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CHAPTER 4.1

Free-Living Saccharolytic Spirochetes: The Genus *Spirochaeta*

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Introduction

The genus *Spirochaeta* includes anaerobic and facultatively anaerobic spirochetes that are indigenous to aquatic environments such as the mud and water of ponds and marshes. These spirochetes occur in nature as free-living forms; that is, their existence does not depend on physical associations with other organisms (Canale-Parola, 1984a).

Spirochaeta cells are helically shaped (Fig. 1–12) and possess the typical ultrastructural features of spirochetes (Canale-Parola, 1984b; Fig. 13). The outermost structure of the cells is an "outer membrane," or "outer sheath," which encloses the coiled cell body ("protoplasmic cylinder") consisting of the cytoplasm, the nuclear region, and the peptidoglycan-cytoplasmic membrane complex (Fig. 13). Organelles ultrastructurally similar to bacterial flagella are located in the area between the outer membrane and the protoplasmic cylinder (Fig. 13). These organelles are essential components of the motility apparatus of spirochetes (Paster and Canale-Parola, 1980) and are usually called "periplasmic flagella." Other names used to designate these motility organelles are "periplasmic fibrils," "axial fibrils," "axial filaments," and "endoflagella."

One end of each periplasmic flagellum is inserted near a pole of the protoplasmic cylinder, while the other end is not inserted (Fig. 13A). Individual periplasmic flagella extend for most of the length of *Spirochaeta* cells so that those inserted near opposite ends overlap in the central region of the organism (Fig. 13A). The *Spirochaeta* cell illustrated in Figure 13 has two overlapping periplasmic flagella, each inserted near a cell pole in a "1-2-1" arrangement. With one exception, all the known *Spirochaeta* species have two periplasmic flagella per cell. The exception is the large spirochete *Spirochaeta plicatilis*, which has as many as 18–20 periplasmic flagella inserted near each end of the protoplasmic cylinder (Blakemore and Canale-Parola, 1973).

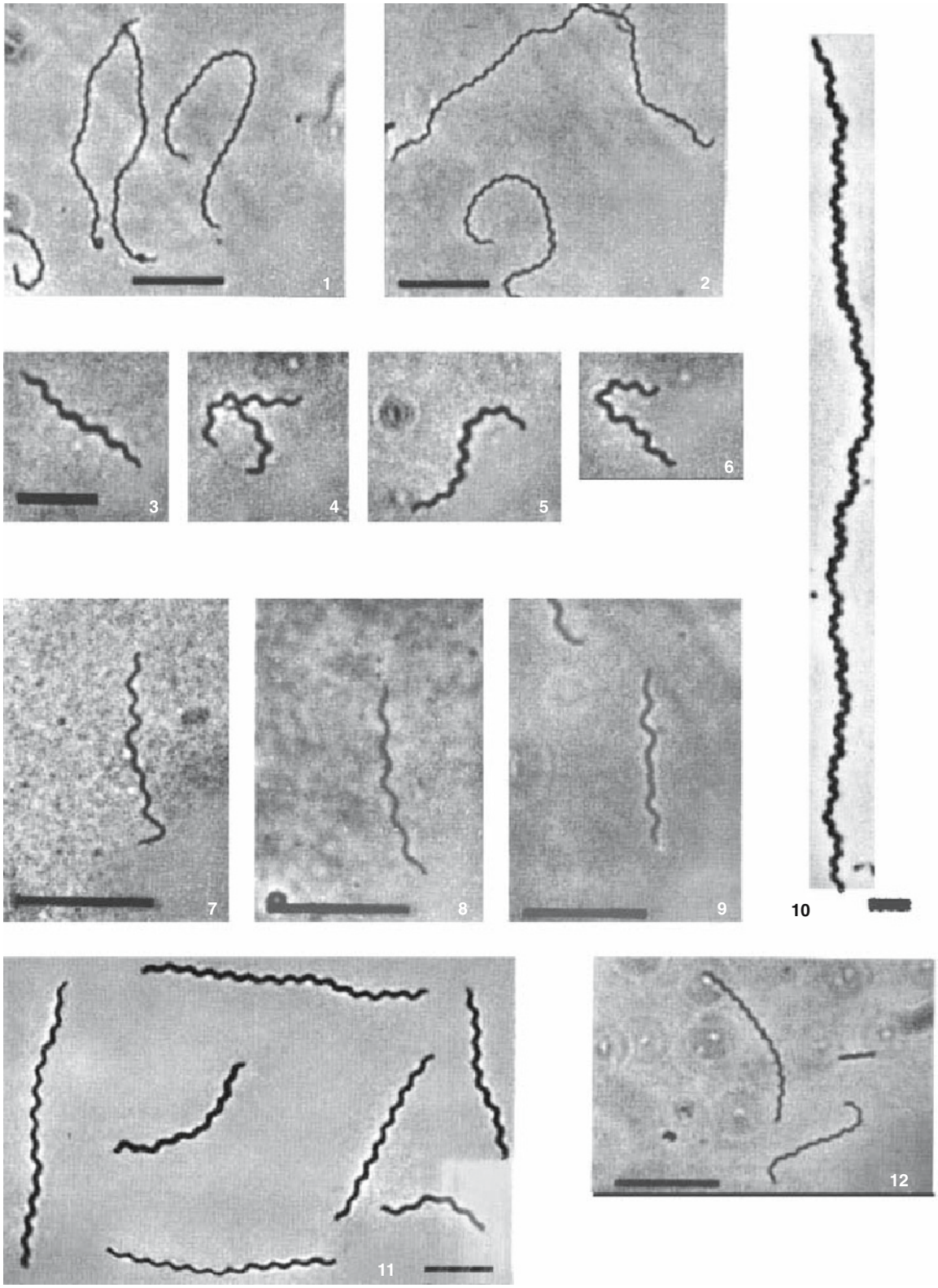
In contrast to flagella of other bacteria, the periplasmic flagella of spirochetes are permanently wound around the cell body and are entirely endocellular (Fig. 13B). Thus, the motility mechanism of spirochetes is different from that of other bacteria, which have flagella that function in direct contact with the external environment and are not wound around the cell body.

Phylogeny

Based on 16S rRNA gene sequence comparisons, most members of the genus *Spirochaeta* form one of the nine phylogenetic clusters of the spirochetes (Fig. 14). All spirochetes are presently classified in the class or phylum Spirochaetes in the order Spirochetales and are divided into three families. The first family, Spirochaetaceae, contains species of the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, "*Spironema*" and *Treponema*. The second proposed family, Serpulinaceae, contains the genus *Brachyspira* (*Serpulina*). The third family, Leptospiraceae, contains species of the genera *Leptonema* and *Leptospira*.

Novel *Spirochaeta* species, or phylotypes, which have not yet been cultivated in vitro, have been identified. For example, several phylotypes from sulfide-rich mud (Tanner et al., 2000) and one associated with the gutless marine oligochete *Olavius loisae* (Dubilier et al, 1999) clearly fall within the genus *Spirochaeta* (Fig. 14). Based on microscopic observations of aquatic and marine sediments, it is apparent that a significant population of additional "uncultivable" spirochetes, including the type species of the genus, *Spirochaeta plicatilis*, remains to be identified.

As shown in Fig. 14, the free-living, obligately anaerobic spirochetes from freshwater environments, *Spirochaeta stenostrepta*, *Spirochaeta zuelzerae* and *Spirochaeta caldaria*, are more closely related to members of the genus *Treponema* than to members of the genus *Spirochaeta*. The phylogenetic clustering of these species has been confirmed by single-base signature analysis, i.e., the sequences of these species possess more



Figs. 1–12. Phase contrast photomicrographs of living *Spirochaeta* cells. Wet mount preparations. Figs. 1 and 2, *Spirochaeta stenostrepta* strain Z1 (DSM 2028, ATCC 25083). Bars = 10 μm. (From Canale-Parola et al., 1968); Figs. 3–6, *Spirochaeta litoralis*, strain R1 (DSM 2029, ATCC 27000). Bar = 5 μm. (From Hespell and Canale-Parola, 1970b); Figs. 7–9, *Spirochaeta aurantia* subsp. *aurantia*, strain J1 (DSM 1902, ATCC 25082), irregularly coiled (Figs. 7 and 9) and regularly coiled (Fig. 8) cells. Bars = 10 mm. (From Breznak and Canale-Parola, 1969); Fig. 10, *Spirochaeta plicatilis*. Bar = 10 μm. (From Blakemore and Canale-Parola, 1973); Fig. 11, *Spirochaeta halophila*, strain RS1 (ATCC 29478). Bar = 5 μm. (From Greenberg and Canale-Parola, 1976); Fig. 12, *Spirochaeta zuelzerae*, strain ATCC 19044. Bar = 10 μm. (From Canale-Parola et al., 1968.)

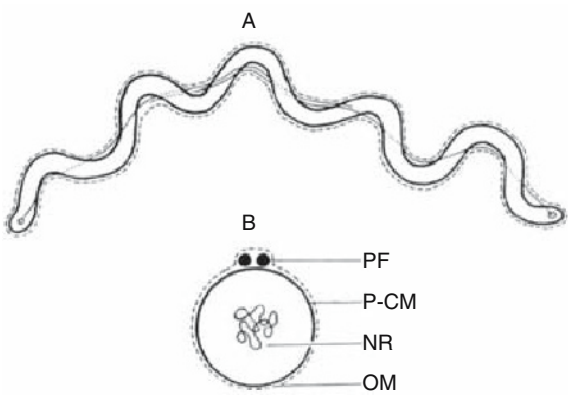


Fig. 13. (A) schematic representation of a *Spirochaeta* cell. The outermost broken line indicates the outer membrane (outer sheath). The protoplasmic cylinder is represented by the area delimited by the solid line adjacent to the outermost broken line. The cell has two periplasmic flagella indicated by the solid-dotted thin lines wound around the protoplasmic cylinder. The insertion points of the periplasmic flagella are represented by the small circles near the ends of the cell. (B) schematic representation of a cross section through a *Spirochaeta* cell. PF, periplasmic flagella; P-CM, peptidoglycan-cytoplasmic membrane complex; NR, nuclear region; OM, outer membrane (outer sheath).

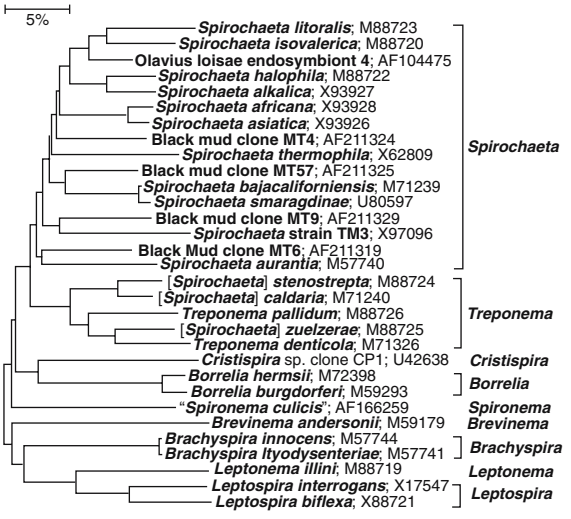


Fig. 14. Phylogeny of the genus *Spirochaeta*. The phylogenetic position of known species and phylotypes of *Spirochaeta* is shown relative to other genera of spirochetes as based on 16S rRNA gene sequence comparisons. GenBank accession numbers for the 16S rRNA sequences of the species tested are shown. Similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (1969). The neighbor-joining method (Saitou and Nei, 1987) was used for phylogenetic tree construction. TREECON,™a software package for the Microsoft Windows environment, was used for the construction and drawing of evolutionary trees (Van de Peer et al., 1994). The scale bar represents a 5% difference in nucleotide sequence determined by taking the sum of all of the horizontal lines connecting two species. Vertical distance has no meaning.

bases found in the sequences of the treponemes than in the sequences of *Spirochaeta* species (Paster et al., 1991). It was speculated that these "*Treponema*-like" free-living spirochetes might represent transitional species, i.e., descendants of the precursors to host-associated treponemes (Paster et al., 1991). However, it is possible that the source of these species may be hosts that release them with their feces. Consequently, it would be worthwhile to screen host environments for these species using specific DNA probes or oligonucleotide primers in polymerase chain reactions (PCRs).

Taxonomy

Fourteen species of *Spirochaeta* are presently known (Table 1). One of these, *Spirochaeta plicatilis*, has not been grown in pure culture, but its ultrastructure and some of its ecological characteristics have been described (Blakemore and Canale-Parola, 1973). Nine species (*Spirochaeta stenostrepta*, *Spirochaeta litoralis*, *Spirochaeta zuelzerae*, *Spirochaeta isovalerica*, *Spirochaeta bajacaliforniensis*, *Spirochaeta thermophila*, *Spirochaeta caldaria*, *Spirochaeta smaragdinae* and *Spirochaeta asiatica*) are obligate anaerobes, and two species (*Spirochaeta alkalica* and *Spirochaeta africana*) are aerotolerant anaerobes. Two other species, *Spirochaeta aurantia* and *Spirochaeta halophila*, are facultative anaerobes and characteristically produce carotenoid pigments when growing aerobically (see Identification). Most species of *Spirochaeta* are mesophilic, growing at optimum temperatures ranging from 15 to 40°C. However, the thermophilic species, *Spirochaeta thermophila* and *Spirochaeta caldaria*, both from thermal springs, have optimum growth temperatures of 66–68 and 48–52°C, respectively (Table 1).

Two subspecies of *Spirochaeta aurantia* are known (Table 1). One of these (subsp. *stricta*) is characterized by significantly narrower coils than the other (subsp. *aurantia*), and its DNA possesses a slightly lower G+C content (Breznak and Canale-Parola, 1975; Canale-Parola, 1984a).

Spirochaeta stenostrepta, *Spirochaeta zuelzerae*, *Spirochaeta caldaria* and *Spirochaeta aurantia* are freshwater species, whereas *Spirochaeta litoralis*, *Spirochaeta isovalerica*, *Spirochaeta bajacaliforniensis* and *Spirochaeta thermophila* are marine species (Table 1) and require sodium ion (Na^+) concentrations ranging from 200 to 480 mM for optimal growth (Aksenova et al., 1992; Fracek and Stolz, 1985; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b). *Spirochaeta halophila* was isolated from a high-salinity pond and grows optimally when 750 mM NaCl, 200 mM $MgSO_4$

Table 1. Characteristics of the fourteen species of the genus *Spirochaeta*.

Species	Size[a] (μm)	Relationship to O_2	Optimum growth temperatures (°C)	Habitat	G+C content DNA (mol%)[b]
1. *Spirochaeta stenostrepta*	0.2–0.3 × 15–45	OA	30–37	Freshwater	60.2
2. *Spirochaeta litoralis*	0.4–0.5 × 5–7	OA	Near 30	Marine	50.5
3. *Spirochaeta zuelzerae*	0.2–0.4 × 8–16	OA	37–39	Freshwater	56.1
4. *Spirochaeta isovalerica*	0.4 × 10–15	OA	15–35	Marine	63.6–65.6
5. *Spirochaeta bajacaliforniensis*	0.1–0.2 × 15–45	OA	36	Marine (laminated sediment)	50.1
6. *Spirochaeta thermophila*	0.2–0.25 × 16–50	OA	66–68	Marine (hot springs)	52.0
7. *Spirochaeta caldaria*	0.2–0.3 × 15–45	OA	48–52	Freshwater (hot springs)	45.0
8. *Spirochaeta smaragdinae*	0.3–0.5 × 5–30	OA	37	Oil field, injection water	50.0
9. *Spirochaeta asiatica*	0.2–0.25 × 15–22.5	OA	35	Alkaline lake	49.2
10. *Spirochaeta alkalica*	0.4–0.5 × 9–18	AT	33–37	Alkaline lake	57.1
11. *Spirochaeta africana*	0.25–0.3 × 15–30	AT	30–37	Alkaline lake	56.1
12. *Spirochaeta aurantia*[c]					
subsp. *aurantia*	0.3 × 10–20	FA	25–30	Freshwater	62.2–65.3
subsp. *stricta*	0.3 × 10–20	FA	25–30	Freshwater	61.2
13. *Spirochaeta halophila*	0.4 × 15–30	FA	35–40	High salinity	62
14. *Spirochaeta plicatilis*[T]	0.75 × 80–250	Unknown	Unknown	Unknown	Unknown

Abbreviations: O_2, molecular oxygen; OA, obligate anaerobic; AT, aerotolerant; FA, facultative anaerobe; and [T], type species.
[a]Lengths indicated are of the majority of cells. Shorter and longer cells are also present in cultures.
[b]Buoyant density determinations, except for species 4, 6, 7, and 9–11 (determined by thermal denaturation method) and species 8 (determined using HPLC).
[c]Wavelength of the cell: subsp. *aurantia* cells, 2.0–2.8 mm; subsp. *stricta* cells, 1.1–1.5 mm.

and 10 mM $CaCl_2$ are present in the medium (Greenberg and Canale-Parola, 1976). Other halophilic species include *Spirochaeta asiatica*, *Spirochaeta alkalica* and *Spirochaeta africana*, which were isolated from the sediments of alkaline lakes and require Na^+ concentrations ranging from 850 to 1,200 mM for optimal growth (Zhilina et al., 1996). The latter three species are also alkaliphilic and growth does not occur below pH 8. *Spirochaeta smaragdinae*, isolated from a production water sample collected from an offshore oil field, requires at least 170 mM NaCl and grows optimally with 850 mM NaCl (Magot et al., 1997).

Habitat

Species of *Spirochaeta* occur, grow, and persist as free-living organisms in a variety of aquatic environments, such as the water, sediments, and muds of ponds, marshes, lakes, rivers, and oceans. Numerous strains of *Spirochaeta* have been isolated from various freshwater environments (Canale-Parola, 1984a; Pohlschroeder et al., 1994) and from marine muds collected in Pacific and Atlantic coastal regions (Aksenova et al., 1992; Fracek and Stolz, 1985; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b). Using 16S rRNA phylogenetic analyses to investigate the microbial diversity of sulfur-rich black mud marine sediments, Tanner et al. (2000) reported the presence of several novel spirochete phylotypes in samples from hypersaline and brackish marshes. Moreover, Weber and Greenberg (1981) determined that *Spirochaeta* cells are present in salt marshes at densities ranging from 104 to 106 per gram (wet weight) of the top 1 cm of sediment.

Selective isolation procedures did not yield spirochetes from deep-sea sediments and water from the Sargasso Sea (3,630 m) and the Puerto Rico Trench (8,140 m; Harwood et al., 1982b). These results indicate that although anaerobic and facultatively anaerobic free-living spirochetes are common in marine coastal environments, they are not widely distributed in deep-sea regions. However, Harwood et al. (1982b) isolated an obligately anaerobic spirochete from a sample collected near a deep-sea (2,550 m) hydrothermal vent at the Galapagos Rift tectonic spreading center, and they observed bacteria with morphologies typical of spirochetes in surface scrapings from mussels present near a vent. The occurrence of spirochetes in this deep-sea region may be ascribed to vent area environmental conditions, which are favorable to the growth of various bacteria. Magot et al. (1997) isolated *Spirochaeta smaragdinae* from a production water sample collected from an offshore oil field of Congo, Africa, and speculated that this

spirochete might be indigenous to the deep subsurface waters of oil fields.

Three alkaliphilic *Spirochaeta* species have been isolated from the sediments of alkaline lakes, including *Spirochaeta alkalica* and *Spirochaeta africana* from Lake Magadi, East African Rift, Kenya, and *Spirochaeta asiatica* from Lake Khatyn in Tuva, Central Asia (Zhilina et al., 1996). These spirochetes grow at pHs of >9, and growth does not occur below pH 8. Moreover, these species are halophilic, requiring Na^+ concentrations ranging from 850 to 1,200 mM for optimal growth (Zhilina et al., 1996). Another halophilic species, *Spirochaeta halophila*, has been isolated from a high-salinity pond located on the Sinai shore of the Gulf of Aqaba (Greenberg and Canale-Parola, 1976).

Most species of *Spirochaeta* are mesophilic, growing at optimum temperatures ranging from 15 to 40°C (Canale-Parola, 1984a). However, thermophilic spirochetes have been isolated from various hot springs in geographically widely separated locations. Obligately anaerobic thermophilic spirochetes isolated from New Zealand thermal springs grow optimally at 45 to 50°C (Patel et al., 1985). Aksenova et al. (Aksenova et al., 1990; Aksenova et al., 1992), and Rainey et al. (1991) isolated (from a marine hot spring on the beach of Shiashkotan Island, Kuril Islands, Kamchatka, Russia, and a brackish hot spring on Raoul Island, Kermadec Archipelago, New Zealand) two strains of thermophilic cellulolytic spirochetes, which they named *Spirochaeta thermophila* (Aksenova et al., 1992). *Spirochaeta thermophila* type strain grew between 40 and 73°C with a growth optimum of 66 to 68°C. Strains of *Spirochaeta caldaria*, which were isolated from hot springs in Oregon and Utah, USA, grew optimally between 48 and 52°C (Pohlschroeder et al., 1994). In addition, based on 16S rRNA gene sequence analyses, spirochete phylotypes have been identified in samples from a hot spring (Octopus Spring) in Yellowstone National Park, USA (Weller et al., 1992). Thus, it is likely that thermophilic spirochetes are widely distributed in thermal spring waters.

Isolation

Selective Procedures

Anaerobic and facultatively anaerobic spirochetes are readily isolated from natural environments by means of selective procedures and usually grow abundantly in ordinary laboratory media. Anaerobic growth yields of the isolates range from 2×10^8 to approximately 10^{10} cells/ml, but commonly are 6×10^8 to 8×10^8 cells/ml (Breznak and Canale-Parola, 1975; Canale-Parola, 1973; Greenberg and Canale-Parola, 1976; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970a; Hespell and Canale-Parola, 1970b). Cell population doubling times in anaerobic cultures vary from 2.2 to 12 h, depending on the species and the growth conditions. Aerobically grown cultures yield from 0.7×10^9 to 1.2×10^9 spirochetes/ml, with doubling times of 2 to 4 h (Breznak and Canale-Parola, 1975; Canale-Parola, 1973; Greenberg and Canale-Parola, 1976).

A procedure in which the antibiotic rifampin (rifampicin) serves as a selective agent is quite effective for the isolation of free-living spirochetes (genus *Spirochaeta*) from natural environments (Harwood et al., 1982b; Patel et al., 1985; Stanton and Canale-Parola, 1979; Weber and Greenberg, 1981). This procedure, which is described below, is based on the observation that spirochetes in general are naturally resistant to rifampin (Leschine and Canale-Parola, 1986; Stanton and Canale-Parola, 1979). Thus, spirochetes such as *Spirochaeta stenostrepta* and *Spirochaeta aurantia* grow in the presence of as much as 100 to 200 μg of rifampin per ml of medium (Leschine and Canale-Parola, 1986), whereas the growth of many other bacteria is inhibited. The resistance of spirochetes to rifampin is probably due to the low affinity of their RNA polymerase for the antibiotic (Allan et al., 1986; Leschine and Canale-Parola, 1986).

Other enrichment procedures used in the isolation of *Spirochaeta* species are based either on one or both of the following selective factors: 1) the ability of spirochetes to pass through filters that retain most other bacteria, and 2) the migratory movement of spirochetes through agar media (Canale-Parola, 1973; Canale-Parola, 1984a). These selective procedures enrich for species of *Spirochaeta* measuring less than 0.5 μm in diameter (Table 1).

In the enrichment-by-filtration procedure, which is described in detail below, separation of *Spirochaeta* species from most of the microorganisms present in mud or water is achieved by techniques involving filtration through cellulose ester filter discs (e.g., Millipore filters) having an average pore diameter of 0.3 or 0.45 μm. Spirochetes pass through these filter discs because of their relatively small cell diameter (Table 1) and probably also because their motility apparatus enables them to swim freely in liquids as well as to move in contact with solid surfaces.

The enrichment-by-migration procedure uses the ability of spirochetes to move through agar gels or media containing as much as 1 to 2% (w/v) agar. This movement or migration occurs primarily within the agar gel, i.e., below the surface of the agar medium. In contrast, flagellated bacteria usually cannot carry out translational

movement through gels or media containing agar at the above-mentioned concentrations, although several exceptions have been reported (Greenberg and Canale-Parola, 1977b). Apparently, the cell coiling of spirochetes is important for their translational motion through agar gels, inasmuch as this type of movement is impaired in mutant spirochetes lacking the cell-coiling characteristic of the parental strain (Greenberg and Canale-Parola, 1977c).

Migration of spirochetes through agar media results from the unique motility mechanism of these bacteria (Canale-Parola, 1977; Canale-Parola, 1978), as well as from chemotaxis toward the energy and carbon source (Breznak and Canale-Parola, 1975; Greenberg and Canale-Parola, 1977a). The role of chemotaxis in the migration of saccharolytic spirochetes through agar media has been studied (Breznak and Canale-Parola, 1975). When these spirochetes are inoculated in the center of glucose-containing agar medium plates, they grow using this sugar as their energy source. Utilization of the sugar by the spirochetes gives rise to a glucose concentration gradient that moves away from the center of the plate as more of this carbohydrate is metabolized by the spirochetes. Because the spirochetes exhibit chemotaxis toward glucose and are able to move through the agar gel, they migrate into the areas of higher glucose concentration within the gradient. Thus, the spirochetal population follows the outward movement of the gradient and migrates toward the periphery of the plate. This behavior results in the formation of a growth "veil" or "ring" of spirochetes for which glucose serves both as the energy source for growth and as the chemoattractant (Breznak and Canale-Parola, 1975; Canale-Parola, 1973). The veil or ring increases continuously in diameter during incubation and may reach the outer edge of the plate. The migration rate of the spirochetal population is greatest in agar media containing low substrate concentrations (e.g., 0.02% glucose). In these media the substrate becomes rapidly depleted in the region where spirochetes are growing, and the spirochetal population moves toward the outer zone of higher substrate concentrations at a relatively fast rate (Breznak and Canale-Parola, 1975).

In procedures for the isolation of *Spirochaeta* species from natural environments, the chemotactic behavior and the ability of these bacteria to move through agar gels have important selective functions. In a typical isolation procedure, a small, shallow cylindrical hole is made through the surface of an agar medium containing a low concentration of carbohydrate. Rifampin may be included in the medium as an additional selective agent for spirochetes. The medium may be in a Petri dish or a small bottle. A tiny drop of pond water, or of any other material in which spirochetes have been observed, is placed within the hole. The chemotactic, saccharolytic spirochetes in the inoculum multiply and form a growth veil that extends outwardly through the agar medium. Thus, the spirochetes in the veil move away from contaminants, which remain mainly in the vicinity of the inoculation site. Spirochetal cells from the outermost edge of the veil are used to obtain pure cultures by conventional methods, such as streaking on agar medium plates. Isolation procedures involving chemotaxis and movement through agar gels are described below.

Selective isolation techniques have not been developed for the large *Spirochaeta* species, such as *Spirochaeta plicatilis*.

Enrichment by Migration

When other bacteria vastly outnumber spirochetes in the inoculum, it is advisable to begin the isolation procedure with an enrichment-by-migration step (the principle has been discussed above). A suitable medium (EBM agar medium) for this enrichment has the following composition in g/100 ml distilled water: yeast extract (Difco), 0.1; Trypticase (BBL Microbiology Systems), 0.1; L-cysteine, HCl, 0.05; resazurin, 10^{-4}; and agar (Difco), 1. For the enrichment and isolation of marine spirochetes, a mixture of seawater (70 ml) and distilled water (30 ml) is used instead of plain distilled water. The pH of the medium is adjusted to 7.2. After autoclaving, cellobiose is added as a sterile solution to a final concentration of 0.01 g/100 ml medium. Rifampin (filter-sterilized solution) may be used as a selective agent (0.5 mg per 100 ml of medium).

The medium is pre-reduced (Hungate, 1969) and is dispensed into narrow-necked 60-ml glass bottles. During this step and the steps that follow, the medium is maintained in an anaerobic atmosphere by delivering a stream of oxygen (O_2)-free nitrogen (N_2) into the bottles. The bottles containing the medium are sealed with neoprene rubber stoppers and are placed in a press to hold the stoppers in place during autoclaving. The sterilized medium is allowed to cool to approximately 50°C, and cellobiose and, if desired, rifampin are added. The complete medium is solidified at a 45° angle so that bottle slants are obtained. Then, a small (5-mm wide, 6-mm deep), cylindrical well is melted halfway down each slant by heating the tip of a thin metal rod and touching the agar with it. Alternatively, the well can be made by aspirating some of the agar medium with a sterile Pasteur pipette connected to a vacuum apparatus. All further manipulations are made either while maintaining an N_2 atmosphere within the bottles (Hungate, 1969)

or with the bottles placed within an anaerobic chamber and the rubber stoppers replaced by cotton stoppers.

Before inoculation, any liquid that oozed from the agar medium is removed by suction from the well and the lower part of each slant. The bottle slants are inoculated by carefully placing a small volume of inoculum (e.g., mud) into the well, and they are incubated at 30°C. In successful enrichments, the spirochetes form a characteristic, semitransparent growth veil that extends down into the agar medium and diffuses out toward the periphery of the slant, away from contaminating organisms growing in and near the well. The veil usually is visible after 4 to 7 days of incubation. Cells from the edges of the growth veil are used to obtain pure cultures of the spirochetes by means of a procedure involving serial dilutions in a rifampin-containing medium (see below).

Isolation in Rifampin-Containing Media

Serial dilutions in melted, rifampin-containing agar (RIM) medium can be used to isolate spirochetes from materials in which they are present in relatively large numbers (e.g., directly from mud or from the outer edges of growth veils in the bottle slants mentioned above). The spirochete-containing inoculum is serially diluted in tubes of melted (45°C), pre-reduced RIM medium. This medium is identical to the EBM agar medium described above except that the final concentration of cellobiose is 0.2% (w/v), rifampin (0.5 mg/100 ml of medium) is added, and the agar (Bacto, Difco) concentration is 0.8% (w/v). The medium is dispensed and sealed in anaerobic culture tubes containing an N_2 atmosphere (Hungate, 1969).

Spirochete colonies within the RIM agar medium are recognizable inasmuch as they are spherical and because, as a result of cell migration through the agar medium, they appear as "transparent bubbles," "veil-like growth with a dense center," or "cotton ball-like growth" (Paster and Canale-Parola, 1982; Stanton and Canale-Parola, 1979; Weber and Greenberg, 1981). To obtain pure cultures, the serial dilution step is repeated at least twice, using cells from spirochete colonies that developed within the RIM agar medium.

Procedures involving serial dilutions in rifampin-containing agar media have been used by Weber and Greenberg (1981) and by Patel et al. (1985) to isolate spirochetes from salt marsh sediments and hot springs, respectively.

Selective Enrichment by Filtration

A filtration technique has been used in the isolation of *Spirochaeta stenostrepta* (Canale-Parola et al., 1967; Canale-Parola et al., 1968), *Spirochaeta litoralis* (Hespell and Canale-Parola, 1970b), and the facultatively anaerobic *Spirochaeta halophila* (Greenberg and Canale-Parola, 1976). In all cases, the source of the spirochetes was black mud that had the characteristic smell of hydrogen sulfide (H_2S). *Spirochaeta stenostrepta* was isolated from a mud sample collected from a fresh-water pond, *Spirochaeta litoralis* from marine mud, and *Spirochaeta halophila* from the mud of Solar Lake, a high-salinity pond located on the Sinai shore of the Gulf of Aqaba. A filtration technique also was used in the isolation of the alkaliphiles, *Spirochaeta asiatica*, *Spirochaeta alkalica* and *Spirochaeta africana*, from the sediments of alkaline lakes located in the East African Rift region and central Asia (Zhilina et al., 1996).

Medium for Isolating *Spirochaeta stenostrepta*

Distilled water	875 ml
Glucose	5.0 g
Peptone	2.0 g
Yeast extract	0.3 g
Vitamin B_{12}	10^{-5} g
Phosphate solution	15 ml
Salts solution	100 ml
Sulfide solution	10 ml

The phosphate solution is prepared by dissolving 30 g of KH_2PO_4 and 70 g of K_2HPO_4 in 1,000 ml of distilled water.

To prepare the salts solution, 0.2 g of ethylenediamine tetraacetic acid are dissolved in 800 ml of distilled water by heating. The pH of the resulting solution is adjusted to 7.0 with 2.5% KOH. Then the following additions are made: $MgSO_4 7H_2O$, 2.0 g; $CaCl_2 2H_2O$, 0.75 g; $FeSO_4 7H_2O$, 0.1 g; trace elements solution (below), 5.0 ml. Finally, the volume of the salts solution is adjusted to 1,000 ml with distilled water. The sulfide solution (2 g of $Na_2S 9H_2O$/100 ml distilled water) is autoclaved separately and added shortly before inoculating the (precooled to 30–35°C) sterile medium. After sterilization by autoclaving, a precipitate is present in the medium, but it disappears as the medium cools. The final pH of the complete medium is 6.9–7.0.

Trace elements solution: a separate solution of each salt listed below, in the amount indicated. Heating may be required to dissolve some of the salts. The pH of the sodium molybdate and sodium vanadate (Na_2MoO_4 and $NaVO_3$) solutions is adjusted to a value below 7. The potassium iodide (KI) solution is added to the aluminum chloride ($AlCl_3$) solution and mixed by stirring. Then the other solutions are added, one at a time, to this mixture, with stirring, in the order in which they are listed below.

$AlCl_3 \cdot 6H_2O$	0.50 g
KI	0.25 g
KBr	0.25 g
LiCl	0.25 g
$MnCl_2 \cdot 4H_2O$	3.50 g
H_3BO_3	5.50 g
$ZnCl_2$	0.50 g
$CuCl_2 \cdot 2H_2O$	0.50 g
$NiCl_2 \cdot 6H_2O$	0.50 g
$CoCl_2 \cdot 6H_2O$	0.50 g
$SnCl_2 \cdot 2H_2O$	0.15 g
$BaCl_2 \cdot 2H_2O$	0.15 g
$Na_2MoO_4 \cdot 2H_2O$	0.25 g
$NaVO_3$	0.05 g

The volume of the final mixture is adjusted to 1,800 ml by adding distilled water and to pH 3–4 with HCl. A fine, white precipitate replaces a yellow precipitate after a few days. The solution should be mixed thoroughly immediately before it is used and may be stored at room temperature. This trace element solution is a modification of a solution described by Pfennig (1965).

Isolation of *Spirochaeta stenostrepta* (Canale-Parola et al., 1967) Black mud, from which a strong odor of H_2S could be detected, was suspended in aqueous sodium sulfide (0.02% Na_2S9H_2O). The slurry was filtered through Whatman No. 40 filter paper, and the filtrate was subjected to filtration through sterile cellulose ester filter discs (Millipore, pore diameter 0.45 μm). Each 1-ml aliquot of the resulting filtrate was added aseptically to a 60-ml glass stoppered bottle, half filled with sterile isolation medium. The bottles were then completely filled with medium, stoppered without trapping air bubbles, and incubated at 30°C (each bottle was covered with a sterile 50-ml beaker). After 5 to 7 d of incubation, the microbial population in many of the bottles consisted predominantly of thin spirochetes. Pure cultures were obtained by use of dilution shake cultures (the medium was covered with sterile paraffin) or by plating serial dilutions and incubating the plates in the absence of O_2 (Bray dishes). The isolation medium solidified with 1.5% agar was used. Spirochetal colonies appeared after 5 to 6 d. After isolation, the organisms were also grown in Florence flasks filled with a medium GYPT containing (g/100 ml distilled water): glucose, 0.5; yeast extract and peptone, 0.2 each; and sodium thioglycolate, 0.05. The pH of this medium was adjusted to 7.0–7.3 before sterilization. The spirochetes were maintained in paraffin-layered stab cultures of medium GYPT containing 1.5% agar at 5°C and transferred monthly. Subsurface colonies of *Spirochaeta stenostrepta* in agar media are white, spherical, and characteristically fluffy in appearance.

Spirochaeta litoralis and *Spirochaeta halophila* were isolated by a procedure similar to that used for *Spirochaeta stenostrepta*, except that different isolation media were used and the incubation temperatures were 22–23°C for *Spirochaeta litoralis* and 37°C for *Spirochaeta halophila* (Hespell and Canale-Parola, 1970b; Greenberg and Canale-Parola, 1976). Furthermore, one volume of the mud used as the source of *Spirochaeta litoralis* was suspended in five volumes of isolation medium before filtration through filter paper.

Isolation of *Spirochaeta litoralis* (Hespell and Canale-Parola, 1970b) The isolation medium for the marine spirochete included (g/100 ml of distilled water): tryptone (Difco), 0.3; yeast extract (Difco), 0.05; and NaCl, 2.0. To this mixture 2 ml of 1 M potassium phosphate buffer (pH 7.4) and 0.2 ml of a salt solution (see below) were added. The pH of the medium was adjusted to 7.3 with KOH before sterilization. Immediately before inoculation, the medium was supplemented with 2 ml of a sterile glucose solution (25 g per 100 ml distilled water) and with 0.5 ml of a sterile Na_2S9H_2O solution (10 g per 100 ml of distilled water). The final pH of the medium ranged from 7.4 to 7.5.

The salts solution contained (g/75 ml of distilled water): tetrasodium ethylenediamine tetraacetate, 1; $CaCl_22H_2O$, 3.75; $MgCl_26H_2O$, 12.5; and $FeSO_47H_2O$, 0.5. To 75 ml of this salt mixture, 25 ml of a trace elements solution (see Medium for Isolating *Spirochaeta stenostrepta*) was added.

After cloning, *Spirochaeta litoralis* was grown routinely in the isolation medium. Subsurface colonies of *Spirochaeta litoralis* in agar media are white to cream-colored and resemble those of *Spirochaeta stenostrepta*.

Maintenance procedures: Broth cultures of *Spirochaeta litoralis* in test tubes remained viable for 3 d at 30°C. Agar stabs grown at 30°C and then kept either at 5 or 30°C were no longer viable after approximately one week, whether they were layered with paraffin or not. Viable cells were not recovered from lyophilized preparations.

Spirochaeta litoralis was satisfactorily maintained by using "depression" cultures (Canale-Parola and Wolfe, 1960) prepared by using 1-liter Erlenmeyer flasks, each containing 800 ml of isolation medium to which 2 g of agar per 100 ml were added. These cultures, when incubated for 2 d at room temperature and then at 15°C, remained viable for at least three months.

Isolation of *Spirochaeta halophila* (Greenberg and Canale-Parola, 1976) The isolation medium contained 0.2 g of peptone (Difco) and 0.4 g of yeast extract (BBL) per 97 ml of an inorganic salt solution which had the

following composition: $CaCl_2 \cdot 2H_2O$, 0.04 M; NaCl, 0.85 M; and $MgSO_4 7H_2O$, 0.2 M. In preparing the salt solution, salts were added in the order in which they are listed to avoid formation of a precipitate. After adjusting the pH to 7.5 with KOH, the medium was sterilized by autoclaving. The volume of the medium was brought to 100 ml by adding separately sterilized solutions of glucose and $Na_2S 9H_2O$ to final concentrations of 0.5% and 0.05%, respectively.

Spirochaeta halophila was grown routinely at 37°C in ISM broth (Greenberg and Canale-Parola, 1975), which differed from the isolation medium because maltose replaced glucose, the $Na_2S 9H_2O$ was omitted, and the composition of the salt solution was changed (to $CaCl_2$, 0.01 M; NaCl, 0.75 M; and $MgSO_4$, 0.2 M). Furthermore, BBL yeast extract was replaced by Difco yeast extract, which supported higher growth yields of our isolate.

Spirochaeta halophila was maintained by storing ISM agar (ISM broth containing 0.75 g agar per 100 ml) plate cultures at 5°C. The cultures were transferred monthly.

Colonies of *Spirochaeta halophila* are pigmented when growing aerobically but lack pigmentation under anaerobic growth conditions. Colonies grown in air on ISM agar plates were red and appeared circular when viewed from above. A portion of each colony grew above the surface of the medium and part of the colony extended into the agar. Areas of diffuse growth were present at the periphery of the colonies. Generally, colonies measured 2–6 mm in diameter after 5 days at 35°C but were smaller or larger depending on the number of colonies on the plate and the length of incubation. Cells streaked on ISM agar plates and grown anaerobically in Bray dishes formed colonies that developed below the surface of the agar medium and were white, spherical and diffuse.

Cultivation of most of the anaerobic species of *Spirochaeta* described here does not require the use of stringent anaerobic procedures (e.g., the Hungate technique). Reducing agents, such as sodium thioglycolate or L-cysteine, are commonly added to the growth media, and other conventional techniques for the growth of anaerobes are used, as discussed elsewhere (Canale-Parola, 1973).

Enrichment by Filtration and Migration

Many strains of *Spirochaeta aurantia* have been isolated by means of a selective technique that combines filtration of the inoculum through a cellulose ester filter disk with migration of the spirochetes in agar media (Breznak and Canale-Parola, 1969; Canale-Parola, 1973; Breznak and Canale-Parola, 1975).

Isolation of *Spirochaeta aurantia* by Filtration and Migration

Ingredient	Medium HE	Medium PEP
Distilled water	50 ml	100 ml
Peptone	0.1 g	0.5 g
Yeast extract	0.1 g	0.05 g
K_2HPO_4	—	0.01 g
Hay extract	50 ml	—
Agar	1 g	1 g

The pH of medium HE is adjusted to 6.5 before sterilization. To prepare the hay extract, 0.5 g of dried barn hay are boiled in 100 ml of distilled water for 10 min. The boiled mixture is filtered using Whatman No. 40 filter paper. The filtrate is the hay extract.

The inoculum, consisting either of pond or marsh water or of a water-mud slurry, was prefiltered through Whatman No. 40 filter paper to remove large particles. Then, the enrichment cultures were prepared by depositing one or two drops of the filtrate near the center of each of a number of sterile cellulose ester filter disks (47-mm disk diameter, 0.3- or 0.45-μm pore diameter, Millipore) previously placed on the surface of isolation medium plates. One filter disk had been placed on each plate, approximately in the center. The cultures were incubated at 30°C for 12–24 h to allow spirochetes in the inoculum to move through the filter disk onto the surface of the medium. Then the filter disks were removed aseptically from the plates, and incubation of the plate cultures was continued. Spirochetes that had passed through the filter disks grew and migrated through the agar medium, forming semitransparent growth veils that diffused toward the edge of the plates. Spirochetal growth veils usually developed in 5–10 days.

Generally, 10–20% of the plate enrichments were successful (i.e., yielded growth veils) when spirochetes were present in the inoculum, as determined by light microscopy. Analyses showed (Breznak and Canale-Parola, 1975) that the total carbohydrate content of a batch of isolation medium HE was 40 mg/100 ml and the glucose content 5 mg/100 ml. Medium PEP contained 6 mg total carbohydrate and less than 1 mg glucose per 100 ml. As discussed previously, low carbohydrate concentrations, such as those in isolation media HE and PEP, are used to enhance the rate of spreading of the spirochetal growth veil through the agar medium.

It should be noted that bacteria other than spirochetes may form subsurface spreading growth veils in the enrichment plates. Most common among these veil-forming bacteria are *Aquaspirillum* (*Spirillum*) *gracile* (Canale-Parola et al., 1966) and *Serpens flexibilis* (Hespell, 1977). *Serpens flexibilis* cells are flexible, Gram-negative rods that have bipolar as well as lateral flagella.

Pure cultures of *Spirochaeta aurantia* are obtained by streaking cells from the outer edge of the growth veil onto isolation medium plates or growth medium plates.

A suitable growth medium for *Spirochaeta aurantia* contains (g/99 ml distilled water): glucose, 0.2; yeast extract, 0.2; and Trypticase (BBL), 0.5. The pH is adjusted to 7.5 before sterilization. When desired, agar (1 g) is added. After autoclaving and allowing the medium to cool, 1 ml of sterile 1 M potassium phosphate buffer (pH 7) is added.

Growth of some *Spirochaeta aurantia* strains is either partially or totally inhibited in media containing agar (Difco) concentrations higher than 1% (w/v). Thus, viable cell counts usually are higher when performed using plates of media containing 0.75 or 1% agar than when the cells are grown in media including agar at higher concentrations. *Spirochaeta aurantia* cells grown in media containing agar at concentrations higher than 1% frequently are aberrant in morphology. Many usually long, poorly coiled, filamentous cells are present, as well as an abundance of spherical bodies (Breznak and Canale-Parola, 1975).

Surface, aerobic colonies of *Spirochaeta aurantia* in growth medium (agar, 1%) are light orange to orange, round or nearly round, with slightly irregular edges, and measure 1–4 mm in diameter after 4–7 days at 30°C. The colonies grow mostly within the agar medium, just under the surface, but many have a slightly raised central portion. One strain (Vinzent strain) was found to produce both this type of colony and a "pinpoint" type of colony, measuring approximately 0.5 mm in diameter and growing primarily on the surface of the agar medium (Breznak and Canale-Parola, 1975).

Surface, anaerobic colonies of *Spirochaeta aurantia* (growth medium, 1% agar) are similar in morphology to the aerobic ones, but are not pigmented. Subsurface anaerobic colonies are white and spherical.

Spirochaeta aurantia may be maintained on slants of growth medium at 5°C. These stock cultures are transferred monthly.

Identification

At present, free-living, anaerobic and facultatively anaerobic, helical bacteria that possess the ultrastructural features typical of spirochetes (e.g., periplasmic flagella and outer sheath) are classified in the genus Spirochaeta. However, phylogenetic analyses have clearly indicated that some species identified as *Spirochaeta* using these criteria—namely, the free-living, obligately anaerobic spirochetes from freshwater environments, *Spirochaeta stenostrepta*, *Spirochaeta zuelzerae* and *Spirochaeta caldaria*—are more closely related to members of the genus *Treponema* than to other members of the genus *Spirochaeta* (see Phylogeny).

Differentiation among the obligately anaerobic species (Table 1, species 1–9; see Taxonomy) may be based, in part, on determinations of carbohydrate fermentation end products. *Spirochaeta stenostrepta*, *Spirochaeta litoralis*, *Spirochaeta isovalerica* and *Spirochaeta bajacaliforniensis* form acetate, ethanol, carbon dioxide (CO_2) and molecular hydrogen (H_2) as major products of glucose fermentation, whereas *Spirochaeta zuelzerae*, *Spirochaeta thermophila* and *Spirochaeta caldaria* produce acetate, lactate, CO_2, H_2, and, in some cases, small amounts of succinate (Aksenova et al., 1992; Canale-Parola, 1984a; Fracek and Stolz, 1985; Pohlschroeder et al., 1994). *Spirochaeta asiatica* produces acetate, ethanol, and lactate, but not H_2, during glucose fermentation (Zhilina et al., 1996). In addition, *Spirochaeta isovalerica* forms small amounts of isovalerate, 2-methylbutyrate and isobutyrate as fermentation end products when growing in media containing L-leucine, L-isoleucine and L-valine, as well as a fermentable carbohydrate (see Physiology; Harwood and Canale-Parola, 1983). *Spirochaeta smaragdinae* is the only species of *Spirochaeta* known to reduce thiosulfate or sulfur (but not sulfate) to H_2S and produces acetate, lactate, CO_2, and H_2S in the presence of thiosulfate and ethanol, lactate, CO_2, and H_2 in its absence (Magot et al., 1997).

Spirochaeta stenostrepta, *Spirochaeta zuelzerae* and *Spirochaeta caldaria* are freshwater anaerobes, whereas *Spirochaeta litoralis*, *Spirochaeta isovalerica*, *Spirochaeta bajacaliforniensis* and *Spirochaeta thermophila* were isolated from seawater environments and have salt requirements typical of marine bacteria (Aksenova et al., 1992; Fracek and Stolz, 1985; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970b; Pohlschroeder et al., 1994). Another anaerobic species, *Spirochaeta asiatica*, was isolated from the sediments of an alkaline lake and requires Na^+ concentrations ranging from 500 to 1,000 mM for optimal growth (Zhilina et al., 1996).

This latter species is also alkaliphilic, and growth does not occur below pH 8. *Spirochaeta smaragdinae*, an obligate anaerobe isolated from a production water sample collected from an offshore oil field, requires at least 170 mM NaCl and grows optimally with 850 mM NaCl (Magot et al., 1997). *Spirochaeta thermophila* and *Spirochaeta caldaria*, both isolated from hot springs, are thermophilic anaerobes with optimum growth temperature of 66–68 and 48–52°C, respectively (Aksenova et al., 1992; Pohlschroeder et al., 1994). Further characteriza-

tion of the obligate anaerobes should take into account interspecific differences in G+C content of the DNA and in cell size (Table 1).

Spirochaeta alkalica and *Spirochaeta africana* (Table 1, species 10 and 11) are aerotolerant species that survive two or three transfers in a glucose-containing medium incubated in air; however, aerobic growth is weak as compared to growth under anaerobic conditions (Zhilina et al., 1996).

These species may be distinguished by their ion requirements. *Spirochaeta africana* requires NaCl for growth, whereas *Spirochaeta alkalica* will grow in media supplemented with equimolar sodium carbonate and bicarbonate (Na_2CO_3 + $NaHCO_3$) in place of NaCl (Zhilina et al., 1996).

Salt requirements constitute an important basis of distinction between the facultative anaerobes *Spirochaeta aurantia* and *Spirochaeta halophila* (Table 1, species 12 and 13). The latter species is halophilic and has an absolute requirement for relatively high concentrations of Na^+, Cl^-, Ca^{++} and Mg^{++} (Greenberg and Canale-Parola, 1976), whereas *Spirochaeta aurantia* strains have been isolated only from freshwater environments and do not exhibit special salt requirements (Breznak and Canale-Parola, 1969; Breznak and Canale-Parola, 1975).

Pigment production is a characteristic of the aerotolerant and facultatively anaerobic species (Table 1, species 10–13). Zhilina et al. (1996) reported that the cell mass of the aerotolerant species *Spirochaeta alkalica* and *Spirochaeta africana* is orange. When growing aerobically, the facultative anaerobes *Spirochaeta halophila* and *Spirochaeta aurantia* produce carotenoid pigments. The major pigment of *Spirochaeta halophila* (strain RS1) is 4-keto-1′,2′-dihydro-1′-hydroxytorulene, whereas *Spirochaeta aurantia* (strain J1) produces mainly 1′, 2′-dihydro-1′-hydroxytorulene (Greenberg and Canale-Parola, 1975). Nonpigmented mutants of *Spirochaeta halophila* (Greenberg and Canale-Parola, 1976) and of *Spirochaeta aurantia* (B. J. Paster and E. Canale-Parola, unpublished data) have been isolated.

Cells of *Spirochaeta aurantia* subspecies *stricta* are more tightly coiled than those of *Spirochaeta aurantia* subspecies *aurantia* (Table 1, footnote c).

Criteria used for the identification of *Spirochaeta plicatilis* (Table 1, species 7) are its large size (Table 1) and its characteristic morphology and motility (Blakemore and Canale-Parola, 1973).

Preservation

Cells of *Spirochaeta* species can be preserved in a viable condition for several years by maintaining them at the temperature of liquid nitrogen. Conventional methods are used to prepare liquid nitrogen stock cultures of spirochetes (Canale-Parola, 1973).

Physiology

Motility and Chemotaxis

Three main types of motion are observed in species of *Spirochaeta*: 1) translational motion; 2) rotation of the cell around its longitudinal axis; and 3) flexing motion (Canale-Parola, 1978). In addition to swimming freely in liquid environments, some *Spirochaeta* are able to "creep" or "crawl" on solid surfaces, a movement resembling that of the gliding bacteria (Blakemore and Canale-Parola, 1973). Species of *Spirochaeta* retain their translational motion in environments of relatively high viscosity, e.g., 500 centipoise (Greenberg and Canale-Parola, 1977c). In comparison, flagellated bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa* and *Aquaspirillum serpens*, are immobilized at viscosities of or above 60 centipoise (Greenberg and Canale-Parola, 1977b).

The motility of a strain of *Spirochaeta aurantia* in liquid environments has been described as follows (Greenberg and Canale-Parola, 1977a; Greenberg et al., 1985): The spirochete usually swims in nearly straight lines (runs) and appears to spin about its longitudinal axis as it progresses through the liquid. From time to time the cell reverses swimming direction, the anterior end of the cell becoming the posterior end. Occasionally, the spirochete stops running, flexes, and then resumes its translational motion. However, upon resuming its translational motion, the spirochete usually alters the direction of its movement, and the previously leading cell end may or may not become the trailing cell end. Thus, *Spirochaeta aurantia* performs three kinds of behavior (runs, flexes, and reversals), in contrast to *Escherichia coli*, which performs two kinds (runs and tumbles). During runs, *Spirochaeta aurantia* cells have an average linear speed of approximately 16 μm/s (Fosnaugh and Greenberg, 1988). Flexes last from a fraction of a second to several seconds. The average frequency of reversals in cell populations is approximately 0.31 reversals/5 s (Fosnaugh and Greenberg, 1988).

A model that interprets the motile behavior of *Spirochaeta aurantia* has been proposed (Berg, 1976; Greenberg et al., 1985). According to this model, the two periplasmic flagella of *Spirochaeta aurantia* rotate, each driven by a motor at the insertion end. Rotation of the flagella in one direction (looking at the cell head on; see Fig. 13B, Introduction) causes the periplasmic cylin-

der and the outer membrane to move in directions opposite to each other. Owing to its helical shape, the cell rotates about its longitudinal axis and moves along it. Runs occur when the flagellar motor at one cell end rotates clockwise (CW) while the motor at the other cell end rotates counterclockwise (CCW). When both motors switch direction of rotation at the same time, a reversal takes place. A flex is generated during asynchronous switching, i.e., when only one motor switches direction so that both motors are rotating CW or CCW. Asynchronous switching causes the cell ends to twist in opposition to each other, and a flex occurs.

Spirochaeta aurantia exhibits chemotaxis toward D-glucose, D-xylose, cellobiose, and various other sugars, but not toward amino acids (Greenberg and Canale-Parola, 1977a). Many of the chemoattractants also serve as carbon and energy sources for growth of *Spirochaeta aurantia*. *Spirochaeta aurantia* cells grown in a chemostat at very low concentrations of an attractant that serves as a carbon and energy source exhibit an enhanced chemotactic response toward that attractant and are able to sense concentrations of the attractant much lower than those sensed by cells grown in the presence of excess attractant (Terracciano and Canale-Parola, 1984). Most likely, the ability to regulate its chemosensory system provides *Spirochaeta aurantia* with competitive advantages in natural environments deficient in nutrients.

Fosnaugh and Greenberg (1988) carried out an analysis of the motility and chemotaxis behavior of *Spirochaeta aurantia*. They observed that a population of *Spirochaeta aurantia* cells spent, on average, 66% of the time swimming smoothly (runs), 33% of the time flexing, and 1% of the time in reversals. After addition of an attractant (D-xylose, 10 mM final concentration), there was an increase in smooth swimming, a decrease in flexing, and a complete suppression of reversals. From 1.5 to 2 min after addition of the attractant, the population resumed its unmodified behavior. On the basis of their observations and of the above-mentioned motility model for *Spirochaeta aurantia*, Fosnaugh and Greenberg (1988) postulated that a mechanism for communication between the two flagellar motors is present in this spirochete and that a motor-switch-synchronizing device is also operating.

Fermentation Products and ATP-Yielding Pathways

Under anaerobic conditions, several species of *Spirochaeta* that have been cultured (Table 1, see Taxonomy) ferment carbohydrates with formation of acetate, ethanol, CO_2, and H_2 as major end products (Canale-Parola, 1984a; Fracek and Stolz, 1985; Harwood and Canale-Parola, 1983). *Spirochaeta zuelzerae*, *Spirochaeta thermophila* and *Spirochaeta caldaria* produce acetate, lactate, CO_2 and H_2 from carbohydrates (Aksenova et al., 1992; Canale-Parola, 1984a; Pohlschroeder et al., 1994), and *Spirochaeta zuelzerae* also produces small amounts of succinate (Canale-Parola, 1977; Canale-Parola, 1984a; Veldkamp, 1960). *Spirochaeta alkalica* and *Spirochaeta africana* produce acetate, ethanol, lactate and H_2, and *Spirochaeta asiatica* produces acetate, ethanol and lactate, but not H_2, during glucose fermentation (Zhilina et al., 1996). *Spirochaeta smaragdinae* is the only species of *Spirochaeta* that has been shown to reduce thiosulfate and sulfur to H_2S, producing acetate, lactate, CO_2 and H_2S in the presence of thiosulfate and producing ethanol, lactate, CO_2 and H_2 in its absence (Magot et al., 1997).

Spirochaeta isovalerica (Table 1) ferments (in addition to carbohydrates) L-leucine, L-isoleucine and L-valine, forming isovaleric, 2-methylbutyric and isobutyric acids, respectively, as end products (Harwood and Canale-Parola, 1983). However, *Spirochaeta isovalerica* requires a fermentable carbohydrate for growth. When *Spirochaeta isovalerica* is grown in medium containing both glucose and the three above-mentioned amino acids, only a relatively small fraction of the total amount of available amino acids is fermented (Harwood and Canale-Parola, 1981a; Harwood and Canale-Parola, 1981b). Under these growth conditions, the ATP derived from amino acid catabolism is estimated to be 4 to 5% of the total ATP formed. Fermentation of the amino acids in the absence of glucose does not support measurable growth of *Spirochaeta isovalerica*, but serves to generate ATP, which is utilized as a source of maintenance energy by the spirochete when fermentable carbohydrates are not available (Harwood and Canale-Parola, 1981a; Harwood and Canale-Parola, 1983). In addition to the branched-chain fatty acids, amino acid catabolism by *Spirochaeta isovalerica* yields small quantities of isobutanol and isoamyl alcohol (Harwood and Canale-Parola, 1981b; 1983).

All *Spirochaeta* species whose metabolic pathways have been studied (species 1, 2, 4, 6, 12 and 13 in Table 1; see Taxonomy) catabolize glucose to pyruvate via the Embden-Meyerhof pathway (Aksenova et al., 1992; Canale-Parola, 1984a; Harwood and Canale-Parola, 1983). Anaerobic metabolism of pyruvate yields acetyl-CoA, CO_2 and H_2 via a clostridial-type clastic reaction (Canale-Parola, 1977; Greenberg and Canale-Parola, 1976). Acetate is formed from acetyl CoA in reactions catalyzed by phosphotransacetylase and acetate kinase. A double reduction involving aldehyde and alcohol dehydrogenases

is responsible for ethanol production from acetyl CoA (Canale-Parola, 1977).

When growing aerobically, the two facultatively aerobic species (*Spirochaeta aurantia* and *Spirochaeta halophila*) oxidize glucose incompletely, with formation of CO_2 and acetate as major end products. Aerobically, both oxidative phosphorylation and substrate-level phosphorylation are utilized by the two species to generate ATP (Breznak and Canale-Parola, 1972b; Greenberg and Canale-Parola, 1976). The tricarboxylic acid cycle either is absent or plays a minor catabolic role in these two species.

Rubredoxin was detected in cell extracts of the obligate anaerobes *Spirochaeta stenostrepta* and *Spirochaeta litoralis* and of the facultative anaerobe *Spirochaeta aurantia* (Breznak and Canale-Parola, 1972a; Hespell and Canale-Parola, 1973; Johnson and Canale-Parola, 1973). Rubredoxin was isolated from extracts of both aerobically and anaerobically grown cells of *Spirochaeta aurantia*. Ferredoxin was present in cell extracts of anaerobically grown *Spirochaeta aurantia*, but was not found in aerobically grown cells of this bacterium (Johnson and Canale-Parola, 1973).

Ecology

Species of *Spirochaeta* are free-living bacteria indigenous to a variety of aquatic environments, such as the water, sediments and muds of ponds, marshes, lakes, rivers and oceans (Canale-Parola, 1984a). In these environments, *Spirochaeta* cells display a wide range of motility behaviors. When *Spirochaeta* cells swim through liquid environments, they may perform rotatory, locomotory and flexing movements (Canale-Parola, 1978). Also, spirochetes may move through environments of viscosity high enough to impede the progress of most flagellated bacteria (Harwood and Canale-Parola, 1984). Moreover, some *Spirochaeta* are able to move on solid surfaces by "creeping" or "crawling," a movement resembling that of the gliding bacteria (Blakemore and Canale-Parola, 1973). Complex physiological and behavioral adaptations, many of which involve one or more types of spirochaetal motility behavior, enable *Spirochaeta* species to persist in their environments and to compete successfully with other organisms for available nutrients (Harwood and Canale-Parola, 1984).

All species of *Spirochaeta* that have been cultivated are saccharolytic, and they usually lack the ability to utilize compounds other than carbohydrates as oxidizable substrates for growth. Various pentoses, hexoses, disaccharides and polysaccharides, such as starch, are used as carbon and energy sources (Aksenova et al., 1992; Canale-Parola, 1984a; Fracek and Stolz, 1985; Greenberg and Canale-Parola, 1976; Magot et al., 1997; Pohlschroeder et al., 1994; Zhilina et al., 1996). Even though free-living spirochetes are commonly observed in natural anaerobic environments in which plant material, containing cellulose and hemicelluloses, is biodegraded (Canale-Parola, 1978; Harwood and Canale-Parola, 1984; Leschine, 1995), only *Spirochaeta thermophila* has been reported to ferment cellulose and xylan (Aksenova et al., 1992). It may be inferred that, in their habitats, which frequently are rich in decaying plant material, *Spirochaeta* species usually ferment soluble sugars released into the environment by the enzymatic activities of other microorganisms that depolymerize plant polysaccharides. Pohlschroeder et al. (1994) reported that *Spirochaeta caldaria*, a thermophilic spirochete from a freshwater hot spring, forms stable, cellulose-degrading cocultures with the cellulolytic bacterium, *Clostridium thermocellum*. Cellulose is degraded more rapidly in cocultures than in monocultures of *Clostridium thermocellum*, suggesting that species of *Spirochaeta* may enhance cellulose breakdown in natural environments (Leschine, 1995).

Both facultatively and obligately anaerobic species of *Spirochaeta* grow abundantly when the energy source available to them is cellobiose, a major product of cellulose depolymerization, which is produced by the extracellular cellulase systems of some microorganisms (Breznak and Canale-Parola, 1969; Breznak and Canale-Parola, 1975; Greenberg and Canale-Parola, 1976; Harwood and Canale-Parola, 1983; Hespell and Canale-Parola, 1970a; Hespell and Canale-Parola, 1970b; Leschine, 1995; Pohlschroeder et al., 1994). Furthermore, free-living spirochetes, such as *Spirochaeta aurantia*, exhibit a strong tactic response toward very low cellobiose concentrations (Greenberg and Canale-Parola, 1977a) and possess regulatory systems that enable them to enhance their chemotactic responses when attractants that serve as energy sources are present at very low concentrations in the environment (Terracciano and Canale-Parola, 1984). Inasmuch as cellobiose levels in environments in which cellulose is degraded are likely to be quite low (Smith et al., 1973), a strong tactic response to very low concentrations of cellobiose may confer an important selective advantage on the spirochetes among cellobiose-utilizing microorganisms.

In addition to their ability to regulate their chemosensory apparatus in response to low substrate concentrations, some species of *Spirochaeta* have developed other strategies to survive in environments lacking or nearly depleted of energy sources. One of these survival strategies

has been studied in *Spirochaeta isovalerica*, a saccharolytic anaerobe that does not utilize amino acids as fermentable substrates for growth, but catabolizes small amounts of L-leucine, L-isoleucine and L-valine with the formation of isovalerate, 2-methylbutyrate and isobutyrate, respectively, as end products (Harwood and Canale-Parola, 1981b; Harwood and Canale-Parola, 1983; Harwood and Canale-Parola, 1984). Although these amino acids are not used by *Spirochaeta isovalerica* as fermentable substrates for growth, their fermentation serves to generate ATP, which is utilized by the spirochetes as a source of maintenance energy. This process allows cells to survive during periods of growth substrate starvation (Harwood and Canale-Parola, 1981a; Harwood and Canale-Parola, 1981b; Harwood and Canale-Parola, 1982a). Other starvation-survival strategies utilized by *Spirochaeta* species may involve ATP generation through metabolism of endogenous RNA (Canale-Parola and Kidder, 1982; Harwood and Canale-Parola, 1984) or metabolism of intracellular polyglucose storage granules (Kropinski et al., 1988).

The large spirochete, *Spirochaeta plicatilis*, has been observed within masses of *Beggiatoa* trichomes in samples of black, sulfide-rich marsh mud covered with a layer of marsh water (Blakemore and Canale-Parola, 1973). In this study, the spirochetes were seen to swim freely among the tangled *Beggiatoa* trichomes, and they crept on or otherwise moved in contact with the surface of the trichomes. The close association with *Beggiatoa* suggested a chemotactic response by the large spirochete toward metabolites produced by the sulfur bacteria. As the level of sulfide generated by biological activities in the mud became low, gradual lysis of the *Beggiatoa* trichomes was observed. This lytic process coincided with a dramatic increase in the number of large spirochetes (Blakemore and Canale-Parola, 1973). Possibly, substances released by the lysing *Beggiatoa* were used as growth substrates for *Spirochaeta plicatilis*.

Applications

Thus far, few applications have been suggested for species of *Spirochaeta*. Pohlschroeder et al. (1994) reported that cellulose degradation by *Clostridium thermocellum* is enhanced in the presence of *Spirochaeta caldaria*, a thermophilic spirochete from a freshwater hot spring. Possibly, species of *Spirochaeta* might be employed in processes for the direct bioconversion of cellulose-containing wastes to fuels such as H_2 or ethanol.

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