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Spirochaeta bajacaliforniensis sp. n. from a microbial mat community at Laguna Figueroa, Baja California Norte, Mexico

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Abstract. A new anaerobic spirochete was isolated from anaerobic muds beneath the laminated sediment in the evaporite flat at Laguna Figueroa, Baja California Norte, Mexico. The organism is a member of the stratified microbial community involved in the deposition of the laminated sediments in the lagoon. The size of the spirochete is 0.3 by 30 μm, with a wave amplitude of 0.5 μm and a wavelength of 1.25 μm. The periplasmic flagella have a 1-2-1 arrangement. The outer membrane of the modified Gram-negative cell wall (the sheath) is irregularly crenulated and has a sillon. The growth medium contained yeast extract, trypticase, cellobiose, sodium thioglycolate and at least 20% natural seawater. Chemically defined artificial seawater media did not support growth. Optimal growth occurred with a seawater concentration of 80% at 36° C and a pH of 7.5. Glucose was fermented to acetate, ethanol, carbon dioxide and hydrogen. The guanine + cytosine content of the DNA was 50 mol %. The spirochete body reacts positively to antibodies raised against eukaryotic brain tubulin protein. On the basis of its free-living anaerobic habitat, its unique morphological and physiological characteristics and G+C ratio, it is proposed that this isolate be considered a new species and named *Spirochaeta bajacaliforniensis*.

Key words: *Spirochaeta bajacaliforniensis* – Spirochete – Laguna Figueroa – Microbial mats – prePhanerozoic Aeon – Tubulin-like protein

Introduction

A new species of the genus *Spirochaeta* was isolated from anaerobic mud beneath the laminated sediment at North Pond, Laguna Figueroa, Baja California Norte, Mexico (Margulis et al. 1980). A regular member of a stratified microbial mat community involved in the deposition of the laminated sediments (Margulis et al. 1980, 1983; Stolz 1983, 1984), this spirochete is only one of the several new microorganisms which have been isolated and characterized (Giovannoni and Margulis 1981; Gong-Collins and Read in press; Margulis et al. 1980, 1983; Read et al. 1983).

Laguna Figueroa is a hypersaline lagoon, 3 km wide and 16 km long, which is separated from the Pacific Ocean by a barrier dune and beach (Horodyski et al. 1977; Margulis et al. 1980). At the salt-marsh/evaporite flat interface laminated sediments are deposited by a stratified microbial community dominated by *Microcoleus chthonoplastes* (Horodyski et al. 1977). Studied for over a decade, the fine grained laminated sediments and associated microbial community have been compared with carbon-rich banded cherts from the Archean Aeon (Horodyski 1977; Horodyski et al. 1977; Margulis et al. 1980, 1983; Stolz 1983, 1984; Stolz and Margulis 1984). Microfossils resembling extant bacteria, primarily cyanobacteria, have been found in cherts associated with laminated sediments and stromatolites as old as 3.4 billion years (Knoll and Golubic 1979; Walsh and Lowe in press). The distinctive colony morphologies and high preservation potential of extant mat communities make interpretation of their fossil record possible (Golubic 1973; Francis et al. 1978a, b; Margulis et al. 1983).

In addition to its ecological and paleoecological significance, this spirochete possesses tubulin-like proteins. The serial endosymbiosis theory (SET), which states that eukaryotic organelles (i.e. mitochondria and chloroplasts) evolved from freeliving prokaryotic ancestors, also suggests that undulipodia (cilia and eukaryotic flagella), the organelles of motility comprised of microtubules arranged in the "9+2" array, evolved through a symbiosis with spirochetes that contained microtubules composed of tubulin proteins (Margulis 1981). Microtubules have been observed in very large spirochetes symbiotic in termites (Margulis et al. 1978). None of these large spirochetes could be isolated (To 1978). This spirochete, designated as strain BA-2, was the first bacterium in pure culture to react positively with antitubulin antibody (Margulis et al. 1980). The spirochete had to be characterized as a prerequisite for analysis of its tubulin-like proteins (Fracek et al. in preparation).

The characterization of this organism, which could not be assigned to any previously described species, is presented here. The spirochete has been named *Spirochaeta bajacaliforniensis* (strain BA-2) after the geographical location from where it was isolated.

Material and methods

Isolation. The organism was isolated from a mud sample collected at Laguna Figueroa using the well-plate technique of Canale-Parola (1973, 1977) and a pure culture was obtained by serial dilution (Margulis et al. 1980).

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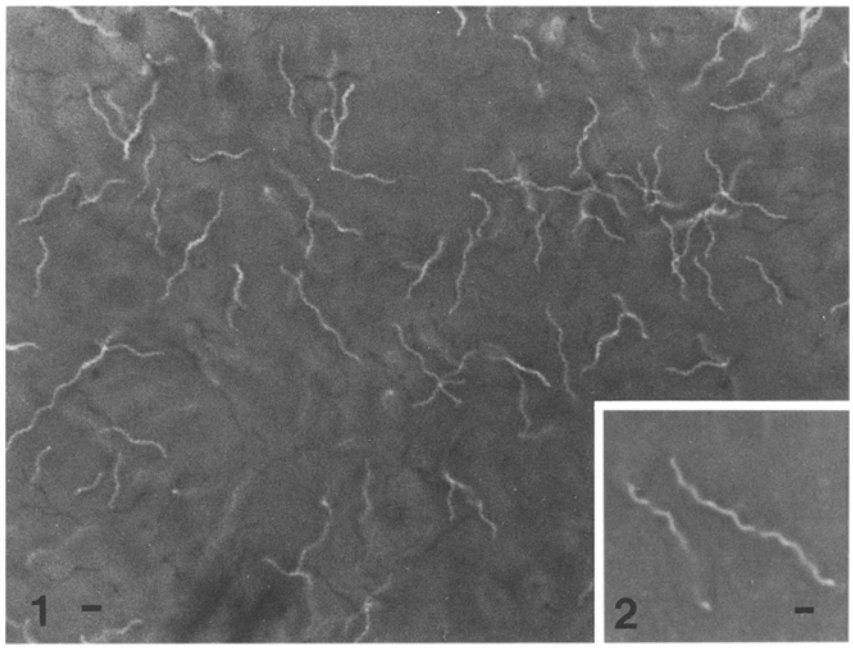


Figs. 1, 2
Spirochaeta bajacaliforniensis, phase contrast light micrographs
Figure 1, bar 5 μm; Figure 2, bar 1 μm

Media and growth conditions. The medium contained 0.2 g cellobiose (Sigma), 0.2 g trypticase (BBL), 0.1 g yeast extract (Difco), and 0.1 g sodium thioglycolate, 80 ml of filtered natural seawater and 20 ml of glass distilled water. The pH was adjusted to 7.5 with 10 *M* KOH and the medium autoclaved. A 1 ml inoculum was added to 19 ml of this medium in a Kimax (13 × 150 mm) screw top culture tube. All cultures were incubated at 30° C, unless otherwise indicated, and subcultured every 3 or 4 days.

To test the spirochete's requirements for natural seawater and NaCl, the medium was prepared with artificial seawater media used for *Spirochaeta litoralis* (Canale-Parola et al. 1967; Hespell and Canale-Parola 1970), other artificial seawater mixtures (Altman and Dittmer 1964) and commercially available artifical seawater mixtures ("Instant Ocean", Aquarium Systems, Mentor, OH, USA). The medium was also prepared varying the amounts of natural seawater and without seawater but with added NaCl.

To study the nutritional characteristics of the spirochete, the medium was prepared without cellobiose and only 0.1 g trypticase and 0.1 g yeast extract. Carbon compounds (0.2 % w/v) were added after autoclaving and the pH was adjusted to 7.5. The medium was then filter sterilized using a Millipore filtration unit with a 0.22 μm average pore size.

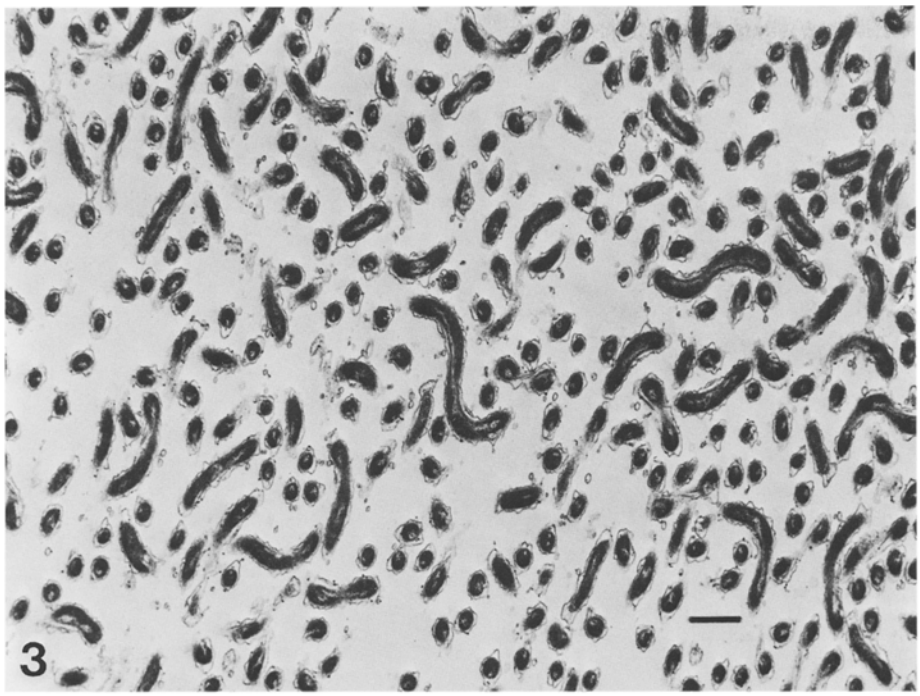
Growth measurements. Growth yields and generation time were determined turbidimetrically with a Perkin-Elmer Hitachi 200 Spectrophotometer at 650 nm. Turbidity was correlated with cell number by use of a standard curve relating optical density to direct cell counts determined by a hemocytometer. Cells were counted after three days of incubation.

Fermentation end products. Fermentation end product analysis was done at the Marine Biological Laboratory at Woods Hole, MA. Log phase cultures growing on cellobiose were pelleted at 4° C for 15 min at 10,000 rpm on a Sorvall RC2-B centrifuge. The supernatant fluid was tested for volatile and non-volatile fatty acids using the methods described in Holdeman and Moore (1975).

Light microscopy. Differential interference microscopy was done with a Nikon Biophot microscope. A Zeiss RA microscope was used for darkfield microscopy. For fluorescence studies, a Nikon Fluorphot microscope equipped with a mercury/xenon epifluorescence lamp, phase contrast, and bright field condensors was used.

Transmission electron microscopy. Two methods were used for negative staining. A drop of bacteria (about 10^9 cells/ml) was placed on a carbon coated grid (200 mesh). The grid was then either stained for 1 min with 2% uranyl acetate and rinsed with distilled water or treated with 1% sodium deoxycholate for 3 min, washed in distilled water, stained for 45 s with 1% ammonium molybdate (pH 7), and then rinsed in distilled water. The first method was used to observe intact spirochetes. The second method, using sodium deoxycholate to lyse the cell, was used in an attempt to find "cytoplasmic tubules", such as those observed in various *Treponema* species (Hovind-Hougen 1976).

For thin sections the spirochetes were fixed with 2.5% glutaraldehyde for 2 h by directly adding 0.7 ml of 70% glutaraldehyde to screw cap tubes containing 20 ml of growth medium with log phase cells. The cells were pelleted at 4,000 x *g* for 15 min at room temperature and then washed twice with filtered sterile seawater. The bacteria were postfixed with 1% osmium tetroxide in seawater for 2 h, rinsed twice with seawater and stain en bloc overnight with 0.5% uranyl acetate in distilled water. The cells were dehydrated in an ethanol series followed by propylene oxide, and embedded in Spurr's low viscosity embedding medium (Stolz 1983). Sections were made with 1/4 inch glass knives on a Sorvall MT-2 ultramicrotome and stained with 1% uranyl acetate in distilled water for 20 min and 1% lead citrate in distilled water for 15 min. All specimens were observed with a Joel 100 B electron microscope at 60 KV.



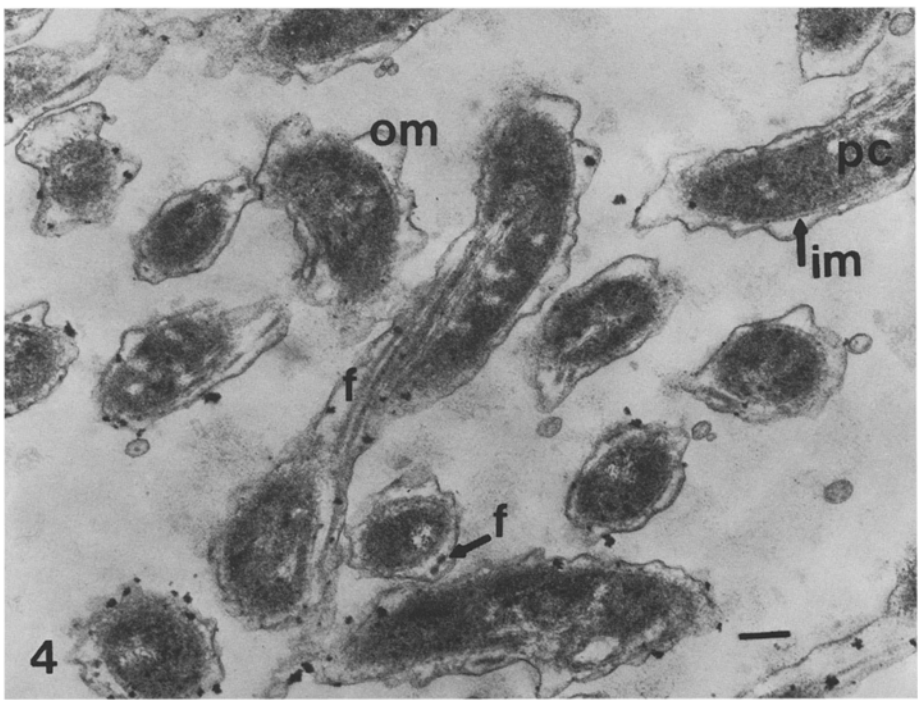


Fig. 3
Spirochaeta bajacaliforniensis, transmission electron micrograph ×15,000

Fig. 4
Spirochaeta bajacaliforniensis. *f* flagella, *im* inner membrane, *om* outer membrane, *pc* protoplasmic cylinder. Transmission electron micrograph. ×75,000

Guanine + cytosine ratio. The guanine + cytosine content of the DNA was determined by cesium chloride density centrifugation.

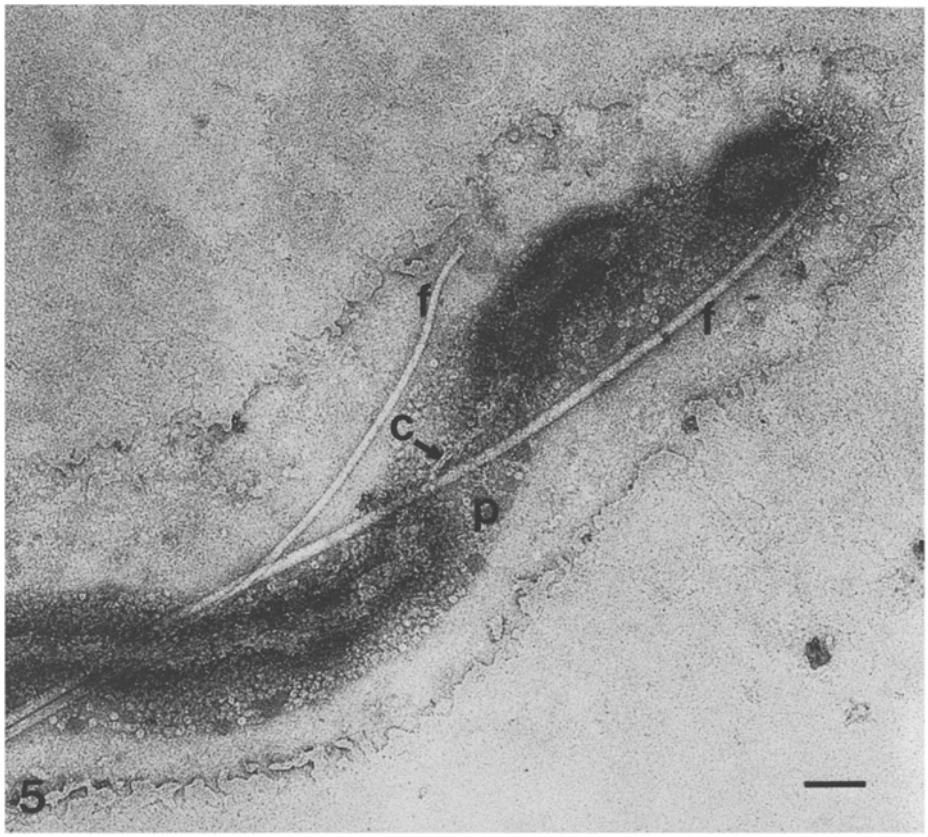
Tubulin assay. The presence of tubulin-like protein was assayed in whole cells using indirect immunofluorescence to tubulin antibody (Shelanski et al. 1973). Tubulin-like proteins were isolated and purified using cold-warm cycling and gel electrophoresis (Fracek 1984).

Other experimental procedures. Catalase was tested with 3% hydrogen peroxide on colonies grown on 2% agar medium under an atmosphere of 80% N_2, 17% CO_2 and 3% H_2 (Hungate 1969).

Results

Morphology

The average size of *Spirochaeta bajacaliforniensis* was 0.2–0.3 μm in diameter. Single cell length varied from 15 to 45 μm (Fig. 1). A few cells occasionally exceeded 300 μm in length. The coiling was regular with an amplitude of 0.5 μm and an approximate wavelength of 1–1.5 μm (Fig. 2). Live



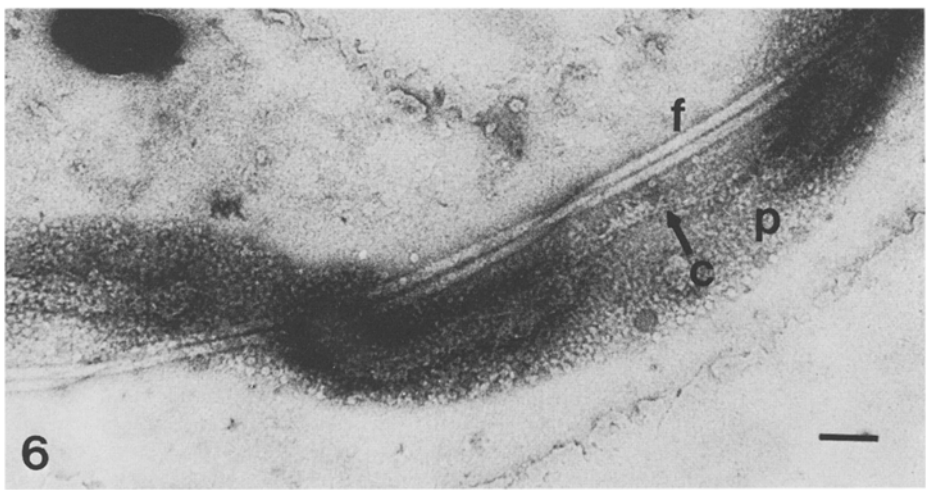


Fig. 5
Spirochaeta bajacaliforniensis negative stain deoxycholate-treated preparations showing flagella *f* at distal end of cell protoplasmic cylinder pattern *p* and possible cytoplasmic fibers *c*. ×75,000

Fig. 6
Spirochaeta bajacaliforniensis negative stain deoxycholate-treated preparations showing overlapping flagella *f*, protoplasmic cylinder pattern *p* and possible cytoplasmic fibers *c*. ×78,000

spirochetes exhibited all the usual forms of spirochetal movement: translational motility, flexing, creeping and crawling (Canale-Parola 1978; Johnson 1981). Two additional cell morphologies, long rods and spheres, were observed in cultures growing under unfavorable conditions and in cultures older than three days. Spherical forms have been reported for other spirochetes (Greenberg and Canale-Parola 1976; Breznak and Canale-Parola 1975).

The identification of this organism as a spirochete was confirmed by transmission electron microscopy (Fig. 3). The central protoplasmic cylinder bounded by the inner membrane, the periplasmic space containing the periplasmic flagella, and the outer membrane are shown in Fig. 4. The ratio of the protoplasmic cylinder (PC) diameter to the overall diameter of the cell has been used as a taxonomic characteristic (Margulis et al. 1981). In *S. bajacaliforniensis* it was approximately 2:3 as determined from transverse sections in electron micrographs (PC over the average diameter = 0.66 ± 0.05). Negatively stained cells (Figs. 5 and 6) and transverse thin sections (Fig. 7) indicated the typical 1-2-1 arrangement of the flagella found in *Spirochaeta*. Each flagellum was inserted via a basal disc at one end (Fig. 8) and ended subterminally at the other end of the protoplasmic cylinder (Fig. 5). Three periplasmic flagella were occasionally seen in cells, presumably just prior to division (Fig. 9). Irregular crenulations characterized the outer membrane (Figs. 9 and 10) and a sillon was evident (Figs. 7 and 9). The sillon is a point of contact between the protoplasmic

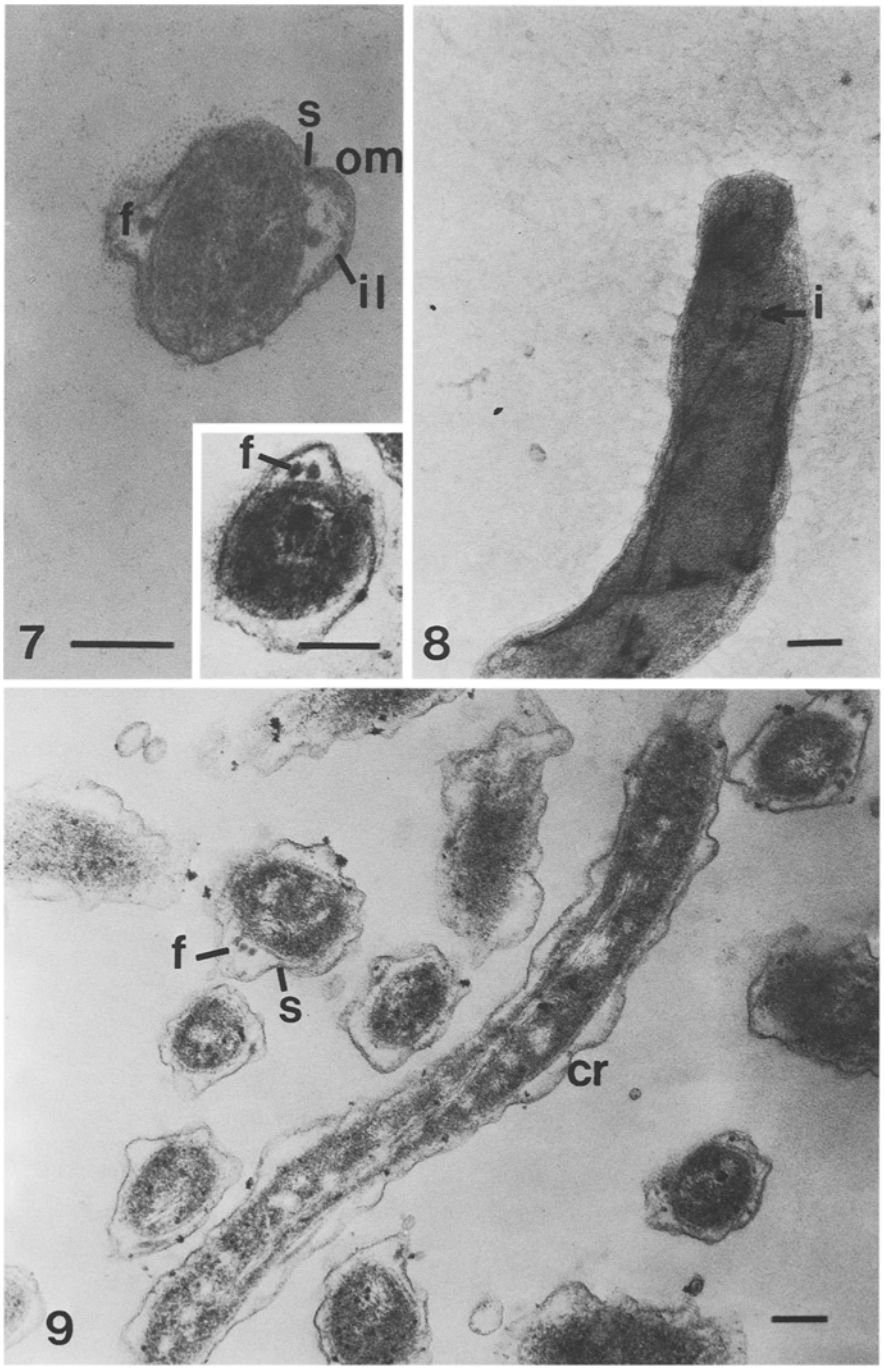


Fig. 7
Spirochaeta bajacaliforniensis transverse thin section showing 1-2-1 arrangement of flagella. *f* flagella, *s* sillon, *om* outer membrane, *il* inner layer of outer membrane. ×255,555, inset ×120,000

Fig. 8
Subterminal flagellar insertion *i*, negative stain. ×150,000

Fig. 9
Three flagella *f* can be seen in *S. bajacaliforniensis* prior to division. *s* sillon, *c* possible cytoplasmic fibrils

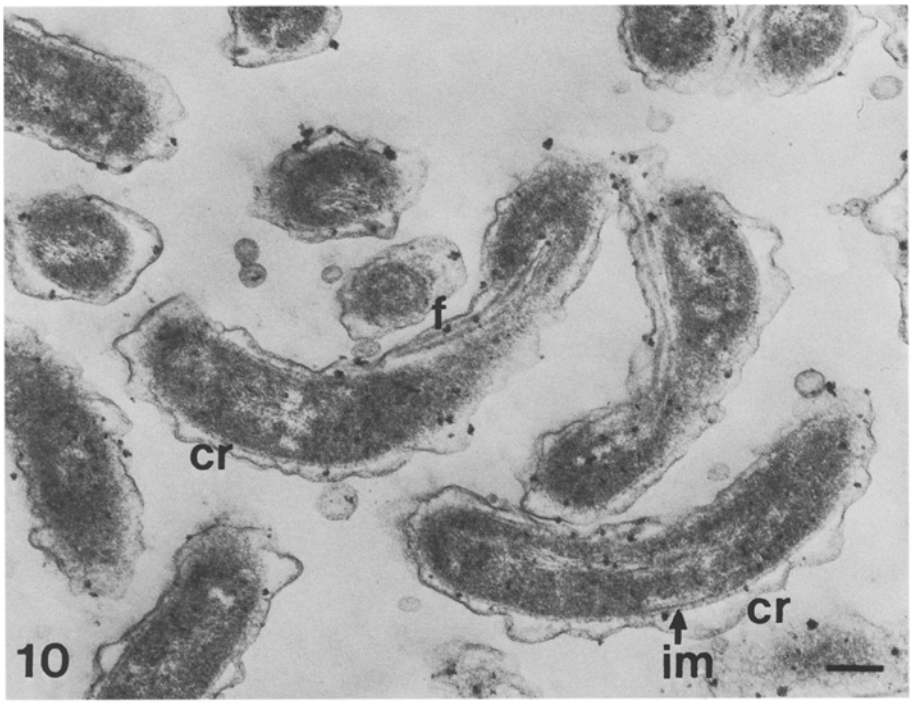
cylinder, the inner membrane and the outer membrane (Margulis et al. 1981). Cytoplasmic fibers were also seen in some micrographs (Figs. 5, 9 and 11). A tightly packed hexagonal pattern characterized the outer surface of the protoplasmic cylinder (Figs. 5 and 6). There was also an additional layer on the inside of the outer membrane (Fig. 7), a feature characteristic of both *Pillotina* and *Clevelandina* (Margulis et al. 1981).

Cultural characteristics

S. bajacaliforniensis was strictly anaerobic. The spirochete could grow on solid medium with an anaerobic atmosphere or in liquid medium with a reducing agent (sodium thioglycolate or cysteine). Cultures grown on agar under an atmosphere of 80% N_2, 17% CO_2 and 3% H_2 (Hungate 1969) produced diffuse spherical white colonies that tended to penetrate the agar rather than grown the surface. Cultures grown in liquid medium had a milky appearance and there was no observable stratification within the culture tubes.

Growth occurred over a wide range of pH. In was vigorous between pH 6.0 and 9.0 with an optimum at 7.5. Little or no growth occurred at or below pH 5 and at or above pH 10.

Optimum growth occurred at 36° C. Growth did not occur below 15° C above 44° C. The spirochete could be



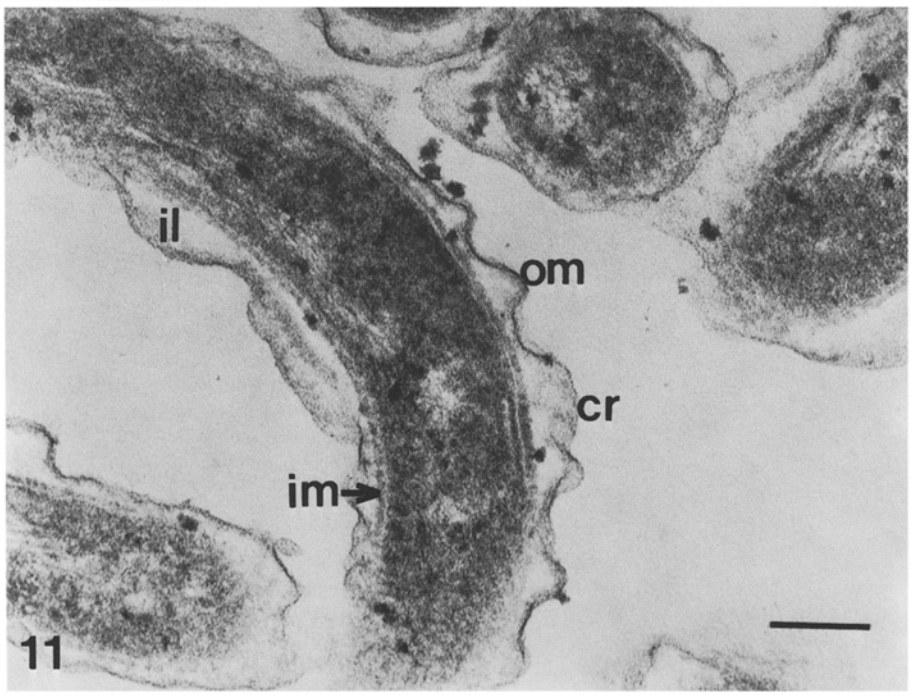


Fig. 10
Spirochaeta bajacaliforniensis longitudinal thin section showing irregular crenulations *cr*, inner membrane *im* and flagella *f*. ×75,000

Fig. 11
Spirochaeta bajacaliforniensis longitudinal thin section showing inner layer of outer membrane *il* and possible cytoplasmic fibrils *c*. ×150,000

stored for over three years at −70°C in 50% glycerol and 50% standard medium.

The shortest generation time for *S. bajacaliforniensis* was approximately 12 h.

Nutrition

S. bajacaliforniensis grew routinely on medium containing yeast extract, trypticase and cellobiose. When the concentrations of trypticase and yeast extract were at or below 0.02% and 0.01% respectively and cellobiose was omitted, no growth occurred. Twenty-six carbon compounds were tested and the results are presented in Table 1. Like most members of the genus *Spirochaeta* (Canale-Parola 1977, 1981; Johnson 1977) *S. bajacaliforniensis* can utilize many carbohydrates for growth (Table 1).

Marine nature of S. bajacaliforniensis

Attempts to replace natural seawater with different artificial seawaters (Altman and Dittmer 1964), including "Instant Ocean" (Aquarium Systems, Mentor, OH, USA), and *S. litoralis* medium (Canale-Parola et al. 1967; Hespell and Canale-Parola 1970), were unsuccessful. *S. bajacaliforniensis*

was able to grow on soil extract medium which contained at least 0.12 *M* NaCl (D. Bermudes, personal communication). By adding additional NaCl to the natural seawater medium the spirochete was found to grow at salinities between 0.3 and 0.72 *M* NaCl with an optimum at 0.48 *M* NaCl. Media containing seawater concentrations less than 20% when supplemented with artificial seawater and NaCl (0.6 *M* final concentration) also did not support growth.

Fermentation end products

The major products of glucose fermentation were acetate, ethanol, carbon dioxide and hydrogen. No test for formate was performed.

Table 1. Utilization of carbon compounds

Compound added	Yield (cells/ml × 10^8)
None	0.39
Arabinose	1.10
Cellobiose	1.30
Galactitol	0.75
Fructose	0.93
Galactose	0.91
Gluconate	0.86
Glucose	0.93
Inulin	0.73
Lactose	0.70
Malate	0.88
Maltose	0.83
Mannitol	1.10
Pyruvate	1.24
Rhamnose	0.91
Sorbose	0.62
Trehalose	1.15

The following carbon sources were tested but yielded growth at or below the level of the control: ethanol, alpha-keto glutaric acid, glycerol, lactate, mannose, methanol, raffinose, soluble starch, glucitol, sucrose, and xylose

Guanine + cytosine content

Analysis of *S. bajacaliforniensis* DNA by cesium chloride density centrifugation revealed a buoyant density which corresponded to 50.1 mol % G + C.

Tubulin-like proteins

Whole cells of the spirochete fluoresced when treated with antitubulin antibody by indirect immunofluorescence (Margulis et al. 1978; Fracek 1984). In addition, two tubulin-like proteins, identified by techniques used to isolate and purify eukaryotic tubulins, were extracted from the spirochete (Fracek 1984).

Discussion

The organism described here has been placed into the genus *Spirochaeta* because it is free-living, anaerobic and possesses typical *Spirochaeta* morphology (Holt and Canale-Parola 1968; Canale-Parola et al. 1968, Johnson 1981). Electron micrographs revealed the irregularly crenulated outer membrane, helical protoplasmic cylinder, periplasmic space and two overlapping periplasmic flagella. Cytoplasmic fibrils, which may be related to similar structures seen in treponemes (Eipert and Black 1979; Hovind-Hougen and Birch-Andersen 1971), were evident in several micrographs (Figs. 9 and 11). They were smaller and far less well defined than the 21 nm microtubules found in the far larger termite spirochetes which are members of the family Pillotaceae (Margulis et al. 1978, 1981).

S. bajacaliforniensis can be distinguished from all the other species of *Spirochaeta* on the basis of morphology, seawater and NaCl requirement and G + C ratio (Table 2). *S. plicatilis*, for which the G + C ratio is unknown, is wider (0.75 μm) and contains a bundle of periplasmic flagella (Blakemore and Canale-Parola 1973). *S. litoralis* has a similar G + C ratio (50.5), but is wider (0.4 – 0.5 μm) and shorter (5.0 – 7.0 μm) than *S. bajacaliforniensis*. *S. litoralis* has both larger amplitute (0.7 – 1 μm) and wavelength (2.0 – 2.7 μm) (Hespell and Canale-Parola 1970). In addition, the

Table 2. Comparison of species of the genus *Spirochaeta*

Species	Size (μm)	Relationship to O_2	Optimum temperature range	G + C content of DNA (mol %)	Environment	Periplasmic fibril arrangement	Optimal NaCl concentration	Optimal pH
S. plicatilis	0.75 × 80 – 25	Unknown	Unknown	Unknown	FW and M	18 to 25	Unknown	Unknown
S. halophila	0.4 × 15 – 30	FA	35 – 40	62	M	1-2-1	0.75	7.5
S. aurantia								
var. *aurantia*	0.3 × 10 – 20	FA	30	65.3 – 62.2	FW	1-2-1	–	7.0
var. *stricta*	0.3 × 10 – 20	FA	30	61.2	FW	1-2-1	–	7.0
S. stenostrepta	0.2 – 0.3 × 15 – 45	A	30 – 37	60.2	FW	1-2-1	–	7.0
S. zuelzerae	0.2 – 0.4 × 8 – 16	A	37 – 39	56.1	FW	1-2-1	–	
S. litoralis	0.4 – 0.5 × 5 – 7	A	30	50.5	M	1-2-1	0.35	7.5
S. bajacaliforniensis[a]	0.2 – 0.3 × 15 – 45	A	36	50.1	M	1-2-1	0.48	7.5

A = Anaerobe; FA = Faculatative anaerobe; FW = Fresh water; M = Marine; – = Information not available in the literature

[a] Blakemore and Canale-Parola 1973; Breznak and Canale-Parola 1969, 1975; Canale-Parola 1981; Canale-Parola et al. 1968; Greenberg and Canale-Parola 1975, 1976; Holt and Canale-Parola 1968; Joseph 1972

optima for salt concentration and temperature are different (0.35 *M*/0.48 *M*; 30° C/36° C).

All attempts to grow *S. bajacaliforniensis* in the defined medium for *S. litoralis* were unsuccessful. *S. bajacaliforniensis* and *S. litoralis* can both utilize arabinose, cellobiose, fructose, galactose, glucose, inulin, lactose, maltose, pyruvate, rhamnose and trehalose (Table 1; Hespell and Canale-Parola 1970, 1973). Those utilizable carbon sources which are mutually exclusive are mannitol, malate and sorbose for *S. bajacaliforniensis* (Table 1), and sucrose, mannose, raffinose and xylose for *S. litoralis* (Hespell and Canale-Parola 1970).

The significance fo free-living spirochetes in nature is considered to be their saccharolytic behavior (Hespell and Canale-Parola 1970). *S. bajacaliforniensis* has shown to be no exception and probably contributes to the breakdown of carbohydrates produced by the phototrophic bacteria and other members of the microbial community at Laguna Figueroa. The extent of sequence homology, if any, of the tubulin-like proteins of *S. bajacaliforniensis* with the eukaryotic microtubule proteins (alpha and beta tubulin) is under investigation (personal communication, B. Obar).

Description

Spi.ro.chae'ta. Gr. n. *spira* a coil; G. n. *chaeta* hair; M. L. fem. n.; *Spirochaeta* coiled hair. ba.ja.ca.li.for.ni.en'sis. L. adj. *bajacaliforniensis* from Baja California; named for the geographical location from where it was isolated.

Single, helical cells, 0.2–0.3 μm by 15–45 μm.

Shorter (10 μm) and longer (up to 300 μm) cells may occur. Highly motile. The amplitute is 0.5 μm and the wavelength is 1.0–1.5 μm. Two subterminally inserted periplasmic flagella are present in a 1-2-1 arrangement. The ratio of protoplasmic cylinder diameter to the cell diameter is 2:3. The surface of the protoplasmic cylinder has a characteristic polygonal pattern. Subsurface colonies are fluffy, white and spherical.

Strictly anaerobic. Chemoorganotrophs: strictly fermentative metabolism, carbohydrates are the fermentable substrates. Arabinose, cellobiose, galactitol, fructose, galactose, gluconate, glucose, inulin, lactose, malate, maltose, mannitol, pyruvate, rhamnose, sorbose and trehalose are fermented. Ethanol, alpha-keto glutaric acid, glycerol, lactate, mannose, methanol, raffinose, soluble starch, glucitol, sucrose and xylose are not fermented. Products of glucose fermentation are acetate, ethanol, carbon dioxide and hydrogen. Catalase negative.

Grow in media containing at least 20% seawater and 0.12 *M* NaCl. Reducing agent needed in liquid medium. An 80% N_2, 17% CO_2 and 3% H_2 atmosphere needed for solid medium.

Temperature optimum: 36° C. Do not grow below 25° C or above 44° C. Survive frozen in 1:1 medium/glycerol at −70° C. Optimum growth at pH 7.5.

The GC content of the DNA is 50.1% (buoyant density).

Isolated from anaerobic sulfide-rich mud underlying the laminated sediment of the microbial mats at North Pond, Laguna Figueroa, Baja California Norte, Mexico.

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