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# Phylogenetic Position of the Spirochetal Genus *Cristispira*

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**Comparative sequence analysis of 16S rRNA genes was used to determine the phylogenetic relationship of the genus *Cristispira* to other spirochetes. Since *Cristispira* organisms cannot presently be grown in vitro, 16S rRNA genes were amplified directly from bacterial DNA isolated from *Cristispira* cell-laden crystalline styles of the oyster *Crassostrea virginica*. The amplified products were then cloned into *Escherichia coli* plasmids. Sequence comparisons of the gene coding for 16S rRNA (rDNA) insert of one clone, designated CP1, indicated that it was spirochetal. The sequence of the 16S rDNA insert of another clone was mycoplasmal. The CP1 sequence possessed most of the individual base signatures that are unique to 16S rRNA (or rDNA) sequences of known spirochetes. CP1 branched deeply among other spirochetal genera within the family *Spirochaetaceae*, and accordingly, it represents a separate genus within this family. A fluorescently labeled DNA probe designed from the CP1 sequence was used for in situ hybridization experiments to verify that the sequence obtained was derived from the observed *Cristispira* cells.**

*Cristispira* cells are large spirochetes found in a specific ecological niche, primarily in the crystalline style of bivalve mollusks (4, 9, 16, 18, 24). The crystalline style is a long, tapered, gelatinous rod that aids in the digestion of food particles. *Cristispira* organisms were first observed from digestive tracts of oysters over 100 years ago (5). Because of the large size (1 to 3 μm by 30 to 180 μm) of the helical organism, it was first mistakenly identified as a trypanosome. Electron microscopy studies later confirmed that *Cristispira* cells possess the ultrastructural features typical of spirochetes, i.e., periplasmic flagella encompassed by an outer sheath (16, 24, 29). *Cristispira* organisms differ from most other known spirochetes in that they have a bundle of hundreds of periplasmic flagella, the so-called "crista," that wraps around the cell (4).

By comparison of the sequences of 16S rRNA or the genes coding for 16S rRNA (rDNA), the spirochetes are divided into two major phylogenetic groupings (7, 10, 21). The first grouping, which corresponds to the family *Spirochaetaceae*, contains species of the genera *Treponema*, *Spirochaeta*, *Borrelia*, *Serpulina* (and *Brachyspira*), and *Brevinema*. The second grouping, which corresponds to the family *Leptospiraceae*, contains species of the genera *Leptospira* and *Leptonema*. However, the phylogenetic relationship of the genus *Cristispira* to these other spirochetes is unknown, primarily since it cannot be grown in vitro.

The phylogenetic identity of organisms that cannot be cultivated can be determined by sequencing of cloned 16S rRNA genes that are amplified directly from environmental samples (8, 19). These procedures have been used to deduce the identity of many unknown cultivable and not-yet-cultivable bacterial species from free-living and host-associated sources (2, 3, 6, 13, 20, 27, 30). In this study, similar techniques were utilized to determine the phylogenetic position of the genus *Cristispira*. In situ hybridization experiments with a fluorescently labeled oligonucleotide probe were then used to verify that the cloned 16S rRNA gene was derived from *Cristispira*-like organisms present in crystalline style material.

(A preliminary report on the phylogeny of the genus *Cristispira* was previously reported [22].)

## MATERIALS AND METHODS

**Isolation of bacterial DNA.** Crystalline styles were removed from oysters (*Crassostrea virginica*) that were freshly harvested by commercial fisherman at Woods Hole, Mass. Intact styles were placed into 1-ml samples of sterile-filtered seawater in ampoules and frozen immediately in liquid nitrogen. The samples were maintained frozen at −70°C until use. Bacterial DNA was extracted by suspension of a crystalline style laden with *Cristispira* cells (as determined by phase-contrast microscopy) in 200 μl of buffer (10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 50 mM KCl)–10 μl of 20% sodium dodecyl sulfate (SDS)–200 μl of phenol. Approximately 0.1 ml of 0.1-mm-diameter acid-washed glass beads was then added to the suspension. The sample was shaken for 4 min with a Minibeater apparatus (Biospec Products, Bartlesville, Okla.) and then centrifuged for phase separation. DNA was precipitated with cold absolute ethanol and dissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). One microliter was used for PCR mixtures.

**16S rRNA gene amplification.** Universally conserved bacterial primers were used for amplification. These primers contained *Sal*I and *Bam*HI restriction sites (32) which facilitated the cloning experiments described below. Conditions for gene amplification consisted of 30 cycles at 94°C for 1 min, 42°C for 2.5 min, and heating to 72°C for 3 min. The PCR products were separated on a 0.8% agarose gel. The band of the correct size (approximately 1,500 bp) was eluted with Gene-Clean (Bio 101, Inc., Vista, Calif.).

**Cloning protocol.** PCR products were digested overnight and were ligated into a pGEM-4 vector (Promega, Madison, Wis.), followed by transformation of *Escherichia coli* HB101 cells. Clones were grown overnight on ampicillin plates. Plasmid DNA was recovered by a modified alkaline lysis procedure as previously described (26).

**Sequencing and analysis of data.** Spirochetal 16S rDNA inserts were sequenced by the Sanger dideoxy chain termination technique with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Programs for entry of data, editing, sequence alignment, secondary structure comparison, similarity matrix generation, and phylogenetic tree construction were written in Microsoft QuickBASIC

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TABLE 1. Similarity matrix

| Bacterium | % Similarity and % difference compared with[a]: | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *T. pallidum* | *T. phagedenis* | *T. denticola* | *T. bryantii* | *T. succinifaciens* | *T. pectinovorum* | *T. saccharophilum* | *S. aurantia* | *S. litoralis* | *S. halophila* | *S. bajacalifforniensis* | *Cristispira* clone CP1 | *B. anserina* | *B. hermsii* | *B. burgdorferi* | *Borrelia* rabbit sp. | *B. andersonii* | *S. hyodysenteriae* | *S. innocens* | *L. illini* | *L. biflexa* | *L. interrogans* |
| *Treponema pallidum* | | 90.8 | 89.0 | 83.7 | 81.9 | 84.0 | 82.5 | 83.7 | 82.4 | 82.2 | 83.6 | 77.0 | 80.4 | 79.7 | 79.6 | 79.1 | 76.5 | 77.1 | 77.0 | 76.4 | 75.7 | 76.9 |
| *Treponema phagedenis* | 9.8 | | 93.1 | 85.4 | 81.9 | 86.2 | 84.3 | 83.6 | 82.7 | 82.8 | 82.9 | 77.8 | 79.5 | 78.6 | 79.0 | 78.6 | 77.7 | 78.1 | 78.1 | 76.5 | 75.5 | 76.5 |
| *Treponema denticola* | 11.8 | 7.2 | | 86.1 | 81.5 | 86.0 | 84.4 | 82.8 | 81.9 | 82.3 | 83.0 | 77.2 | 80.0 | 79.0 | 79.7 | 79.3 | 76.9 | 77.8 | 77.8 | 75.9 | 76.1 | 76.1 |
| *Treponema bryantii* | 18.3 | 16.3 | 15.3 | | 85.2 | 88.3 | 84.0 | 82.1 | 82.0 | 82.5 | 82.1 | 75.0 | 81.2 | 80.3 | 79.8 | 79.6 | 75.6 | 77.5 | 77.4 | 77.0 | 75.4 | 75.9 |
| *Treponema succinifaciens* | 20.7 | 20.8 | 21.2 | 16.5 | | 84.0 | 83.2 | 80.0 | 78.3 | 79.3 | 79.3 | 70.6 | 75.4 | 74.8 | 73.8 | 73.7 | 76.3 | 73.7 | 73.7 | 75.6 | 73.7 | 74.4 |
| *Treponema pectinovorum* | 18.0 | 15.2 | 15.5 | 12.7 | 18.0 | | 86.3 | 82.8 | 81.8 | 80.5 | 79.5 | 75.0 | 80.0 | 79.1 | 78.9 | 78.4 | 76.2 | 76.0 | 75.7 | 75.7 | 74.6 | 74.0 |
| *Treponema saccharophilum* | 20.0 | 17.6 | 17.5 | 17.9 | 19.0 | 15.2 | | 80.2 | 80.2 | 79.5 | 79.7 | 73.5 | 77.2 | 76.8 | 76.7 | 76.6 | 75.4 | 75.3 | 75.5 | 73.7 | 73.4 | 72.1 |
| *Spirochaeta aurantia* | 18.4 | 18.5 | 19.5 | 20.5 | 23.2 | 19.5 | 23.0 | | 85.2 | 85.6 | 85.0 | 77.6 | 83.1 | 82.4 | 81.5 | 81.2 | 77.0 | 76.5 | 76.3 | 77.6 | 78.1 | 78.5 |
| *Spirochaeta litoralis* | 20.1 | 19.7 | 20.7 | 20.6 | 25.6 | 20.8 | 22.9 | 16.5 | | 86.7 | 85.7 | 79.1 | 82.6 | 81.9 | 81.9 | 81.7 | 77.8 | 76.0 | 76.0 | 77.5 | 77.6 | 77.4 |
| *Spirochaeta halophila* | 20.4 | 19.5 | 20.2 | 19.9 | 24.2 | 22.6 | 23.9 | 15.9 | 14.7 | | 87.6 | 78.1 | 81.8 | 81.7 | 81.4 | 81.0 | 78.3 | 78.9 | 78.8 | 77.4 | 77.9 | 78.3 |
| *Spirochaeta bajacalifforniensis* | 18.5 | 19.5 | 19.3 | 20.4 | 24.3 | 23.9 | 23.6 | 16.7 | 15.8 | 13.6 | | 78.9 | 82.4 | 82.1 | 81.1 | 80.7 | 78.0 | 77.3 | 77.4 | 78.8 | 78.9 | 79.4 |
| *Cristispira* clone CP1 | 27.5 | 26.3 | 27.2 | 30.5 | 37.3 | 30.4 | 32.7 | 26.6 | 24.5 | 25.9 | 24.8 | | 79.2 | 78.2 | 79.4 | 79.2 | 74.2 | 73.5 | 73.5 | 73.5 | 74.0 | 73.7 |
| *Borrelia anserina* | 22.7 | 24.0 | 23.3 | 21.7 | 29.8 | 23.3 | 27.1 | 19.2 | 19.8 | 20.8 | 20.0 | 24.4 | | 98.5 | 96.5 | 96.0 | 76.2 | 76.2 | 76.1 | 75.8 | 77.8 | 77.7 |
| *Borrelia hermsii* | 23.7 | 25.2 | 24.6 | 22.8 | 30.7 | 24.4 | 27.7 | 20.0 | 20.8 | 20.9 | 20.4 | 25.7 | 1.5 | | 96.6 | 96.0 | 75.4 | 75.2 | 75.1 | 74.8 | 76.3 | 76.3 |
| *Borrelia burgdorferi* | 23.8 | 24.7 | 23.6 | 23.5 | 32.2 | 24.8 | 27.8 | 21.2 | 20.7 | 21.4 | 21.7 | 24.1 | 3.6 | 3.4 | | 98.9 | 75.8 | 75.9 | 75.6 | 74.9 | 77.3 | 76.8 |
| *Borrelia* rabbit sp. | 24.5 | 25.2 | 24.2 | 23.8 | 32.4 | 25.5 | 28.0 | 21.7 | 21.0 | 21.9 | 22.3 | 24.3 | 4.1 | 4.1 | 1.1 | | 75.1 | 75.7 | 75.4 | 74.3 | 76.7 | 76.2 |
| *Brevinema andersonii* | 28.2 | 26.5 | 27.6 | 29.6 | 28.5 | 28.7 | 29.8 | 27.4 | 26.3 | 25.6 | 26.0 | 31.6 | 28.6 | 29.8 | 29.2 | 30.3 | | 75.1 | 75.2 | 75.8 | 75.8 | 75.7 |
| *Serpulina hyodysenteriae* | 27.3 | 25.9 | 26.3 | 26.8 | 32.4 | 28.9 | 29.9 | 28.2 | 29.0 | 24.8 | 27.1 | 32.6 | 28.6 | 30.2 | 29.1 | 29.4 | 30.3 | | 99.5 | 75.8 | 75.9 | 75.5 |
| *Serpulina innocens* | 27.4 | 25.9 | 26.4 | 26.9 | 32.4 | 29.3 | 29.7 | 28.5 | 28.9 | 24.9 | 26.9 | 32.6 | 28.8 | 30.3 | 29.5 | 29.8 | 30.1 | 0.5 | | 76.2 | 75.9 | 75.7 |
| *Leptonema illini* | 28.3 | 28.1 | 29.0 | 27.5 | 29.5 | 29.4 | 32.3 | 26.6 | 26.8 | 26.9 | 25.0 | 32.7 | 29.2 | 30.7 | 30.5 | 31.5 | 29.2 | 29.3 | 28.6 | | 82.1 | 84.1 |
| *Leptospira biflexa* | 29.3 | 29.6 | 28.8 | 29.9 | 32.4 | 31.0 | 32.8 | 25.9 | 26.7 | 26.2 | 24.7 | 32.0 | 26.4 | 28.4 | 27.0 | 27.9 | 29.3 | 29.0 | 29.0 | 20.4 | | 89.5 |
| *Leptospira interrogans* | 27.7 | 28.3 | 28.8 | 29.1 | 31.3 | 32.0 | 34.8 | 25.4 | 26.9 | 25.7 | 24.1 | 32.4 | 26.4 | 28.5 | 27.8 | 28.6 | 29.4 | 29.7 | 29.3 | 17.8 | 11.4 | |

[a] Numbers above the diagonal represent percentages of similarity and those below the diagonal are percentages of difference corrected for multiple base changes.

for use on IBM PC-AT and compatible computers. Our sequence database contains approximately 500 sequences determined in our laboratory and 400 published sequences from other laboratories. We also have access to the Ribosomal Database Project (Urbana, Ill. [14]), which currently contains about 3,000 bacterial 16S rRNA sequences. Similarity matrices were constructed from aligned sequences by using only those sequence positions for which data were shown for 90% of the strains. Similarity matrices were corrected for multiple base changes by the method of Jukes and Cantor (11). The neighbor-joining method of Saitou and Nei (25) was used for phylogenetic tree construction.

**Fixation of bacterial cells for in situ hybridization experiments.** Cells were fixed in 3% paraformaldehyde as previously described (28) and kept at −20°C in storage buffer (50% ethyl alcohol, 10 mM Tris [pH 7.5], 0.1% Nonidet P-40) until use.

**Oligonucleotide probes.** A *Cristispira*-specific DNA probe, designated as probe CP1, was designed from the 16S rRNA sequence data. The probe had the sequence 5′ GTACGATTCGCTTGACCTCTC-3′ (corresponding to base positions 1268 to 1285 according to the *E. coli* numbering system). The specificity of the probe was tested by use of the CHECK-PROBE program at the Ribosomal Database Project (14) against the ribosomal database (release 3.1) and our own database of ribosomal sequences. In addition, a probe targeting most of the bacterial domain, Eub338 (28), was used as a universal probe. Probe CP1 was labeled at the 3′ end with fluorescein during the synthesis of the oligonucleotide (23). Probe Eub338 was labeled by synthesis of the oligonucleotide with a 5′-aminolinker (Aminolink 2; Applied Biosystems, Foster City, Calif.) which subsequently was used as a coupling substrate for lissamine-rhodamine B-sulfonyl chloride (Molecular Probes, Eugene, Oreg.). The oligonucleotides were subsequently purified by reverse-phase liquid chromatography as previously described (12).

**Whole-cell hybridization.** Samples were spotted onto six-well Teflon slides coated with poly-L-lysine (Sigma Chemical, St. Louis, Mo.) and hybridized by addition of 10 μl of hybridization solution (100 mM NaCl, 50 mM $NaPO_4$ buffer, 0.1% SDS, 5 mM EDTA, 25 ng of each probe) to each well. The slides were kept in a humid chamber for 16 h at 37°C (1). The slides were rinsed in $H_2O$, incubated in 100 ml of prewarmed washing solution (100 mM NaCl, 50 mM $NaPO_4$ buffer, 0.1% SDS, 5 mM EDTA) for 15 min at 37°C, rinsed in distilled water, and air dried. Prior to microscopic analysis, the slides were mounted in Citifluor (Citifluor Ltd., London, United Kingdom).

**Microscopy and image analysis.** An Axioplan epifluorescence microscope (Carl Zeiss) was used to visualize the cells. The microscope was equipped with a 100-W mercury burner. Filter sets 10 and 15 (Carl Zeiss) were used to visualize fluorescein and lissamine-rhodamine B, respectively, and a narrow-bandpass filter (BP 590/10) (Oriel Corp., Stratford, Conn.) was used in combination with filter set 15. For differential interference contrast and fluorescence microscopy, a ×63/1.25 Plan Neofluor (Carl Zeiss) oil objective was used. The microscope was fitted with a slow-scan charge-coupled-device camera for capturing digitized images. The charge-coupled-device camera was a CH250 camera (Photometrics, Tucson, Ariz.) with a KAF 1400 chip (pixel size, 6.8 by 6.8 μm) operated at −40°C and read out in 12 bits (4,096 intensity levels) at a rate of 200 kHz. The integration times for the charge-coupled-device camera were 1 and 4 s for the fluorescein and lissamine-rhodamine B, respectively. For image analysis, the bit range of interest was linearly scaled to 8 bit files in PMIS software (version 2.11; Photometrics) and exported to Photoshop (Adobe) for final analysis. A disk operating system-based 486 computer was used as a controller for the charge-coupled-device camera, and a Macintosh Quadra 950 was used to run Photoshop.

**Nucleotide sequence accession number.** The 16S rRNA gene sequence of *Cristispira* clone CP1 is available for electronic retrieval from the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number U42638. The accession numbers of other spirochetal sequences that were used for phylogenetic analysis have been previously published (7, 10, 21).

## RESULTS AND DISCUSSION

Nearly complete sequences of the 16S rRNA gene fragments of two of the clones were determined. The sequence of one clone (1,491 bases), designated CP1, was clearly spirochetal. It possessed most of the individual base signatures that are unique to the 16S rRNA sequences of known spirochetes (7, 21). A similarity matrix of these sequences is shown in Table 1. A dendrogram constructed from these data is shown in Fig. 1. On the basis of percent similarity, CP1 branched deeply among other spirochetal genera of the family *Spirochaetaceae*, and it clearly represents a separate genus. These results denote the first evidence that the genus *Cristispira* is genetically related to other spirochetes.

The sequence of the 16S rRNA insert of the other clone represented a putative new species of the genus *Mycoplasma*. The new species was most closely related to *Mycoplasma mobile* (31), a species isolated from fish. It was not surprising that

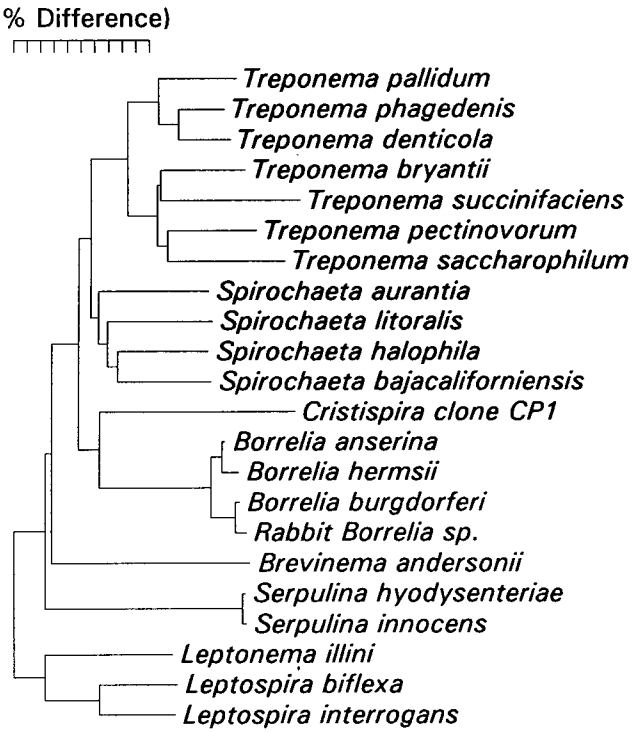


FIG. 1. Phylogenetic position of *Cristispira* clone CP1. The dendrogram was constructed from 1,410 base comparisons. The scale bar represents a 10% difference in nucleotide sequence as determined by measurement of the lengths of horizontal lines connecting two species.

mycoplasmas were detected by the procedures used in this study. Typically, the only bacteria observed in crystalline style contents are the genus *Cristispira* and, often, "*Spirillum ostrea*," a narrow spirillum with tufts of flagella inserted at each end (16, 18) (although "*S. ostrea*" was not observed in the styles used for this study). Because of the small size of mycoplasmas, they would not be visible by light microscopy. However, unidentified mycoplasmas have been observed in electron micrographs of crystalline style contents (15). Since a major component of the crystalline style is lysozyme (17), the environment would select for lysozyme-resistant organisms, including the cell wall-less mycoplasmas.

Probe CP1 hybridized only with *Cristispira*-like organisms observed in crystalline style preparations (Fig. 2, right panel). Cells that hybridized with the CP1 probe were loosely coiled and measured approximately 1 by 30 μm (Fig. 2). However, the rhodamine-labeled Eub338 probe did not hybridize with *Cristispira* cells. Closer examination of the sequence of CP1 indicated that the target region for the Eub338 probe had a 4-base mismatch. These base changes are uncommon in sequences of most species of the bacterial domain (14). Consequently, it was not surprising that the Eub338 probe did not hybridize with *Cristispira* cells. Since no other bacteria were observed in the microscopic field (Fig. 2, left panel), hybridization was not detected (thus, a figure was not included). In a separate study, probe Eub338 did hybridize with bacteria including not-yet-cultured spirochetes from the guts of termites (20). The in situ hybridization results indicated that the amplified DNA from crystalline style material was indeed derived from the observed *Cristispira* cells.

Although the genus *Cristispira* has resisted in vitro cultivation for over 100 years, we demonstrated that this large spiro-

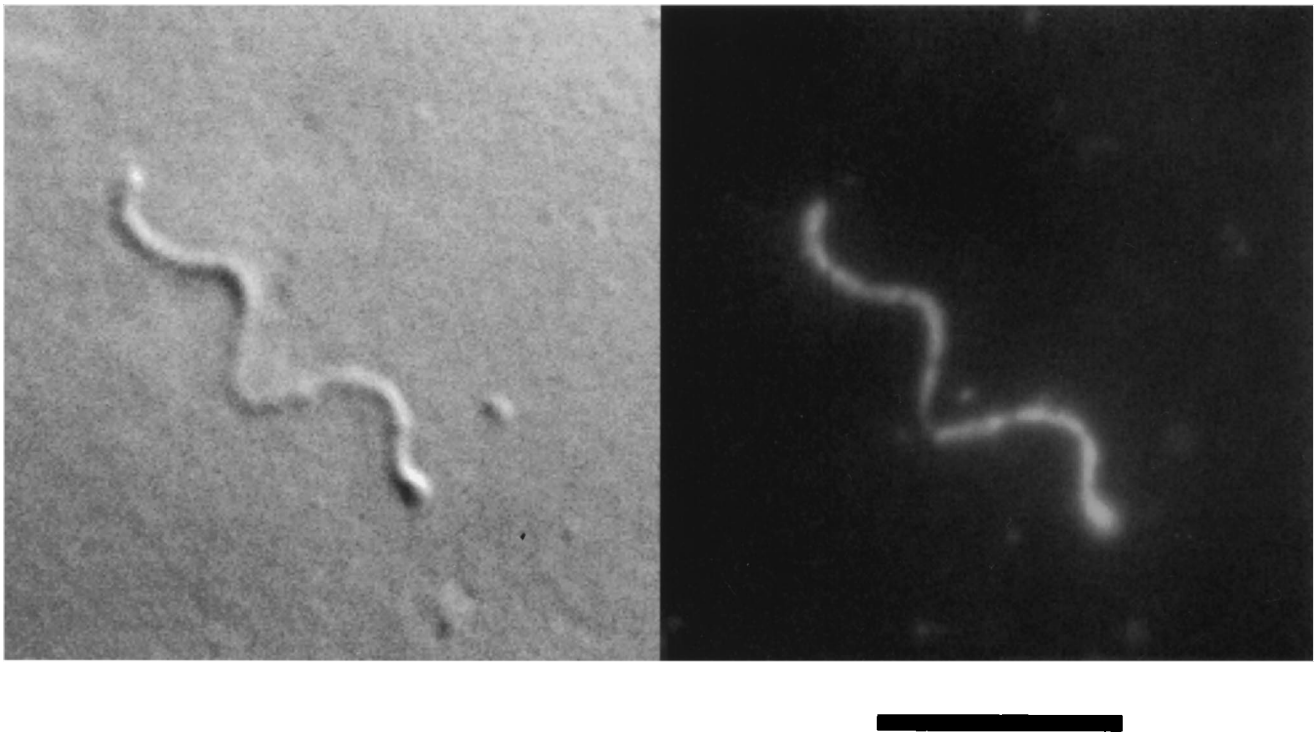

FIG. 2. In situ hybridization of *Cristispira*-like organisms in crystalline style material. The micrographs display the same viewing area visualized by differential interference contrast microscopy (left panel) and fluorescence microscopy (right panel). In the right panel, in situ hybridization with the CP1 probe labeled with fluorescein verified that the sequence obtained was derived from the observed *Cristispira* cells. Bar, 20 μm.

chete represents a genus separate from other known spirochetal genera within the family *Spirochaetaceae*. However, additional studies will be necessary to determine whether more than one species of the genus *Cristispira* is present in *C. virginica* and whether additional species of the genus *Cristispira* or other genera of *Cristispira*-like spirochetes are present in the crystalline styles and digestive tracts of other mollusks.

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