Enrichment and Attempted Isolation of *Cristispira* from the Oyster.

An Individual Project for the MBL Microbial Diversity Course.

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Abstract

Cristispira are spirochete-like organisms found in the digestive tracts of many bivalve mollusks. The name *Cristispira* is derived from the presence of large bundles ("cristi") of periplasmic flagella observed in microscopic cross-sections. Cells can be from 1-3 um in diameter, up to 500 um in length, and possess a spirochete-like ("spira") helical morphology. Although *Cristispira* are found concentrated in high numbers in several bivalve mollusks, they have not be cultured in the laboratory. As an individual project for the 1998 MBL Microbial Diversity course, I attempted to enrich for, and ultimately isolate, *Cristispira* in pure culture. Three different buffer conditions were tested (MES, MOPS, HCO_3^-) in liquid and methylcellulose. It was discovered that *Cristispira* can survive for up to one week in 2% methylcellulose prepared in seawater, buffered at pH 5.7 with MES. A medium successfully used to isolated termite-hindgut spirochetes was also used in an attempt to isolate *Cristispira*. However, under a variety of conditions such as pH, temperature, H_2/CO_2, microaerophilic conditions, and different buffers, *Cristispira* was failed to survive longer than a few hours in the termite-spirochete medium. Each individual supplement in the termite-associated spirochete medium was examined for its effect on the mortality of *Cristispira*. Preliminary findings indicate that, unlike most spirochetes, *Cristispira* is sensitive to the antibiotics rifamycin SV and phosphomycin which bind prokaryotic RNA polymerase and inhibit cellular transcription. These findings provide incite into methods of cultivation that may be employed in future attempts to enrich for and isolate *Cristispira*.

Introduction

Some of the largest spirochete-like organisms in nature are found as symbionts in mollusks and arthropods. The largest of these spirochetes, *Cristispira*, was observed over 100 years ago in the bivalve mollusk oyster (Certes, 1882). *Cristispira* is localized to the digestive tract of the oyster and is exclusively found in the crystalline style (Breznak, 1984). The crystalline style is an opaque mucoproteinacious (10% solids) tapered rod (fig. 1) that is thought to serve as a "pestle" to pulverize food against the stomach of the oyster (Judd, 1979). Styles can readily dissolve and reform along with the associated absence or presence of *Cristispira*. *Cristispira* possesses a characteristic spirochete helical morphology and can be from 1 - 3 um in diameter and from 30 - 500 um long (fig. 4 & 5). Periplasmic flagella are observed in cross-sections of *Cristispira* however, unlike most spirochetes, *Cristispira* contains a bundle ("cristi") of 100 or more flagella at both ends of the cell.

Phylogenetic analysis of 16S rDNA clones obtained for the crystalline style of oysters confirms morphological classification of *Cristispira* as a member of the Spirochete eubacterial division, and a deeply branching member of the genus *Spirochaetaceae* (Paster, 1996);(fig. 3). Fluorescent in situ hybridization of DNA extracts from oyster crystalline styles indicates that the large spirochete-like organisms are the predominant OTU in the *Spirochaetaceae* recovered in cloning experiments (Paster, 1996).

The crystalline style readily dissolves upon storage in the laboratory, therefore to observe *Cristispira* it is best to use freshly harvested oysters. However, the presence of a crystalline style does not guarantee the presence of *Cristispira*. Oysters collected from the same location at the same time of the day may not all contain crystalline styles and therefore *Cristispira*. Oysters collected from both aerobic (beach surface) and anaerobic (anaerobic mud or deep water) locations can contain *Cristispira* (Margulis & Hinkle, 1991). *Cristispira* can be maintained in the laboratory from 2 - 24 hours in seawater, however no axenic or pure culture of *Cristispira* exists in the laboratory.

Previous attempts to enrich for and isolate *Cristispira* during the MBL Microbial Diversity course were met with limited success (Lazar, 1995; Kreiling 1992). Jill Kreiling attempted to isolate *Cristispira* using a modified *Leptospira* medium buffered with citric acid (pH 5.7) and amended with bovine serum albumen, N-acetylglucoseamine, cholesterol, and β-hydroxybutyrate. A two percent solution of methylcellulose was used as a growth substrate and both aerobic and anaerobic (N_2/CO_2, 80/20) were examined. Observation of these cultures by Kreiling revealed only non-motile and presumably dead *Cristispira* cells. Attempts by Sara Lazar in 1995 were also met with limited success, but several important observations were recorded. Different concentrations of methylcellulose (0.1 - 2.0 %) revealed that *Cristispira* cells survived longest in 2% methylcellulose made up in seawater. Attempts to amend methylcellulose/seawater with mucin, yeast extract, peptone, and casamino acids resulted in rapid contamination of cultures. However, analysis of oyster digestive tract revealed a pH of between 5.5 and 6.0. The longest survival of *Cristispira* occurred in 1%

methylcellulose and 50% seawater, and spanned around 10 days prior to the culture being overtaken by other bacteria.

As a member of the "Midwestern Molecular School" of microbiology, I attempted to isolate *Cristispira* to acquire skills in classical, or "Delft-ian," aerobic and anaerobic culturing techniques. This is not to say that I wasn't fascinated by the motility and impressive size of *Cristispira* observed in the crystalline styles. Culturing techniques successfully employed in the isolation of spirochetes from the termite hindgut were used in an attempt to isolate *Cristispira* from oysters. Additional culture conditions were built upon previous observations and experimentation by past students in the Microbial Diversity course.

Materials & Methods

Sampling: a.k.a. Shucking Oysters

The key to obtaining crystalline styles from oysters is to plan ahead! Oysters can be obtained from the Marine Resource Center (MRC) at MBL within a days or two notice at a cost of $2.10 apiece. All media should be prepared and aliquoted prior to obtaining oysters since the crystalline style readily dissolves (and *Cristispira* along with it) once out of their natural habitat and in the laboratory. To open an oyster, use a shucking knife, a pair of gloves, and have a towel handy (things get slippery in a hurry!). Place the oyster on solid surface with the flat side up, insert the shucking knife into the indentation at the end of the oyster (umbo) and slowly twist (fig 2). Once your knife is into the oyster, run the blade along the underside of the flat shell and break the muscle holding the shell together. With the umbo facing away from you, use a razor blade to make a 1 -2 cm length (0.5 cm deep) incision in the tissue on the far left side of the oyster (fig 2). Using your thumb, gently press down the oyster tissue towards the incision and the crystalline style (opaque yellow-green; fig. 1) should exit from the incision if it is present. Remove the style with pair of sterile forceps and place in an 1.5 mL polypropylene tube containing 2% methylcellulose in seawater buffered with MES at pH 5.7. Remember that all oysters do not contain styles, and all styles do not contain oysters. For example, on one occasion I shucked 18 oysters and obtained 6 crystalline styles, 2 of which contained *Cristispira*.

Mortality of Cristispira in Different Buffers

Four different buffer conditions were examined for their ability to support *Cristispira*: 2% methylcellulose in seawater buffered with MES (pH 5.7), 2% methylcellulose in Widdel medium buffered with MES (pH 5.7), Widdel medium and bicarbonate buffer (pH 7.0), Widdel medium and MOPS buffer (pH 7.0), and plain unamended seawater. Methylcellulose (Sigma) was prepared by dissolution with constant stirring for 1 - 2 hrs at room temperature. Solutions of methylcellulose become insoluble at high temperatures while autoclaving, but become soluble once the solution returns to room temperature.

Crystalline styles were removed asceptically from fresh oysters (generally 1 hr post-harvesting) and placed in sterile 1.5 mL polypropylene tubes containing 1.0 mL of the buffer conditions under examination. The styles were allowed to dissolve at room

temperature (21°C - 23°C) and were then homogenized by repeatedly pipetting up and down with a 1.0 mL pipettor. Small aliquots (~5 uL) of each buffer/style suspension were removed periodically for microscopic examination.

Enrichment of Cristispira in Termite Hindgut Spirochete Media.

Recently, several spirochetes have been successfully cultured from the termite hindgut in the laboratory of John Breznak (1998). These termite associated spirochetes were demonstrated to be acetogenic under atmospheric conditions of hydrogen and carbon dioxide (80%/20%). Two media constituents were critical to the enrichment and isolation of termite associated spirochetes: rumen fluid and yeast autolysate. In attempt to isolate the mollusk-associated spirochete *Cristispira,* media conditions established for termite-associated spirochetes were employed.

Two different media formulations were utilized in an attempt to enrich for *Cristispira* sp. and other putative spirochetes. Both media used the supplements listed below, but one formulation used seawater as a base while the other used Widdel basal salts. Different pH values were achieved by the use of carbonate and sulfonic acid buffers as described below. Non-liquid media was combined with either 2% methylcellulose or 1.8% low melting temperature agarose (Seakem LE, FMC Bioproducts) to provide a substrate for motility. The table below (Table 1) describes the different media compositions and temperatures at which enrichments were established.

Table 1: Media Composition of *Cristispira* Enrichments

Medium Name	Salts Base	Supplements	Motility Substrate	Buffer	pH	Temp.	Atmosphere
CSP1-A	Widdel	Yes	agar	MES	5.7	15°C	O_2
CSP1-B	Widdel	Yes	liquid	MES	5.7	15°C	O_2
CSP1-C	Widdel	Yes	2% Me-Cell.	MES	5.7	22°C	O_2
CSP1-C	Widdel	Yes	2% Me-Cell.	MES	5.7	15°C	H_2/CO_2
CSP1-C	Widdel	Yes	2% Me-Cell.	MES	5.7	22°C	H_2/CO_2
CSP1-C	Widdel	Yes	2% Me-Cell.	MES	5.7	15°C	O_2
CSP1-D	Widdel	Yes	agar	MOPS	7.0	15°C	O_2
CSP1-D	Widdel	Yes	liquid	MOPS	7.0	15°C	O_2
CSP1-E	Widdel	Yes	agar	HCO_3^-	7.0	15°C	H_2/CO_2
CSP1-E	Widdel	Yes	liquid	HCO_3^-	7.0	15°C	H_2/CO_2
SW+	Seawater	Yes	2% Me-Cell.	MES	5.7	22°C	O_2
SW+	Seawater	Yes	2% Me-Cell.	MES	5.7	15°C	O_2
SW+	Seawater	Yes	2% Me-Cell.	MES	5.7	22°C	H_2/CO_2
SW+	Seawater	Yes	2% Me-Cell.	MES	5.7	15°C	H_2/CO_2

Widdel and Pfennig Basal Salts Media

Medium Component	Amount
KH_2PO_4	0.2 g/L
NH_4Cl	0.25 g/L
NaCl	20 g/L
$MgCl_2\ 6H_2O$	3.0 g/L
KCl	0.5 g/L
$CaCl_2\ 2H_20$	0.15 g/L
Na_2SO_4	1 mM

Widdel medium was prepared with the basal salts composition listed in the table above, autoclaved, and cooled under a constant stream of N_2/CO_2 (80%/20%). Medium was dispensed into 120 mL serum bottles with an equal volume of liquid and gas phase of N_2/CO_2 (80%/20%) and sealed with a butyl rubber stopper and aluminum crimp top.

Buffers

Buffer Name	pKa	Final Conc.
MES (2-[N-morpholino]ethanesulfonic acid)	6.1 at 25°C (5.5-6.7 range)	20 mM
MOPS (3-[N-moprholino]propanesulfonic acid)	7.2 at 25°C (6.5-7.9 range)	20 mM
Sodium Bicarbonate	6.4 at 25°C (6.0-8.0 range)	2.5 g/L

The sulfonic acid buffers were autoclaved with the medium, while the bicarbonate was autoclaved separately as a 8.3% stock in a serum vial under a headspace of carbon dioxide (100%). Procedurally, place 5 g of sodium bicarbonate in 60 mL gdH_2O in a serum vial containing an equal volume of headspace and seal with a crimp-top stopper, autoclave.

Heat Instable Supplements

Pfennig's Trace Elements (SL10)	1 mL/L of 1000x stock
Vitamin Solution (Widdel & Pfennig)	1 mL/L of 1000x stock
Vitamin B_{12}	1 mL/L of 1000x stock
Rumen Fluid (prepared by Jared Leadbetter 3/96)	2% final conc.
Yeast Autolysate (see below for preparation)	2% final conc.
rifamycin SV (water soluble version)	200 ug/mL final conc. (2% stock)
phosphomycin	100 ug/ml final conc. (1% stock)
N-acetylglucoseamine (NAG)	10 mM final conc.
Lysozyme	10 ug/mL final conc. (4% stock)

All heat sensitive medium constituents were filtered through a 0.22 μm cutoff syringe filter (Millipore Aerodisc) and stored at 4°C.

Motility Substrate

Two different solid substances were used in an attempt to provide spirochetes, including *Cristispira*, a substrate for motility:

1. Seakem LE (FMC Bioproducts) Low Melting Temperature Agarose. Low melting temperature agarose (18°C - 26°C gelling temperature) was chosen to minimize any temperature shock encountered by living cells during inoculation into agar shakes. Balch tubes were filled with 2% Seakem LE agarose and 6 mL of Widdel medium was added while tubes were maintained at approximately 30°C. Hungate technique was employed to maintain anaerobic conditions when necessary.
2. Methylcellulose (Sigma). A 2% solution of methylcellulose was prepared in seawater (from tap in lab) and buffered with 20 mM MES at pH 5.7. Methylcellulose is soluble under these conditions, but becomes reversibly insoluble during autoclaving.

Effects of Individual Supplements on the Mortality of Cristispira

Cristispira did not survive for longer than 2 hours in all media formulations or substrate conditions described above (described in Results section, below), therefore the effects of each medium ingredient was tested individually. The longest survival of *Cristispira* outside the crystalline style was over one week on 2% methylcellulose in seawater buffered at pH 5.7 with MES (abbreviated as SMC). Therefore, these conditions were chosen to test the effect the each medium ingredient on the growth and motility of *Cristispira* obtained from freshly harvested oysters.

Crystalline styles were asceptically removed from freshly harvested oysters and allowed to dissolve in 1.0 mL of unamended SMC for approximately 1 - 2 hrs in a sealed 1.5 mL polypropylene tube. Each tube was homogenized by repeated pipetting and examined for *Cristispira* using phase contrast microscopy at 400X magnification. Tubes containing no *Cristispira* were discarded and the remaining tubes were pooled into a sterile 15 mL polypropylene Falcon tube. Aliquots of 250 uL were used to inoculate 1.5 mL polypropylene tubes containing 1.0 mL of SMC plus each amendment. All "Heat Instable Amendments" listed in the table above were tested at the listed concentration with the exception of the antibiotics. Rifamycin SV was tested at 50, 100, 200 ug/mL and phosphomycin was tested at final concentrations of 25, 50, and 100 ug/mL. Samples of approximately 10 uL were removed periodically to examine the motility and integrity of *Cristispira* cells.

Microscopic Examination of Cristispira.

Crystalline styles from freshly harvested oysters were dissolved in 1.0 mL of sterile seawater and the resulting suspension was utilized for microscopic examination. Aliquots of this suspension were stained with both DAPI and Live/Dead stain and examined using fluorescence microscopy (Zeiss Axioplan 10). Photographic images of samples were captured used an Optronics 3 CCD digital camera and MetaMorph software. Additional manipulations of images were performed using Adobe Photoshop 4.0 and printed on an Epson Stylus Color 600 printer.

Results

Mortality of Cristispira in Different Buffers

The greatest duration of *Cristispira* survival occurred in 2% methylcellulose in seawater buffered at pH 5.7 with MES. Approximately 75% of *Cristispira* were motile after 3 days and around 50% after 5 days. After 7 days only 10 - 20% of visible cells were motile, however several other motile rods and cocci were present in high numbers. Regardless of buffer conditions (type and pH), motile *Cristispira* cells were observed for only 12 - 18 hrs in liquid conditions without methylcellulose. As previously observed by Krieling (1992) and Lazar (1996), *Cristispira* are motile for only a short duration in liquid medium. There appears to be some need for a viscous medium for motility and survival. In a liquid medium of low viscosity, *Cristispira* "spin their wheels," so to speak, and quickly lyse. It is quite easy to detect lysed cells with ruptured outer membranes and protruding periplasmic flagellar tufts under phase contrast microscopy at 400X magnification (fig 4). The survival of *Cristispira* in 2% methylcellulose in seawater confirmed earlier observations by Lazar (1996) and indicated that MES is a suitable buffer for maintenance and possibly growth. It is undetermined whether *Cristispira* may also use the cellulose as a source of carbon and energy. Future experimentation could focus on whether other compounds may be used for a "motility substrate" such as Ficoll, glycerol, or low percentage agarose.

Enrichment of Cristispira in Termite Hindgut Spirochete Media.

Cultures were typically inoculated in the late afternoon with fresh samples and then examined the following morning for signs of survival or growth. None of the media compositions tested supported the growth or survival of *Cristispira* or other spirochete-like organisms. The Widdel basal salts or seawater were not inhibitory to *Cristispira* when examined by themselves (see above), therefore it is likely that some particular constituent of the heat instable supplements was responsible for the rapid death of *Cristispira*. Alternatively, the addition of a number of amendments could have raised the osmolarity of the medium to a lethal level. Results from these enrichments indicated that 2% methylcellulose in seawater (MES pH 5.7) should serve as a base to which individual amendments are added in low concentrations. Preliminary results from such experiments are discussed below, although the long term results were limited by the length of the course.

Effects of Individual Supplements on the Mortality of Cristispira

Experiments that examined the effects of individual supplements on *Cristispira* mortality were limited by the amount of available inoculum (Table 2). In this line of experiment, 18 large oysters were obtained of which 6 possessed styles. Of the 6 styles collected, only 2 styles contained *Cristispira* at low densities. Microscopic examination of each sample condition generally revealed between 0 and 4 *Cristispira* per field at 400x magnification (5 uL samples). Despite the small number of *Cristispira* under

examination, these experiments provided a preliminary indication that the antibiotics rifamycin SV and phosphomycin are inhibitory to *Cristispira*. Mortality rates under all other conditions were generally inconclusive due to the small number of *Cristispira* present (Table 2).

Table 2 - Effects of supplements on *Cristispira* mortality

Supplement	Final Concentration	Result
Pfennig's Trace Elements (SL10)	1 mL/L	-
Vitamin Solution (Widdel & Pfennig)	1 mL/L	ND
Vitamin B12	1 mL/L	ND
Rumen Fluid	2% final conc.	ND
Yeast Autolysate	2% final conc.	+
rifamycin SV (water soluble version)	200 ug/mL	-
rifamycin SV	100 ug/mL	-
rifamycin SV	50 ug/mL	-
phosphomycin	100 ug/mL	-
phosphomycin	50 ug/mL	-
phosphomycin	25 ug/mL	-
N-acetylglucoseamine (NAG)	10 mM	ND
Lysozyme	10 ug/mL	ND

(+) = motile *Cristispira* present
(-) = lysed *Cristispira* present
ND = insufficient number of cells

Discussion

While experimentation was generally limited by the amount of inoculum, several important observations were obtained that provide valuable information for future isolation attempts of *Cristispira*. First, it is clear that a correlation must be established between the presence of crystalline styles and the conditions from which oysters are harvested. Several factors have been suggested to influence the formation of the crystalline styles, including time of day, filter feeding, tides, and the size of oysters. In future studies it would be best to find a site to examine oysters for the presence of *Cristispira* under a variety of conditions listed above. In this way, oysters could be collected at the same time, under similar conditions, which would increase the likelihood of finding large numbers of oysters containing actively motile *Cristispira*. (Collection of oyster would also save large sums of money charged to the course from the MRC!)

Secondly, the experimentation described above indicates that the antibiotics rifamycin SV and phosphomycin are lethal to *Cristispira*. These antibiotics bind to prokaryotic RNA polymerases and prevent transcription, however they are not inhibitory to all cultured spirochetes. Apparently this is not the case with *Cristispira*. The effects of other antibiotics should also be explored as they could inhibit the growth of bacteria other than *Cristispira* in enrichment cultures.

Lastly, it was demonstrated that the sulfonic acid buffer MES is not inhibitory to *Cristispira* and can support survival up to one week. Future studies should utilize 2% methylcellulose prepared in seawater using MES as a buffer at pH 5.7. It is unclear whether low melt agarose is inhibitory to *Cristispira*, therefore this motility substrate also deserves further attention.

References

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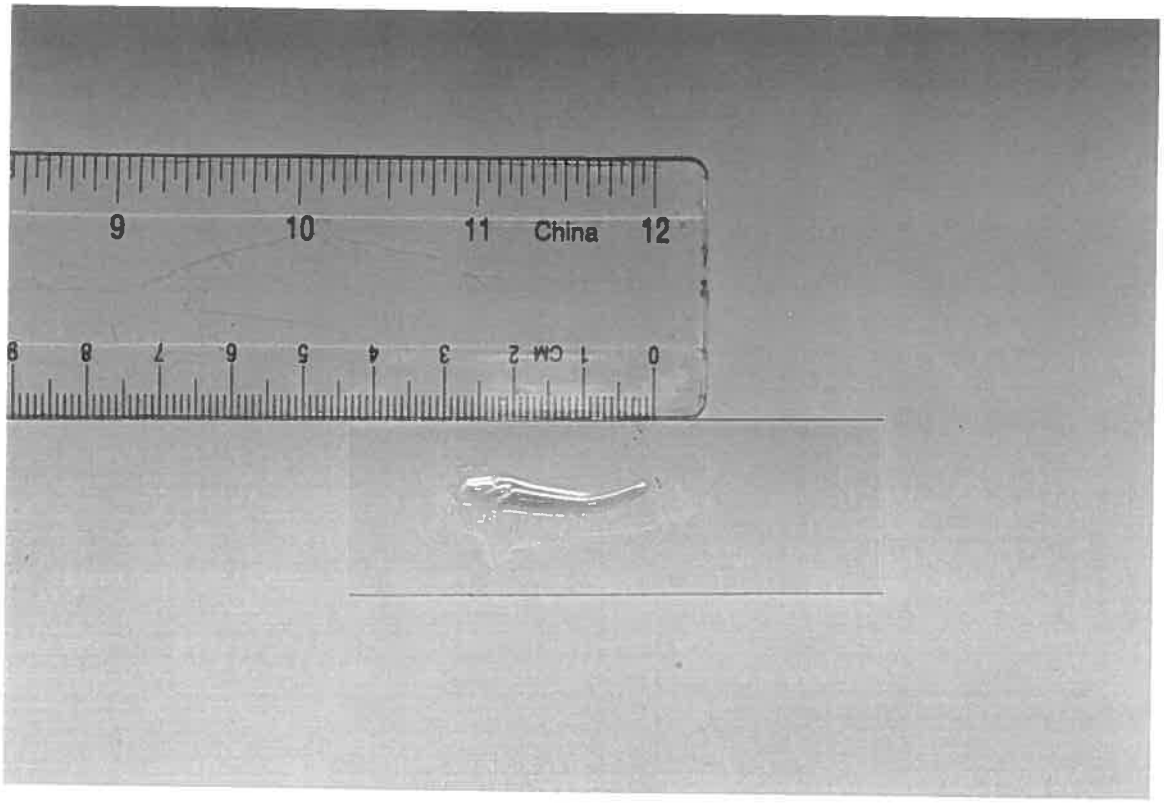
Figure 1 (top) & 2 (bottom)



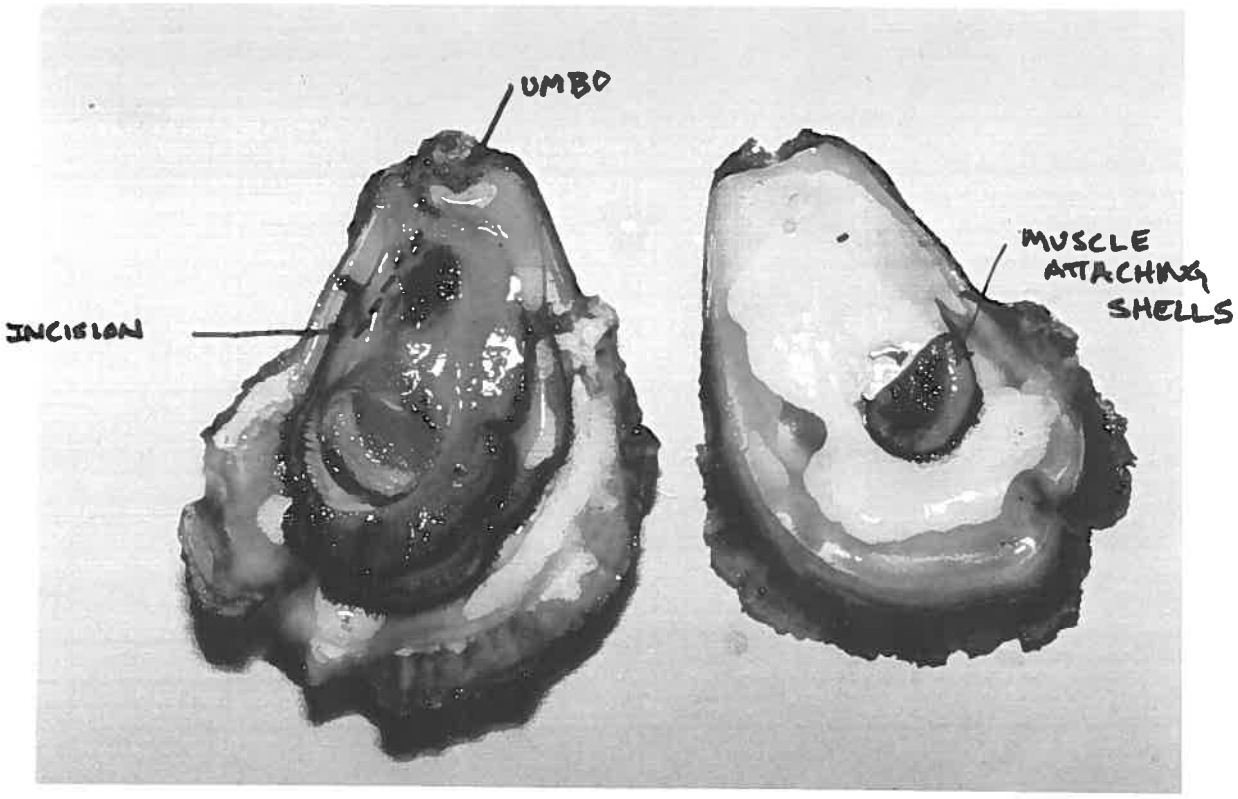


Figure 3

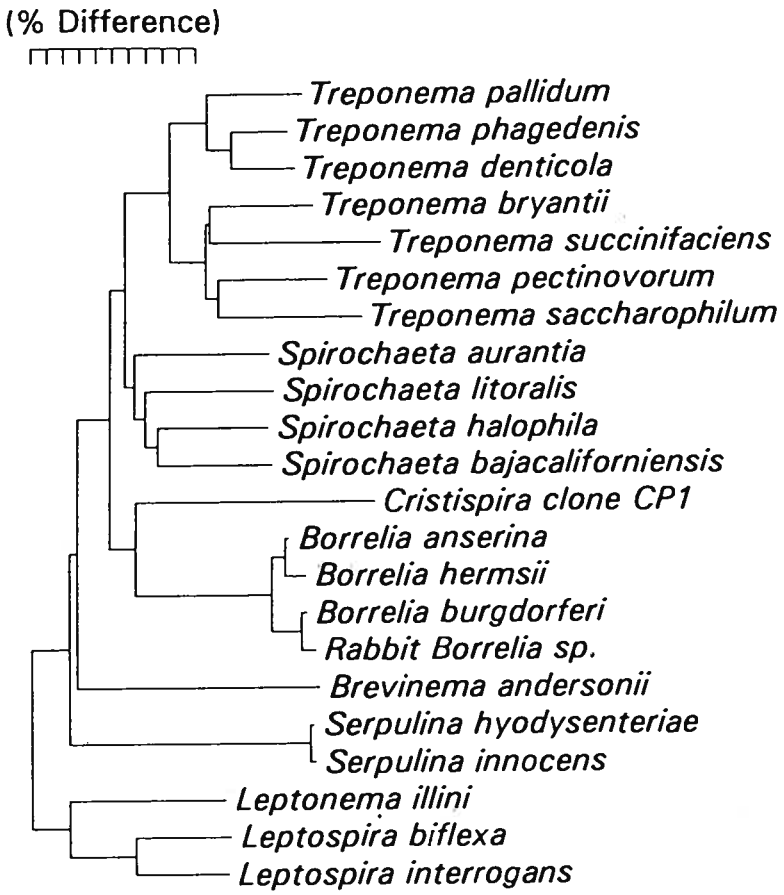
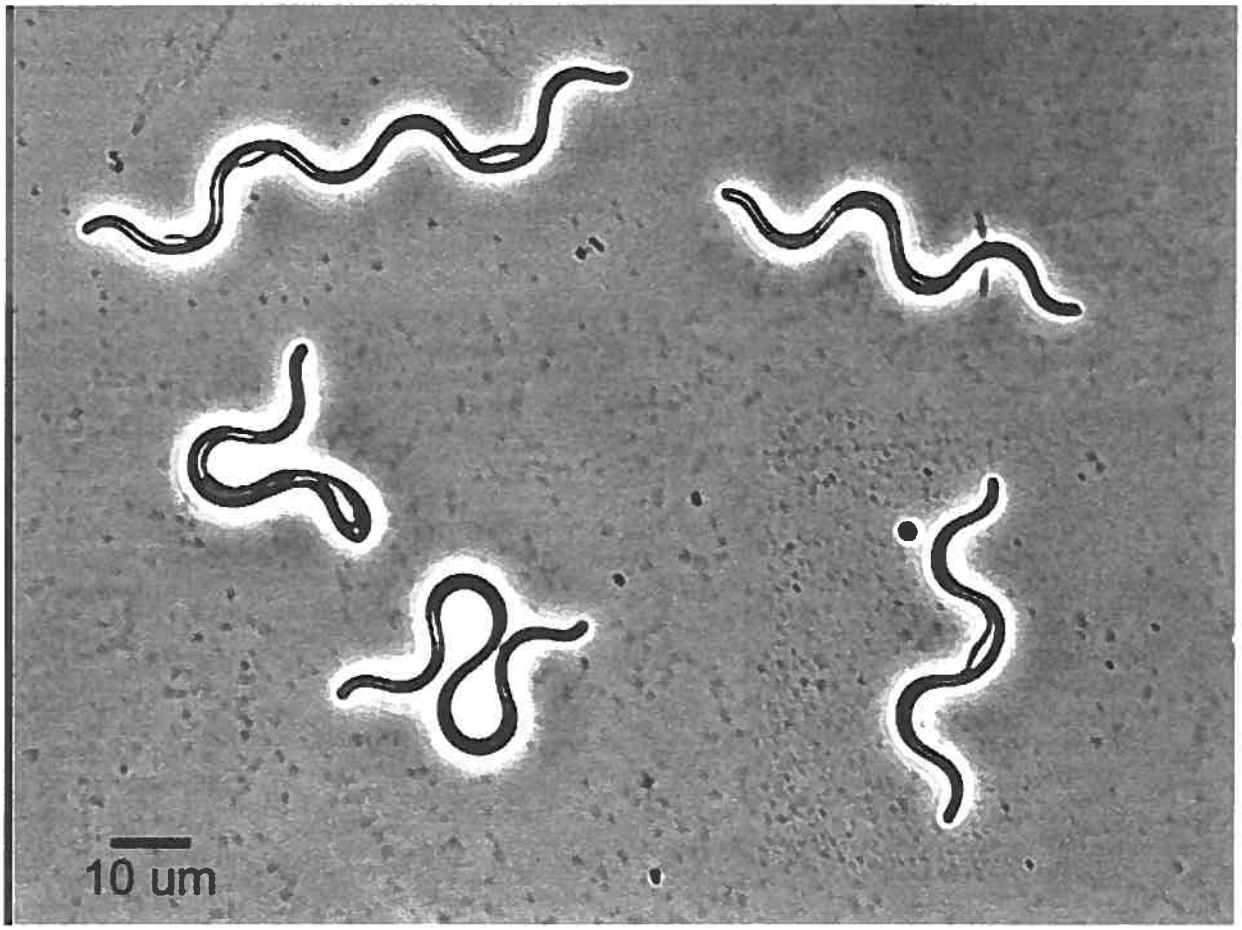


FIG. 1. Phylogenetic position of *Cristispira* clone CP1. The dendrogram was constructed from 1,410 base comparisons. The scale bar represents a 10% difference in nucleotide sequence as determined by measurement of the lengths of horizontal lines connecting two species.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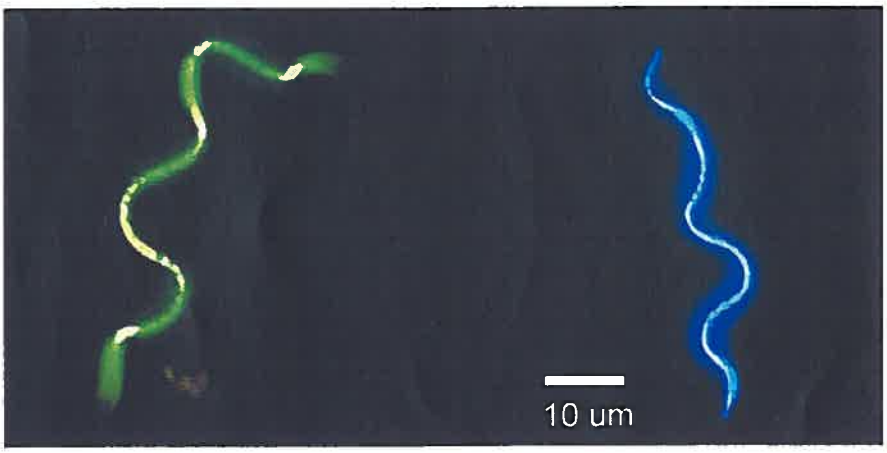
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