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Spirochaeta litoralis Sp. n., a Strictly Anaerobic Marine Spirochete

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Summary. A strictly anaerobic spirochete was isolated from a sample of marine mud. The organism possessed two axial fibrils entwined with the regularly coiled protoplasmic cylinder. An outer envelope or sheath enclosed both protoplasmic cylinder and axial fibrils. The spirochete grew in chemically defined media containing glucose, amino acids or NH_4Cl, sulfide, NaCl, vitamins, coenzyme A, and inorganic salts. A reducing agent, such as sodium sulfide or L-cysteine, as well as exogenous supplements of biotin, niacin and coenzyme A were required for growth. Pantothenate replaced coenzyme A as an exogenous growth factor, but the resulting cell yields were low. The spirochete grew in media prepared with sea water, but not in fresh water media containing less than 0.05 M NaCl (optimum concentration 0.35 M). Both Na^+ and Cl^- were required. Carbohydrates served as fermentable substrates. Amino acids, sugar alcohols, tricarboxylic acid cycle intermediates, and other organic acids and alcohols were not fermented. Glucose was fermented to ethyl alcohol, acetate, CO_2, H_2, and small amounts of lactate, formate and pyruvate. The guanine + cytosine content of the DNA of the spirochete was 50.5 moles-% (buoyant density). It is proposed that the marine isolate be considered a new species and that it be named *Spirochaeta litoralis*.

The widespread occurrence of spirochetes in nature has been amply documented (see review by Starr and Skerman, 1965). Spirochetes exist in association with hosts as diverse as mammals, insects and molluscs, and are present as free-living organisms in aquatic environments. The properties of free-living spirochetes isolated from fresh water environments and those of the host-associated forms have been the subject of numerous investigations. On the other hand, marine spirochetes have received little attention, even though their existence has been known for many years (Zuelzer, 1911).

The present paper deals with the isolation and characterization of a free-living, anaerobic marine spirochete that could not be identified with any hitherto described species. In view of its occurrence in seashore mud, it has been named *Spirochaeta litoralis* (strain R1).

Methods

Isolation of the Spirochete. The organism was observed in a sample of black (sulfide-containing) marine mud collected from a Luquillo Beach sandbar, near San Juan, Puerto Rico. Isolation was accomplished by a method similar to that described by Canale-Parola *et al.* (1967) for anaerobic spirochetes, except that a different isolation medium was used (see below) and incubation was at room temperature (22° to 23° C). One volume of the mud used as the source of the organism was suspended in five volumes of isolation medium before proceeding with the filtration steps.

Media and Growth Conditions. The isolation medium for the marine spirochete included (g per 100 ml distilled water): tryptone (Difco), 0.3; yeast extract (Difco), 0.05; and NaCl, 2.0. To this mixture 2 ml of 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 dist. water) and with 0.5 ml of a sterile $Na_2S \cdot 9\,H_2O$ solution (10 g per 100 ml dist. water). The final pH of the medium ranged from 7.4 to 7.5.

The salts solution contained (g per 75 ml dist. water): tetrasodium ethylenediamine tetraacetate, 1; $CaCl_2 \cdot 2\,H_2O$, 3.75; $MgCl_2 \cdot 6\,H_2O$, 12.5; $FeSO_4 \cdot 7\,H_2O$, 0.5. To 75 ml of this salt mixture 25 ml of a modified Pfennig's trace element solution (Canale-Parola *et al.*, 1967) were added.

Spirochaeta litoralis was grown routinely in the isolation medium. A modified isolation medium, used when increased cell yields were desired, contained higher concentrations of the potassium phosphate buffer (0.06 M) and of yeast extract (0.25 g per 100 ml).

Spirochaeta aurantia strains *J1* and *M1* were grown in maltose broth (Breznak and Canale-Parola, 1969). *Spirochaeta stenostrepta* strain *Z1* was grown on medium GYPT (Canale-Parola *et al.*, 1967); *Spirochaeta (Treponema) zuelzerae* ATCC 19044 in a medium containing (g per 100 ml dist. water): glucose, 0.2; yeast extract, 0.1; peptone, 0.2; $CaCl_2 \cdot 2\,H_2O$, 0.004; $MgSO_4 \cdot 7\,H_2O$, 0.05; $NaHCO_3$, 0.1; and KH_2PO_4, 0.1. Each of the two latter compounds was added as a separately sterilized solution.

A chemically defined medium was used in studying the nitrogen and vitamin requirements of *S. litoralis*. This medium had the same composition as the isolation medium except that the yeast extract was replaced by a growth factor mixture (1 ml per 100 ml of medium) and the tryptone either by an amino acid mixture (5 ml per 100 ml of medium) or by a single nitrogen source such as L-asparagine or $(NH_4)_2SO_4$ (0.4 g of either per 100 ml of medium).

The growth factor mixture was composed of (mg per ml): coenzyme A (added to the mixture immediately before use), 1.0; nicotinic acid, nicotinamide adenine dinucleotide, nicotinamide adenine dinucleotide phosphate, calcium pantothenate, lipoic acid, and para-aminobenzoic acid, 0.1 each; riboflavin, thiamine · HCl, pyridoxine · HCl, pyridoxal phosphate, pyridoxal · HCl, inositol, and folic acid, 0.05 each; biotin and vitamin B_{12}, 0.02 each. The growth factor mixture was prepared by dissolving the components in 0.05 M HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) and adjusting the pH of the solution to 7.5 with KOH prior to sterilization by filtration through a sterile 0.22 μm cellulose ester filter disc (Millipore Corporation).

The amino acid mixture consisted of the L-isomers of the following amino acids (μmoles/ml): alanine and aspartic acid, 6.0 each; asparagine, arginine, and methionine, 3.0 each; cystine, 0.5; glycine, 3.5; glutamic acid, 17.5; histidine, 2.8;

isoleucine, 7.5; leucine, 11.5; lysine, 7.7; phenylalanine, 4.5; proline, 15.0; threonine, 5.8; tryptophan, 1.0; tyrosine, 2.5; valine, 9.1; and serine, 5.0. The amino acids were dissolved in 0.005 M HEPES and the pH of the solution was adjusted to pH 7.0 with KOH prior to sterilization by filtration through a sterile 0.22 μm cellulose ester filter disc.

The strictly anaerobic spirochetes were grown in 15×125 mm test tubes each containing approximately 10 ml of medium and in Florence flasks filled with medium. *S. aurantia* was grown as described by Breznak and Canale-Parola (1969). Incubation of all spirochetes studied was at 30° C, unless indicated otherwise.

In experiments designed to study the nutritional characteristics of *S. litoralis* (e.g., when testing for fermentable substrates, nitrogen sources, vitamin and inorganic ion requirements) the cultures were incubated in an argon or nitrogen atmosphere to prevent oxidation of medium components during long lag phases of growth occurring when the cells were transferred to media different from those in which they were previously grown. Anaerobic environments were also provided for the cultures when testing the NaCl tolerance of *S. stenostrepta* and *S. zuelzerae*. In the nutritional studies all compounds under test were sterilized separately and added to the medium immediately before inoculation. Growth yields were determined after the third transfer in the test medium.

Preservation of Cultures. Broth cultures of *S. litoralis* in test tubes remained viable for three days 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.

S. 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 days at room temperature and then at 15° C, remained viable for at least three months.

Longer viability (minimum 18 months) resulted when the cells were stored at the temperature of liquid nitrogen (— 196° C). To prepare liquid nitrogen stock cultures, cell pellets were suspended in five times their volume of freshly made isolation medium, which had been sterilized and then supplemented with dimethyl sulfoxide (5 ml per 100 ml of medium; Aldrich Chem. Corp.). 1 ml samples of the suspension were syringed aseptically into sterile ampoules (A. H. Thomas Co., Philadelphia, Pa., U.S.A., catalogue no. 1071-D) which were sealed with a torch. The ampoules were then placed into metal holders or "canes" (Shur-Bend Mfg.; No. Minneapolis, Minn., U.S.A.) and immersed in 95% ethyl alcohol contained in a 500 ml graduated cylinder. The ampoules in the ethyl alcohol bath were cooled to — 90° C in an ultra low temperature freezer (Revco Inc. Deerfield, Mich., U.S.A.). The cold ampoules, still in their holders, were finally placed in a Linde liquid nitrogen refrigerator (type LR-35-9; Union Carbide Corp., Speedway, Indiana, U.S.A.).

S. aurantia, *S. stenostrepta*, and *S. zuelzerae* were maintained as previously described (Breznak and Canale-Parola, 1969; Canale-Parola *et al.*, 1967) as well as in liquid nitrogen.

Growth Measurements. Growth yields were determined turbidimetrically and, where appropriate, by direct cell counts using a Petroff-Hausser bacterial counter. Turbidimetric measurements were made by means of a Klett-Summerson photoelectric colorimeter equipped with a 660 nm filter. Colorimetric readings were converted to cell numbers by using a standard curve relating the readings to direct cell counts.

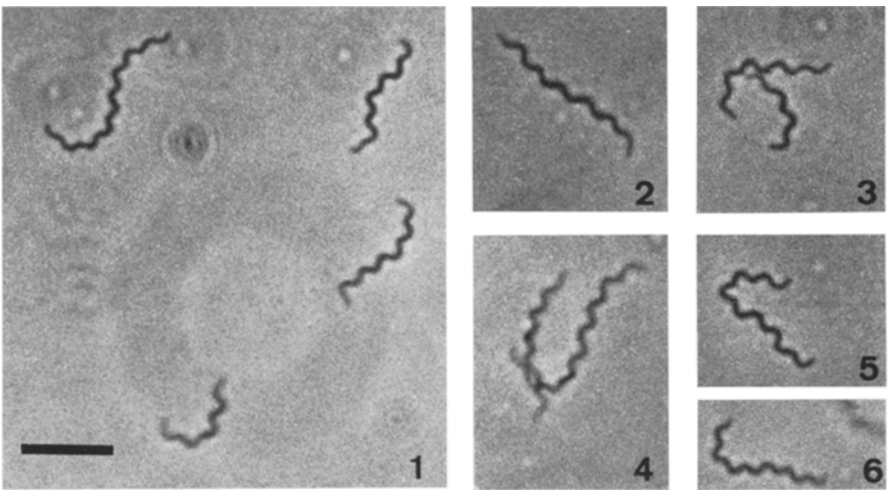


Fig. 1—6. Living cells of *Spirochaeta litoralis* R 1. Wet mount preparations, phase contrast. Moving cells displayed both straight (Fig. 2) and flexed (Fig. 1) contours. Slowly moving and stationary cells were frequently bent or curled to various degrees (Fig. 3—6). The magnification bar indicates 5 μm

Analyses of Fermentation Products. Fermentation products were determined by the methods of Neish (1952) and as described previously (Canale-Parola *et al.*, 1967, 1968; Breznak and Canale-Parola, 1969). Hydrogen sulfide was assayed by the method of Fogo and Popowsky (1949).

Microscopy. A Zeiss GFL phase-contrast microscope was used for light microscopic observations. Photographs were taken on Kodak Plus-X Pan film, using a Leitz Mikas camera attachment and a series M Leica camera body.

Negatively stained specimens for electron microscopy were prepared by the following procedure. A drop of broth culture was placed onto a formvar-coated, carbon reinforced copper grid (300 mesh) and the excess fluid was drawn off with a piece of filter paper. The specimen was stained by addition to the grid of a drop of 1% (w/v) aqueous solution of phosphotungstic acid (pH 7.0), and the excess stain was removed with filter paper. This step was repeated in order to dilute and remove the growth medium constituents and to complete the staining of the cells. It should be noted that *S. litoralis* cells are very fragile and are readily disrupted by staining compounds used for electron microscopy, such as phosphotungstic acid.

Samples for thin sectioning were prepared by prefixing the cells for 1 h at 4°C in phosphate-buffered (pH 7.5, 0.05 M) glutaraldehyde solution (5%, w/v). The cells were postfixed with osmium tetraoxide according to the method of Kellenberger *et al.* (1958) and were then dehydrated and embedded as described by Holt and Canale-Parola (1967). Thin sections were stained with a 2% (w/v) aqueous uranyl acetate solution and then stained with Reynolds' lead citrate solution (Reynolds, 1963).

All specimen preparations were examined in a Philips EM 200 electron microscope operating at 60 Kv and equipped with a 30 μm objective aperture.

Guanine + Cytosine Content of the DNA. The guanine + cytosine (GC) content of the DNA of *S. litoralis* was determined by Dr. M. Mandel from estimation of the buoyant density in CsCl (Mandel, 1966).

Other Experimental Procedures. Nitrite formed by cells growing in the isolation medium supplemented with 0.15% (w/v) KNO_3 was determined as described by Conn *et al.* (1957).

Results

Morphology

Cells of *S. litoralis* measured 0.4 to 0.5 by 5.5 to 7.0 μm during exponential growth in the isolation medium. Cells in which binary fission was near completion were approximately twice as long. The coiling of the cells was regular and compact (Fig. 1—6), except in the early lag and in the stationary growth phases, during which the cells were loosely coiled. The spirochete was vigorously motile, displaying translational movement as well as rotation around the longitudinal axis of the cell and the flexing movements exhibited by other spirochetes (Zuelzer, 1911; Canale-Parola *et al.*, 1968; Breznak and Canale-Parola, 1969). Morphological aberrations such as cytoplasmic protrusions and spherical bodies (DeLamater *et al.*, 1951; Canale-Parola *et al.*, 1967) were common in stationary phase cultures and under adverse growth conditions (e.g. incubation at 37°C).

Electron microscopy of *S. litoralis* cells revealed morphological features similar to those which have been observed in other spirochetes (Pillot and Ryter, 1965; Ryter and Pillot, 1965; Holt and Canale-Parola, 1968). Two axial fibrils were present, one inserted near one end of the protoplasmic cylinder and the other near the opposite end (Fig. 7 and 8). The two fibrils appeared to overlap for most of their length. At the insertion end of the axial fibrils, disc-like structures resembling those of *S. stenostrepta* (Holt and Canale-Parola, 1968) were present (Fig. 10). Occasionally, cells were observed which had two axial fibrils inserted at one end of the protoplasmic cylinder and a third fibril inserted at the opposite end (Fig. 11). An outer sheath or cell envelope surrounded both the axial fibrils and the protoplasmic cylinder (Fig. 8 and 9). In negatively stained preparations this sheath exhibited a polygonal substructure (Fig. 8).

Colonies on agar plates of isolation medium (1% agar, w/v) incubated in argon were 2 to 5 mm in diameter, round, partially subsurface with a raised center, and cream-colored. A diffuse clear area in the medium surrounded each colony (the opaqueness of the medium was probably due to formation of elemental sulfur from the added Na_2S). Colonies developing within the agar medium in plate or tube cultures were spherical, fluffy, and measured 1 to 5 mm in diameter. In tube cultures the agar gel was extensively split as a result of gas evolution.

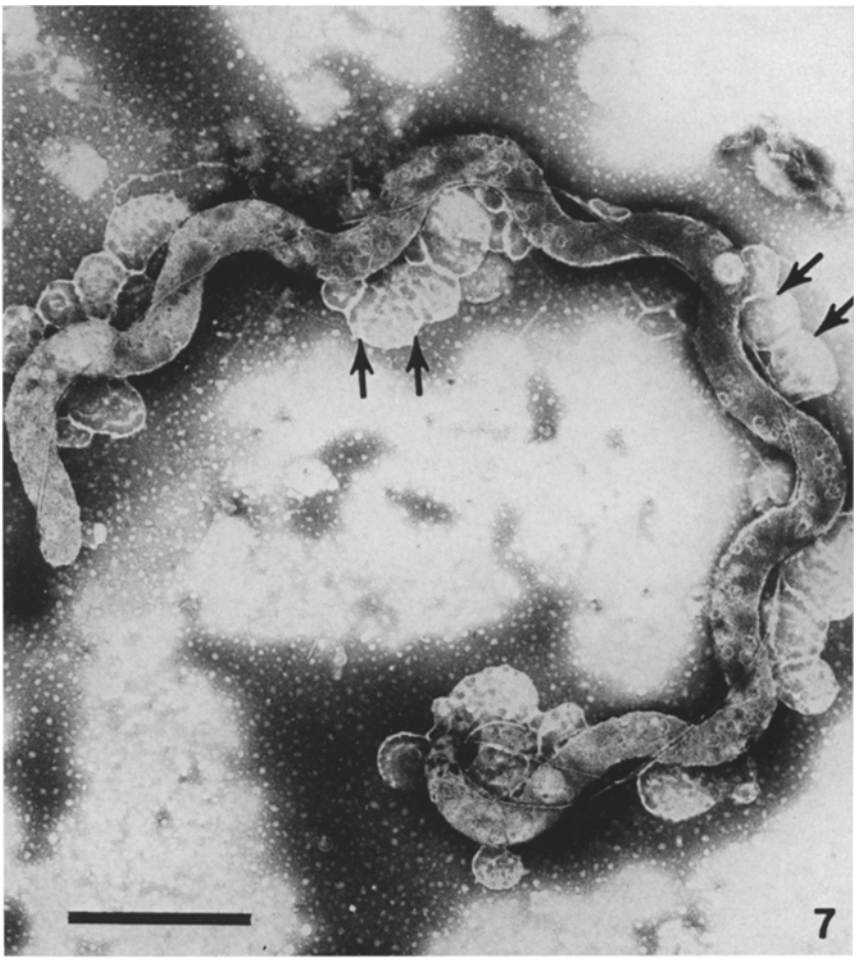


Fig. 7. Electron micrograph of a cell of *S. litoralis* R1. Negative stain preparation. The coiling of the protoplasmic cylinder has been preserved. The overlapping axial fibrils are visible. The outer sheath is largely disrupted (arrows). The magnification bar indicates 1 μm

Cultural Characteristics

S. litoralis was strictly anaerobic and sensitive to atmospheric oxygen. A reducing agent, such as sodium sulfide or L-cysteine, was required for growth in all media used. Highest cell yields in the isolation medium (6 to 9×10^8 cells/ml) were obtained in the presence of 0.05 to 0.125 g per 100 ml of $Na_2S \cdot 9H_2O$. At similar concentrations, L-cysteine per-

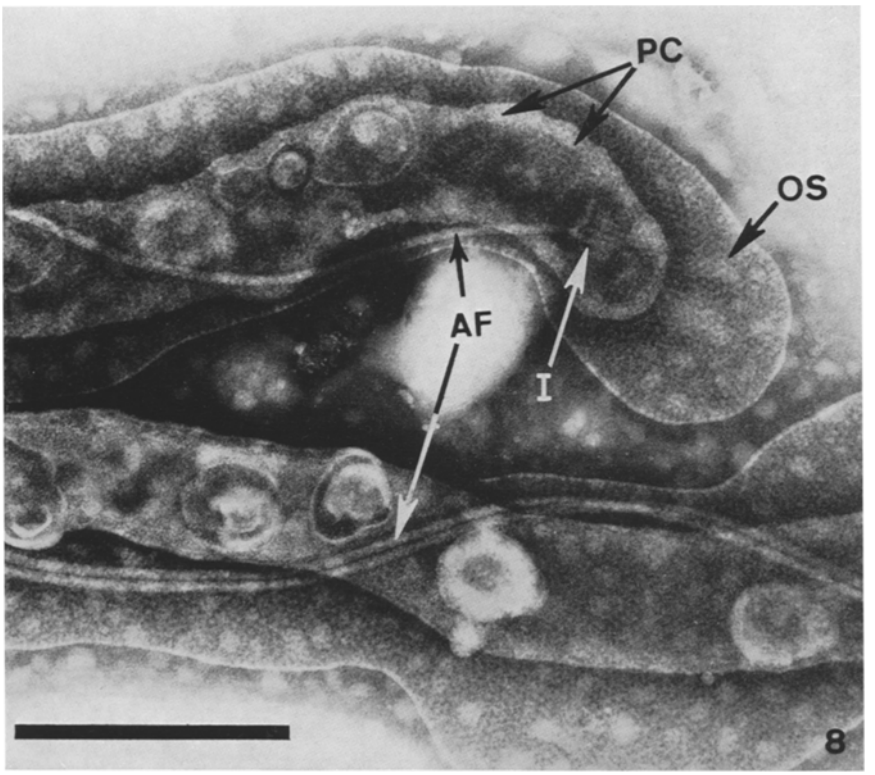
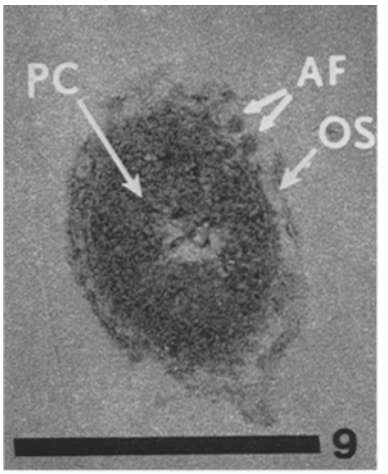
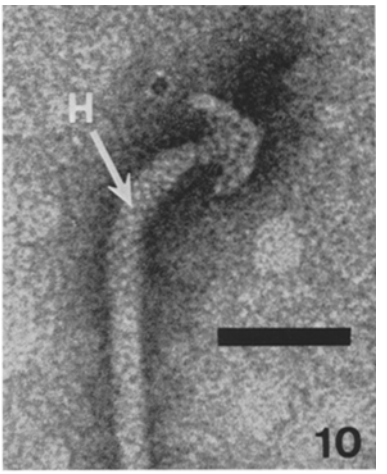


Fig. 8. Electron micrograph of the terminal and central portion of a *S. litoralis* cell. Negative stain preparation. The subterminal insertion *I* of one of the axial fibrils *AF* into the protoplasmic cylinder *PC* is apparent. The axial fibrils overlap in the central portion of the cell. The outer sheath *OS* appears to have a polygonal substructure. The magnification bar indicates 0.5 μm

mitted less growth (3 to 4×10^8 cells/ml), and when sodium thioglycolate or L-ascorbic acid were used the growth yield was much smaller (1 to 7×10^7 cells/ml).

The final growth yield of *S. litoralis* was affected by the initial pH and by the buffer content of the medium. In the isolation medium the highest yields resulted when the initial pH was between 7 and 7.5. At a phosphate buffer concentration of 0.06 M the isolation medium yielded slightly over 10^9 cells per ml. At lower or higher phosphate concentrations the amount of growth decreased. A modified isolation medium, which contained 0.06 M phosphate buffer and 0.25% (w/v) yeast extract, supported growth yields approaching 10^{10} cells per ml. In contrast,





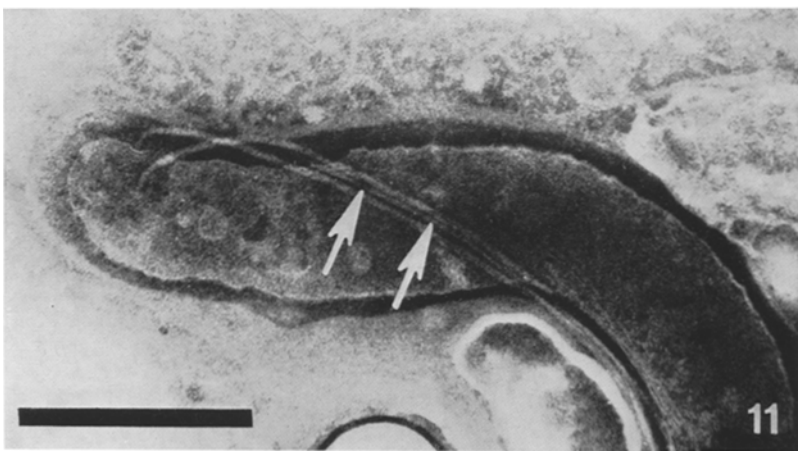


Fig. 9. Electron micrograph of a transverse thin section through a cell of *S. litoralis*. The overlapping axial fibrils *AF*, located between the protoplasmic cylinder *PC* and the partially disrupted outer sheath *OS*, are visible. The magnification bar indicates 0.5 μm

Fig. 10. Electron micrograph of the insertion end of an isolated axial fibril. *S. litoralis* R1. Negative contrast. The hooked portion *H*, near the disc-shaped insertion apparatus, exhibits a honeycomb-like substructure. The magnification bar indicates 0.1 μm

Fig. 11. Electron micrograph of one end of an atypical *S. litoralis* cell. Negative contrast. This cell has three axial fibrils. Two fibrils are inserted at the cell end shown in this micrograph, and part of the third fibril is visible (arrows). The magnification bar indicates 0.5 μm

S. aurantia and *S. stenostrepta* did not grow in media with 0.06 M potassium (or sodium) phosphate buffer. Phosphate buffer concentrations as low as 0.02 M partially inhibited the growth of *S. stenostrepta*, but did not affect the cell yields of *S. aurantia*.

In the modified isolation medium the shortest generation time and the highest growth yields of *S. litoralis* were obtained at 30°C (2.2 h; 9.9×10^9 cells/ml). At 25 or 35°C the generation time was longer (3.5 h), whereas at 15°C the growth rate was very low. At 5 and at or above 40°C no growth was observed. At 37°C the generation time was 5.5 h and a high number of spherical bodies and other morphological aberrations were present.

Growing cells of *S. litoralis* evolved a relatively small amount of hydrogen sulfide, which was probably formed from the reduced sulfur compound added to the medium (e.g., sulfide or cysteine).

Nutrition

S. litoralis did not grow in isolation medium from which glucose was omitted. Media without added sugar but containing higher concentrations of yeast extract supported slight growth (1 to 5×10^6 cells/ml) probably because of the presence of small quantities of carbohydrates in the yeast extract. Substrates utilized by the organism included a variety of pentoses, mono- and disaccharides, as well as carbohydrates of higher molecular weight (Table 1). Sugar alcohols, tricarboxylic acid cycle intermediates, various organic acids and alcohols were not utilized. Lactate and pyruvate supported low levels of growth. It is apparent from these results that the nutritional pattern of *S. litoralis* resembles that of fresh water anaerobic spirochetes which are eminently saccharolytic (Canale-Parola *et al.*, 1968).

S. litoralis grew in a chemically defined medium containing glucose, amino acids, various growth factors, NaCl, sulfide, and a supplement of inorganic salts (see Methods). This medium supported yields of approximately 5.5×10^8 cells/ml. When L-asparagine was supplied as the only amino acid the growth yield was lower (2.2×10^8 cells/ml); ammonium sulfate could also serve as the sole nitrogen source (3×10^8 cells/ml). Nitrate was used either poorly or not at all as a nitrogen source by *S. litoralis*.

Experiments in which growth factors were omitted from the chemically defined medium indicated that *S. litoralis* grew in a medium containing niacin, thiamine, biotin and calcium pantothenate as the only growth factors (Medium 1, Table 2). When pantothenate was omitted from the latter medium no growth occurred (Medium 2, Table 2), but if coenzyme A was added instead of pantothenate greater growth

Table 1. *Utilization of carbon compounds by* Spirochaeta litoralis

Compound added[a]	Yield (cells/ml)	Compound added[a]	Yield (cells/ml)
None	0.01×10^8	Maltose	1.5×10^8
D-glucose	10.0×10^8	Cellobiose	2.8×10^8
D-mannose	1.4×10^8	Lactose	2.4×10^8
D-galactose	2.1×10^8	Sucrose	7.2×10^8
D-fructose	2.4×10^8	Trehalose	1.0×10^8
D-arabinose	2.8×10^8	Inulin	3.7×10^8
L-arabinose	2.6×10^8	Raffinose	2.9×10^8
D-xylose	2.6×10^8	Li lactate	0.14×10^8
L-fucose	3.9×10^8	K pyruvate	0.18×10^8
L-rhamnose	0.3×10^8		

Compounds not supporting growth: L-sorbose, D-ribose, L-xylose, D-lyxose, L-lyxose, L-arabitol, dulcitol, glycerol, mannitol, i-erythritol, ribitol, D-sorbitol, xylitol, D-arabitol, inositol, pectin, α-ketoglutarate, oxaloacetate, K gluconate, Na citrate, Na succinate, Na fumarate, malate, NaK tartrate, Tween 80, allantoin, orotic acid, uric acid, glucosamine, ethanol, and methanol.

[a] 0.3 g added per 100 ml modified isolation medium minus glucose, except Tween 80 (sorbitan mono-oleate polyoxyethylene) which was added at a final concentration of 0.05% (v/v).

Table 2. *Vitamin and coenzyme A requirements of* Spirochaeta litoralis

Medium[a]	Vitamins omitted	Coenzyme A added (10 µg/ml)	Growth yield (cells/ml)
1	None	no	6×10^7
2	Ca pantothenate	no	no growth
3	Ca pantothenate	yes	2.2×10^8
4	Ca pantothenate, biotin	yes	no growth
5	Ca pantothenate, niacin	yes	no growth
6	Ca pantothenate, thiamine	yes	4×10^7
7	All	no	no growth

[a] The basic medium from which vitamins were omitted and to which coenzyme A was added, as indicated in this Table, was the chemically defined medium (see Methods) containing 0.4 g $(NH_4)_2SO_4$ as the nitrogen source and the following vitamins instead of the growth factor mixture (µg/ml of medium): calcium pantothenate, 10; niacin and thiamine, 1.0 each; and biotin, 0.5. When concentrations of coenzyme A and of pantothenate lower than those indicated were used the resulting cell yields were similar to those listed in this Table.

yields resulted (Medium 3, Table 2) than in the medium containing the above-mentioned four vitamins as the only growth factors (Medium 1, Table 2). When biotin or niacin were omitted no growth was observed (Media 4 and 5, Table 2), and the omission of thiamine resulted in low

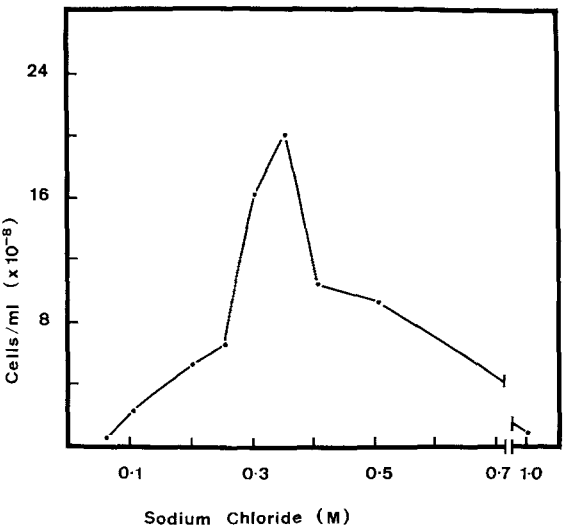


Fig. 12. Growth response of *S. litoralis* R1 to different NaCl concentrations. The organism was grown in modified isolation medium including the concentrations of NaCl indicated

growth yields (Medium 6, Table 2). These results indicated that *S. litoralis* requires for growth exogenous supplements of biotin, niacin, and coenzyme A. Pantothenate replaces coenzyme A as an exogenous growth factor, but the cell yields are drastically decreased. The presence of thiamine in the medium is stimulatory for growth.

The Marine Nature of *S. litoralis*

Experiments were conducted to determine whether *S. litoralis* R1 was indeed a marine bacterium or whether its presence in sea water mud was of a transitory nature.

A criterion which may be used to distinguish between terrestrial and marine bacteria is that the latter possess the ability to survive and grow in the sea. It has been suggested that those bacteria which, on initial isolation from marine environments, grow in suitable media prepared with sea water but not on corresponding fresh water media may be considered as being marine bacteria (MacLeod, 1965).

Various investigators have studied the response of marine bacteria to certain inorganic ions (see review by MacLeod, 1965). It has been found that, generally, marine bacteria have a highly specific, stable requirement for Na^+ for growth. Such organisms grow only in media to which sodium ions have been added, and many among those which

have been examined require Na^+ concentrations ranging from 0.2 to 0.3 M for optimal growth. However, a specific requirement for Na^+ is not restricted to marine bacteria, but has been detected in a number of bacteria isolated from non-marine environments. Some marine bacteria grow poorly or fail to grow in media which are devoid of or contain low concentrations of halide ions, such as Cl^- or Br^-.

S. litoralis grew readily in sea water media. The isolation medium prepared with sea water, and not including the NaCl supplement, supported growth (approximately 3×10^8 cells/ml). When fresh water was used instead of sea water in this medium, and no NaCl was added, the organism did not grow. Generally media prepared with a mixture of seven volumes of sea water to three volumes of distilled water supported higher growth yields than media prepared with undiluted sea water. *S. litoralis* cells remained viable (as determined by observing their motility) and multiplied to a small extent for at least 16 days at 30° C in sea water supplemented with glucose (0.5%, w/v) and $Na_2S \cdot 9H_2O$ (0.05%, w/v).

Thus, it may be concluded that *S. litoralis* has the ability to survive and grow in sea water environments. Furthermore, the organism does not grow in media prepared with fresh water containing less than 0.05 M NaCl; for optimal growth a concentration of 0.35 M NaCl was required (Fig. 12). Sodium chloride could not be replaced by Na or K citrate, NaK tartrate, Na_2SO_4, $NaNO_3$, NaI, NaBr, KCl, KBr, KI, KNO_3, LiCl, $(NH_4)_2SO_4$, $MgCl_2$, or $MnCl_2$. When any of these salts was added singly to the isolation medium in place of NaCl and in concentrations ranging from 0.1 to 0.5 M, *S. litoralis* failed to grow. These data indicated that *S. litoralis* has a specific requirement for Na^+ and Cl^-.

In contrast, strains of *S. aurantia*, *S. stenostrepta* and *S. zuelzerae*, which were isolated from fresh water environments, did not require added NaCl in their respective growth media. In fact, the incorporation of 0.10 M NaCl in the growth medium prevented or greatly inhibited growth of *S. aurantia* and *S. stenostrepta*. Growth of *S. zuelzerae* did not seem to be affected by this concentration of NaCl.

In conclusion, the presence of *S. litoralis* in sea water mud, its ability to survive and grow in sea water environments, as well as its specific requirements for Na^+ and Cl^- indicate that this organism is indeed a marine bacterium.

Fermentation of Glucose by *S. litoralis*

The major products of glucose fermentation by growing cells or cell suspensions of *S. litoralis* were ethyl alcohol, acetate, CO_2 and H_2 (Table 3). Small amounts of lactate, formate, and pyruvate were present in the fermented medium. Succinate, acetone, diacetyl, acetoin, and

Table 3. *Products of glucose fermentation by* Spirochaeta litoralis

Products	Amount[a]	
	growing cells[b]	cell suspensions[c]
Ethyl alcohol	109.5	140.5
Acetate	37.5	57.0
CO_2	127.5	201.8
H_2	74.0	74.4
Lactate	6.5	trace[d]
Formate	2.8	trace[d]
Pyruvate	0.3	trace[d]
Carbon recovery	88%[e]	99.5%
Oxidation-reduction balance	0.88	1.14

[a] Expressed as μmoles of product per 100 μmoles of glucose fermented.

[b] In modified isolation medium including 0.05% (w/v) L-cysteine as the reducing agent.

[c] Added per Warburg vessels (μmoles): K phosphate buffer (pH 7.0), 200; dithiothreitol, 10; NaCl, 1000; and glucose, 200; to a final volume of 3.2 ml. Cells per vessel: 1.5×10^{11}. In argon atmosphere at 30° C for 4 h.

[d] Less than 1 μmole.

[e] Includes glucose carbon incorporated into cell material (14% of glucose carbon utilized).

2,3-butanediol were not detected. Glucose was used by growing cells both as an energy and a carbon source since, in the presence of uniformly labelled C^{14}-glucose, approximately 14% of the glucose carbon utilized was incorporated into cell material.

Guanine + Cytosine (GC) Content of DNA

Analysis of *S. litoralis* DNA by cesium chloride gradient centrifugation revealed a buoyant density of 1.709 g/cm^3, corresponding to 50.5 moles percent guanine + cytosine.

Discussion

A recently proposed working classification scheme assigns the obligately and facultatively anaerobic, free-living spirochetes to the genus *Spirochaeta* (Canale-Parola *et al.*, 1968). According to this scheme, the marine bacterium described in this report belongs to the genus *Spirochaeta*.

A comparison of the properties of our marine isolate with those of recognized *Spirochaeta* species indicates that, although there are numerous similarities, the marine spirochete cannot be identified with any of the existing species of this genus. In some respects the marine spiro-

chete resembles *S. stenostrepta* and *S. zuelzerae*. All three of these organisms are obligate anaerobes and ferment sugars. However, cells of *S. stenostrepta* are thinner and at least 3 to 8 times longer than those of the marine spirochete. Furthermore, the GC content of *S. stenostrepta* DNA (60.2 moles-%, buoyant density) is considerably higher than that of the marine organism. *S. zuelzerae*, although more similar in size to the marine spirochete, differs from the latter because it forms succinate (but not ethanol) as a fermentation product, requires a CO_2 supplement for growth, and has a higher GC content in its DNA (56.1 moles-%, buoyant density). *S. plicatilis*, and a similar marine strain (*S. plicatilis marina*) which was described but not cultivated by Zuelzer (1911), are strikingly different from our marine spirochete because of their large size.

The facultatively anaerobic, pigment-forming *S. aurantia* does not appear to be as closely related phylogenetically to our marine isolate as the strictly anaerobic species of *Spirochaeta*. In addition to physiological and morphological dissimilarities, *S. aurantia* has a much higher GC content in its DNA (66.8 moles-%, buoyant density) than the marine spirochete. This situation again emphasizes the necessity for a revision of the classification of the facultatively and strictly anaerobic free-living spirochetes. As pointed out by Canale-Parola *et al.* (1968) scrutiny of the properties of these organisms indicates that the obligate anaerobes should be assigned to one genus and the facultative anaerobes to another. However, revision of the existing classification of these organisms is hampered by the lack of information on *S. plicatilis* which, in accordance with the International Code of Nomenclature of Bacteria and Viruses, is considered to be the type species of the genus. *S. plicatilis*, described in 1838 by Ehrenberg, has not been isolated in pure culture and essentially nothing is known of its properties (e.g., in nature the organism has been observed in anaerobic environments, but it is not certain whether it is an obligate anaerobe).

Finally, it should be pointed out that the marine spirochete we isolated, unlike all previously described species of *Spirochaeta* which were cultivated, requires for growth the addition of Na^+ and Cl^- to the medium. As already mentioned in the "Introduction", we propose that our marine isolate be considered a new species and be named *Spirochaeta litoralis*. The derivation of the specific name and a description of the organism are included below.

In recent years the isolation of two spirochetes from marine environments has been briefly reported (Veldkamp, 1965; Attebery, 1969). Because of the limited information available on these organisms, a comparison with *S. litoralis* and other species of *Spirochaeta* is not feasible at present.

An interesting property of *S. litoralis* is its requirement of coenzyme A for growth. Although pantothenate, a component of the coenzyme A molecule, replaces the latter as a growth factor, optimum growth of the organism occurs in the presence of coenzyme A. Generally, bacteria which exhibit a growth response to externally supplied pantothenate show little or no growth response to exogenous coenzyme A, probably because of impermeability of the cell envelopes to the coenzyme or because of their inability to degrade it. An exception is *Acetobacter suboxidans*, which has a greater growth response to coenzyme A than to pantothenate (see Koser, 1968). Steinman *et al.* (1954) reported that exogenous coenzyme A is required for growth by an oral spirochete they studied (strain S-69), and that it cannot be replaced by pantothenate.

As we will report in a forthcoming publication, broken cell preparations as well as cell-free extracts of *S. litoralis* contain extremely low concentrations of coenzyme A, compared with extracts of other bacteria, such as *S. stenostrepta* and *Clostridium pasteurianum*. This indicates a deficiency or inadequacy in the organism's coenzyme A-synthesizing ability.

It is conceivable that low levels of coenzyme A, which is a relatively stable compound (Buyske *et al.*, 1954), are available, in natural environments, to free-living bacteria. Likewise, bacteria existing in association with a living host may have ready access to coenzyme A, since this molecule is manufactured and utilized by the host for its metabolism. Provided that coenzyme A can penetrate their cells, bacteria present in coenzyme A-containing environments need not possess the enzymes necessary for the synthesis of this compound. On the other hand, bacteria impermeable to coenzyme A may have the ability to break down this compound extracellularly and utilize fragments of the molecule for coenzyme A biosynthesis. Thus, these bacteria could survive and grow even though they may lack enzymes needed to synthesize a portion of the coenzyme A molecule.

The striking growth response of *S. litoralis* to externally supplied coenzyme A, and the low levels of endogenous coenzyme A are not readily interpreted in evolutionary terms. It is possible that *S. litoralis* (or its ancestor) previously existed in association with a host upon which it depended for its supply of coenzyme A, and that later the spirochete became adapted to a free-living existence. However, it is equally possible that *S. litoralis* is a relatively primitive, free-living bacterium which has not yet fully developed its coenzyme A-synthesizing mechanisms.

The saccharolytic behavior of *S. litoralis* is similar to that of other free-living spirochetes that were studied, and reinforces the conclusion that their main chemical role in nature is the degradation of carbohy-

drates. This does not exclude the possible occurrence of anaerobic free-living spirochetes that decompose other compounds.

Description of *Spirochaeta litoralis*

Spi.ro.chae'ta. Gr. n. *spira* a coil; Gr. n. *chaeta* hair; M.L. fem. n. *Spirochaeta* coiled hair. li.to.ra'lis. L. adj. *litoralis* of the shore.

Single, helical cells, 0.4 to 0.5 by 5.5 to 7.0 μm.

The coiling of the cells is regular and tight during the exponential phase of growth. Two axial fibrils are present, one inserted near one end of the protoplasmic cylinder and the other near the opposite end. The two fibrils overlap for most of their length and are wound together with the protoplasmic cylinder. Both the protoplasmic cylinder and the axial fibrils are enclosed by a sheath or outer cell envelope. Motile. Spherical bodies, generally 2 to 3.5 μm in diameter, are present in stationary growth phase cultures and under unfavorable growth conditions (i.e., in the presence of O_2). Subsurface colonies in agar media are spherical, fluffy, cream-colored, 1 to 5 mm in diameter. Surface colonies (anaerobic) are round, partially growing within the agar medium, cream-colored, 2 to 5 mm in diameter. Gram-negative.

Obligate anaerobes. Chemoorganotrophs: strictly fermentative metabolism, carbohydrates are the fermentable substrates. D-arabinose, L-arabinose, L-fucose, L-rhamnose, D-fructose, D-galactose, D-glucose, D-mannose, cellobiose, lactose, maltose, sucrose, trehalose, inulin, raffinose are fermented. D-lyxose, L-lyxose, D-ribose, L-xylose, ethanol, methanol, pectin, α-ketoglutarate, oxaloacetate, citrate, succinate, fumarate, malate, gluconate, tartrate, allantoin, uric acid, orotic acid, glucosamine, sugar alcohols, and amino acids are not fermented. Products of glucose fermentation (strain R1, cell suspensions, μmoles/100 μmoles glucose): ethanol, 140.5; acetate, 57; CO_2, 201.8; H_2, 74.4; and trace amounts of lactate, formate, and pyruvate. Nitrite is not accumulated in the medium by cells growing in the presence of nitrate. Catalase negative.

Exogenous supplements of biotin, niacin, and coenzyme A are required for growth. Coenzyme A may be replaced by pantothenate, but the resulting cell yields are low. An exogenous supplement of thiamine is stimulatory for growth. Inorganic ammonium salts or amino acids serve as nitrogen sources. A reducing agent (sulfide or cysteine) is required for growth in laboratory media. Grow in media prepared with sea water but not with fresh water unless NaCl is added (minimum concentration, 0.05 M; optimum, 0.35 M). Have specific requirements for Na^+ and Cl^-.

Temperature optimum: 30°C. Grow slowly at 15, but do not grow at 5 or 40°C. Optimum growth yields result when the initial pH of the medium is between 7 and 7.5.

The GC content of the DNA is 50.5 moles-% (strain R1, buoyant density).

Isolated from sulfide-containing marine mud.

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