of chloramphenicol at a concentration sufficient to inhibit incorporation of ^{14}C-labeled amino acids (added after deflagellation) into the regenerated flagella. When cells were cultured with labeled amino acids prior to deflagellation, the flagella regenerated in the presence of chloramphenicol were strongly labeled. These results suggested the existence of a functional pool of preformed flagellin in *A. serpens* that could be assembled into flagella in the absence of protein synthesis. This hypothesis was supported by studies of a conditional mutant that was nonflagellated when grown at 45 C. When grown with ^{14}C-labeled amino acids at 45 C and then transferred to broth (at 30 C) containing chloramphenicol, labeled flagella were formed, and a great reduction of radioactive intracellular antibody-precipitable flagellin occurred concomitantly. The mutant therefore appeared to be capable of producing a pool of intracellular flagellin at 45 C but could not allow assembly of the flagellin into flagella.

TACTIC RESPONSES OF SPIRILLA

The directed motility of bacteria in response to various stimuli continues to fascinate modern investigators just as it did those of 50 to 95 years ago. Bacterial tactic responses in general have been reviewed by Weibull (190) and Berg (10), and the following discussion is restricted to investigations of chemoheterotrophic spirilla.

Aerotaxis

The ability of certain spirilla in wet mounts to accumulate in a narrow band somewhat removed from the edge of the cover slip was first described by Engelmann (60) and later by Massart (123) and Jennings and Crosby (94). Beijerinck (7) also noted that spirilla suspended in a tube of water or semisolid agar would form a band some distance below the surface of the medium. This behavior indicated that spirilla were repelled by too little oxygen (in the central portion of wet mounts or in the depths of a tube) as well as by too much oxygen (at the edge of wet mounts or at the surface of a medium). Wells and Krieg (191) used this response as a clue toward obtaining suitable growth of pure cultures of the microaerophile *S. volutans* (Fig. 9). However, the formation of "spirillum bands" does not appear to be restricted to microaerophilic spirilla, since spirilla capable of growing well under an air atmosphere may also form them (102).

Using a defined motility medium, Caraway and Krieg (27) studied the aerotactic response of *S. volutans*. In capillary tubes containing an oxidizable carbon source and inoculated heavily at one end, the spirilla rapidly created an oxygen gradient as the result of their respiration. Some of the cells (those nearest the gradient) then moved quickly away from the main mass of the inoculum, forming a band that migrated down the length of the capillary and eventually

reached the liquid-air interface at the opposite end. The band displayed a well-defined trailing edge and a diffuse leading edge and, as it migrated, left an anaerobic region behind it. Although *S. volutans* forms microaerophilic bands in wet mounts (191), in capillary tubes the bands reached the air interface without stopping short of it. This inconsistency might have been due to the high density of spirilla in the band, which could permit oxygen uptake at a rate sufficient to keep the level of dissolved oxygen low, even at the interface. The rate of migration of the band along the capillary was correlated closely with the respiratory rate of the spirilla for the oxidizable substrate provided. Substrates that were oxidized rapidly allowed high rates of migration (e.g., 0.98 mm/min when succinate was present). Because the cells exhibited no detectable endogenous oxygen uptake, band formation failed to occur in medium containing no oxidizable substrate. In the presence of an oxidizable substrate, respiratory inhibitors such as KCN prevented band formation (but allowed motility). 2,4-Dinitrophenol at a 10^{-4} M concentration stimulated oxygen uptake, but no band formation occurred even though the cells were motile and coordinated. This observation indicated that an oxygen gradient alone was not the only factor governing aerotactic band formation. Sodium arsenate at a concentration of 0.03 M caused only slight decreases (5%) in oxygen uptake with succinate as the carbon source but, like 2,4-dinitrophenol, completely prevented formation of aerotactic bands without preventing motility (B. H. Caraway, Ph.D. thesis, Virginia Polytechnic Institute and State Univ., Blacksburg, 1972). Because both 2,4-dinitrophenol and arsenate, and also anaerobic conditions, caused decreases in the levels of intracellular ATP, Caraway suggested that the energy of ATP might be required for aerotaxis in *S. volutans*. The involvement of ATP in the chemotaxis of *E. coli* has been indicated by Larsen et al. (105). The function of the ATP might be to provide a supply of *S*-adenosylmethione, which has been implicated in the chemotaxis of *E. coli* (1, 2).

Geotaxis

In 1891, Massart (123) studied the response of spirilla to gravitational stimuli. He used spirilla cultured in infusions prepared by macerating in seawater various kinds of animal and vegetable debris collected at a beach. Although not clearly stated, different spirilla presumably predominated in different infusions. Two spirilla, designated A and C, appeared morphologically similar but behaved differently in response to gravitational stimuli. When capillary tubes were filled with the seawater infusions and kept horizontal, the spirilla accumulated at one or the other end, presumably by an aerotactic response. (Since the tubes were filled with a homogeneous suspension, it is not clear why they did not accumulate at both ends.) When the tubes were held vertically, however, a different situation occurred. *Spirillum* A invariably migrated to the top of the tube, and when the tube was subsequently inverted the spirilla again migrated to the top within 10 to 15 min. In contrast, *Spirillum* C invariably accumulated at the bottom of the tube, and when the tube was inverted they again accumulated at the bottom. This was not due to passive falling of dead cells since the spirilla were still actively motile. Thus, *Spirillum* A was said to be negatively geotactic, whereas *Spirillum* C was said to be positively geotactic. These results seem convincing, but no attempts have ever been made to confirm them. Considering that seawater was used, it is possible that a difference in the bouyant density of the two spirilla might have played a role.

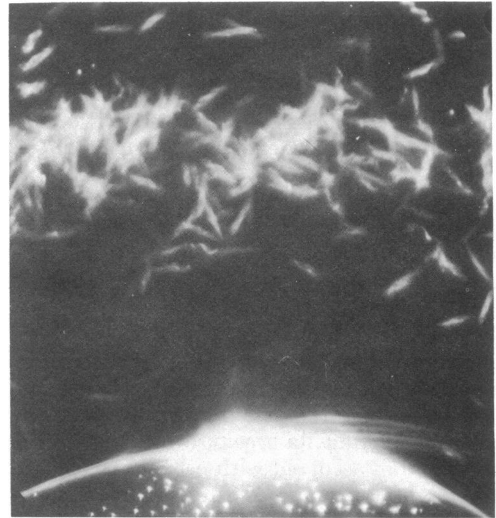


FIG. 9. *Portion of an aerotactic band of Spirillum volutans showing accumulation of the spirilla at a distance from the edge of an air bubble. Cells within the band are highly motile and reverse direction frequently. The wet mount was prepared from a pure dialysis-sac culture in Pringsheim soil medium. ×170.*

Thermotaxis

The effect of thermal stimuli on *S. volutans* was studied by Metzner (129) in 1919 by use of wet mounts containing an electrically heated

wire. Immediately after the current was switched on, spirilla could be observed swimming toward the wire and collecting near it. The spirilla swam straight towards the wire and then, after touching it, swam along one side of it. Cooler regions at a short distance from the wire seemed to repel the spirilla, causing them to reverse and reenter the warm zone. With increased temperature of the wire, the spirilla would become motionless as they swam into the heated zone. After spirilla had accumulated around a warm wire, cooling of the wire led to a rapid back-and-forth swimming near the wire, and eventually the spirilla became distributed again throughout the medium. A general rather than local cooling of the medium also induced the back-and-forth motility. Although Metzner ascribed the attraction of the spirilla toward a heated wire to a thermal stimulus, the results might alternatively have been caused by a change in the level of dissolved oxygen in the medium. It seems possible that an initially high level of dissolved oxygen (occurring immediately after preparation of the wet mounts) might have been decreased locally by heating of the wire. The attraction of the spirilla to the heated zone might therefore have been in response to a lower (and more favorable) oxygen level. Upon cooling of the wire, the rapid back-and-forth motility might have been caused by the repellent effect of the increasing level of oxygen near the wire and also the high oxygen levels already existing farther away from the wire.

Chemotaxis

In 1891, Massart (123) studied the response of marine spirilla to a gradient of NaCl. By using drops of infusions (see previous section on geotaxis) to which a few crystals of NaCl were added near one edge, he found that two morphologically similar spirilla (A and C) were repelled by the high salt concentration, eventually accumulating at the opposite side of the drop. When a drop of infusion was placed near a drop of distilled water and the two drops were connected by a narrow canal, the spirilla also were repelled by the decrease in salt concentration near the canal, again accumulating at the opposite side of their drop. A third kind of spirillum, morphologically different from A and C and which was designated B, appeared to be indifferent to both high and low concentrations of salt.

The tactic responses of *S. volutans* to certain chemical compounds were studied by Metzner (129) in 1920. Crystalline compounds were placed at the edge of wet mounts and allowed to diffuse inward. In the case of liquids such as chloroform, a drop of the liquid and a drop of spirilla were placed near each other on a slide; after the cover slip was affixed, a dividing line occurred between the two materials under the cover slip. In the case of NaCl, the spirilla were repelled sharply just as they began to enter the zone of concentrated NaCl, with flagellar reorientation occurring. A few individuals, however, exhibited dual-tail uncoordination for a short but noticeable time (the front flagellar fascicle changed from the head to the tail configuration, while the rear fascicle remained in the tail configuration). Such cells thus remained motionless for a short time but then swam away from the NaCl zone when the tail fascicle assumed the head configuration. Metzner interpreted the motionless periods as reflecting the time required for transmission of a signal from the anterior end of the cell to the posterior end. Such motionless periods were seen only rarely, however. Krieg et al. (101) found, using freshly prepared wet mounts of *S. volutans* cultured in PSS medium in which the spirilla were reversing direction frequently, that both fascicles would reorient their configuration in apparently simultaneous fashion (or at least within 0.06 s).

With further diffusion of NaCl under the cover slip and, consequently, a more gradual concentration gradient, Metzner noted that many spirilla could swim for some distance into the gradient and would then exhibit dual-tail uncoordination (as discussed previously in this review). In the case of chloroform, ether, or acetone, no repellent effect ever occurred, but the spirilla exhibited dual-head uncoordination. Certain toxic metals (e.g., $CuSO_4$) also failed to repel the spirilla; here the spirilla would swim into the gradient and suddenly cease all flagellar action. However, Krieg et al. (101) found that when *S. volutans* was suspended in highly dilute solutions of copper salts dual-tail uncoordination would occur.

Chemotaxis has been studied more recently in *A. serpens* and other bacteria by Doetsch and Seymour (55) by use of a capillary tube method. After a light suspension of cells was drawn up, one end of the capillary tube (the control end) was sealed with plain agar, whereas the other end was sealed with agar containing a metal salt. *A. serpens* formed bands or clouds that migrated away from several of a variety of metal salts, indicating negative chemotaxis. Because buffering of the test agar plugs or the bacterial suspension retarded the responses, it appeared likely that the cells were migrating away from an acid gradient. Similar results were obtained by Seymour and Doetsch (160) for basic gradients. Seymour and Doetsch also

found positive chemotaxis to occur (i.e., accumulation of the bacteria in a mass or cloud immediately adjacent to the test plug) with many monosaccharides and a few disaccharides or glycosides. In such cases, pH gradients were not involved. These results are especially interesting in view of the inability of *A. serpens* to catabolize carbohydrates.

CELL WALL AND PERIPLASMIC SPACE OF SPIRILLA

Profile of the Cell Wall in Thin Sections

Electron microscopy of thin sections of *A. serpens* in 1957 by Chapman and Kroll (32) served to establish the existence of a cytoplasmic membrane and cell wall, but details of the wall structure could not be seen with the existing methods. Improved methods of fixation, staining, and sectioning enabled Murray and Birch-Andersen (135) in 1963 to detect a thin, dense, taut layer that was stretched over the surface of the cytoplasmic membrane, yet separated from the latter by a fairly constant gap. Outside of the thin layer was the wavy, three-layered lipopolysaccharide-containing "wall membrane" characteristic of gram-negative bacteria. The contrast of the thin layer was greatly enhanced by staining with such metal salts as lanthanum nitrate or lead tartrate (134, 136), and, through the use of lysozyme, the layer was subsequently identified as mucopeptide (136) (Fig. 10). Murray (134) also detected an additional cell wall layer outside of the lipopolysaccharide-containing wall membrane (Fig. 11). This layer corresponded to the structured external surface layer seen by other methods and discussed later in this review.

Cell division in *A. serpens* was studied by Steed and Murray (171) by use of thin sections. When cultured at 30 C, *A. serpens* showed a constrictive division in which the cells appeared to be pinched in the middle and septa were not seen. However, septa were seen in cells grown at 45 C and fixed at either 45 or 20 C, suggesting that growth at supraoptimal temperatures might upset the synthesis of cell components in such a way as to "trap" the process of cell division for a time at the septum stage. However, cells grown and fixed at 30 C

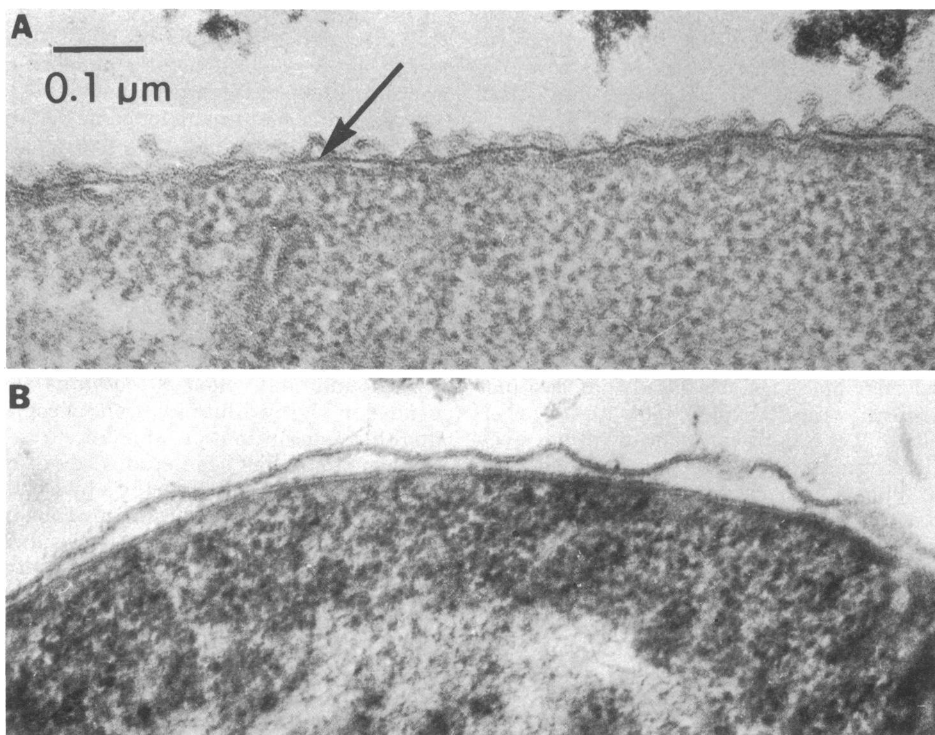


FIG. 10. *(A) Section of normal, intact Aquaspirillum serpens, showing the wavy, outer, trilaminar, wall membrane overlying the dense, thin, inner layer (mucopeptide) of the cell wall (arrow). (B) Companion preparation of a spheroplast formed by lysozyme-EDTA treatment. The thin, dense, inner layer is missing. (Reproduced by permission of the National Research Council of Canada from:. R. G. E. Murray, P. Steed, and H. E. Elson, Can. J. Microbiol. 11:547-560, 1965)*

also showed septa provided that the veronal acetate buffer in the Ryter-Kellenberger fixative was diluted, indicating that the toxicity of the fixing environment was also a factor. Cell division began by a centripetal growth of plasma membrane enclosing a newly synthesized layer of mucopeptide.

The mucopeptide layer then thickened and delaminated into two layers (Fig. 12), and the eventual severance of the sister cells was achieved by the growth and invagination of the outer layers of the cell wall.

Mucopeptide and the Shape of Spirilla

One of the most characteristic features of spirilla is their possession of a rigid helical shape. Presumably, this is a function of the mucopeptide layer since isolated murein sacculi retain the characteristic cell shape (99). What attributes of this layer allow some bacteria to the cocci, others to be rods, and others to be helical are not known, but certain clues exist. It is also not known why the spirilla of a given species are either all clockwise helices or all counterclockwise helices (180).

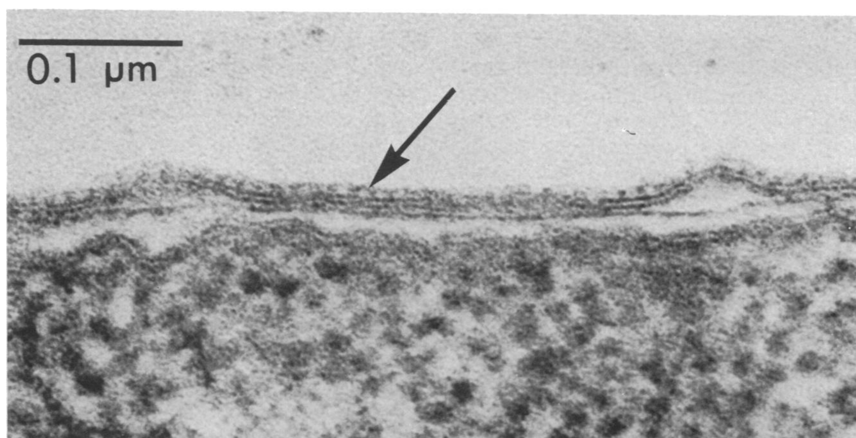


FIG. 11. *Thin section of Aquaspirillum serpens showing the outermost protein layer (arrow), lying outside of the trilaminar, lipopolysaccharide, wall membrane. (Courtesy of R. G. E. Murray, Department of Microbiology and Immunology, University of Western Ontario, London, Canada.)*

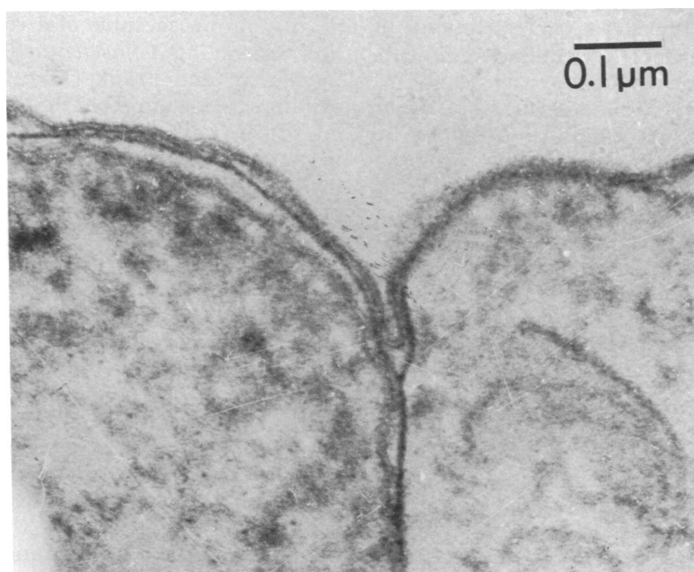


FIG. 12. *Lanthanum-stained section of Aquaspirillum serpens from a 6-h-old culture grown and prefixed at 45 C. The mucopeptide layers of the septum have split apart, whereas the outer layers of cell wall have not yet participated in separating the daughter protoplasts. (Reproduced by permission of the National Research Council of Canada from: P. Steed and R. G. E. Murray, Can. J. Microbiol. 12:263-270, 1966)*

The tendency of some spirilla to become nearly straight rods on prolonged serial transfer or through selection of mutants (139, 180, 196; see also Fig. 16 and 19 of reference 83) is well known. In the case of marine spirilla, Watanabe (185) noted that, upon cultivation with various levels of NaCl (from 1 to 6%), the wavelength became longer and the cells became straighter as the salt concentration was decreased. Using *A. serpens*, Kolenbrander and Ensign (99) found that 54% of the diaminopimelic acid (DAP) molecules in the peptidoglycan were involved in cross-linkage between tetrapeptides, an unusually high value compared with other gram-negative bacteria. In a comparison of the peptidoglycans from a parent strain and those from bacilliform mutants of *Rhodospirillum rubrum*, Newton (139) found 44% of the DAP in the peptidoglycan of the parent to be involved in cross-linkage, whereas only 29 to 36% was so involved for the mutants. Spiral revertants yielded a value of 43 to 47% of the DAP. When the parent strain was cultivated in the presence of sublethal amounts of ampicillin, which inhibits cross-linking, the cells grew predominantly as long chains of bacilli. Moreover, mutants grown in the presence of 0.2% D-alanine were converted into markedly curved forms, and these were found to have ca. 42% of their DAP involved in cross-linkage. These results, as well as the finding of much lower levels of L-alanine dehydrogenase (inducible by D-alanine) in the bacilliform mutants, suggested that the intracellular levels of D-alanine might affect the degree of cross-linkage in the peptidoglycan and, consequently, the curvature of the cells.

In 1838, Ehrenberg (59) described *Spirillum* species as rigid rather than flexible; indeed, he was tempted to consider spirilla as "armored animals" and noted that they retained their shape and size upon drying. Although spirilla are certainly more rigid than spirochetes, they do appear to have a surprising degree of elasticity. In 1786 Müller (133) observed that "*Vibrio*" *serpens* (undoubtedly a spirillum) tended to become straighter during rapid motion. Williams (194) noted that other early investigators of the genus *Spirillum*, such as Cohn, Migula, and Giesberger, had made similar observations, and the accuracy of these observations has been supported by the cinephotomicrographic studies of spirilla made by Pijper et al. (145). Additional and convincing evidence for flexibility has more recently been obtained by Isaac and Ware (84), who embedded spirilla in strips of glycerol-gelatin. The strips were subjected to stretching while the embedded cells were viewed under the microscope. Cells of *Bacillus megaterium* could be stretched to about 1.25 times their original length before detachment from the gel by cavitation occurred, and cells of *E. coli* could be stretched to about twice their original length. The spirilla, however, were first seen to uncoil into straight rods, which could be stretched further until the organisms were up to three times their original length. During this stretching, the cytoplasm separated into three or more segments. When the tension was released, the cell walls and cell contents returned to their original size and shape. The stretching could be repeated several times for a given cell. No spirillum was seen to break except where signs of cell division had previously been observed.

The flexibility of spirilla led Williams and Rittenberg (198) to suggest that spirochetes might possibly have evolved from spirilla, and the similarity of the axial fibrils of spirochetes to bacterial flagella has been noted by several investigators (reviewed by Smibert [165])—they have even been referred to as "endoflagella." It is conceivable that through mutation or by accident the flagella of a spirillum might have been formed between the mucopeptide and the lipopolysaccharide wall membrane, giving rise to an aerobic organism morphologically resembling a spirochete such as *Leptospira*. There is little genetic evidence, however, to support this hypothesis, since the DNA base composition of leptospires is quite different from that of aquaspirilla.

The murein sacculus of a rod-shaped spirillum resembling *A. fasciculus* (78) was isolated by Martin and Frank (113). Sodium dodecyl sulfate was used to separate the outermost layers of the wall (structured protein layer [see below] and lipopolysaccharide-containing wall membrane) and cell contents from the mucopeptide. The latter was found to contain muramic acid, glucosamine, DAP, glutamic acid, and alanine in a ratio of roughly 1:1:1:1:2. Similar results were obtained for the peptidoglycan of *A. serpens* by Kolenbrander and Ensign (99), who used Pronase in addition to sodium dodecyl sulfate to remove residual protein. Since Buckmire and Murray (20) demonstrated that sodium dodecyl sulfate effectively removes the outer structured protein layer in *A. serpens*, and also since Martin et al. (114) were unable to demonstrate covalently linked protein in murein sacculi, it is possible that the residual protein found by Kolenbrander and Ensign may have been of intracellular origin. The DAP of *A. serpens* was found to be the *meso* type, characteristic of most gram-negative bacteria.

Treatment of the peptidoglycan with myxobacter AL-1 protease, which cleaves the tetrapeptides from their muramic acids, revealed that the average chain length of the polysaccharide backbone chains was 99 hexosamines (99). The purified murein sacculi prepared by Kolenbrander and Ensign (99) from *A. serpens* contained ca. 13% PHB. This was of intracellular origin, however, since PHB granules could be seen inside the sacculi.

An interesting alteration of the appearance of the highly purified sacculi of *A. serpens* was reported by Preusser (147) to occur upon treatment with 0.012 M uranyl acetate at pH 4.5. Viewed by electron microscopy, a progressive contraction occurred along the length of the sacculi, eventually resulting in the appearance of electron-dense rings and separate, ring-shaped sacculus segments. Contraction of the diameter of the sacculi was quite small compared with that for the length. The action of uranyl ions in binding to, and cross-linking, free carboxyl groups in collagen suggested that these ions might also be able to cross-link the free carboxyl groups of the peptides on adjacent glycan chains of bacterial murein, pulling the glycan chains closer together. Lengthwise contraction, but not contraction across the diameter of the sacculus, could result if a suitable anisotropic arrangement of the murein lattice was present.

Recently, a three-dimensional model of a monolayer of the murein of *A. serpens* was proposed by Formanek et al. (62) on the basis of isopycnic density-gradient centrifugation, infrared spectrophotometry, and X-ray diffraction of purified sacculi. The model proposed that the tetrapeptide chains on the *N*-acetylmuramic acid residues have a 2.2_7 helical conformation. It further proposed that the tetrapeptide chains are linked to the glycan chain in such a way as to form two hydrogen bonds with the sugar residues and to cause an angle of ca. 150° between the glycan and peptide chains.

Outermost Layers of Cell Walls

During examination of shadowed preparation of whole cells and especially isolated walls from a rod-shaped *Spirillum* resembling *A. fasciculus*, Houwink (78) observed a monolayer of apparently spherical particles arranged in a hexagonal pattern on the outer surface of the walls. This structured layer could be removed by treatment with SDS (113). A similar regular macromolecular surface array (RS layer) was described by Murray (134) for *A. serpens* (Fig. 13). Each subunit appeared to be cogwheel-shaped with a central hole and six projections. The projections appeared to couple with the projection of adjoining subunits to form Y-shaped linkers (see also reference 22). The RS layer appeared to be organized on a thin, unstructured, triplet backing layer (20).

Sodium dodecyl sulfate, phenol, guanidine, dimethyl sulfoxide, and water saturated with phenol were found by Buckmire and Murray (20) to be highly effective in removing the RS layer from the walls of intact cells, and urea was also moderately effective. Purified water-soluble preparations could be obtained after dialysis of guanidine-solubilized RS layers (20). Incubation of such preparations with cell wall fragments from which the layer had been previously removed resulted in reassembly of the RS layers onto these templates (21). No such in vitro reassembly occurred in the absence of either cell wall fragments or of Ca^{2+}. Buckmire and Murray (22) found that Ca^{2+}, Sr^{2+}, Mg^{2+}, Na^{+}, and K^{+} were equally effective when used at high concentration in permitting in vitro assembly on templates, but at lower concentrations Na^{+} and K^{+} were ineffective and Mg^{2+} was only partially effective. When RS layers that had self-assembled on templates in the presence of high concentrations of cations were subsequently diluted in water, little release of protein occurred if Ca^{2+} or Sr^{2+} had been used; however, if Mg^{2+} had been used there was a partial release of the protein, and with the monovalent cations most of the protein was released. These results suggested that, although initial assembly of the RS layer was dependent on ionic strength, stability depended on specific cations and presumably involved formation of strong "salt" linkages between the protein and the underlying membrane.

The purified RS preparations obtained by Buckmire and Murray (20, 21) contained ca. 98% protein. Although 2% carbohydrate also appeared to be present, this may have been an artifact (21). The RS protein was homogeneous by ultracentrifugation, gel electrophoresis, chromatography with Sephadex, and sucrose density gradient centrifugation (21). The purified RS protein appeared to be a trimer, with guanidine causing depolymerization into the monomeric form. From amino acid analyses, the molecular weight of the monomer was 48,000. Aspartic acid, alanine, threonine, and glycine were present in the highest molar amounts, and the protein was highly acidic. No cysteine or cystine was present.

One function of the RS layer of *A. serpens* is to protect the spirilla against parasitism by *Bdellovibrio* (F. L. A. Buckmire, Bacteriol. Proc., p. 43, 1971). Removal of the RS layer

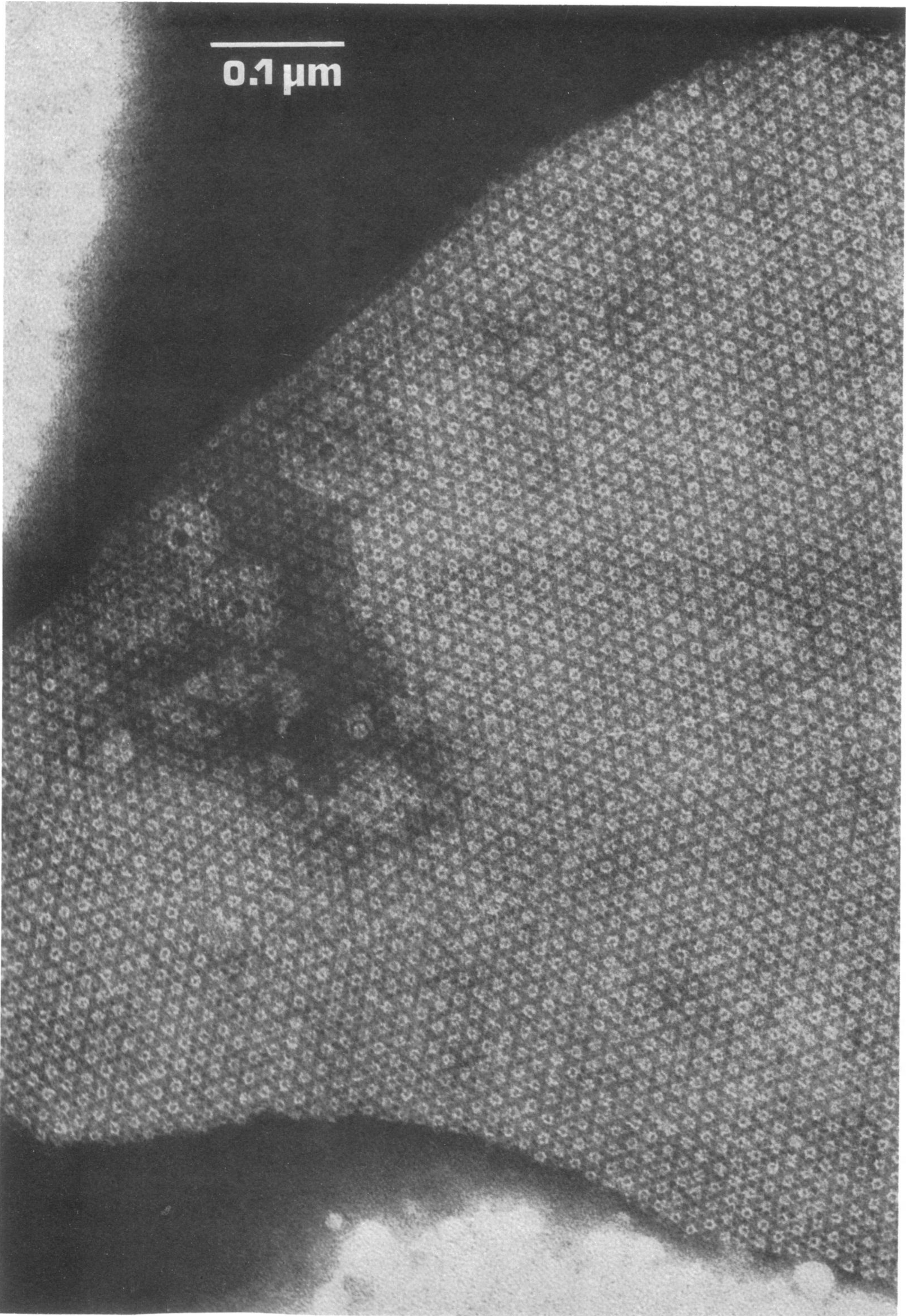


FIG. 13. *Electron micrograph of a cell wall fragment of Aquaspirillum serpens showing the outermost protein layer (regular macromolecular surface array). Negatively stained with 1% phosphotungstate. (Courtesy of R. G. E. Murray, Department of Microbiology and Immunology, University of Western Ontario, London, Canada)*

rendered the spirilla susceptible to attack by *Bdellovibrio* but, when the RS protein was allowed to reassemble on the denuded cell surface in its original form, the spirilla again became resistant. The RS layer apparently masked receptor sites on the cell surface for attachment of the parasite. Another possible function of the RS layer was suggested by Martin et al. (114): namely, that the layer might be able to confer a degree of rigidity to the wall membrane of *A. serpens*. This suggestion is especially attractive in view of the apparent absence of lipoprotein covalently linked to the murein sacculi of *A. serpens* (114); such lipoprotein in *Enterobacteriaceae* is thought to function by linking the wall membrane to the murein layer. It is pertinent that *A. serpens* VHA has been shown to undergo lysis towards the end of growth in aerated cultures, this being prevented by incorporation of calcium salts in the medium (20). Since Ca^{2+} is required for synthesis of the RS layer, this layer might well be a factor in resistance to lysis. On the other hand, strain VHL (a variant which, like VHA, was derived from the parent strain VH initially isolated by C. F. Robinow in 1957) is incapable of forming an RS layer even with Ca^{2+} (19), yet survives without it.

A. putridiconchylium possesses a more complex macromolecular array than does *A. serpens* (11). Two structured layers formed an RS layer having a complex pattern. The outer layer was a linear array of subunits overlying an inner tetragonal array of larger subunits. Some fragments of the RS showed predominantly the former (Fig. 14) and some showed predominantly the latter (Fig. 15). By use of optical diffraction combined with image filtering and reconstruction, as well as linear and rotary integration techniques, the relation of the outer and inner structured layers was determined. A diagrammatic model is illustrated in Fig. 16.

Although first discovered in spirilla, RS layers are not restricted to this group and have been found in a variety of gram-positive and gram-negative bacteria (11). As noted by Murray (134), such almost crystalline arrays occurring in a cell wall induce some fascinating speculations, not the least interesting of which is how such surfaces can be increased to allow growth.

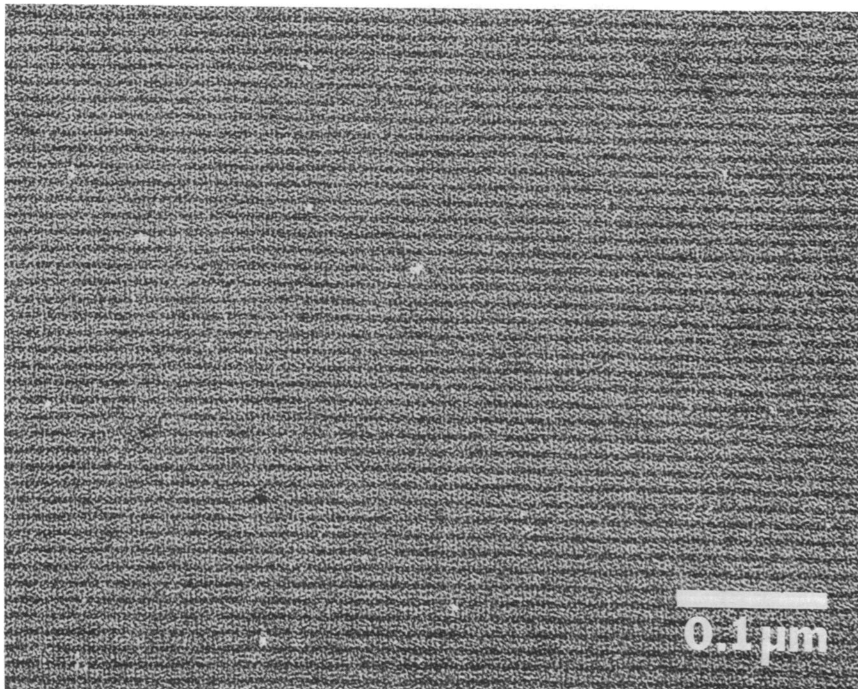


FIG. 14. *Portion of an RS fragment (regular macromolecular surface array) from Aquaspirillum putridiconchylium, showing mainly the outermost of the two layers comprising the fragment. Compare with Fig. 15. (Reproduced by permission of the American Society for Microbiology from: T. J. Beveridge and R. G. E. Murray, J. Bacteriol. 119:1019–1038, 1974)*

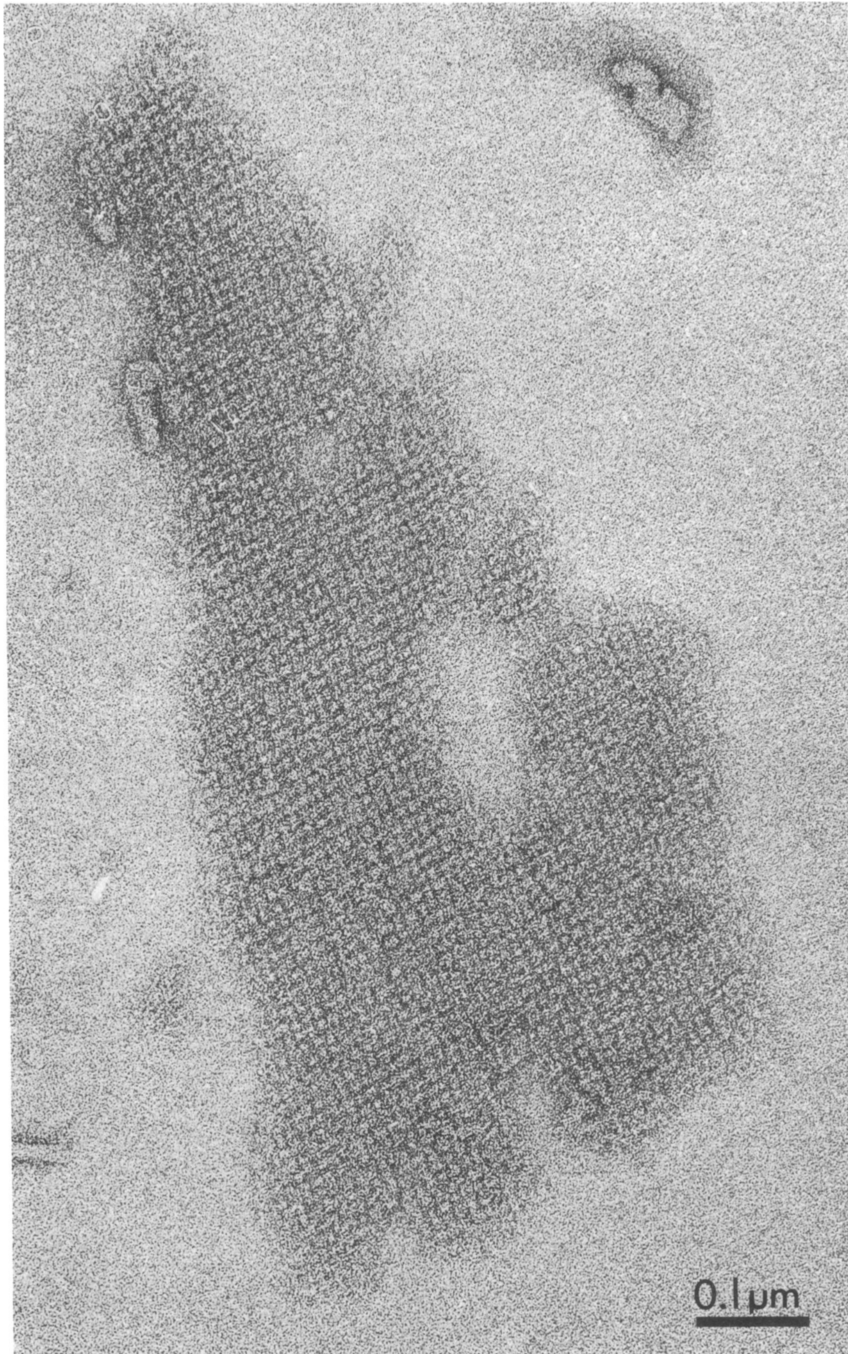


FIG. 15. *Portion of an RS fragment (regular macromolecular surface array) from Aquaspirillum putridiconchylium, showing mainly the innermost of the two layers comprising the fragment. Compare with Fig. 14. (Reproduced by permission of the American Society for Microbiology from: T. J. Beveridge and R. G. E. Murray, J. Bacteriol. 119:1019-1038, 1974)*

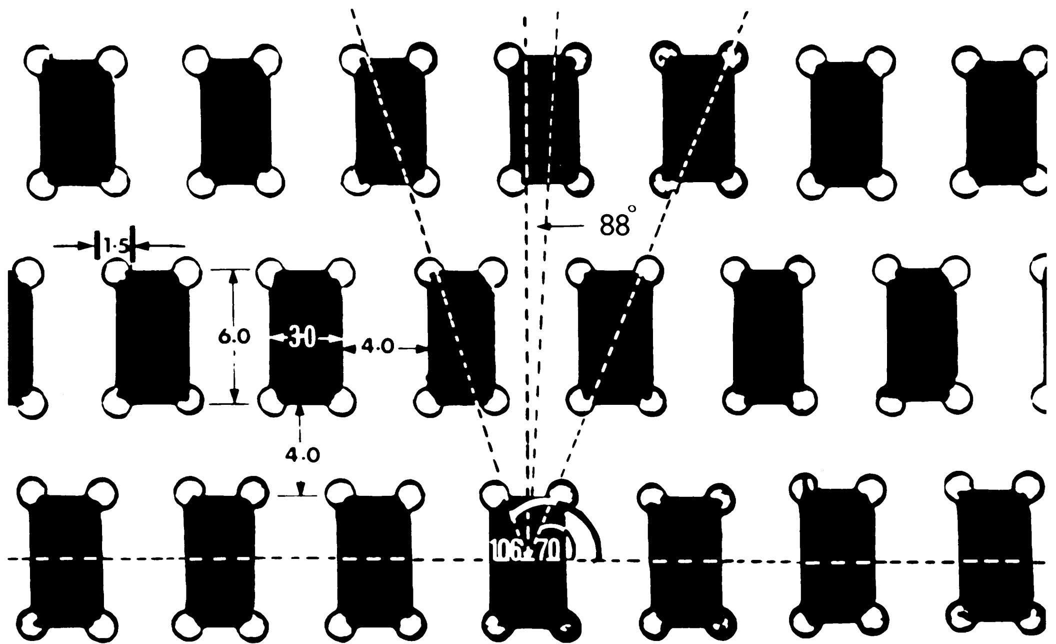


FIG. 16. *Diagrammatic model, drawn to scale, of the RS layer of Aquaspirillum putridiconchylium. The RS layer consists of an inner structured layer (ISL) and an outer structured layer (OSL). The ISL units have been drawn as rectangular blocks; the OSL units have been drawn as simple spheres. To aid interpretation, the OSL units have not been filled in. The alignment angles and dimensions (in nanometers) have been added. The longitudinal (linear) axis runs from the left to the right of the page. (Reproduced by permission of the American Society for Microbiology from: T. J. Beveridge and R. G. E. Murray, J. Bacteriol. 119:1019-1038, 1974)*

Growth of Cell Walls

Williams (192) presented two hypotheses to account for the mode of growth of the cell wall in spirilla. (i) The main growth of the wall is from a growing point at one pole. As a spirillum elongates and eventually divides into two daughter cells, one daughter cell would possess most of the old wall, whereas the other would contain mostly newly synthesized wall. (ii) Cell elongation occurs by the intercalation of new wall at various points, or even diffusely, along the length of the cell. After cell division, both daughter cells would contain approximately equal amounts of new walls. By observing increases in distance between the cell inclusions (probably PHB granules) of growing and dividing cells of *A. anulus,* Williams concluded that hypothesis ii applied. Wagner (184) studied cell surface synthesis in *A. serpens* by staining the surface with fluorescent antiserum prepared against whole cells. After being washed to remove excess antibodies, the spirilla were allowed to grow in a nutrient medium. Any new surface formed after the initial labeling would be nonfluorescent. Such nonfluorescent regions first appeared at the cell poles, followed by formation of multiple nonfluorescent zones along the length of the cells. A small fluorescent patch remained at the tip of the cell poles. Nonfluorescent zones appeared at regions of cell division. Somewhat different results were obtained by McElroy et al. (127), who applied a similar immunofluorescence procedure to *S. volutans.* Here, a uniform decrease in cell wall fluorescence occurred with increasing time. This indicated that in *S. volutans* a diffuse intercalation of new cell surface into old was occurring, whereas in *A. serpens* new cell surface appeared to be formed at only a relatively few sites. Cells of *S. volutans* retained one bright polar region after the rest of the cell had become nonfluorescent, but eventually this too faded. This correlated well with similar results obtained by E. H. Beachey and R. M. Cole (Bacteriol. Proc., p. 27, 1966) for *E. coli;* it was suggested that the polar surface was also formed by diffuse intercalation but at a slower rate than the rest of the cell surface.

In immunofluorescence experiments such as those described above, in which antiserum prepared against whole cells is used, it is difficult

to know which cell wall layers are being observed – presumably, only the more superficial layers, such as the RS or lipopolysaccharide layers. Although it is not known whether *S. volutans* possesses an RS layer, *A. serpens* does possess such a layer, and the use of antiserum prepared against the purified protein of this layer (21) could enable one to study the synthesis of RS layers specifically.

With respect to RS layer growth at the polar regions of spirilla, Murray (134) has noted that, although the hexagonal packing of the RS layer of *A. serpens* can uniformly cover the cylindrical portion of the cells, the poles can be expected to show discontinuities marked by six pentagonal arrangements of subunits at each hemispherical end. Pentagonal arrangements were observed occasionally by Murray (134).

Lipopolysaccharide Layers

The lipids and lipopolysaccharides of the cell envelope of *A. serpens* were examined recently (33). Loosely bound lipids, other than PHB (of cytoplasmic origin), comprised ca. 12% of the envelopes. The major lipid component was phosphatidylethanolamine, with diphosphatidylglycerol, phosphatidylglycerol, and lysophosphatidylethanolamine also occurring. Small amounts of nonpolar lipids also were present. Lipopolysaccharide was tightly bound and could be removed only after digestion of the preparations with Pronase. The lipopolysaccharide of *A. serpens,* although similar in many respects to lipopolysaccharides isolated from other gram-negative organisms, differed in certain respects from the majority of these; e.g., 2-keto-3-deoxyoctonic acid was not detected. This compound usually provides an acid-labile link between lipid A and polysaccharide moieties, and it is perhaps significant that unusually vigorous conditions were required to cleave lipid A from polysaccharide in *A. serpens.* 2-Keto-3-deoxy-octanoic acid could be detected in lipopolysaccharides from *A. itersonii* and *A. peregrinum.* The lipid A of *A. serpens* differed from enterobacterial lipid A in that 3-hydroxydodecanoic acid was the *N*-acylating acid rather than 3-hydroxytetradecanoic acid. This situation resembled that occurring in *Pseudomonas aeruginosa* and *P. alcaligenes,* but the range of other acids present in *A. serpens* more closely resembled that for *P. alcaligenes.* After removal of lipid A, the polysaccharides of *A. serpens* could be separated into a core polysaccharide and a very-low-molecular-weight side-chain polysaccharide. The latter was partially characterized as *N*-acetylglucosaminyl-(1 $\rightarrow$ 4)-rhamnose.

Periplasmic Space

Garrard (66) was able to cause the release of three proteins (soluble cytochrome *c*, alkaline phosphatase, and ribonuclease) in high yield from cells of *A. itersonii* by converting the cells to spheroplasts, using a mixture of lysozyme, ethylenediaminetetraacetate (EDTA), and tris-(hydroxymethyl)aminomethane (Tris). Tris-EDTA alone caused nearly quantitative release of the three proteins without visibly affecting cell morphology, although cell viability was severely affected. The three proteins together comprised 6% of the total cell protein and, since only 9% of the total cell protein was released from the cells by the Tris-EDTA treatment, a selective release was indicated. The proteins were assumed to be localized in the surface layers of the cell, possibly in the periplasmic space or loosely bound to the cytoplasmic membrane. The three proteins were released differentially as a function of time or of EDTA concentration, suggesting that each protein was bound to the cell surface in a different manner. The differential release might be attributable to the differential affinity of EDTA for cations involved in the binding.

DNA OF SPIRILLA

DNA Base Composition

The G+C content of the DNA of members of *Spirillum, Aquaspirillum,* and *Oceanospirillum,* as determined by various investigators, is indicated in Table 6.

Although the thymine + guanine content of double-stranded DNA represents a molar fraction of 0.5, there is no requirement that this value applies to either of the two strands. If one strand was richer in thymine and/or guanine than the other, it should be denser in an alkaline CsCl gradient. Primrose (148) reported that, when denatured DNA from strain SP5 of *A. itersonii* was centrifuged to equilibrium in such a gradient in an analytical ultracentrifuge, two components present in equal amounts but differing in buoyant density by 0.009 g/cm^3 were observed. The two peaks could not be distinguished in a preparative ultracentrifuge, however. One of the components appeared to bind a polyribonucleotide [random co-polymer, poly(A, C)] selectively, and it was possible to achieve separation by use of a column of kieselguhr coated with poly(A, C). When material from both column fractions was cross-annealed, the melting profile of the product was characteristic of a mixture of single- and double-stranded DNA, and it yielded a melting point

TABLE 6. *DNA base compositions for members of Spirillum, Aquaspirillum, and Oceanospirillum*

Genus and species	Strain	G+C (mol%)	Method[a]	Reference
Spirillum				
S. volutans	ATCC 19554	38	T_m	82
	ATCC 19553	38	T_m	82
		36	Bd	39
Aquaspirillum				
A. serpens	ATCC 11335	51[b]	T_m	83
	ATCC 12638	50	T_m	29, 83
		49	Bd	39
	ATCC 15278	50	T_m	29, 83
	NCIB 9011	54	Ch	158
	NCIB 8658	49	Ch	158
	VH, VHL, VHA, VHS, Victoria, St. Rhodes	50–51	T_m	83
A. bengal	ATCC 27641	51	T_m	102
A. putridiconchylium	ATCC 15279	52	T_m	29, 83
A. giesbergeri	ATCC 11334	58	T_m	83
		57	T_m	29
	NCIB 8230	57	T_m	83
Aquaspirillum sp.	ATCC 12289	58	T_m	83
A. anulus	ATCC 19259	58	T_m	29, 83
	NCIB 9012	59	T_m	83
A. sinuosum	ATCC 9786	57	T_m	83
		58	T_m	29
A. peregrinum	ATCC 15387	62	T_m	83
		60	T_m	29
A. itersonii	ATCC 12639	62	T_m	83
		60	T_m	29
		61	Bd	39
	ATCC 11331	62	T_m	83
		61	T_m	29
		64	T_m	43
		65	Bd	43
	NCIB 9071	57	Ch	158
	Strain Sp-5	60	T_m	148
		62	Bd	148
A. polymorphum	NCIB 9072	62	T_m	29, 83
A. metamorphum	ATCC 15280	63[2]	T_m	83
A. delicatum	ATCC 14667	63	T_m	29, 83
A. dispar	ATCC 27510	65	T_m	83
	ATCC 27650	63	T_m	83
A. aquaticum	ATCC 11330	65	T_m	83
		64	T_m	43
		65	Bd	43
A. gracile	ATCC 19624	65	T_m	83
		64–65	T_m	29
	ATCC 19625	65	T_m	83
		64	T_m	29
	ATCC 19626	65	T_m	83
		64	T_m	29
Oceanospirillum				
O. minutulum	ATCC 19193	42	T_m	83
		44	T_m	29
	ATCC 19192	42	T_m	83
		43	T_m	29
O. japonicum	ATCC 19191	45	T_m	83
		46	T_m	29
O. beijerinckii	ATCC 12754	47	T_m	83
		46	T_m	29
O. maris	ATCC 27509	46	T_m	83
	ATCC 27649	45	T_m	83
	ATCC 27648	45	T_m	83

TABLE 6—*Continued*

Genus and species	Strain	G+C (mol%)	Method[a]	Reference
O. linum	ATCC 11336	48	T_m	83
		49	T_m	29
	ATCC 12753	48	T_m	83
		50	T_m	29
	NCIB 55	51	Ch	158
Genus uncertain				
"*Spirillum lunatum*"	ATCC 11337	63	T_m	83
		61	T_m	29

[a] T_m, Thermal denaturation; Bd, buoyant density; Ch, chemical analysis.

[b] Carney et al. (29), using strains supplied by our laboratory, reported that ATCC 11335 (*A. serpens*) and 15280 (*A. metamorphum*) had a G+C value of 63 and 50%, respectively. This was just the reverse of what our laboratory had reported previously (83). We have examined our original preparations of DNA for these two strains, as well as new preparations from our stock cultures, and have obtained values of 51 and 63% for ATCC 11335 and 15280, respectively, confirming our previous results.

(T_m) identical to that of the native DNA. When material from each fraction was self-annealed, the melting profile was characteristic of single-stranded DNA. The selective binding of poly(A, C) by one kind of strand indicated that it was richer in guanine and/or thymine than the other strand.

BACTERIOPHAGES FOR SPIRILLA

In 1971 Clark-Walker and Primrose (36) provided what appears to be the only report of a bacteriophage for a spirillum (although Clark-Walker [35] had earlier demonstrated the spontaneous induction of a defective bacteriophage in *A. itersonii*). The phage was isolated from raw sewage in Australia, although the host strain of *A. itersonii* had originally been obtained from Lake Erie, U.S.A. The phage was specific for only this strain and failed to attack a number of other spirilla, even locally isolated strains.

Although the phage produced plaques on agar plates, it was not possible to obtain lysis of broth cultures; consequently, preparations of the phage for subsequent purification were made from plate lysates. The particles of purified virus were 63 nm in diameter and icosahedral in shape. Although they did not appear to possess a tail, one vertex seemed to have a different structure than the others. By buoyant density determinations, the base composition of the double-stranded DNA was calculated to be 53 mol% G+C. With an electron microscope the DNA was seen a linear molecule 13.25 μm long; from this length a molecular weight of 2.6×10^7 was calculated.

COCCOID BODIES AND LIFE CYCLES

In 1956 Williams and Rittenberg (197) photographed the formation of coccoid bodies, which they termed "microcysts," in a marine spirillum, *S. lunatum*. (This organism was quite different than that presently occurring in the type strain ATCC 11337, since the latter does not require seawater and does not form coccoid bodies. The organism described by Williams and Rittenberg was undoubtedly a typical *Oceanospirillum*.) Coccoid body formation generally occurred after active cell division had ceased. Three main modes of formation were observed. (i) Two spirilla became entwined, and after both had eventually become motionless they appeared to fuse together (Fig. 17). The organisms then became shorter and thicker, and a protuberance was formed at the point of fusion. The protuberance gradually enlarged, absorbing the organisms, to form the coccoid body. Frequently, more than one coccoid body was formed from a pair of entwined spirilla. (ii) The spirilla became shorter and thicker. A protuberance could arise from the center of the cell (Fig. 18), which would gradually enlarge and absorb the rest of the cell, or protuberances could arise at both ends of a cell, enlarge, and merge into a single body. (iii) The spirilla could also merely undergo a gradual shortening and rounding, ending up as a coccoid body (Fig. 19).

When 4-week-old cultures that were completely in the coccoid body stage were transferred to fresh medium, germination by unipolar or bipolar emergence for the majority of coccoid bodies occurred within 16 h (Fig. 20). No shedding of a coat was evident, and the coccoid body appeared merely to be absorbed into the emerging spirillum.

The occurrence of such coccoid bodies in large numbers in old cultures of spirilla has taxonomic importance (83, 180, 198) and has been described in the following organisms: *A. itersonii, A. peregrinum, A. polymorphum, A. fas-*

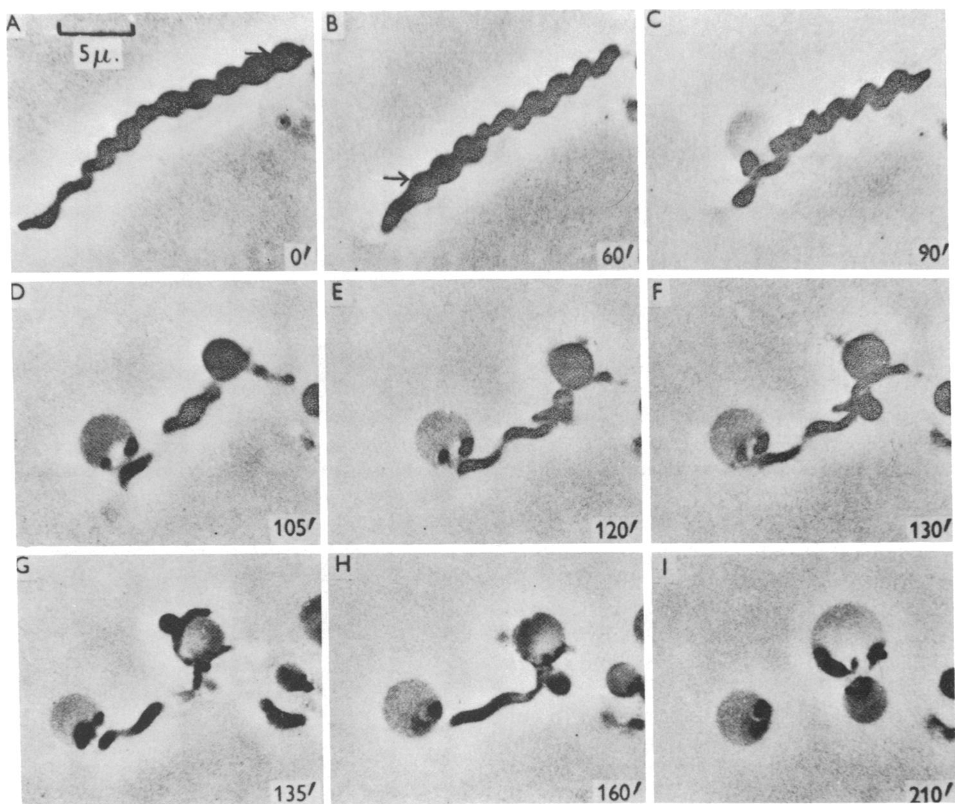


FIG. 17. *Coccoid body formation after fusion. Arrows shown upper and lower points of fusion. From a 26-h-old broth culture of Spirillum lunatum. (Reproduced by permission of Cambridge University Press from: M. A. Williams and S. C. Rittenberg, J. Gen. Microbiol. 15:205–209, 1956)*

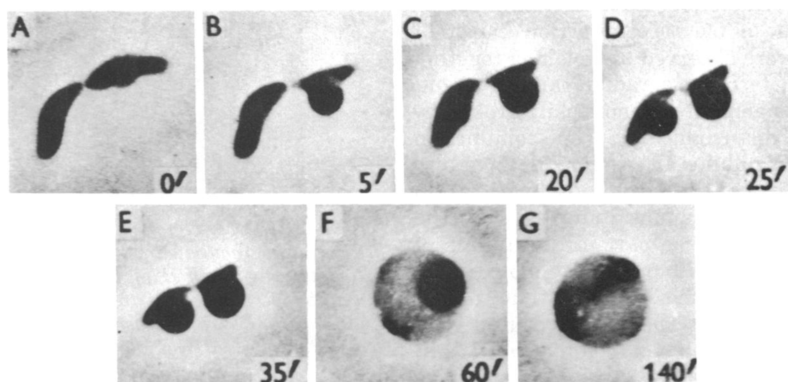


FIG. 18. *Coccoid body formation from a protuberance from the middle of a crescent-shaped form. The coccoid bodies from the contiguous organisms have fused. From a 30-h-old broth culture of Spirillum lunatum. (Reproduced by permission of Cambridge University Press from: M. A. Williams and S. C. Rittenberg, J. Gen. Microbiol. 15:205–209, 1956)*

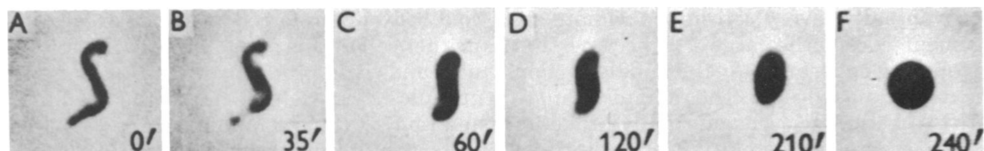


FIG. 19. *Coccoid body formation by shortening and rounding. From a 24-h-old broth culture of Spirillum lunatum. (Reproduced by permission of Cambridge University Press from: M. A. Williams and S. C. Rittenberg, J. Gen. Microbiol. 15:205–209, 1956)*

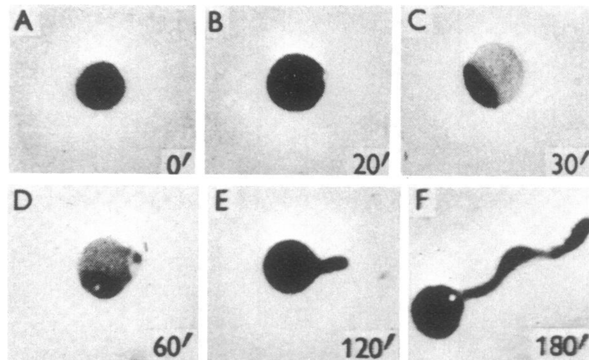


FIG. 20. *Unipolar germination of a coccoid body. Coccoid bodies were transferred from a 4-week-old culture into fresh medium; the sequence started after 10 h in new culture. (Reproduced by permission of Cambridge University Press from: M. A. Williams and S. C. Rittenberg, J. Gen. Microbiol. 15:205-209, 1956)*

ciculus, and all members of *Oceanospirillum* except *O. japonicum* (83, 180, 181). Coccoid body formation also occurs in other helical bacteria besides spirilla, such as *Campylobacter fetus* (140, 166), *Vibrio* (4, 61, 161), and *Desulfovibrio* (111).

The morphological changes occurring in cultures of *O. japonicum* were studied in detail by Watanabe (187). Using a vital-staining procedure (dilute methylene blue), Watanabe found that young cells possessed an evenly staining protoplasm but, in 40- to 48-h-old cultures, stained granules could be seen in the cells. At this stage, a large unstained region at the extreme ends of the cells could be seen; this region was referred to as a "conjunction capsule." Many cells were observed to be joined together at the poles, forming a radiate arrangement, and this arrangement was not easily broken by mechanical disturbance. Such "conjunction capsules" and radiate arrangements have also been photographed in *A. serpens* by Terasaki (179). Although Watanabe indicated that the polar flagella played no part in the linking of the cells, Terasaki (179) presented photographs of flagella stains which indicated that radiate arrangements in *A. serpens* were caused by a mechanical intertwining of flagella (Fig. 21). In *O. japonicum*, one unusually large spirillum, about twice as wide as usual, seemed to be always present in the radiate arrangement (187). This large cell, or "stalk cell," was thought to keep the radiate arrangement stable, but there was little evidence to support this. Eventually, every cell in the radiate arrangement except the large cell became thin with tapering ends, and only the middle portion of the cells stained deeply. The radiate arrangement then became looser and finally broke up into free organisms exhibiting a slow spiral movement. A protuberance appeared at the middle of some of the cells, and as it enlarged it absorbed much of the protoplasm of the cell. Eventually this coccoid body separated from its mother cell; because of this separation, the coccoid body was referred to as an "exocyst." The mother cell at this point contained mostly granules and little protoplasm, and upon lysis the granules were released into the medium. In addition to the formation of exocysts, "endocysts" were formed by highly motile cells in the

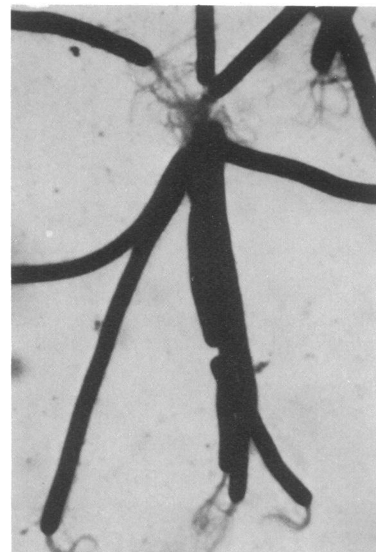


FIG. 21. *Radiate arrangement in Aquaspirillum serpens ATCC 15278 taken from a 24-h-old nutrient broth culture incubated at 25 C. Stained by the flagella staining method of Löffler. ×2,000. (Reproduced by permission of the Suzugamine Women's College from: Y. Terasaki, Bull. Suzugamine Women's Coll. Nat. Sci. 15:9-17, 1970)*

medium. These cells, which did not aggregate into a radiate arrangement, gradually became thicker and rounded up to form a coccoid body in the manner described by Williams and Rittenberg (197). The formation of both types of cysts required ca. 5 to 6 days of incubation. Both types of cysts could germinate when transferred to fresh medium. Another point of interest described by Watanabe (185) was the formation of $CaCO_3$ crystals by *O. japonicum* when cultured in media containing calcium malate or lactate. This was presumably due to the rise in pH caused by oxidation of the substrates. The most interesting phenomenon of all in this connection was the formation of $CaCO_3$ tubes or sheaths surrounding many of the cells.

Whether or not such terms as "microcyst," "exocyst," or "endocyst" should be used is questionable, since the term "cyst" implies a resting form that has thickened walls and is able to survive under conditions where the vegetative cell cannot; e.g., resistance to drying is characteristic of the microcysts of myxobacteria or the cysts of *Azotobacter*. No evidence of thickened walls can be seen in the phase-contrast photomicrographs of coccoid bodies taken by Williams and Rittenberg (197) or in electron micrographs of coccoid bodies formed by members of *Vibrio* (4, 61) or *Desulfovibrio* (111). Thin sections of coccoid bodies from an organism identified as *A. serpens* revealed no unusual features in the cell walls, although intracytoplasmic membranes occurred in some of the partially empty bodies (13). The possibility that the coccoid bodies formed by the strain of *S. lunatum* used by Williams and Rittenberg (197) might, in fact, have been spheroplasts was investigated by B. L. Kelly (Ph.D. thesis, University of Southern California, Los Angeles, 1958). She prepared spheroplasts by means of penicillin and compared their sensitivity to lysis in distilled water with that for "microcysts" occurring naturally in old cultures (4 to 27 days of incubation). The "microcysts" were quite refractory to lysis compared with the spheroplasts. However, the "microcysts" might essentially be spheroplasts that retained sufficient peptidoglycan to resist lysis. Moreover, studies of the coccoid bodies formed by *Desulfovibrio aestuarii* (111) indicate that resistance to lysis is not necessarily indicative of viability. However, Williams and Rittenberg (197) found by microscopy examination of 4-week-old cultures of *S. lunatum* suspended in fresh medium that the majority of coccoid bodies were capable of germination. Whether this applies to coccoid bodies formed by freshwater spirilla or other species of marine spirilla is not yet known.

Studies with other genera of helical bacteria in which coccoid body formation has been observed may be pertinent to an understanding of the nature of the bodies formed by members of *Aquaspirillum* or *Oceanospirillum*. In *Campylobacter fetus*, resistance to lysis and an ability to germinate have been described for coccoid bodies (140), but it is not clear how long the coccoid bodies can remain viable. Baker and Park (4) recently studied the properties of coccoid bodies formed by a cholera-like *Vibrio*. They found the bodies to be nonviable, and survival of the culture depended on the few rod forms that were still present. The walls of the coccoid bodies contained much less peptidoglycan than those of the rod form, but some was still present. The bodies contained much less ribonucleic acid than the rods, and an examination of the supernatant medium indicated that much DNA had leaked from the bodies. In a study of the coccoid bodies formed by *D. aestuarii*, Levin and Vaughn (111) found a high resistance to osmotic lysis, in contrast to the vegetative cells; yet the coccoid bodies were not viable and, indeed, loss of viability appeared to precede their formation. Phase-contrast microscopy revealed the occurrence of a discrete, peripheral region of cytoplasm in the otherwise empty-appearing bodies. Such an appearance was also evident in photographs taken by Williams and Rittenberg (197) of the coccoid bodies of *S. lunatum*. Thin-sections of *Desulfovibrio* coccoid bodies revealed the occurrence of a protoplast greatly retracted away from the trilaminar cell wall (111). The coccoid bodies of *Vibrio marinus* in thin section revealed the occurrence of at least one, and often three or four, retracted protoplasts, all enclosed by a single trilaminar cell wall (61). The viability of the coccoid bodies of *V. marinus* was not determined.

Possibly the most significant report pertaining to the nature and formation of coccoid bodies in spirilla is that by Clark-Walker (35). Within 6 h after treatment of cultures of three strains of *A. itersonii* with mitomycin or ultraviolet light, large numbers of coccoid bodies were formed. Untreated control cultures exhibited no such formation of bodies at the same time. The bodies were fragile and they lysed on electron microscope grids, allowing internal structures to be negatively stained. Numerous phage tails, and occasionally some rhapidosomes, were evident. Purified preparations of the phage tails revealed many contracted tail sheaths (Fig. 22), and in some cases tail fibers were present. Neither phage heads nor com-

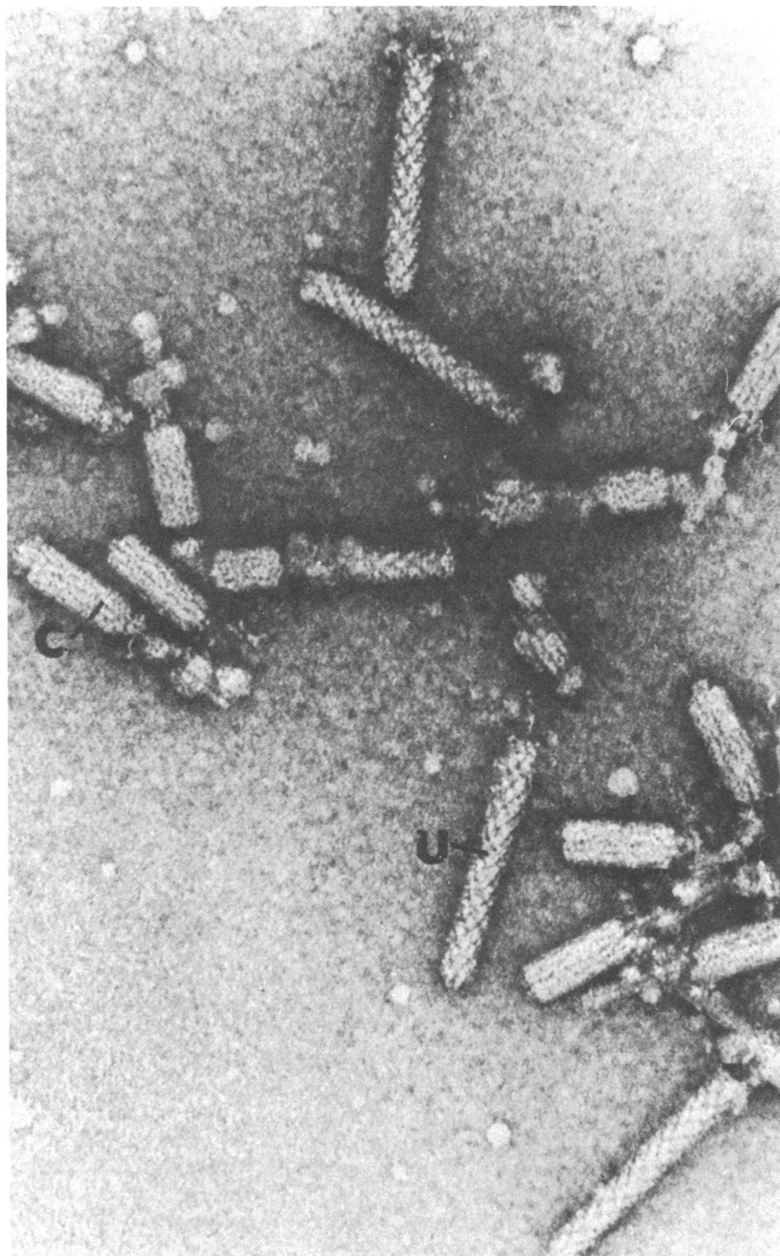


FIG. 22. *Purified phage tails from lysed coccoid bodies of Aquaspirillum itersonii. The coccoid bodies were obtained by treatment of the bacteria with mitomycin. U, Uncontracted phage tail; C, contracted phage tail. ×246,000. (Reproduced by permission of the American Society for Microbiology from: G. D. Clark-Walker, J. Bacteriol. 97:885–892, 1969)*

plete virus particles were observed. The phage preparations were not infectious for the strains of *A. itersonii* from which they had been derived or for other strains of *A. itersonii*. An examination of naturally occurring coccoid bodies (arising in 3- or 4-day-old cultures) rarely revealed phage tails, but many of the coccoid bodies contained rhapidosomes. The possibility that rhapidosomes may be of bacteriophage origin, perhaps polysheaths, has been discussed

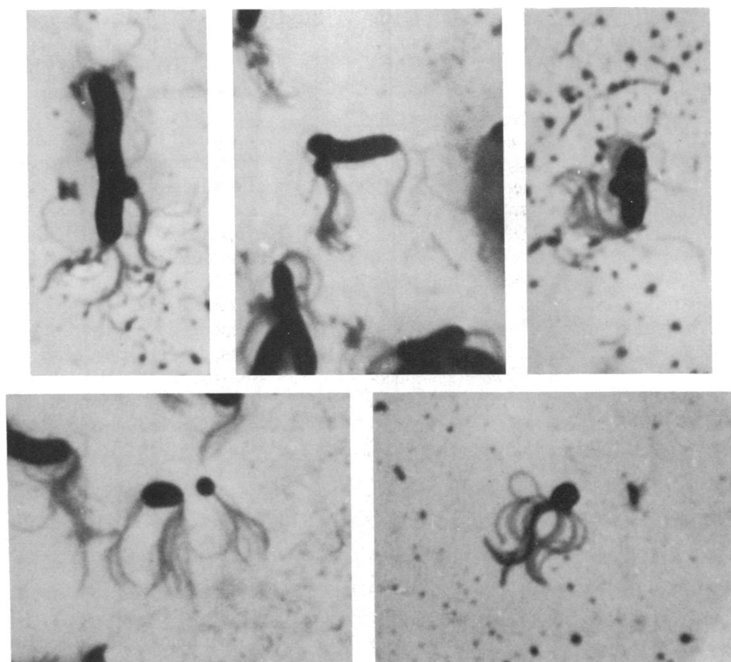


FIG. 23. *Formation of budlike bodies in Aquaspirillum serpens ATCC 15278 from 24-h-old agar slant cultures incubated at 37 C. Cells were stained by the flagella staining method of Löffler. ×2,000. (Reproduced by permission of the Suzugamine Women's College from: Y. Terasaki, Bull. Suzugamine Women's Coll. Nat. Sci. 15:9-17, 1970)*

by Bradley (17). Clark-Walker (35) suggested that the coccoid bodies of spirilla might result from the action of lysozyme produced by the defective phages. The action of the lysozyme from within the spirilla might lead to a softening of the cell wall, resulting in a spherically shaped cell. One way to obtain additional supporting evidence for this proposed role of defective phages would be to derive a strain of *A. itersonii* lacking the ability to produce defective phages when exposed to mitomycin or ultraviolet light. Such a strain should also be incapable of forming coccoid bodies under normal conditions. Unfortunately, all attempts by Clark-Walker to derive a strain lacking defective phages were unsuccessful. Nevertheless, the demonstration of defective phages in *A. itersonii* provides a plausible hypothesis to account for the formation of coccoid bodies by certain spirilla. That it may not be applicable to all coccoid bodies is indicated by the failure of Baker and Park (4) to induce formation of coccoid bodies or phage particles by application of mitomycin to a *Vibrio* capable of forming coccoid bodies in old cultures. Moreover, whether defective phages occur in spirilla other than *A. itersonii* is not yet known.

Budlike Bodies

Spirilla generally multiply by fission but, in a careful study of the morphological changes occurring in cultures of *A. serpens*, Terasaki (179) photographed small budlike bodies that were either free in the medium or attached to one pole or laterally on some of the cells (Fig. 23). These bodies sometimes possessed polar flagella, even when still attached to the mother cell. The bodies were different from the coccoid bodies previously discussed in that they never had a diameter greater than that of the spirilla. The viability of the budlike bodies was not determined.

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