Common bacterial symbionts in clonal cultures of the toxic dinoflagellate *Ostreopsis lenticularis*

By

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ABSTRACT

The marine toxic dinoflagellate *Ostreopsis lenticularis* Fukuyo 1981 is implicated as the major vector in ciguatera fish poisoning in the southwest coast of Puerto Rico. Studies have demonstrated that bacteria play a role in the production of ciguatoxin by the dinoflagellate and that different clonal cultures of *Ostreopsis* harbor different culturable bacterial symbionts. It was recently demonstrated the presence of viable but non culturable (VBNC) species of bacteria associated with *O. lenticularis*. More than 100 associated bacteria from two toxic clonal cultures of *Ostreopsis* (#302, and #303), were examined for common bacterial strains that could be involved in the production of ciguatoxins. DNA extraction, PCR amplification of partial small subunit ribosomal DNA (rDNA), denaturing gradient gel electrophoresis (DGGE) and DNA sequencing were performed. One uncultured bacterium belonging to the genus *Cytophaga* was found to be common to both clones, indicating a close association with the dinoflagellate and its possible involvement in the ciguatoxin production.

RESUMEN

El dinoflagelado tóxico marino *Ostreopsis lenticularis* es considerado el vector principal en envenenamiento por ciguatera en la costa suroeste de Puerto Rico. Estudios han demostrado que las bacterias llevan a cabo un rol en la producción de ciguatoxina por el dinoflagelado y que cada clon contiene diferentes simbiontes cultivables. Recientemente se demostró la presencia de bacterias viables pero no cultivables (VPNC) en *Ostreopsis*. Se estudiaron más de 100 bacterias asociadas con dos cultivos clonales tóxicos de *O. lenticularis* (#302 y #303) para identificar especies que puedan estar involucradas en la producción de la toxina. Se realizó extracción de ADN, amplificación parcial de la sub-unidad pequeña del ADN ribosomal, electroforesis de gel denaturalizante en gradiente y secuenciación del ADN. Una bacteria no-cultivada perteneciente al género *Cytophaga* fue encontrada común en ambos cultivos clonales de *Ostreopsis*. Esto indica una posible asociación de la bacteria con el dinoflagelado y sugiere que *Cytophaga* puede estar implicada en la producción de ciguatoxinas.

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INTRODUCTION

Toxic Marine Microalgae

Microalgae are the major producers of biomass and organic compounds in the ocean. More than 5000 species of marine microalgae are known to date and are separated into six major divisions: *Chlorophyta* (green algae), *Ochrophyta* (yellow algae, golden brown and diatoms), *Haptophyta* (coccolithophorids), *Pyrrhophyta* (dinoflagellates), *Euglenophyta* and *Cyanophyta* (blue-green algae). Among the 5000 species about 300 can proliferate in high numbers to form the so-called red tide and brown tide phenomena (18, 32), and 75 species mainly belonging to the taxa of dinoflagellates and diatoms can produce toxins (1).

Harmful algal blooms can be classified into three major groups, according to the problem they cause: non-toxic species that discolor the water, those that can seriously damage fish gills and species that produce toxins that can find their way through the food chain to humans, causing gastrointestinal and neurological illness.

About 40 microalgal species have the capacity to produce potent toxic secondary metabolites that can find their way through fish and shellfish to humans (65). According to symptoms observed in human intoxications caused by these secondary metabolites it is possible to consider the following illness groups: amnesic shellfish poisoning (ASP), azaspiracid poisoning (AZP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), paralytic shellfish poisoning (PSP), and ciguatera fish poisoning (CFP).

Ciguatera and Toxic Dinoflagellates

Ciguatera is a widespread ichthyosarcotoxaemia with dramatic and clinically important neurological features (56). Annually, more than 50,000 people are affected (18) by ingestion of coral reef fish that have become toxic through diet. This severe form of fish poisoning may present with either acute or chronic intoxication syndromes and constitutes a global health problem (56). Cases have been reported in the past years from

Canada (70), Dominican Republic (3, 70), Europe (46), Hawaii (43) and Puerto Rico (71, 72).

Traditionally, ciguatera was limited to tropical regions where marine fish represent a significant source of food; however modern improvements in refrigeration and transport have increased both commercialization of tropical reef fish and the frequency of fish poisoning in temperate regions of the earth (46, 70). Extensive international commerce in frozen fish means that victims of this intoxication may be encountered in all countries.

The toxin, produced by benthic dinoflagellates, is stable in the tissue of living fish and does them no harm (56). Causative organisms produce maitotoxins (MTXs), okadaic acid (OA), palytoxin (PTX), and ciguatoxins (CTXs) (23, 26). These latter are very potent lipid-soluble neurotoxins that have been isolated from Pacific and Indian Ocean organisms (39, 48) and from the Caribbean (40). The first ciguatoxin was isolated from moray eels, its structure was elucidated in 1989 (48), and more than twenty analogues have been found in fish and in dinoflagellates (47, 48, 79).

Ciguatoxins are tasteless, odorless and relatively heat stable to temperatures employed in cooking. Human victims are the end of the food chain cascade and the intoxication is usually identified by paresthesia, sensory reversal (dysaesthesia) and pruritis (71). Prevention of the intoxication depends on abstinence from eating any tropical reef fish, since there is currently no way to measure the toxins in any seafood prior to consumption.

Ostreopsis lenticularis: A Toxic Dinoflagellate

Dinoflagellates are a very large and diverse group of eukaryotic organisms that play a major role in aquatic food webs of both, fresh water and marine habitats. Based on morphology and cytology, their motile phase is distinctive at the light microscope level by the placement of two dimorphic flagella. These organisms are of great evolutionary importance because of their systematic position, unusual chromosome structure and

composition (58). In fact, they are the only eukaryotes known to lack histones completely (57, 58).

Some toxic members of this group produce phytotoxins that pose a health threat in the form of red tides (58), while others produce toxins that can find their way through the food chain to humans. The source of the diversity of these toxins in the marine food web is not well understood. Also, it is not clear what roles these secondary metabolites might have in the organisms that produce them.

Ostreopsis lenticularis, a 50μm size epiphytic marine toxic dinoflagellate, is commonly found on shallow coastal waters. *Ostreopsis* lacks undulation of cingulum in lateral view and the theca has numerous fine pores. It has been described as one benthic dinoflagellate likely to contribute to the ciguatera syndrome in coral reef communities in French Polynesia (12), the U.S. Virgin Islands (11) and Puerto Rico (71).

Research reports indicated that *Ostreopsis lenticularis* is the dominant dinoflagellate of the epiphytic flora on macroalgae in coral reef environments in Puerto Rico (7). It is also the major vector implicated in ciguatera fish poisoning on the southwest coast of Puerto Rico due to both its reported toxicity and abundance (8, 71).

Ostreopsis toxins include lipid soluble and water soluble entities (69). Palytoxin (PTX), a water soluble compound, is the most toxic of the ciguatera complex and has been implicated in severe fish poisoning (36). It is also reported that *Ostreopsis lenticularis* produces the polyether compound Ostreotoxin (77), and at least two neuroactive compounds that interact with nicotinic cholinergic receptors and voltage dependent Na^+ channels (44).

Factors Involved in Dinoflagellate Blooms and Toxicity

The abundance and toxicity of dinoflagellates might be affected by synergistic interactions among temperature, salinity, rainfall, light intensity and wind patterns. It has been demonstrated that increases in temperature promoted blooms of *Gymnodinium catenatum*, and *Gambierdiscus toxicus* (6, 17, 59). In addition to elevated temperature, *Gyrodinium aureolum* required higher salinity concentrations to cause blooms (51).

Other factors such as wind pattern seem to affect blooms. On the west coast of Florida, weak winds and warm temperatures correlated with blooms of *Karenia brevis* (6).

Temperature appears to be an important factor determining the seasonal trends of some ciguatoxic dinoflagellate species (45). Both lower and higher temperatures influenced the cellular chemical composition of phytoplankton (28). Cultures of the ciguatoxic dinoflagellate *G. toxicus* grown in the laboratory at elevated temperatures (27°C) were more toxic than those grown at lower temperatures (21°C) (9). Elevated temperatures induced peak toxicities in *O. lenticularis*, followed by increased ciguatoxicity frequency in *Sphyraena barracuda* (74).

Toxic Dinoflagellates and Associated Bacteria

Bacteria are universally associated with algae in ocean. It has been reported that *Aeromonas*, *Alteromonas*, *Bacillus*, *Flavobacterium*, *Moraxella*, *Pseudomonas*, *Roseobacter* and *Vibrio* are the bacterial genera most frequently associated with toxic dinoflagellates (6, 73). Some of these bacteria also produce tetrodotoxins (6).

The toxins present in the dinoflagellate might be the result of variable microbial symbionts, environmental conditions, or a combination of both influences (74). The interactions between bacteria and macroalgae are dynamic and important factors in microbial proliferation and survival (72). In experiments by Tosteson *et al* (73) looking at culturable bacteria found that the toxic dinoflagellate *Ostreopsis lenticularis* hosts a variety of symbiotic bacterial flora however; each clone sequesters a variable set of bacterial strains.

Associated bacterial strains have been reported to be involved in the development of toxicity in cultured *O. lenticularis* (29). It was determined that culturable bacteria from the genus *Pseudomonas/Alteromonas* were required for the production of enhanced toxicity in clonal laboratory cultures of *Ostreopsis* cells during the static phase of growth (29, 71). They also reported that the number and diversity of associated bacterial genera was variable among dinoflagellate clones.

Viable but Non-Culturable Bacteria

At the simplest level, bacteria may be classified into two physiological groups: those that can, and those that can not readily be grown to detectable levels *in vitro* (34). The term *viable count* usually refers to the number of individual organisms in a sample that can be grown to a detectable level, *in vitro* by forming colonies on an agar-based medium (34). Roszak and Colwell (61), coined the term viable but nonculturable (VBNC) for those bacterial cells with detectable metabolic function, but not culturable by conventional methods.

Bacterial cells may become unculturable as a consequence of several different processes such as starvation (52, 64, 78), low temperature (30), high pressure, and changes in pH or salinity (67). The so-called VBNC cells are often claimed to have exactly the opposite properties as compared to the culturables: they are metabolically active, but nonculturable (ABNC) (34). In some cases, the likely ecophysiology of an uncultured microorganism can be inferred by considering the physiology of its closest culturable relatives (76). However, this approach cannot be applied to uncultured prokaryotes from newly identified, altogether uncultivated groups (76).

Previous research demonstrated the presence of nonculturable bacterial strains associated with *Ostreopsis* cells in culture. In 2003, Ashton *et al* (5) utilized denaturing gradient gel electrophoresis (DGGE) and found that four out of seven bacterial strains associated with *Ostreopsis lenticularis* were nonculturable; however those strains were not identified.

Molecular Approach

Molecular techniques have been used and are considered to be the best tools to determine the diversity of microorganisms in natural environments since 1980's. Molecular methods have revolutionized the detection and quantification of bacteria in the environment because they circumvent the errors of cultivation (33). The study of

microbial diversity and community analysis (Fig.1) has leapt forward through the use of DNA sequencing (15, 33).

Nucleic acid-based technologies are changing our perspective on the nature and extent of marine microbial diversity. The development of molecular techniques has enabled us to investigate community diversity using the 16S rDNA gene (15). Small subunit (SSU) rRNA has emerged as a reliable tool for phylogenetics because it is present in all living organisms, functionally constant, and highly conserved (55). Although these methods have been conducted in diverse microbial habitats nearly every molecular survey of microbial diversity indicated that cultivated microorganisms apparently represent only a small fraction of naturally occurring microbial diversity (19).

Microbial isolates can be subject to direct analysis, using *in situ* cultivation methods, or nucleic acids can be extracted for analysis using microarrays or hybridization. The gene can also be amplified using the polymerase chain reaction, and evaluated using cloning, pattern analysis and sequencing.

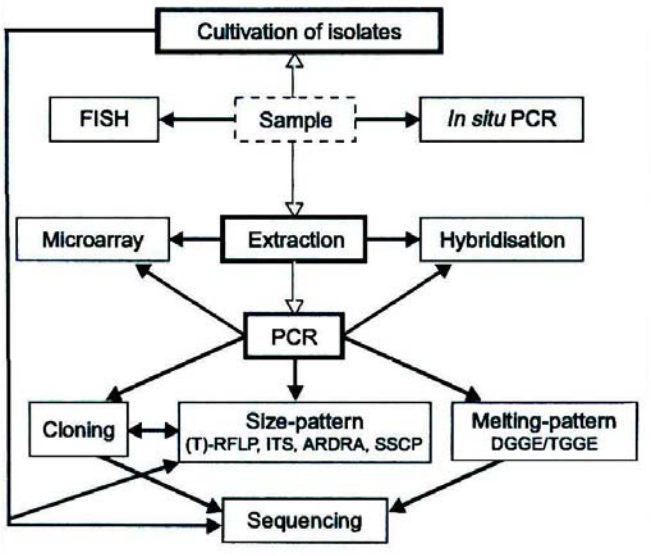

Figure 1. Current approaches for the analysis of microbial diversity, from Dahllöf, 2002 (15).

Research Scope and Objectives

Culturing microorganisms in the laboratory was considered as sufficient proof of viability (13). However, one of the major limitations to research in marine microbial ecology is the inability to isolate and grow in culture the vast majority of marine bacteria which occur in nature (13, 14). Most cells obtained from the ocean and visualized by microscopy are viable but they do not generally form visible colonies in *in vitro* cultivation (21, 78).

Viable but nonculturable (VBNC) cells go undetected unless appropriate methods for detection are employed. These cells will not form colonies on agar media or grow in broth culture (10, 78). Since the standard plate count cannot be used to enumerate VBNC organisms, alternative techniques are needed to enumerate these bacteria.

The application of molecular methods to study the microbial diversity in the marine environment seems to be the most effective means to identify the nonculturable microorganisms. This work utilized molecular biological techniques such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and DNA sequencing to identify the bacterial flora associated with the marine dinoflagellate *Ostreopsis lenticularis* clones #302 and #303. These techniques offer opportunities for the analysis of the structure and species composition (15, 49) of microbial communities as well as the identification of many unculturable microorganisms.

The major objectives of the study were: [1] to isolate and identify the bacterial flora associated with the dinoflagellate *Ostreopsis lenticularis*, and [2] to identify VBNC species associated with *O. lenticularis*. The purpose of identification of the VBNC bacterial strains is to find common bacterial strains that could be involved in the production of ciguatoxins by *Ostreopsis*.

MATERIALS AND METHODS

Dinoflagellate Cultivation

Marine dinoflagellate *Ostreopsis lenticularis* was isolated from the surfaces of macroalgae from an inner reef at the South west coast of Puerto Rico. *Ostreopsis* clones #302 and #303 were established from single dinoflagellate cells and have been maintained in continuous clonal laboratory cultures, in natural seawater based enriched media since 1997.

Clonal cultures were kept in 2.5liter Fernbach flasks at 25-27˚C, in a light/dark cycle of 12:12 hours. The cultures were grown at a light irradiance of 90 Einstein/m^2/sec.

Associated Bacterial Flora

For the identification of dinoflagellate associated bacterial flora, samples of *Ostreopsis lenticularis* cultures were used. The amount of sample was empirically determined based on colony forming units (CFU) of bacteria, and analyzed using molecular techniques such as DNA extraction, polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and sequencing (see below).

Culturable Bacteria

Whole dinoflagellate cells cultures were disrupted by sonication (Megason Ultrasonic Disintegrator) during fifteen minutes and diluted to concentrations of 10^4, 10^5, and 10^6. Inocula of 100µL of the serial dilutions were plated on solid media (Marine Broth + agar) for bacterial growth. Plates were incubated at room temperature for at least 72 hours. Once bacteria were grown, all colonies were removed from the plate by scraping and resuspended in 1mL of DNA grade water. Subsequently, 100µL of this suspension were used for DNA extraction.

Unculturable Bacteria

5mL of dinoflagellate culture were centrifuged at 13,000rpm to pellet the bacteria present in the sample. The pellet was washed with 1mL of DNA grade water, and aliquots of 100µL were taken for DNA extraction.

DNA Extraction

DNA extraction for both, culturable and nonculturable bacteria was performed following the protocol described by Groβkopf *et al* (31). Briefly, this method consisted in boiling the bacterial samples for 10 minutes to cause lysis. After subsequent centrifugation, the supernatant was collected, and transferred to a new tube and used for PCR.

Polymerase Chain Reaction

Nucleic acid detection techniques such as Polymerase Chain Reaction (PCR) have become an efficient tool for the identification of microorganisms. For this work, 2µL of supernatant from the DNA extraction were used for PCR amplifications of the variable V3 region of 16S rDNA gene. Approximately 530 base pairs (bp) of this region were amplified (Fig. 2) using highly conserved primers.

This procedure consisted of 30 cycles of amplification as reported in Rosado & Govind (60) using primers BSF8 and BSR538 corresponding to positions 8, and 538 in *E. coli* numbering system respectively (Table 1). Conditions for the amplification included 30 cycles with denaturing at 95°C for one minute; annealing and extension for one minute at 45°C and 72°C respectively.

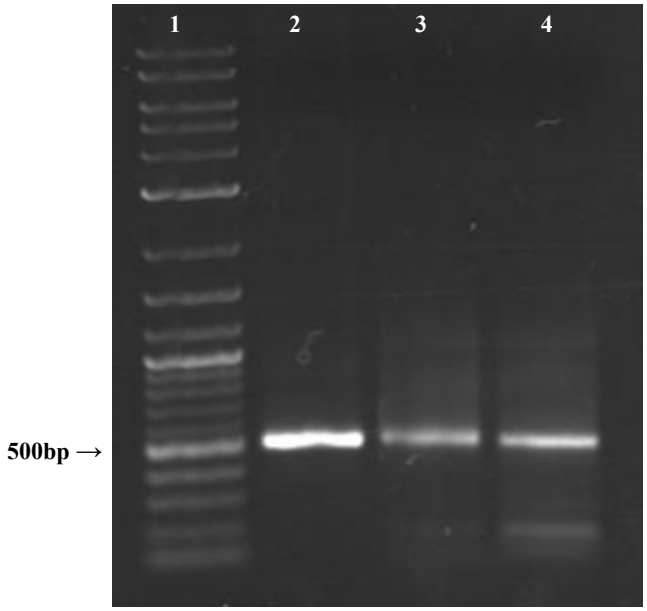

Figure 2. Polymerase Chain Reaction. DNA was extracted from *Ostreopsis lenticularis* associated bacteria and the small sub unit (SSU) 16S rDNA gene was PCR amplified with primers BSF8 and BSR538. Amplicons were checked on 1.0% (v/w) agarose gel electrophoresis containing ethidium bromide. First lane was loaded with a 2Kb ladder as a marker, and second lane with a positive control (previously amplified DNA). Third and fourth lanes were loaded with 2µL (100ng) of #302 and #303 amplicons respectively. Products of approximately 500bp were visualized for each sample.

PCR Clamp

In order to use the PCR amplicons for DGGE, a G-C clamp was included. This sequence of guanines (G) and cytosines (C) acts as a high melting domain preventing the two strands of DNA from becoming a single strand during the running of the DGGE (49). For our experiment, primer 349F (Table 1) with a 40bp GC clamp attached to the 5' end was used for the creation of the GC-clamped amplicons.

Denaturing Gradient Gel Electrophoresis

For our experiments, approximately 226 base pair GC-clamped PCR products were purified on a 1.5% agarose gel using Eppendorf Perfectprep Gel Cleanup Kit (Westbury, New York, USA). Gradient gels were prepared with denaturant gradients of formamide and urea from 45-70% (using 0, and 80% denaturant stock solutions), and polymerized by addition of ammonium persulfate and TEMED. Subsequently, approximately 450ng of the PCR-amplified DNA were loaded onto the 6.5% polyacrylamide gel. Electrophoresis was carried out at 160 volts at 60°C for three hours in 1X TAE buffer. Finally, the gels were removed from the glass plates and stained with SYBR Green I (Molecular Probes, Eugene OR, USA) [Muyzer & Smalla, (50)] for 30 minutes, and visualized under ultraviolet light. The generated bands were analyzed using NIH ImageJ software (http://rsb.info.nih.gov/ij/).

DNA Sequencing and Analysis

PCR products of partial 16S rDNA gene (~530bp) were transformed and cloned into *E. coli* using commercially available vector pCR 2.1 (TA Cloning kit; Invitrogen Carlsbad CA, USA). Transformants were grown in liquid medium containing Ampicillin (LB + Amp) and the plasmids were purified using Eppendorf Fast Plasmid Mini Prep (Westbury, NY, USA). These plasmids were used as templates for sequencing reactions with the ABI Prism® Big Dye ™ Terminator reaction (Applied Biosystems, CA USA) and ABI 310 genetic analyzer using universal primers M-13 forward, and reverse. The DNA sequence data were compared and analyzed using BLASTN (2).

Phylogenetic Analysis

Phylogenetic analyses were performed by evolutionary distance and maximum likelihood using Molecular Evolutionary Genetics Analysis (MEGA) 3 software (37). The phylogenetic trees (Figs 7-12) demonstrate the most robust relationships observed and were determined with the Kimura 2-parameter model for nucleotide change, and

Neighbor-joining. Bootstrap proportions from 500 resamplings were determined by evolutionary distance.

Cloning and Sequencing of Specific 16S rDNA Genes

Multiple sequence alignment was performed with CLUSTAL W (68) and specific regions included in the *Cytophaga* sp. sequence were chosen as forward primers (Table 1). The primer walking was repeated until the sequences overlapped (Fig. 3).

Primer	Sequence	Reference
1	5'GCTTGCTAATTTGCTGAC3'	This work
2	5'CCTACGGGAGGCAGCAGT3'	This work
3	5'ATTAAGTCAGAGGTGAAA3'	This work
4	5'GATACCCTGGTAGTCCAC3'	This work
1492 R	5'GGTTACCTTGTTACGACTT3'	Suzuki *et al* (66)
BSF8	5'AGAGTTTGATCCTGGCTCAG3'	Lane *et al* (38)
BSR538	5'TTACCGCGGCTGCTGGC3'	Rosado & Govind (60)
349F + clamp	5'GGCCCGGGGCGCGCCCCGGGC GGGGCGGGGGCACGGGGGGAAG GCAGCAGTGGGGAAT 3'	Diez *et al* (20)

Table 1. Specific primers used for this study.

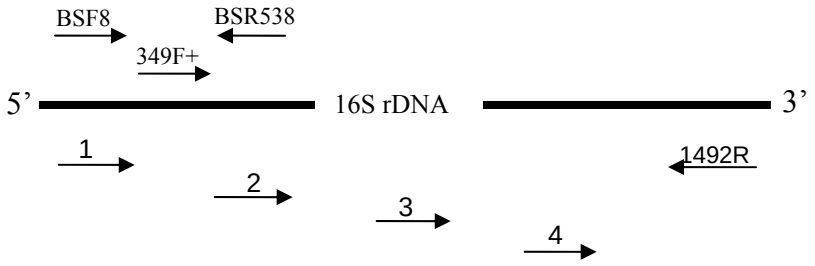

Figure 3. Primers used in this study. The arrows indicate the direction of the sequencing reaction, and in top of the arrows are the names of the corresponding primers. Primers 1 through 4 and 1492R were used for the complete sequence of the 16S rDNA molecule from *Cytophaga* sp.

Nucleotide Sequence Accession Numbers

The 16S rDNA sequences were submitted to GenBank and given accession numbers DQ473616 to DQ473643, and DQ482735 to DQ482737.

RESULTS

Denaturing Gradient Gel Electrophoresis Analysis

DGGE was used as an initial method to evaluate species diversity in the bacterial communities of two clonal cultures of *Ostreopsis lenticularis*. The gel (Fig. 4) showed approximately seven and nine distinct bands from clone #302 cultured and total bacteria respectively.

The ImageJ analysis of the DGGE banding pattern (Fig. 5) demonstrated higher number of bands for both samples: eight bands were detected for cultured samples whereas the analysis for total bacteria showed 13 bands. This analysis also demonstrated that clone #302 has five co-occurring bands (shown in blue), and one pair that migrates closely (lines 7 and 12 in panels A and B respectively).

For *Ostreopsis* clone #303, the DGGE (Fig. 4) showed six and eight distinct bands from cultured and total bacteria samples respectively, while the banding pattern analysis using ImageJ (Fig. 5) demonstrated one less band for the cultured samples. Two common bands were found between cultured and total bacteria (shown in red) and bands 1 and 1in panels C and D migrated closely.

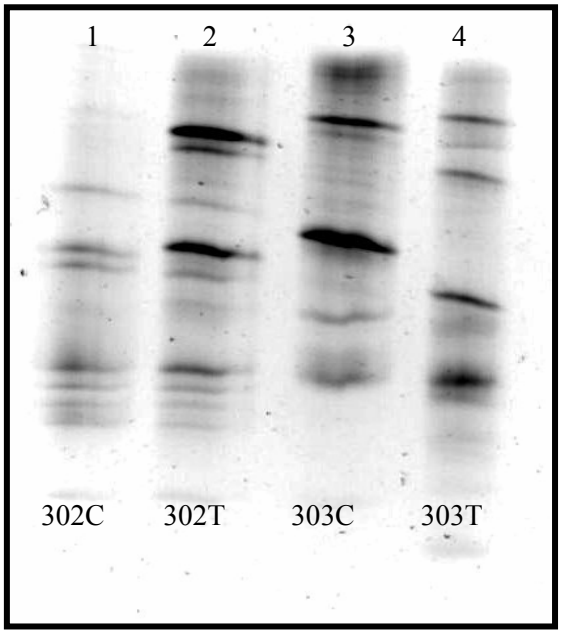

Figure 4. Denaturing gradient gel electrophoresis. Approximately 450ng of the GC-clamped amplicons from *Ostreopsis* associated bacteria were loaded onto the 6.5% polyacrylamide gel. Numbers at the bottom of lanes represent *Ostreopsis* clones, while the letters C and T indicate cultured and total bacteria respectively.

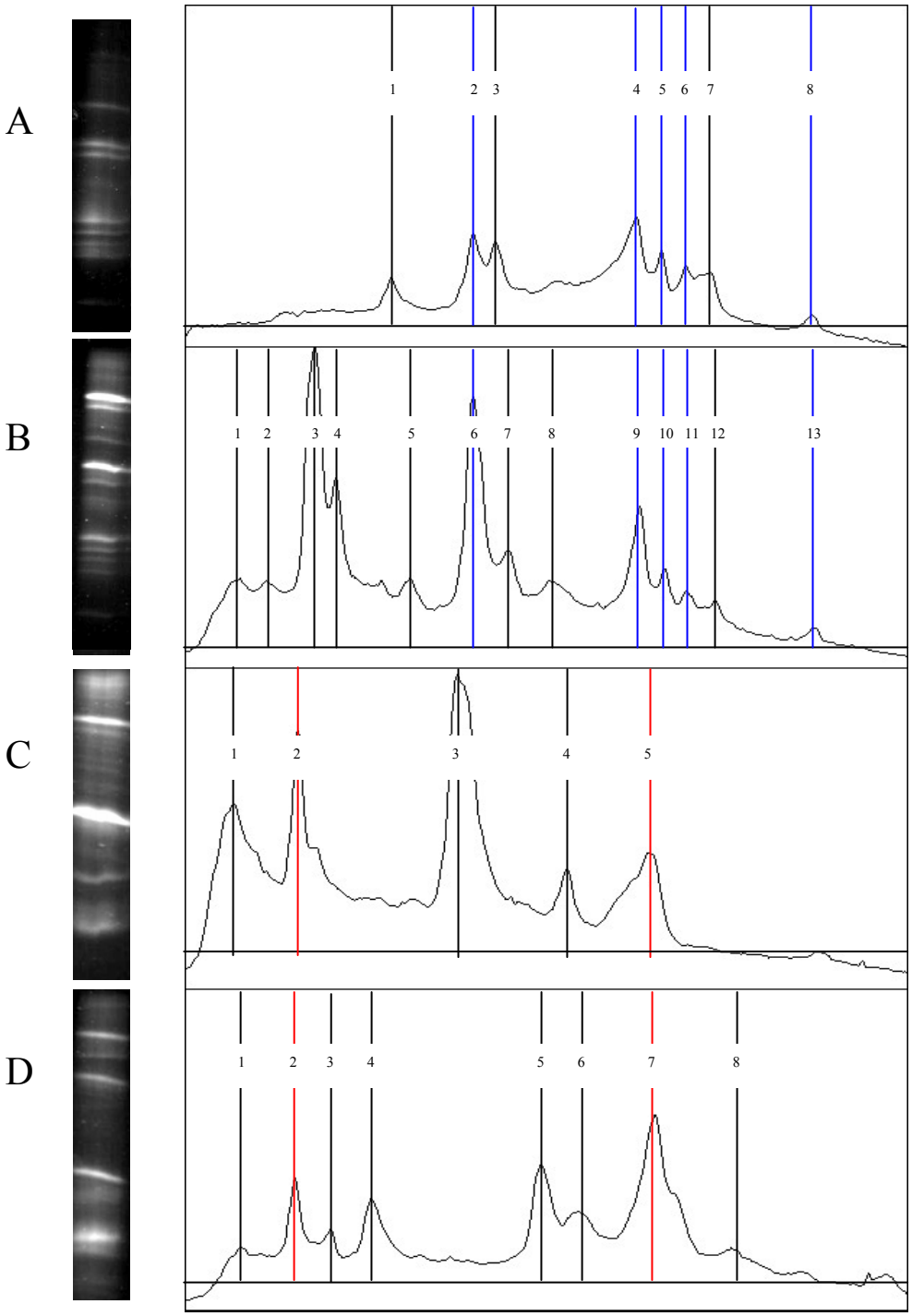

Figure 5. ImageJ analysis of the DGGE. Clones #302 cultured, #302 total, #303 cultured, and #303 total are panels A, B, C, and D, respectively. A DGGE lane is located on the left side of the corresponding ImageJ analysis. Horizontal lines show the background, while vertical lines show individual picks.

The occurrence of common bands between cultured samples was not shown in our DGGE results. However, the ImageJ analysis of total bacteria (Fig. 6) showed that clones #302 and #303 have one band in common, which indicates a possible shared symbiont.

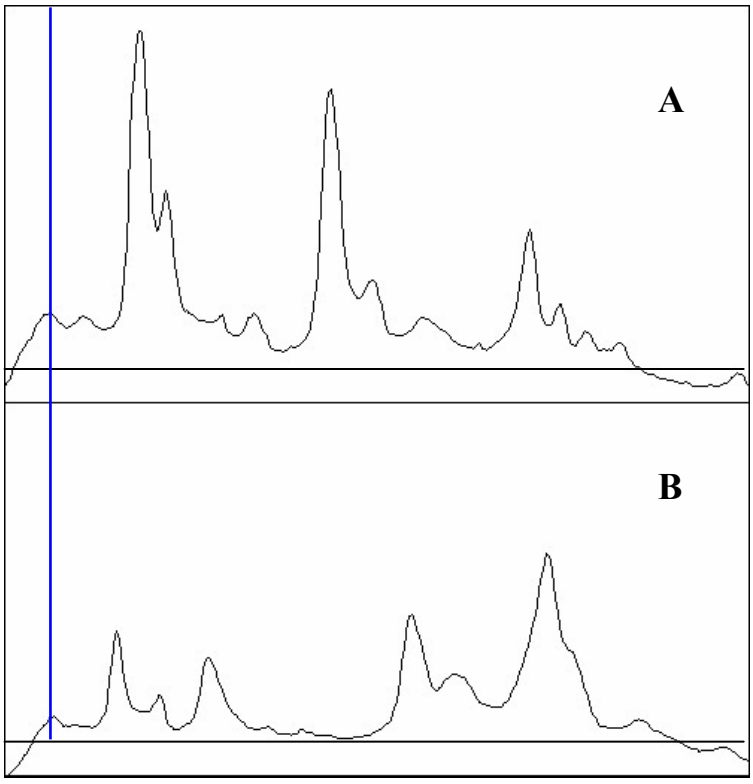

Figure 6. ImageJ analysis of the DGGE from total bacteria. The analysis of uncultured bacteria indicated that clones #302 (A) and #303 (B) have one band in common (shown in blue) which suggests a possible shared symbiont. Horizontal lines show the level of background.

Phylogenetic Analysis

A total of 127 sequences from *Ostreopsis lenticularis* associated bacteria were generated and analyzed phylogenetically using MEGA 3 software (37) (Fig. 7-13). Although there are exceptions to the rule, bacteria are considered to be the same species if they share ≥ 97% 16S rDNA (41, 63). For this work, sequences that showed 98% similarity among them were grouped into clusters.

Clone #302

From *Ostreopsis* clone #302 cultured bacteria (Fig.7), 21 sequences were generated and grouped into five clusters belonging to the gamma division of *Proteobacteria*. According to BLASTN search (2) all of these were represented by members of *Vibrio* spp.

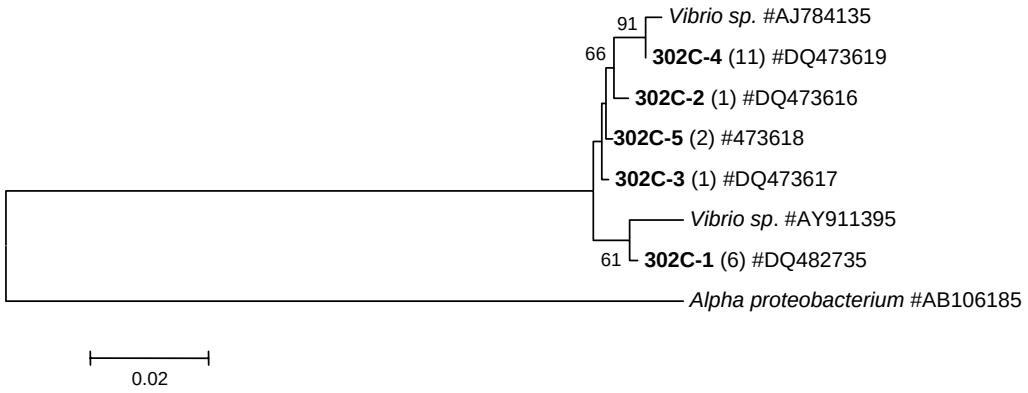

Figure 7. Phylogenetic tree from clone #302 illustrating the placement of 16S rDNA from cultured bacteria samples. The Neighbor-joining tree demonstrates the relationships determined by evolutionary distance calculated with the Kimura 2-parameter model. The scale bar represents .02 substitutions per nucleotide position. The numbers at the nodes indicate the bootstrap values, whereas the numbers on parenthesis reflect sequences included per cluster. Bootstrap values lower than 60 were not included. The sequences from this study are in bold, and those in *italics* are sequences closely related to ours according to BLASTN search with their accession numbers. An alpha proteobacterium was used as outgroup.

From total bacteria (Fig. 8), 41 sequences were obtained and grouped into nine clusters. Fifty-one percent of sequences were classified as members of the *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) complex and represented by the clusters 302T-1 and 302T-3.

The α *Proteobacteria* clade comprised 10% of total bacteria sequences. These were divided into 4 clusters that included members from the genera *Roseobacter* and *Mesorhizobum*.

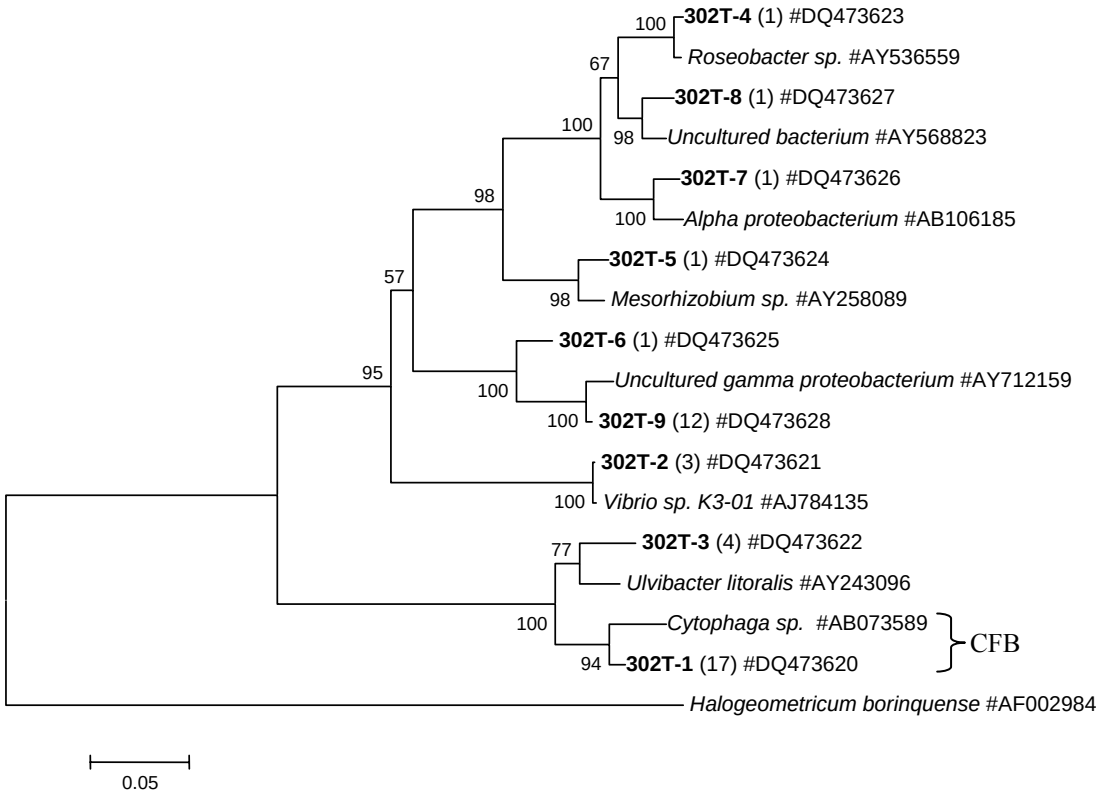

Figure 8. Phylogenetic tree from clone #302 illustrating the placement of 16S rDNA from total bacteria samples. The Neighbor-joining tree demonstrates the relationships determined with the Kimura 2-parameter model. The scale bar represents .05 substitutions per nucleotide position. The numbers at the nodes indicate the bootstrap values, whereas the numbers on parenthesis reflect sequences included per cluster. The sequences from this study are in bold, and those in *italics* are sequences closely related to ours according to a BLASTN search with their accession numbers. An archaea, *Halogeometricum borinquense* was used as outgroup.

A total of 62 sequences from *Ostreopsis* clone #302 associated bacteria (Fig. 9) were obtained and divided into 14 clusters. These were grouped within three major bacterial clades that included: *Proteobacteria* (alpha and gamma), and the CFB complex.

Sixty-six percent of total sequences were represented by the phylum *Proteobacteria*, of which the majority (90%) was γ *Proteobacteria*. This latter was dominated by members of *Vibrio* species from both cultured and total bacteria, and grouped within clusters 302T-2, 302C-1, 302C-2, 302C-3, 302C-4 and 302C-5. From these, 302T-2 and 302C-4 shared a similarity of 100% which suggests they are the same organism.

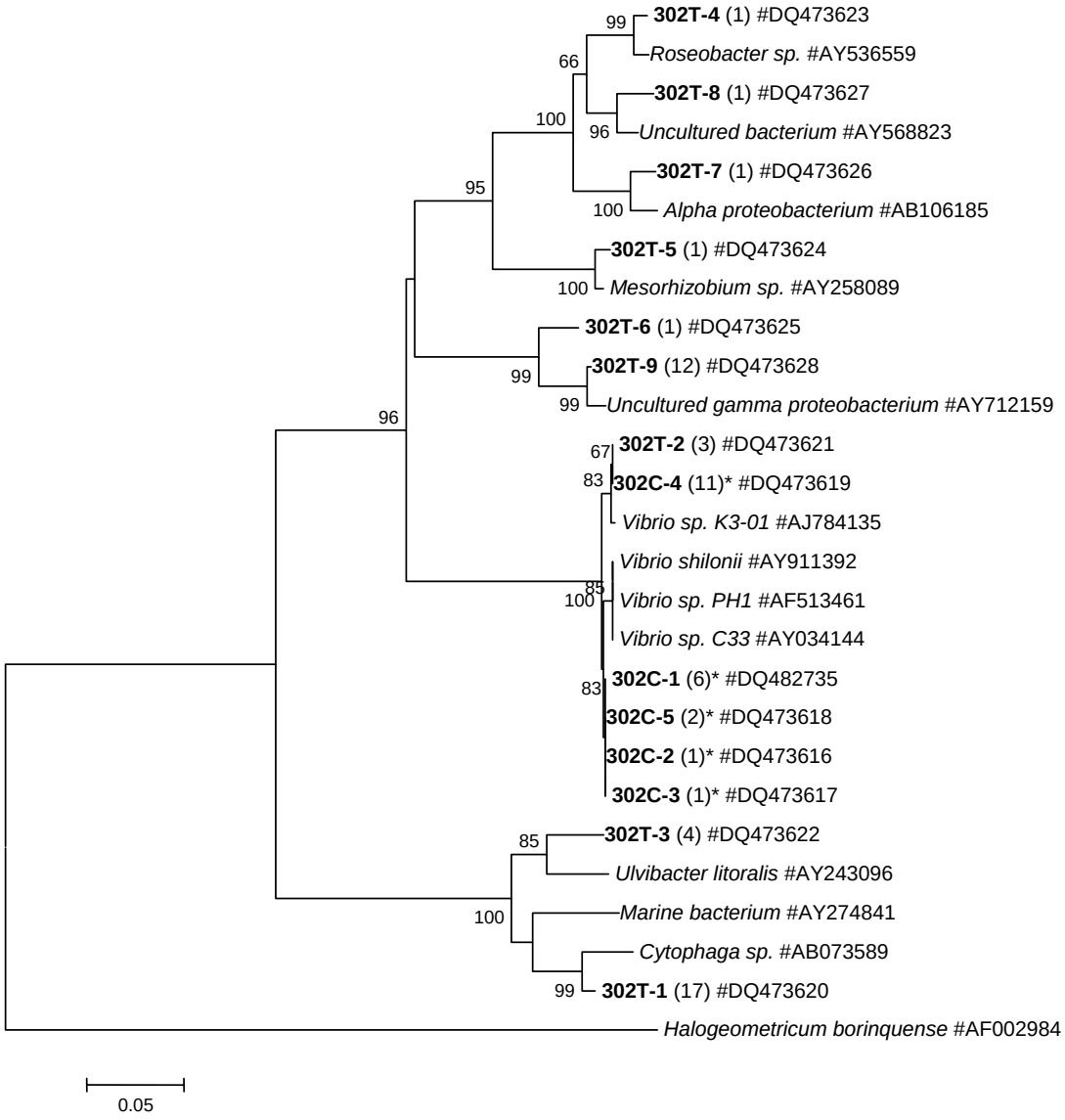

Figure 9. Phylogenetic tree from clone #302 illustrating the placement of 16S rDNA from both cultured and total bacteria samples. The Neighbor-joining tree demonstrates the relationships determined with the Kimura 2-parameter model. The scale bar represents .05 substitutions per nucleotide position. The numbers at the nodes indicate the bootstrap values, whereas the numbers on parenthesis reflect sequences included per cluster. The sequences from this study are in bold, and those in *italics* are sequences closely related to ours according to a BLASTN search with their accession numbers. *Halogeometricum borinquense*, an archaea, was used as outgroup.

Clone #303

For *Ostreopsis* clone #303 cultured bacterial samples, 22 sequences were generated and grouped within five clusters (Fig. 10). The most abundant class (86%) was the γ *Proteobacteria* represented mostly by *Thalassomonas* sp. from cluster 303C-5 which shared homology of 99% with a previously described *T. loyana* (AY643537).

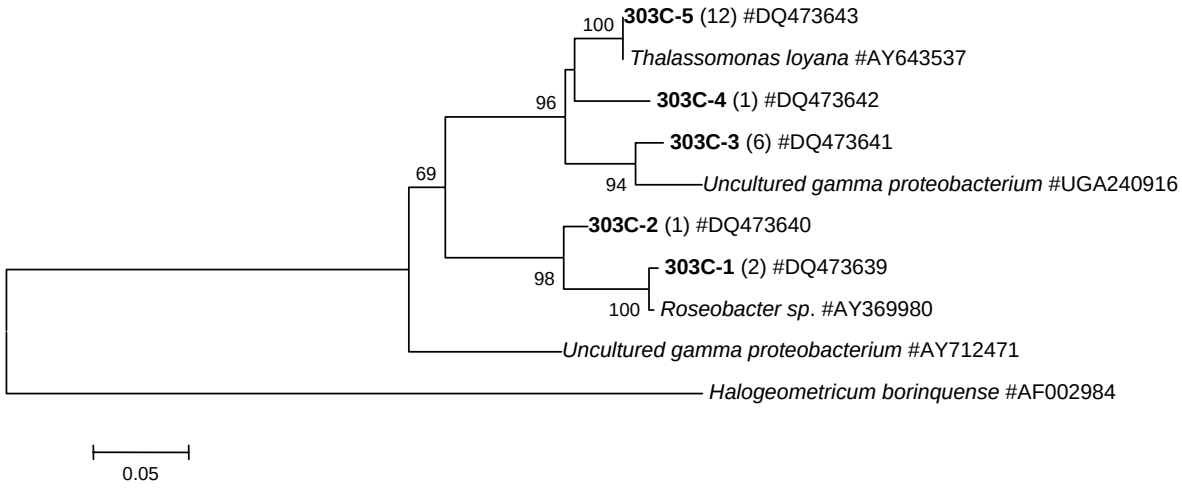

Figure 10. Phylogenetic tree from clone #303 illustrating the placement of 16S rDNA from cultured bacteria samples. The Neighbor-joining tree demonstrates the relationships determined with the Kimura 2-parameter model. The scale bar represents .05 substitutions per nucleotide position. The numbers at the nodes indicate the bootstrap values, whereas the numbers on parenthesis reflect sequences included per cluster. The sequences from this study are in bold, and those in *italics* are sequences closely related to ours according to a BLASTN search with their accession numbers. *Halogeometricum borinquense*, an archaea, was used as outgroup.

For total bacteria analysis, 43 sequences (Fig. 11) were obtained and grouped in 10 clusters. The most abundant (47%) clade and predominant organisms were represented by CFB complex where 100% of symbionts were *Cytophaga*-like bacteria. This clade was followed in abundance by gamma *Proteobacteria* (37%).

Only 16% of the sequences belong to the α *Proteobacteria*. Among these, clusters 303T-3 and 303T-7 presented a homology of 100% with previously described *Erythrobacter aquimaris* (AY461443) and α *Proteobacteria* (AY369982) respectively.

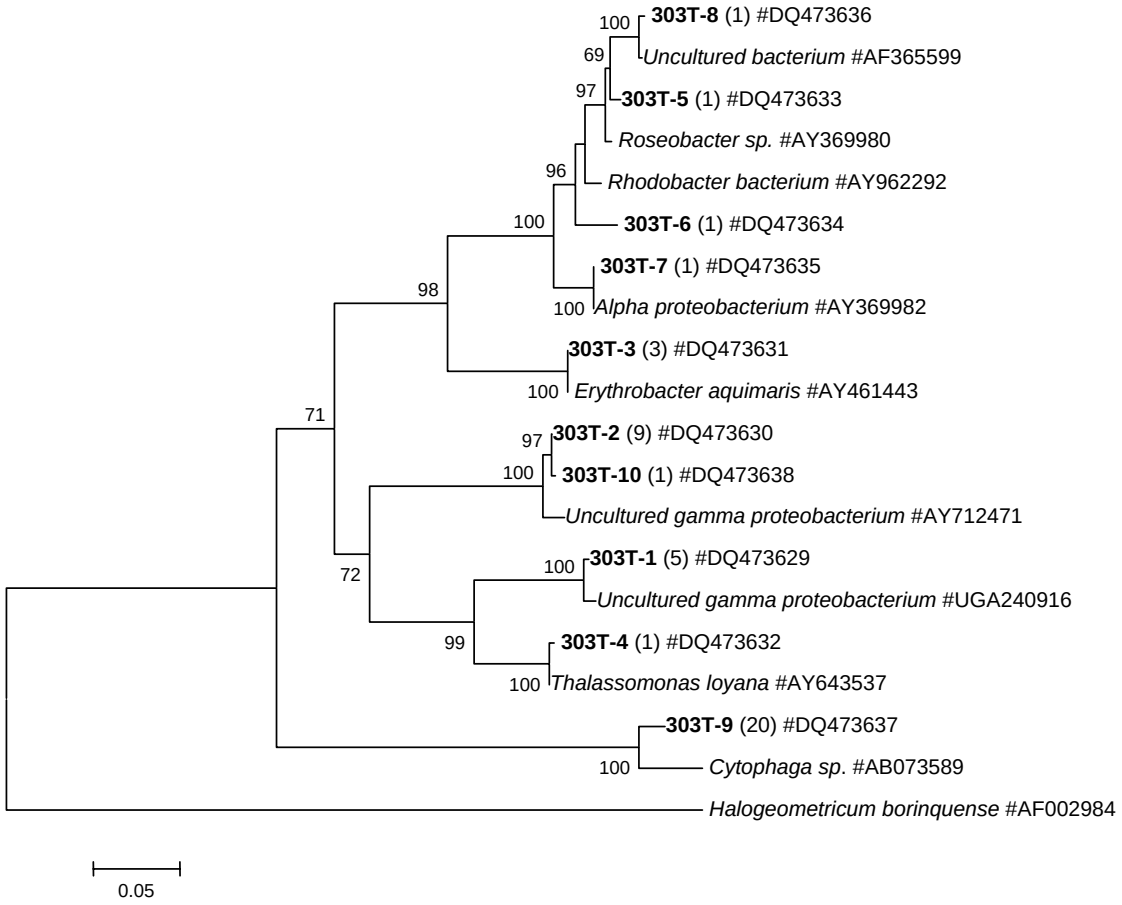

Figure 11. Phylogenetic tree from clone #303 illustrating the placement of 16S rDNA from total bacteria samples. The Neighbor-joining tree demonstrates the relationships determined with the Kimura 2-parameter model for nucleotide change. The scale bar represents .05 substitutions per nucleotide position. The numbers at the nodes indicate the bootstrap values, whereas the numbers on parenthesis reflect sequences included per cluster. The sequences from this study are in bold, and those in *italics* are sequences closely related to ours according to a BLASTN search with their accession numbers. *Halogeometricum borinquense*, an archaea, was used as outgroup.

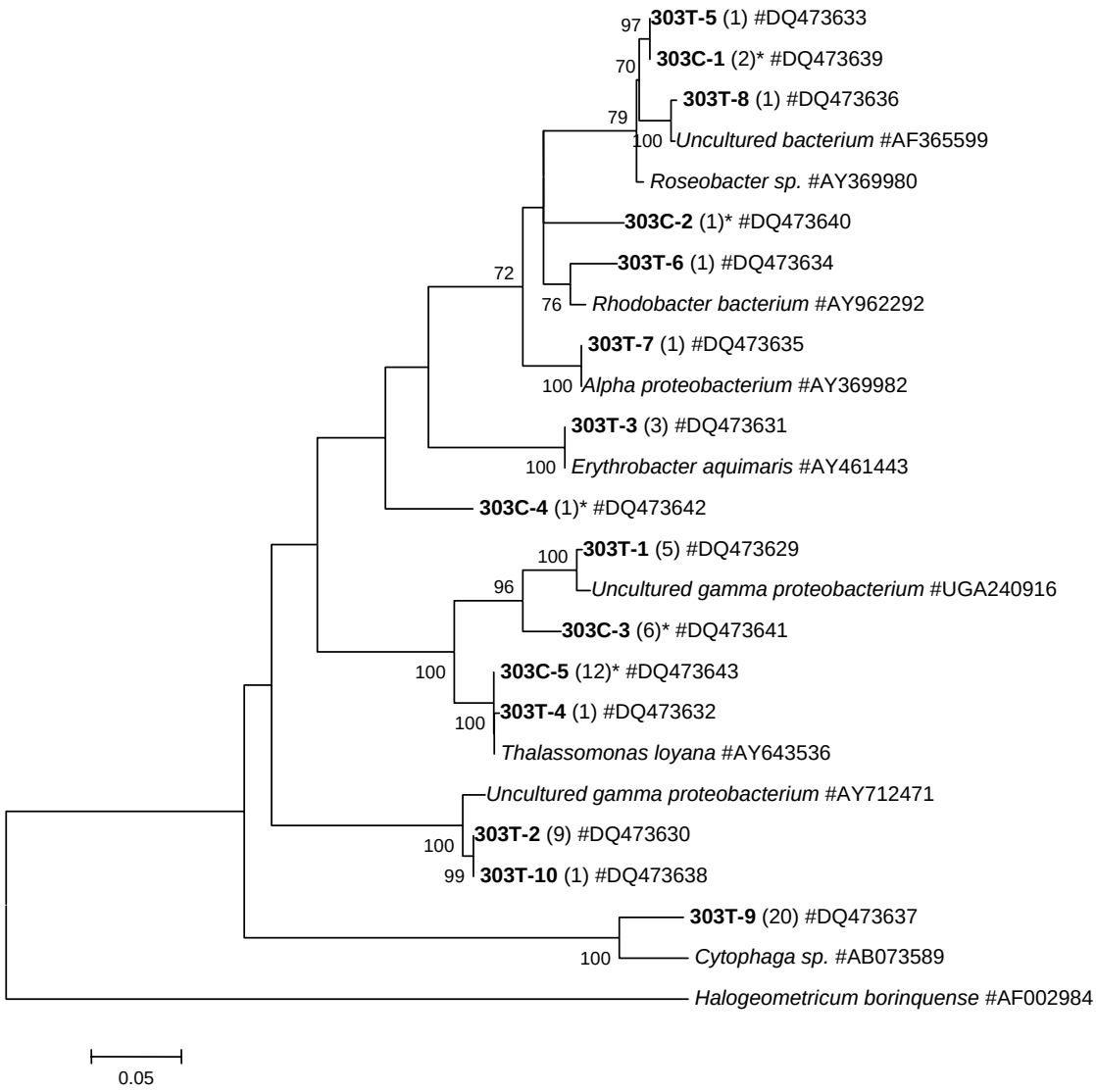

Figure 12. Phylogenetic tree from clone #303 illustrating the placement of 16S rDNA from cultured and total bacteria samples. The Neighbor-joining tree demonstrates the relationships determined with the Kimura 2-parameter model for nucleotide change. The scale bar represents .05 substitutions per nucleotide position. The numbers at the nodes indicate the bootstrap values, whereas the numbers on parenthesis reflect sequences included per cluster. The sequences from this study are in bold, and those in *italics* are sequences closely related to ours according to a BLASTN search with their accession numbers. *Halogeometricum borinquense*, an archaea, was used as outgroup.

From the 65 sequences generated in total from clone #303 associated bacteria (Fig. 12), the predominant clade was the γ *Proteobacteria*. This latter was subcategorized into seven clusters. Eighty-six percent and 37% of the 16S rDNA sequences from cultured and total bacteria respectively, belong to the gamma class. The phylogenetic tree demonstrated that clusters 303C-1 and 303C-5 share 99% similarity with 303T-5 and 303T-4 respectively, suggesting being the same species.

Common Organisms in *Ostreopsis lenticularis*

A library consisting of 127 sequences from partial 16S rDNA was generated from *O. lenticularis* associated bacteria. Sixty-two sequences were obtained from clone #302 and 65 from #303. Members from the α and γ *Proteobacteria* were found in the two clones of our study, and from both cultured and total bacterial samples.

The phylogenetic analysis performed with MEGA 3 software revealed that the predominant clade for both clones was the class γ *Proteobacteria*. Seventy-two sequences (57%) of total sequences belong to this class. However, the predominant clusters (organisms) were members of the CFB complex (Fig. 13) including *Cytophaga* sp. (37 sequences) from clusters 302T-1, and 303T-9 (Fig. 14).

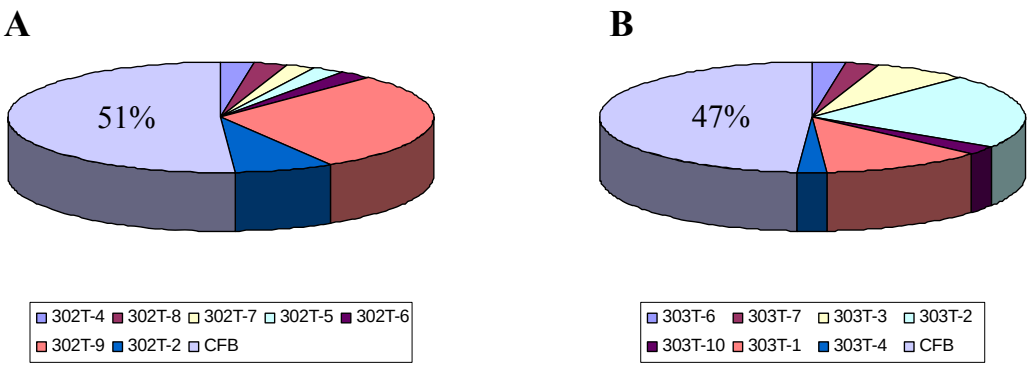

Figure 13. Abundance of total bacteria in two clones of *Ostreopsis lenticularis*. The 16S rDNA sequence with homology to *Cytophaga* sp. appeared in both clones to the level of 50% of total bacteria.

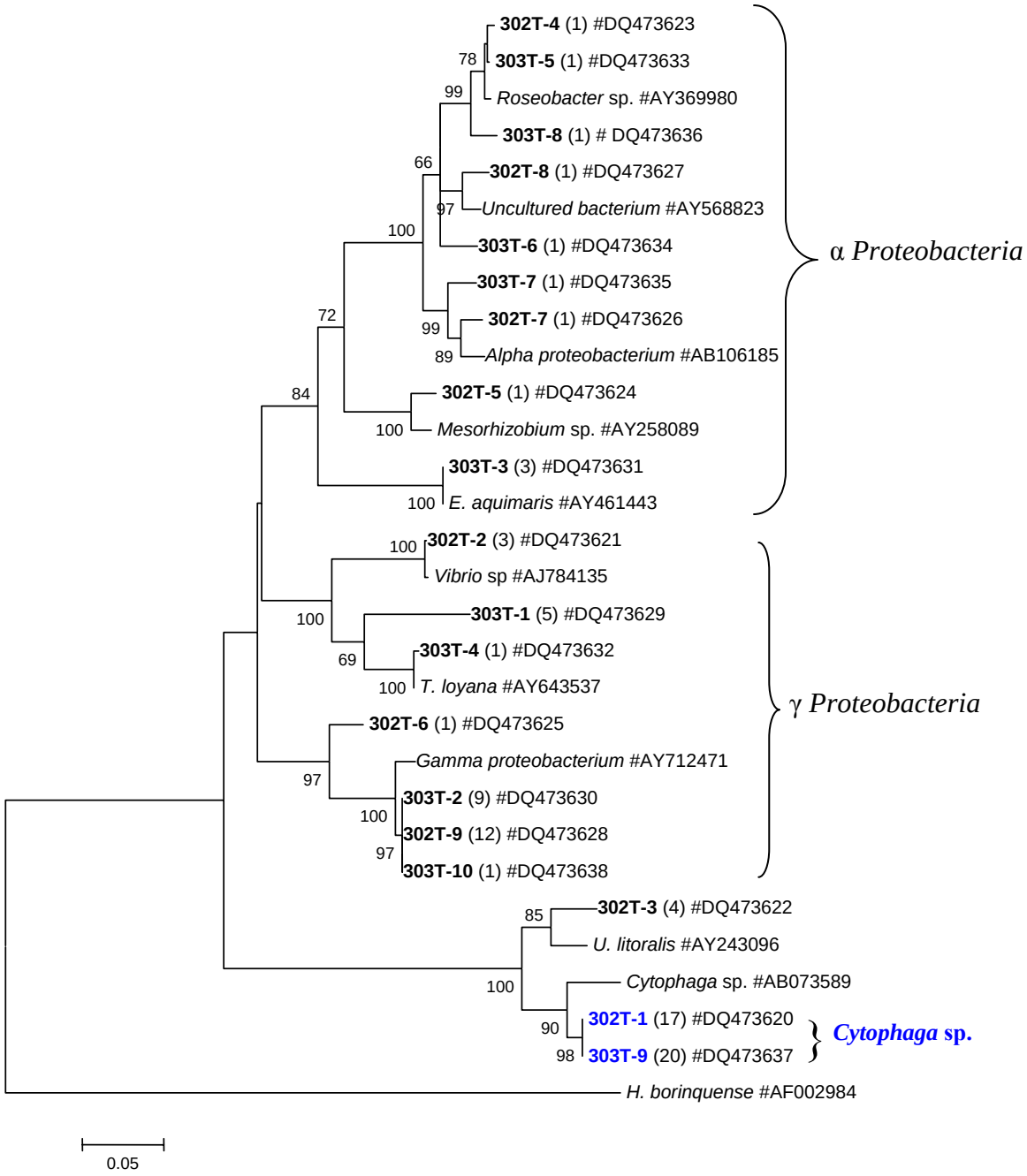

Figure 14. Phylogenetic tree from clones #302 and #303 illustrating the placement of 16S rDNA from total bacteria samples. The Neighbor-joining tree demonstrates the relationships determined with the Kimura 2-parameter model for nucleotide change. The scale bar represents .05 substitutions per nucleotide position. The numbers at the nodes indicate the bootstrap values, whereas the numbers on parenthesis reflect sequences included per cluster. The sequences from this study are in bold, and those in *italics* are sequences closely related to ours according to a BLASTN search with their accession numbers. *Halogeometricum borinquense*, an archaea, was used as outgroup.

Common Bacterial Species

Cytophaga species were found common in both clones. These members from the CFB complex were recovered from total (uncultured) bacterial samples. These bacteria represented by clusters 302T-1 and 303T-9 (shown in blue, Fig. 14) shared a similarity of 100% which suggests they are the same organism. This result is supported by the ImageJ analysis where a co-occurring band between clones was detected (Fig. 6).

Since this *Cytophaga* species was found to be common in both clones, specific primers were designed (see materials and methods). From the primer walking 1,447bp and 1,446bp were amplified for clones #302 and #303 respectively. Both sequences were analyzed using BLASTN and were identified as *Bacteroidetes*. These two partial 16S rDNA sequences share 99% identity (Fig.15) and their closest match in GenBank (AM040105) showed a homology of 95% (not shown).

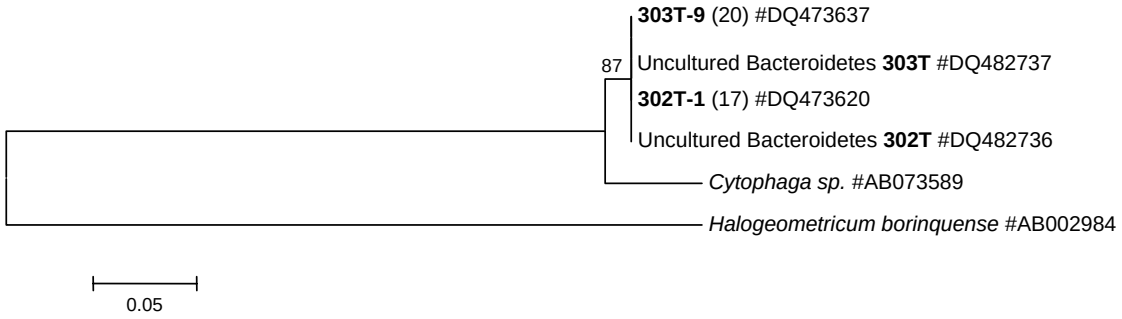

Figure 15. Phylogenetic tree illustrating the placement of 16S rDNA of *Cytophaga* species from clones #302 and #303. The Neighbor-joining tree demonstrates the relationships determined with the Kimura 2-parameter model for nucleotide change. The scale bar represents .05 substitutions per nucleotide position. The numbers at the nodes indicate the bootstrap values, whereas the numbers on parenthesis reflect sequences included per cluster. *Halogeometricum borinquense*, an archaea, was used as outgroup.

DISCUSSION

The principal aim of this project was to determine microbial diversity in two toxic clones of *Ostreopsis lenticularis*. More than 100 bacteria from two clones of *Ostreopsis* were examined for common bacterial strains that could be involved in the production of ciguatoxins. Partial sequences were therefore used that encompass hypervariable regions of the 16S rDNA gene that would satisfy both the requirement for a rapid screen, as well as sufficient taxonomic identity at least to the genus level.

DGGE has the advantage of detecting bacterial species regardless of their culturability. This technique demonstrated the differences between bacterial community compositions as determined by molecular techniques from *O. lenticularis* cultures and cultivation. Different banding patterns were shown for cultured bacteria versus the bacterial DNA isolated from *Ostreopsis* (Fig. 5). Additional bands were observed from total isolation samples compared to those from cultured samples in clones #302 and #303 (5 and 3 bands respectively). The ImageJ analysis from DGGE also showed that total bacteria samples from clone #302 had more diversity with 13 distinct bands while #303 had only 8. Furthermore, lanes from cultured bacteria of clone #302 contained 3 more bands than its counterpart from #303.

The ImageJ analysis of the gel (Fig. 5) gave us the number of bands for each *Ostreopsis* clone used in this study. The DGGE banding patterns were compared to the number of sequences. The ImageJ analysis for clone #302 cultured and total bacteria showed higher numbers of bands than the clusters obtained from sequencing. Since DGGE separates products of the same size based on their nitrogen bases composition (49), each band visualized on the gel should represent a single bacterial species. However multiple bands of 16S rDNA from single species of bacteria have been documented (16, 24, 25). This intraspecies heterogeneity observed in DGGE banding pattern could be the result of the presence of multiple copies of the ribosomal genes and the fact that the gene copies have evolved differently (75). For *Ostreopsis* clone #303 cultured bacteria, the number of clusters and sequences coincided. Total bacteria from this clone, showed fewer bands than the number of species found. This could be due to the co-migration of bands with similar G-C content (24). Even with these limitations

(over and underestimation of the number of bacterial species), we have proven that DGGE is an effective method for the estimation of the number of bacterial species in a sample.

Ostreopsis lenticularis and Associated Bacteria

Bacteria do not produce the ciguatoxins, however they seem to play a role in the production of the toxin by the dinoflagellate. Therefore, if a specific bacterial species is required for *O. lenticularis* to enhance toxicity during stationary phase, it should be present in every culture of *Ostreopsis* and is likely to comprise a large fraction of the bacterial population. Culturable bacteria from genus *Pseudomonas/Alteromonas* have been reported to be necessary for the production of enhanced toxicity in clonal cultures of *Ostreopsis* (29, 71).

Our results demonstrated that γ-*Proteobacteria* bacteria from genus *Alteromonas* appeared in clone #303, but not in clone #302. Previous studies have shown that culturable marine bacteria from seawater fall predominantly within the γ subclass (21, 62). This can be due to the common bacteriological media used in the laboratory that selectively isolate Gram-negative chemoorganotrophs of the γ- *Proteobacteria* (53). Therefore sequences obtained from both culturing and total DNA bacterial samples from clone #303 prove that the presence of *Pseudomonas/Alteromonas* could be more casual than obligate.

Proteobacteria and the *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) group, usually dominate heterotrophic bacterial communities in the ocean (27). From our results the most abundant class was the γ-*Proteobacteria* (57%) followed by the CFB complex (32%). This group was retrieved from uncultured bacteria and represented by *Bacteroidetes* and *Cytophaga* sp. This latter was the most abundant symbiont for each clone. The *Cytophaga* sp. sequences shared 100% homology to each other and their closest match in GenBank, an uncultured bacterium (AM040105), was only 95% homologous to our sequences. It is remarkable that although the CFB group of bacteria is very abundant in the ocean, *Ostreopsis* appears to have an obligate association to only one CFB, namely *Cytophaga* sp. that we found.

Bacteria that belong to the phylum CFB are one of the dominant groups of the many microbial populations that inhabit different marine environments (27, 35, 42) and fresh water ecosystems (54). Members of this group have been reported to induce differentiation in algae (42), cause pathogenicity for fish and shellfish (22) and have been associated with toxic dinoflagellates (4). This all makes the *Cytophaga* sp. that we found a likely candidate to be involved in the production of the toxin by *Ostreopsis lenticularis*. It will be very important then to test for the presence of *Cytophaga* in *Ostreopsis* field samples to verify its close association with the dinoflagellate. This next step will be facilitated by the specific *Cytophaga* primer that we have designed and have shown to be specific for this bacterium.

CONCLUSIONS

We generated 127 sequences from bacterial DNA from two clonal cultures of the marine toxic dinoflagellate *Ostreopsis lenticularis*. In general, bacteria were classified into two phyla and three major classes. The most abundant organisms belong to *Vibrio* spp. from the γ *Proteobacteria* and to *Cytophaga* sp. The latter was the only one found to be common to both clones, and was retrieved from uncultured bacteria.

- Evidently *Pseudomonas/Alteromonas* is not involved in the production of the toxin. Its presence in *Ostreopsis* cultures appears to be more casual than obligate.

- A total of 37 sequences of *Cytophaga* sp. included in clusters 302T-1, and 303T-9 shared 100% homology to each other. This suggests they are the same organism.

- The 16S rDNA sequence with homology to *Cytophaga* sp. appeared in both clonal cultures of *O. lenticularis* to the level of 50% of total bacteria. This shows a possible close association of these bacteria with the dinoflagellate.

- Common species between these two toxic clonal cultures could signify bacteria involved in the ciguatoxin production by *Ostreopsis lenticularis*.

- Such diversity studies can be an effective general technique for the identification of symbiotic association in ocean environments.

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APPENDIXES

APPENDIX 1: Culture Media

Marine Agar (MB+Agar)

37.4g Bacto® Marine Broth 2216l (Difco Laboratories Detroit MI, USA)
18.0g Bacto® Agar (Difco Laboratories Detroit MI, USA)
Procedure:
Suspend the powder in 1L of distilled water and boil for 1-2 minutes. Autoclave at 121-124°C for 15 minutes.

Luria Broth with Ampicillin (LB+Amp)

5g Yeast extract
10g NaCl
10g Tryptone
18g Agar (for solid media)
Procedure:
Suspend the powder in 1L of distilled water and boil for 1-2 minutes. Autoclave at 121-124°C for 15 minutes. Cool media to 50°C and add Ampicillin to a final concentration of 50µg/mL.

Appendix 2: Partial 16S rDNA Sequences

Ostreopsis clone #302

Cultured bacteria

Vibrio sp. #DQ482735
AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGC
GGAAACGAGTTAACTGAACCTTCGGGGAACGTTAACGGCGTCGAGCGGCGGACGGGTGAG
TAATGCCTGGGAAATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCG
CATAATAGCTTCGGCTCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCCAG
GTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTG
AGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGG
CCTTCGGGTTGTAAAGCACTTTCAGCAGTGAGGAAGGCGGGTACGTTAATAGCGTATTCG
TTTGACGTTAGCTGCAGAAGAAGCACCGGCTAACTCCGT

Vibrio sp. #DQ473616
CGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAGTTAACTGAACCTTCGGGGAACGTT
AACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCTGATGTGGGGGAT
AACCATTGGAAACGATGGCTAATACCGCATAATAGCTTCGGCTCAAAGAGGGGGACCTTC
GGGCCTCTCGCGTCAGGATATGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCA
CCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT
GCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCAGTGAGG
AAGGTGGTGTAGTTAATAGCTGCATTATTTGACGTTAGCTGCAGAAGAAGCACCGGCTAA
CTCCGT

Vibrio sp. #DQ473617
ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCCGAGCGGAAACGAGTTAACTGAAC
CTTCGGGGAACGTTAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCC
CTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATAGCTTCGGCTCAA
AGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCCAGGTGGGATTAGCTAGTTGGT
GAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACAC
TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCAC
TTTCAGCAGTGAGGAAGGTGGTGTCGTTAATAGCGGCATCATTTGACGTTAGCTGCAGAA
GAAGCACCGGCTAACTCCGT

Vibrio sp. #DQ473618
ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAGTTAACTGAACC
TTCGGGGAACGTTAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCC
TGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATAGCTTCGGCTCAAA
GAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCCAGGTGGGATTAGCTAGTTGGTG
AGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
GCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACT
TTCAGCAGTGAGGAAGGCGGGTA

Vibrio sp. #DQ473619
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TTCGGGGAACGTTAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCC
TGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATAGCTTCGGCTCAAA
GAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCCAGGTGGGATTAGCTAGTTGGTG
AGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGG
GCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACT
TTCAGTCGTGAGGAAGGTGGTGTAGTTAATAGCTGCATCATTTGACGTTAGCGACAGAAG
AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAAAGCCGAATTCTGGCAGAATTT
CCTTCACACTGGCGGCCCGCTCCGAGCCATGCAT

Uncultured bacteria

Cytophaga sp. #DQ473620
GATGAACGCTAGCGGCAGGCCTAACACATGCAAGTCGAGGGGTAACAGGAATTAGCTTGC
TAATTTGCTGACGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTGCCTTATACTGGG
GGATAGCCTTTAGAAATGAAGATTAATACCCCATAGTATGTGACTGTGGCATCACAGACA
CATTAAAGGTTACGGTATAAGATGAGCATGCGTCCTATTAGCTAGATGGTGTGGTAACGG
CACACCATGGCAACGATAGGTAGGGGCCCTGAGAGGGGGATCCCCCACACTGGTACTGAG
ACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGAGGCAACTC
TGATCCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTTTTATACG
GAAGAAACCCCCTACGTGTAGGGGCTTGACGGTACCGTAAGAATAAGCATCGGCTAACTC
CGTGCCAGCAGCCGCGTAAT

Vibrio sp. #DQ473621
TGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAGTTA
ACTGAACCTTCGGGGAACGTTAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTGGGA
AATTGCCCTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATAATAGCTTC
GGCTCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCCAGGTGGGATTAGCT
AGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
CACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGT
AAAGCACTTTCAGTCGTGAGGAAGGTGGTGTAGTTAATAGCTGCATCATTTGACGTTAGC
GACAGAAGAAGCACCGGCTAACTCCGT

Bacteroidetes #DQ473622
GATGAACGCTAGCGGCAGGCCTAACACATGCAAGTCGAGGGGTAACAGGGATTGCTTGCA
ATCCGCTGACGACCGGCGCACGGGTGCGTAACGCGTATACAATCTACCTTACACTGGGGG
ATAGCCTTTAGAAATGAAGATTAATACCCCATGGTATGTTAATGTGGCATCACATATACA
TTAAAGGTTACGGTGTAAGATGAGTATGCGTCCTATTAGTTTGTTGGTGAGGTAACGGCT
CACCAAGACGACGATAGGTAGGGGCCCTGAGAGGGGGATCCCCCACACTGGTACTGAGAC
ACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGTCAATGGGCGAGAGCCTG
AACCAGCCATGCCGCGTGCAGGAAGACGGCCCTATGGGTTGTAAACTGCTTTTATACAGG
AAGAAACACTCCCTCGTGAGGGAGCTTGACGGTACTGTAAGAATAAGGACCGGCTAACTC
CGT

Rhodobacterales #DQ473623
ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGGAAACGAGTTAACTGAACC
TTCGGGGAACGTTAACGGCCGTCGAGCGGCGGACGGGTTAGTAACGCGTGGGAACGTACC
CAGATCTAAGGAATAGCCACTGGAAACGGTGAGTAATACCTTATACGCCCTTCGGGGGAA
AGATTTATCGGATTTGGATCGGCCCGCGTTAGATTAGATAGTTGGTGGGGTAACGGCCTA
CCAAGTCTACGATCTATAGCTGGTTTTAGAGGATGGTCAGCAACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTGGACAATGGGCGCAAGCCTGAT
CCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTCGTAAAGCTCTTTCGCCAGAGATG
ATAATGACAGTATCTGGTAAAGAAACCCCGGCTAACTCCGTT

Marine bacterium #DQ473624
AACCGAACGCTGGCGGCAGGCTAACACATGCAAGTCGAACGCCCTTCGGGGGAGTGGCAG
ACGGGTGAGTAACGCGTGGGAATCTACCCAGCTCTACGGAATAACTGAGGGAAACTTCAG
CTAATACCGTATACGTCCTTCGGGAGAAAGATTTATCGGAGTTGGATGAGCCCGCGTTGG
ATTAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGG
ATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
AATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTA
GGGTCGTAAAGCTCTTTCGCCTGTGAAGATAATGACGGTAGCAGGTAAAGAAACCCCGGC
TAACTCCGT

Marine bacterium #DQ473625
TACACATGCAAGTCGAGCGAGAAAGGTCTTCGGACTGAGTACAGCGGCGGACGGGTGAGT
AACGCGTAGGAATCTACCCAGTAGTGGGGGACAACTTGGGGAAACTCAAGCTAATACCGC
ATACGCCCTATGGGGGAAAGCGGGGGATCTTCGGACCTCGCGCTATTGGATGAGCCTGCG
TCGGATTAGCTAGTTGGTGGGGTAAAAGCCTACCAAGGCGACGATCCGTAGCTGGTCTGA
GAGGACGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGC
CTTAGGGCTGTAAAGCTCTTTTACCAGGGATGATAATGACAGTACCTGGAGAATAAGCTC
CGGCTAACTCCGT

Marine bacterium #DQ473626
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GAGTAATACCGTATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTGGATTAGCCCGCGTC
TGATTAGATAGTTGGTGGGGTAACGGCCTACCAAGTCGACGATCAGTAGCTGGTTTGAGA
GGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATCTTAGACAATGGGCGCAAGCCTGATCTAGCCATGCCGCGTGAGTGACGAAGGTCT
TAGGATCGTAAAGCTCTTTCGCCAGGGATGATAATGACAGTACCTGGTAAAGAAACCCCG
GGCTAACTCCGT

Marine bacterium #DQ473627
AACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGCACCTTCGGGTGAGCGGCG
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AGTAATACCGCATACGCCCTTCGGGGGAAAGATTTATCGGAGAGGGATCGGCCCGCGTTA
GATTAGGTAGTTGGTGGGGTAACGGCCTACCAAGCCTACGATCTATAGCTGGTTTTAGAG
GACGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATCTTGGACGATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGACGAAGGCCCT
AGGGTCGTAAAGCTCTTTCGCCAGGGATGATAATGACAGTACCTGGTAAAGAAACCCCCG
GCTAACTCCGT

Gamma Proteobacterium #DQ473628

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GTACAGCGGCGGACGGGTGAGTAACGCGTAGGAATCTACCCAGTAGTGGGGGACAACTTG
GGGAAACTCAAGCTAATACCGCATACGCCCTATGGGGGAAAGCGGGGGATCTTCGGACCT
CGCGCTATTGGATGAGCCTGCGTCGGATTAGCTAGTTGGTGGGGTAAAAGCCTACCAAGG
CGACGATCCGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCC
ATGCCGCGTGTGTGAAGAAGGCTCTAGGGTTGTAAAGCACTTTCAGCAGTGAGAAAAAGC
CACCGGATAATACCCGTTGGTCTTGATGTTAACTGTAGAAGAAGCACCGGCTAACTCCGT

Bacteroidetes (whole gene) #DQ482736

GATGAACGCTAGCGGCAGGCCTAACACATGCAAGTCGAGGGGTAACAGGAATTAGCTTGC
TAATTTGCTGACGACCGGCGCACGGGTGCGTAACGCGTATGCAATCTGCCTTATACTGGG
GGATAGCCTTTAGAAATGAAGATTAATACCCCATAGTATGTGACTGTGGCATCACAGACA
CATTAAAGGTTACGGTATAAGATGAGCATGCGTCCTATTAGCTAGATGGTGTGGTAACGG
CACACCATGGCAACGATAGGTAGGGGCCCTGAGAGGGGGATCCCCCACACTGGTACTGAG
ACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGAGGCAACTC
TGATCCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTTTTATACG
GGAAGAAACCCCCCTACGTGTAGGGGCTTGACGGTACCGTAAGAATAAGCATCGGCTAAC
TCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATCATTGGGTTT
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TGCCTTTGATACTGGTTGTCTTGAATCATTGTGAAGTGGTTAGAATATGTAGTGTAGCGG
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CGTAAACGATGGATACTAGCTGTTCGATCTTCGGATTGAGTGGCTAAGCGAAAGTGATAA
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CACAAGCGGTGGAGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCAGGGCTTA
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ACAATGGCCGGTACAGAGGGCAGCCACTGGGTGACCAGGAGCGAATCCTTAAAACCGGTC
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GAAGCTGGGGGTACCTGAAGTCGGTGACCGCAAGGAGCTGCCTAGGGTAAAACTGGTAAC
TGGGGCT

Ostreopsis clone #303

Cultured bacteria

Roseobacter sp. #DQ473639
AACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGCACTCTTCGGAGTGAGCGG
CGGACGGGTTAGTAACGCGTGGGAACGTACCCAGATCTAAGGAATAGCCACTGGAAACGG
TGAGTAATACCTTATACGCCCTTCGGGGGAAAGATTTATCGGATTTGGATCGGCCCGCGT
TAGATTAGATAGTTGGTGGGGTAACGGCCTACCAAGTCTACGATCTATAGCTGGTTTTAG
AGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATCTTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCC
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GGCTAACTCCGT

Rhodobacterales #DQ473640
AACGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAGCGCACTCTTCGGAGTGAGCGG
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TGAGTAATACCTTATACGCCCTTCGGGGGAAAGATTTATCGGATTTGGATCGGCCCGCGT
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AGGATGATCAGCAACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCC
TTCGGGTTGTAAAGCACTTTCAGTAGGGAGGAAAGGTTAGTAGCTAATATCTGCTAGCTG
TGACGTTACCTACAGAAGAAGCACCGGCTAACTCCGT

Marine bacterium #DQ473641
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CTCCCACCTATAGAGAAGCCCAAGTGAGATTAGCTAGTTGGTGAGGTAAAGGCTCACCAA
GGCGACGATCTCTAGCTGTTCTGAGAGGAAGATCAGCCACACTGGAACTGAGACACGGTC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAG
CCATGCCAGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTTGTGAGGAAA
GGTTAGTAGTTAATACCTGCTAGCTGTGACGTTAACAACAGAAGAAGCACCGGCTAACTC
CGT

Alteromonadales #DQ473642
ATTGAACGCTGGCGGCAGGCTTAACACATGCAAGTCGAGCGGAAACGATGATTGCTTGCA
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CAACAGTTGGAAACGACTGCTAATACCGCATAATGTCTACGGACCAAAGGAGGGGATCTT
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ACCAAGGCGACGATCTCTAGCTGGTTTTAGAGGATGATCAGCAACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATCTTGGACAATGGGCGCAAGCCTGA
TCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTCGTAAAGCTCTTTCGCCAGAGAT
GATAATGACAGTATCTGGTAAAGAAACCCCGGCTAACTCCGT

Thalassomonas sp. #DQ473643
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CAACAGTTGGAAACGACTGCTAATACCGCATAATGTCTACGGACCAAAGGAGGGGACCTT
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CGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGA
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GAAAGGTTAGTAGCTAATATCTGCTAGCTGTGACGTTACCTACAGAAGAAGCACCGGCTA
ACTCCGT

Uncultured bacteria

Gamma Proteobacterium #DQ473629
ATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGAACTAGCTTGCT
AGTTGCTGACGAGTGGCGGACGGGTGAGTAATGCTTGGGAATTTGCCTTTAGGAGGGGGA
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CTCCCACCTATAGAGAAGCCCAAGTGAGATTAGATAGTTGGTGAGGTAAAGGCTCACCAA
GGCGACGATCTCTAGCTGTTCTGAGAGGAAGATCAGCCACACTGGAACTGAGACACGGTC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAG
CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTTGTGAGGAAAG
GTTAGTAGTTAATACCTGCTAGCTGTGACGTTAACAACAGAAGAAGCACCGGCTAACTCC
GT

Gamma Proteobacterium #DQ473630
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GTACAGCGGCGGACGGGTGAGTAACGCGTAGGAATCTACCCAGTAGTGGGGGACAACTTG
GGGAAACTCAAGCTAATACCGCATACGCCCTATGGGGGAAAGCGGGGGATCTTCGGACCT
CGCGCTATTGGATGAGCCTGCGTCGGATTAGCTAGTTGGTGGGGTAAAAGCCTACCAAGG
CGACGATCCGTAGCTGGTCTGAGAGGACGATCAGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCC
ATGCCGCGTGTGTGAAGAAGGCTCTAGGGTTGTAAAGCACTTTCAGCAGTGAGAAAAAGC
CACCGGATAATACCCGTTGGTCTTGATGTTAACTGTAGAAGAAGCACCGGCTAACTCCGT

Erythrobacter sp. #DQ473631
AACGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGAACCCTTCGGGGTTAGTGG
CGCACGGGTGCGTAACGCGTGGGAACCTGCCTTTAGGTTCGGAATAACTCAGAGAAATTT
GAGCTAATACCGGATAATGTCTTCGGACCAAAGATTTATCGCCTTTAGATGGGCCCGCGT
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GGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCC
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Alteromonadaceae #DQ473632
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Roseobacter sp. #DQ473633
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Rhodobacterales #DQ473634
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Alpha Proteobacterium #DQ473635
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Marine bacterium #DQ473636
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Cytophaga sp. #DQ473637

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TGATCCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTTTTATACG
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CCGT

Marine bacterium #DQ473638

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CGCGCTATTGGATGAGCCTGCGTCGGATTAGCTAGTTGGTGGGGTAAAAGCCTACCAAGG
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GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCC
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Bacteroidetes (whole gene) #DQ482737

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