Further Molecular Characterization of Microbiota Found in the Internal Stylus of a Bi-valve Mollusk

John R. Spear
Microbial Diversity Course, 1998
Marine Biological Laboratory, Woods Hole, MA

Introduction

An attempt has been made at further characterizing the microbiota found in the gelatinous stylus of the oyster *Crassostrea virginica.* Previous studies (Paster, *et al.*) have examined the relationship and characterization of the unique spirochete found inside the stylus, *Cristispira.* This study attempted to examine the microbiological community of whole, intact styli from a molecular standpoint. Paster *et al.* worked up only one clonal isolate of the 16s rRNA gene *Cristispira* and did not look at other species of bacteria present. Microscopic studies indicate the presence of a number of morphologically different cells, as well as the presence of a spirillum. The goal of the work described herein is to work-up the DNA of whole stylus, sequence interesting clones, attempt to partially identify species present with sequence comparisons to a database, then attempt to design a probe specific to associated species found within the stylus for use with *in situ* hybridization.

Materials and Methods

Fresh oysters were obtained from the Marine Resource Center at the Marine Biological Laboratory. For enrichment work performed by a fellow student, a large number of oysters were required; for the molecular work here, only a few. The oyster stylus was removed from a freshly shucked oyster by cutting away at the lower right hand corner of the meat inside an open oyster (hinge at the bottom, smaller half of shell removed). The stylus was suspended in approximately 300 µl sterile sea water in an Eppendorf tube. In approximately one-half hour the gelatinous sheath of the stylus dissolves and the cells are evenly suspended in solution.

Microscopy of the sample included a wet mount examination under phase contrast at 400x and 1000x with oil. A live/dead stain was used to determine how many cells were viable. Images were digitally acquired on a Zeiss Axioplan microscope.

DNA extraction of the stylus was performed using a phenol:chloroform:isoamyl alcohol / extraction buffer mix along with glass beads in a bead beater tube. The tube was shaken for one minute on a mini beadbeater (DNA lysis procedure). Extracted DNA was suspended in autoclave and filter sterilized water and frozen at -20°C. Samples of this DNA were then used for polymerase chain reaction experiments to amplify the 16s rRNA gene for sequencing and analysis. To amplify the DNA of interest the universal bacterial primers 8-forward and 1492-reverse were used along with Taq beads (Promega). A 0.8% agarose gel was then made in 0.5xTBE buffer, and the PCR products checked for DNA in the 16s size region. Once found, the tube containing a band of interest was cloned using a Blunt Cloning Kit (Invitrogen). The DNA was precipitated to remove any excess Taq enzyme, and the ends of the molecule were modified to be blunt by adding on additional adenine residues as per kit protocol. Bacterial DNA inserts on plasmid DNA were mixed with competent *E. coli* cells and shaken at 37°C for 1 hour. 200 µl of cells were then plated onto one ampicillin plate by spreading and incubated overnight at 37°C. Growth of colonies on the plate indicated successful insertion, but not necessarily 16s rRNA gene containing inserts. Selected colonies were picked from the plate by a sterile pipet tip, touched to a back-up ampicillin plate, then stirred into a PCR prepared tube for PCR amplification. The back-up plate was incubated at 37°C overnight, and then refrigerated. PCR amplification of the cloned colonies was verified by another 0.8% agarose gel for 16s rRNA gene band patterns. If present, 10 µl of PCR product was subjected to restriction fragment length polymorphism analysis (RFLP Procedure) to determine different band patterns and which colonies were candidates for DNA sequencing. Sequencing was done at the Forsythe Dental Center in Boston, MA by an automated DNA sequencer. For checkerboard hybridization comparison with several bacterial probes, the forward primer was replaced with a D.I.G. primer. This PCR product was then given to the checkerboard hybridization experiment.

Results and Discussion

Phase contrast microscopy of the contents of an oyster stylus revealed a number of interesting cell types with varying morphology. At 400x magnification a large number of long, fast swimming *Cristispira* were visible against a background of smaller bacteria. At 1000x-oil a closer view of the spirochete motility is observable along with other cell types. Long, straight motile rods approximately 5µm in length were swimming through the field, as well as a small, fat motile rod, approximately 1 µm long. Of interest was what appeared to be a non-motile fat

rod, 5-10 μm long, with what appeared to be small round appendages attached up and down both sides. These appeared to be a small round coccus, different in morphology than the large rod, but may have been part of the rod itself. Pictures of the cells could not be made at the time. DNA sequencing (below) indicated that *Mycoplasma sp.* may be associated with the stylus. Being of small size, 0.3-0.8 μm, this could have been potentially what was viewed in the microscope. Of non-cell interest was the fact that the oyster stylus had several needle-like, crystalline structures throughout the wet mount. These crystal needles are approximately 10-50 μm long with a wider bottom which has two points, tapering to a top with one sharp point. They are not motile and appear to be a rigid crystalline like material. They were viewed in wet mounts from several different oysters and appear to be part of the stylus.

Using the DNA lysing procedure (DNA lysis procedure) on one oyster stylus, yielded almost no DNA as amplified by PCR and viewed on a gel. Attempts to extract and purify (Wizard Prep, Promega, Inc.) the acquired DNA failed to produce bands as well. A second DNA lysing procedure was performed on the styli from 3 oysters at once. When the styli were suspended in extraction buffer, phenol:chloroform:isoamyl alcohol mix with glass beads and bead beat, the mixture was extremely foamy, with a large, white fluffy protein containing layer between the aqueous and phenol phase. The aqueous layer also contained some of this white protein material, and once separated, was spun at 13,000 rpm for 5 minutes to further separate. PCR amplification of this DNA extract yielded strong bands on an agarose gel. Both universal 8-forward / 1492-reverse and D.I.G.-forward / universal reverse primers were used to get amplified product for cloning and for checkerboard hybridization procedures.

The D.I.G. tagged oyster DNA yielded cross links hybridizations with the following probes: enterics 2-T/some Gamma proteobacter-a faint band; all Alpha proteobacter-a strong band, but for all reactions tested this band was strong, possibly indicating too much probe was present or some other confounding variable; sulfate reducing bacteria (SRB) 2T/some Delta proteobacter-a faint band; sulfate reducing bacteria T/some Delta proteobacter-a faint band; flavobacter-a good band. SRB activity in the stylus could mean the presence of an anaerobic or facultatively anaerobic condition which could also be conducive to an environment suitable for *Mycoplasma sp.* This information indicates that these species may be present in the oyster stylus, but cloning and sequencing of a selected population yielded no conformation for presence of SRB or for *Flavobacteria sp.*

Cloning was done using a Blunt cloning kit (Invitrogen) where the PCR product DNA gets re-precipitated to remove the presence of interfering proteins and excess Taq polymerase. Next the PCR product DNA has to be modified to produce blunt ends by adding more adenine residues, followed by ligation into the Blunt vector and incorporation into competent *E. coli* cells. 200 µl of the cell suspension containing perspective clones were then plated on an ampicillin plate and incubated overnight at 37°C. The plate comes up with about 100 perspective clone colonies, 24 of which are picked for PCR amplification to see if the 16s rRNA gene is present (by size migration down the gel). Of these 24, 18 come up positive, a 75% effectiveness ratio. These 18 clone PCR products were then restriction enzyme digested via a RFLP protocol (RFLP Protocol) and run on a 1.5% low melt agarose gel. The PCR products were incubated at 37°C for four hours with the restriction enzymes, and yielded the gel pattern as seen in Figure 1. Of the 18 products tested it appeared that there were five different band patterns present. These five were then sent to the Forsythe Dental Center for DNA sequence analysis.

Sequence analysis of approximately 500 base pairs of the 1500 base pair size 16s rRNA gene indicated the following results: Woods Hole clone #60 (WH-60) was 87% identical to an unidentified proteobacter; WH-61 was 85% identical to an alpha proteobacter egg clone D42; WH-62 was 98-99% identical to *Cristispira* CP-1; WH-63 had a high 80's% identical to *Mycoplasma pulmonas* as did WH-64. Results from WH-60 are good, but overall inconclusive due to the low percentage of sequence identity and identity to an unknown. WH-61 is of interest because of the relation to the egg clone. This clone came from the Microbial Diversity course at MBL in summer of 1997 out of a squid, *Loligo pealei*, egg sac. The percent identity of relatedness is low but it is of interest that this α proteobacter has now been found in two marine organisms; one from the open ocean in the squid, and one from the shallower ocean in the oyster. WH-62 was 3 base pairs out of 500 sequenced different than the one *Cristispira* clone analyzed in Paster *et al.* which also came from information obtained in the Microbial Diversity course at the MBL. WH-63 and WH-64 had slightly different band patterns on the RFLP gel, but sequenced out to be nearly identical to each other and while having a low percent identity to known mycoplasma's, they appeared to have cloned with the highest frequency, appearing in 8 out of 18 or 44% of the RFLP tested clones. A partial phylogenetic tree showing the position of WH-63 and WH-64 as related to other *Mycoplasma* species is shown in Figure 2. Paster *et al.*

did find an associated *Mycoplasma sp.* in the oyster stylus in 1996, which phylogenetically is similar to the two described here. Paster *et al.* based their data published on one *Cristispira* clone, and with this work a second clone has been obtained. It is true however that only about 25% of cloned colonies that grew were tested. Several more clones could be waiting to be worked up from this procedure.

Fluorescent in-situ hybridization (FISH) was attempted on an oyster stylus which had been dissolved in water. Slides were prepared with cells in dilution series against fluorescently tagged probes for α proteobacter, all bacteria, sulfate reducing bacteria, and enteric bacteria. Examination under phase contrast at 1000x-oil yielded information showing that the probes were taken up in varying quantity, but that the cell preparations were to lumpy, i.e. no even cell suspensions across the slide. No direct quantification of tagged probe uptake could be inferred from the images.

Further Research Possibilities

Viewing a stylus under phase contrast microscopy at 1000x-oil sometimes shows the presence of a spirillum. I was hoping to get a positive clone of the spirillum and sequence information as to where the spirillum might phylogenecticlly correspond. With this information a FISH probe could be designed and cross checked back into a freshly prepared stylus. This could be useful information to determine the spirillum-spirochete ratio and/or diversity information of the oyster stylus.

Design of a FISH probe off of the *Mycoplasma* sequence information could also provide extremely worthwhile information on the presence and number of these high G+C ratio, Gram positive, potentially disease causing bacteria. The FISH probe could then be used to examine other marine bi-valves, edible or otherwise for signs of *Mycoplasma sp.* presence. Being difficult to see under phase contrast microscopy, a FISH approach could lead to a discriminating method of visualization. This could potentially be a screening method for the examination of *Mycoplasma* content in popular organisms of human consumption.

References

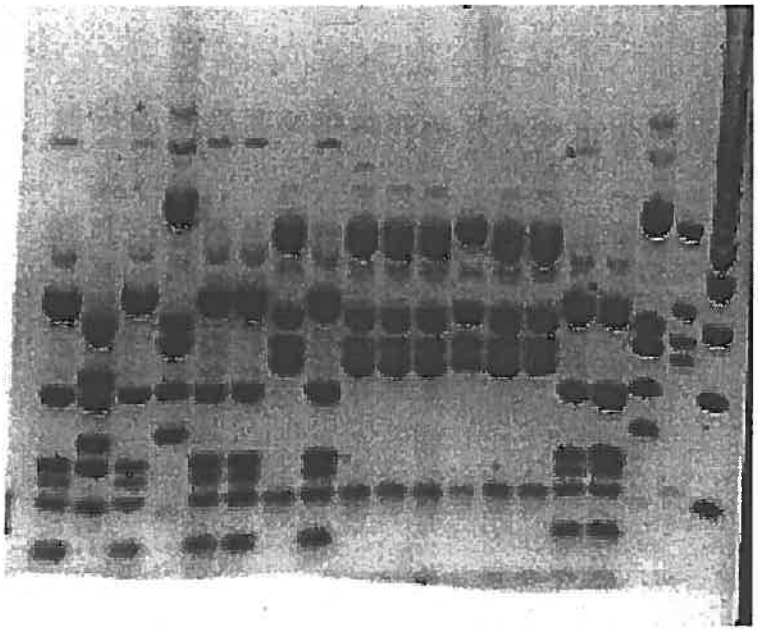
Paster, B.J., *et al.* 1996. Phylogenetic Position of the Spirochetal Genus *Cristispira*. *Applied and Environmental Microbiology*, **62**:3, p. 942-946.

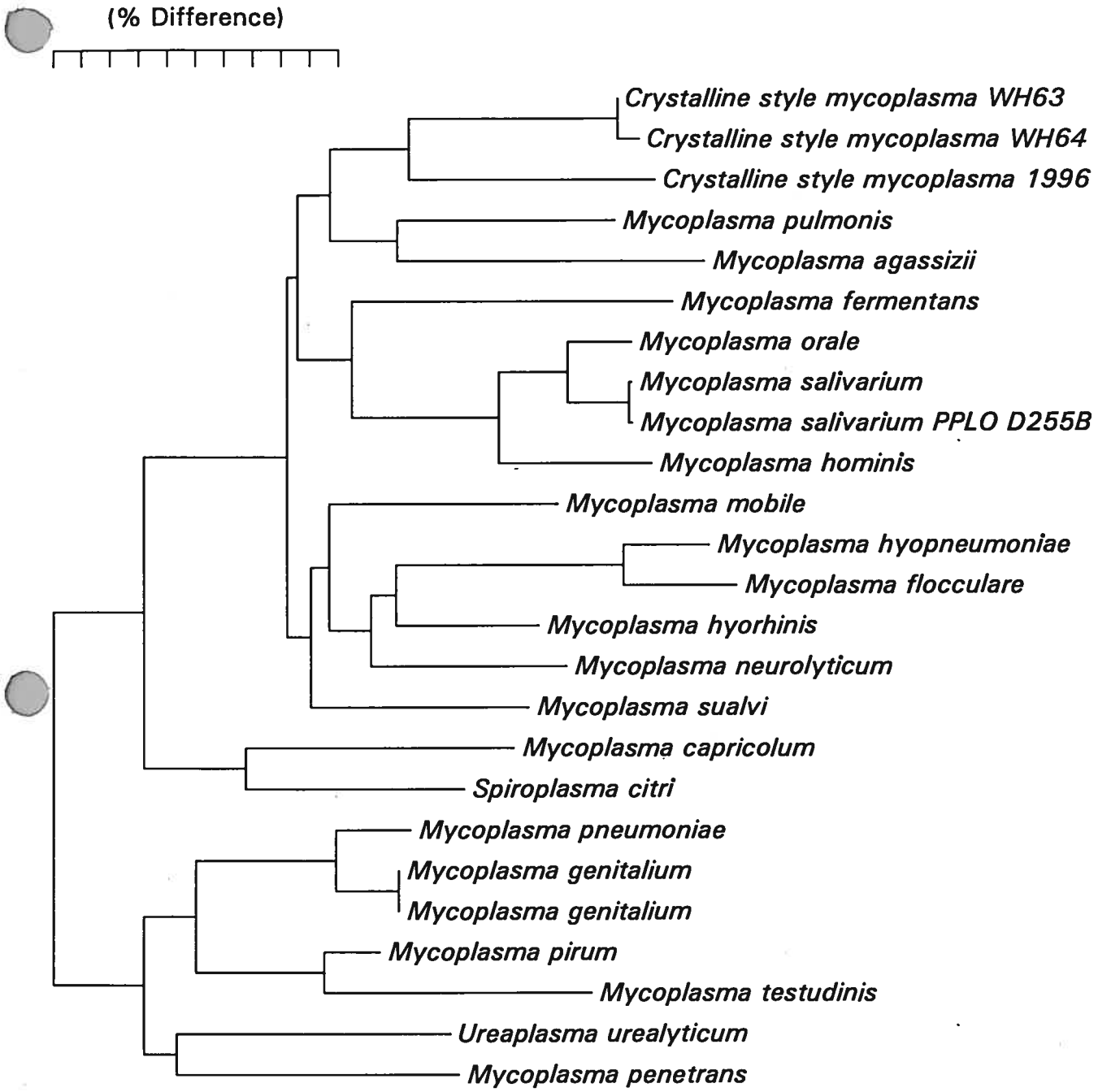
DNA Lysis and PCR Protocol, Microbial Diversity Course, MBL, Woods Hole, MA, 1998.

RFLP Protocol, Microbial Diversity Course, MBL, Woods Hole, MA, 1998.

Schlegel, H. 1993. *General Bacteriology*. Cambridge University Press.

Figure 1: Image of RFLP gel, 1.5% Meta Phor agarose with digested PCR product DNA obtained from oyster styli. Lane 1 is the left most lane and was clone WH-60, lane 2 was clone WH-61, lane four was clone WH-62, lane 7 was clone WH-63, and lane 18 was clone WH-64. Each of these lane band series were thought to be unique and therefore sequenced. The band pattern unique to *Cristispira* is in lane 4, the *Mycoplasma sp.* are in lane 7, and lanes 9-14.




(% Difference)
Crystalline style mycoplasma WH63
Crystalline style mycoplasma WH64
Crystalline style mycoplasma 1996
Mycoplasma pulmonis
Mycoplasma agassizii
Mycoplasma fermentans
Mycoplasma orale
Mycoplasma salivarium
Mycoplasma salivarium PPLO D255B
Mycoplasma hominis
Mycoplasma mobile
Mycoplasma hyopneumoniae
Mycoplasma flocculare
Mycoplasma hyorhinis
Mycoplasma neurolyticum
Mycoplasma sualvi
Mycoplasma capricolum
Spiroplasma citri
Mycoplasma pneumoniae
Mycoplasma genitalium
Mycoplasma genitalium
Mycoplasma pirum
Mycoplasma testudinis
Ureaplasma urealyticum
Mycoplasma penetrans