Insights into the Shipworm (Bivalvia: *Teredinidae*)-Bacterial Symbiosis: Novel Isolates and Micrographic Localization Studies

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Abstract

Insights into the Shipworm (Bivalvia: *Teredinidae*)-Bacterial Symbiosis: Novel Isolates and Micrographic Localization Studies

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Marine bivalves of the family *Teredinidae* (commonly known as shipworms), burrow in wood for shelter and ingest the excavated wood particles as they burrow. The gills of shipworms harbor a dense community of at least four closely related gamma proteobacterial endosymbiont types (Luyten et al. 2006, Distel et al. 2002a) and one of these, *Teredinibacter turnerae*, has been cultivated and studied extensively. *T. turnerae* has the demonstrated capacity to fix N_2 and produce cellulases when grown in culture (Distel et al. 2002b, Waterbury et al. 1983) and a significant proportion of the genome of *T. turnerae* T7901 is dedicated to production of bioactive metabolites (Yang et al. 2009). The majority of cellulose consuming organisms contain cellulolytic microbes in their digestive tracts, yet the cellulase producing endosymbionts of *Lyrodus pedicellatus* are localized to bacteriocytes within the gills (Distel et al. 1991, Distel et al. 2002a). The gill endosymbiotic community has been studied extensively, but the digestive tissues have not been thoroughly investigated.

In this study cultivation and characterization of additional shipworm gill endosymbiont strains was undertaken and resulted in isolated strains covering five different clades of shipworm symbiont types from numerous host species including Philippine shipworm species and the Pacific Northwestern *Bankia setacea*. Metabolic analyses showed that some of these symbionts lack the ability to fix N_2 and the aptitude for degradation of crystalline cellulose varies across strains. *Teredinibacter turnerae* strains have demonstrated the ability to inhibit growth of other symbiont types in culture.

Fluorescence *in situ* hybridizations (FISH) combined with laser scanning confocal microscopy (LSCM) with colony reared *Lyrodus pedicellatus* (Distel et al. 2002a) revealed a pattern of spatial segregation of symbionts within gill bacteriocytes (host cells inhabited by bacteria) according to symbiont phylogeny. In the study presented here FISH with LSCM and general bacterial and symbiont specific 16S rRNA oligonucleotide probes has demonstrated symbiont segregation in the gills of five additional shipworm species. Novel strains predicted to be symbionts based on 16S rRNA gene sequence analysis, were confirmed as residents of shipworm gill tissue through FISH. Strains lacking the ability to fix atmospheric N_2 were not detected in bacteriocytes together with N_2 fixing symbionts based on dual probe FISH and LSCM with oligonucleotide probes specific to cultivated strains lacking the ability to fix N_2 and *T. turnerae*. Strains inhibited by *T. turnerae* strains were not detected in bacteriocytes with *T. turnerae*. While the shipworm gill microbial community had been characterized previously, a micrographic examination of shipworm digestive tissues was lacking. FISH of caecum and intestinal tissues detected very few microbes in the caecum and a rich community of bacteria in the intestines.

The shipworm microbial community is highly specialized for degradation of lignocellulosic substrate and shows high potential for production of bioactive compounds. This unique combination of traits makes the consortium of shipworm symbionts a rich source for discovery of compounds applicable in both medical and cellulosic biofuels technologies.

Chapter 1. An introduction to the xylophagous shipworm symbiosis

What is a shipworm?

The majority of shipworms, xylophagous marine bivalves of the family Teredinidae, are obligate wood-borers that use wood as a source of shelter and food, ingesting the excavated wood particles as they burrow. Due to their voracious appetite for woody substrates, shipworms are thought to play an important role in remineralization of plant matter, carbon cycling and destruction of wooden structures in shallow marine environments (0-150 m), causing greater than one billion dollars of damage annually (Distel 2003). Recently, shipworms have received attention as a source of enzymes applicable in cellulosic biofuels production (Distel et al. 2011).

To date, at least 65 shipworm species have been described comprising 14 genera distributed broadly in temperate to tropical marine and brackish environments across the globe (Distel et al. 2011). Shipworms are very resilient and contain the ability to exist in living, floating, or submerged wood from the inter-tidal zone to depths of up to 150 meters, and to tolerate a wide range of salinities. They have also adapted the ability to degrade a multitude of wood types, thus facilitating their ubiquity in diverse environments. Protandry is common among shipworms, but some species brood their young while others spawn (Turner 1966).

In relation to other bivalve mollusks, shipworms are characterized by an elongated, worm-like body plan with valves (shells) greatly reduced in size and adapted for boring. The valves contain a rasping surface (Figure 1.2d.), which breaks wood into tiny pieces, creating a burrow in which the animal resides as well as

providing a food source for the animal. As a shipworm bores it lays down a calcareous lining to strengthen its burrow. Pallets, calcareous structures, are used to seal off the shipworm tunnel during adverse environmental conditions providing resiliency in the ever-changing shallow marine environment. See Figure 1.1 for photographs of shipworms and Figure 1.2 for an overview of shipworm anatomical features.

Three different types of shipworm anatomy exist, with the greatest variation in digestive system anatomy. The first type is seen in the anomalous, non-wood boring *Kuphus polythalamia*, and is characterized by the lack of a caecum. In the second type a small caecum is present, but woody substrate accumulates in a large anal canal. The third type possesses an enlarged caecum where lignocellulosic material accumulates and is the target of this study (Distel et al. 2011). In shipworms characterized by the third anatomy type, woody substrate is ingested through the mouth, passed through the stomach and accumulated in the caecum. Post-digestion, waste material is transported via the intestine to the excurrent siphon for excretion. Similar to other bivalves, shipworms contain the ability to filter feed. The gills (ctenidium) filter particles from seawater supplied by the incurrent siphon and pass them along the food groove to the mouth and stomach for digestion (Turner 1966).

The challenge of a xylophagous lifestyle

Lignocellulosic biomass is comprised predominately of cellulose (40% by weight of wood), but contains hemicellulose (approximately 30% by weight) and is held together by the highly inert lignin (Dean 1978). The proportion of these three materials varies depending on wood type (Maki et al. 2009). Cellulose is a highly recalcitrant compound, requiring the synergistic activity of at least three cellulase classes for complete breakdown to usable sugars. Endoglucanases (EC 3.2.1.4) cleave

long cellulose strands to smaller units via hydrolysis of β-1,4-glucosidic bonds. Exoglucanases (cellobiohydrolases, EC 3.2.1.91) processively cleave ends created by endoglucanases releasing soluble cellobiose or glucose. Cellobiose molecules are hydrolyzed to glucose by β-glucosidases (EC 3.2.1.21) (Zhang et al. 2006). See Figure 1.3 for details.

Obtaining nutrition from cellulosic substrates is challenging; not only is lignocellulose difficult to break down due to its crystalline structure it is also a poor source of nitrogen. Very few animals subsist on a diet consisting predominantly of recalcitrant cellulosic substrates and even fewer animals are able to produce cellulases themselves. Thus, xylophagous animals typically depend on symbiotic microbes to produce enzymes necessary for efficient digestion of cellulose to simple sugars usable by the host. The hindgut of lower termites and the cow rumen contain diverse microbial communities providing the host with enzymes for cellulose breakdown and supplementing the nitrogen deficiency of the host's cellulose rich diet (Brune and Stingl, 2005, Ohkuma 2003, Hess et al. 2011, Wallace 1994). In contrast, the wood boring marine isopod *Limnoria quadripunctata* contains a sterile caecum and utilizes lignocellulose for nutrition without support of microbes (King et al. 2010).

The mechanism of lignocellulosic degradation and source of usable nitrogen in shipworms has been investigated for decades with much debate over the origin of cellulases detected in digestive tissues. While cellulase activity was detected, evidence of digestive tract microbes was elusive (Greenfield and Lane 1953, Liu and Walden 1970). The isolation of *Teredinibacter turnerae*, a celluloytic nitrogen-fixing bacterium, and localization to bacteriocytes in the gill epithelium of shipworms was a significant breakthrough, leading to the current explanation for the mechanism of

wood degradation in shipworms (Distel et al. 1991, Distel at al. 2002a, Waterbury et al. 1983).

Shipworm endosymbiotic community

Recently, the genome of *T. turnerae* strain T7901 has been sequenced and analysis of genes present shows support for the role of *T. turnerae* in host nutrition. The genome contains a remarkable number of genes devoted to complex polysaccharide degradation, with a significant proportion of glycoside hydrolase genes devoted to degradation of woody plant polysaccharides. In comparison to other notable wood-degrading systems, including *Nausitermes* hindgut metagenome and *T. turnerae*'s closest free-living relative *Saccharophagus degradans*, the overall number of *T. turnerae* glycoside hydrolase genes is not extraordinary, but the specialization for woody substrate is remarkable (Yang et al. 2009). Fifty four percent of glycoside hydrolase (GH) domains in the *T. turnerae* T7901 genome target cellulose or xylan and less than 20% of GH domains are specific to other substrates, including peptidoglycan, laminarin, pectin, and chitin (Yang et al. 2009). See Figure 1.4. The *T. turnerae* genome also includes a complete set of genes for nitrogen fixation (*nif*) and therefore has the capacity to supplement the shipworm's usable nitrogen deficient diet of wood. Another remarkable feature of the *T. turnerae* genome is the large fraction predicted to encode bioactive compounds (Yang et al. 2009). Seven percent of the genome is dedicated to putative secondary metabolite production and is comparable to *Streptomyces coelicolor* and *Streptomyces avermitilis*, known powerhouses of secondary metabolite production (Bentley et al. 2002, Ikeda et al. 2003). Confirming genome evidence, *T. turnerae* in culture has demonstrated the ability to degrade cellulose, (Ekborg et al. 2007, Distel et al. 2003) fix nitrogen, (Distel et al. 2003,

Waterbury et al. 1983), and inhibit growth of other bacteria (Elshahawi and Haygood, unpublished).

T. turnerae is not the only shipworm gill endosymbiont, in fact shipworm gills contain a consortium of closely related endosymbionts. At least four genetically distinct endosymbiont ribotypes have been detected in the gill of a single shipworm specimen (Distel et al. 2002a, Luyten et al. 2006) with *Teredinibacter turnerae* comprising on average 10% of the gill endosymbiont community in the shipworm species *Lyrodus pedicellatus* (Luyten et al. 2006). Current work in the lab of collaborator Dan Distel has focused on analysis of a 16S rRNA clone library of a *Bankia setacea* gill microbial community and shows the presence of multiple closely-related bacterial ribotypes, but lacks a significant population of *Teredinibacter turnerae* (Fung and Distel, unpublished). Although *T. turnerae* is cultivable in the laboratory and has received extensive characterization, the other shipworm endosymbionts have proven resistant to cultivation.

Rationale for the study of shipworm endosymbionts

Bio-based liquid fuel produced from corn or sugarcane (first-generation) biofuels have received criticism due to the direct competition with valuable food sources and the large amounts of water and fertilizer required to produce these feedstocks. Cellulosic biofuels (second generation), which can take advantage of a multitude of feedstocks including yard waste, un-usable by-products of logging, or rice or coconut husks in developing nations, are more sustainable than first generation biofuels however, this technology has progressed at a far slower pace (Gomez et al. 2008). The breakdown of cellulosic feedstock to fermentable sugars has proven a difficult bottleneck in the production of cellulosic biofuels (Sanderson, 2011). The shipworm system is naturally efficient in the breakdown of a wide range of lignocellulosic substrates and thus serves as an ideal target for the discovery of enzymes for the application in second-generation biofuels development.

With the constant evolution of antibiotic resistance in common pathogens a continuing need for novel antimicrobial compounds exists. The majority of bacterially-derived bioactive compounds have been sourced from *Actinomycetes,* leaving groups such as the gamma-proteobacteria understudied. In addition, bioactive compounds from marine organisms are currently underexploited (Piel 2006) and *Teredinibacter turnerae* shows potential, both based on genome content and laboratory experiments, as a target for discovery of secondary metabolites showing activity against other bacteria. The shipworm endosymbiotic community shows potential for discovery of novel antimicrobial compounds.

Understanding the complex physiology of host and endosymbionts will provide an important base for efficient bio-prospecting and subsequent development of

technology. This work will also provide information regarding the ecology of shipworms in the marine environment and their ability to successfully degrade multiple wood types under numerous environmental conditions. The topic of Chapter 3 is cultivation and characterization of additional shipworm symbiont strains. Chapter 4 focuses on spatial localization of symbionts in gill tissue of multiple shipworm species. Interactions amongst cultivated symbionts are presented in Chapter 5. An analysis of microbes in the digestive tract via Fluorescence *in situ* hybridization is presented in Chapter 6.

Chapter 2. Aim of Research

The proposed model for xylophagy in the shipworm symbiosis is characterized by a notable physical separation between the site of lignocellulose accumulation in the caecum and residence in the gill of the symbionts thought to produce cellulolytic enzymes and fix N_2 to forms usable by the host. Molecular and micrographic characterization of the *L. pedicellatus* gill microbial community has revealed the presence of at least four different symbiont types (Distel et al. 2002a, Luyten et al. 2006) and one of these symbionts, *T. turnerae,* has been cultivated (Distel et al. 2002b, Waterbury et al. 1983). While the endosymbionts founding the gills of shipworms have been extensively characterized using micrographic and molecular methods, the shipworm gut microbiota has not.

Cultivation of additional members of the gill endosymbiont community, localization of symbionts in gill tissue of multiple shipworm species, and a micrographic examination of the digestive tract are aims of this study. This work will extend our knowledge of the shipworm symbiosis to additional symbiont strains, shipworm species, and tissues and will provide a more comprehensive understanding of the symbiosis as a whole. Questions addressed in the work presented here include: Can we cultivate shipworm symbiont strains in addition to *T. turnerae*? Do the other symbionts have the same physiological characteristics as *T. turnerae*? Do the shipworm symbionts known through molecular methods in *L. pedicellatus* inhabit gills of additional shipworm species? Is there a significant microbial community in the shipworm digestive tract?

Chapter 3. Isolation and characterization of shipworm gill endosymbionts.

3.1 Introduction

The fact that a vast majority of microbes (99.99% of marine bacteria) have thus far resisted cultivation and can be termed as-yet unculturable is widely accepted amongst microbiologists (Button et al. 1993, Zengler 2009). This "great plate count anomaly" has led to the development of many cultivation-independent methods, such as SSU rRNA sequence analyses useful in the study of microbial communities (Amann et al. 1995). While cultivation-independent analyses provide valuable information describing community composition, there are questions regarding physiology that can only be answered through the cultivation of microbes in the lab (Zengler 2009, Zengler et al. 2002). Common factors leading to the challenge of laboratory cultivation are improper nutrients in the growth media, pH, incubation temperature, oxidative stress, osmolarity, and impatience in detecting growth of slow growing bacteria (Zengler 2009). In this chapter I present attempts to isolate previously uncultivated shipworm symbionts.

The gill endosymbiont community of *Lyrodus pedicellatus* has been studied extensively and is comprised of at least four bacterial ribotypes, one of which is the cultivated *Teredinibacter turnerae* and the others are not yet cultivated (Distel et al. 2002a, Luyten et al. 2006). Analysis of a SSU rRNA clone library from *Bankia setacea* gill also shows multiple bacterial ribotypes closely related to *T. turnerae*, none of which had previously been extensively cultivated in the lab (Fung and Distel, unpublished). The proposed role of shipworm gill endosymbionts in host nutrition is to provide enzymes facilitating digestion of cellulosic substrate and fixing atmospheric

N_2 to a form usable by the host, the latter has been demonstrated *in situ* (Lechene et al. 2007). *Teredinibacter turnerae* has demonstrated the ability to fix nitrogen and produce cellulases in culture, but are all gill endosymbionts able to do this? Thus far the role of the entire symbiotic community has been inferred based upon characteristics of the cultivated representative, *T. turnerae*.

With 65 shipworm species (Distel et al. 2011), all of which are presumed to harbor multiple *T. turnerae*-like gill endosymbionts, we have only examined the tip of the iceberg. Shipworms have adapted to inhabit numerous wood types in varying environments globally and are therefore an ideal target for bio-prospecting enzymes useful in degradation of an assortment of cellulosic feedstocks and bioactive compounds. Work presented in this chapter will focus on cultivation of novel shipworm gill endosymbionts and phylogenetic characterization of isolates.

The SSU (16S) rRNA gene is a useful phylogenetic marker due to its ubiquity and high conservation across all living organisms, thus this gene was sequenced and utilized in phylogenetic analysis of isolates obtained. This will allow us to evaluate our capacity for cultivating novel strains and to guide future efforts in bacterial isolations from shipworm gill tissues. Correlation of phylogenetic relationships of symbiont strains with observations of physiological characteristics will provide information useful in understanding the roles of the non-*T. turnerae* symbiont types in the symbiosis.

3.2 Materials and Methods

Shipworm Collections

Shipworm specimens utilized in this study were collected in Yaquina Bay, OR; Puget Sound, WA; Hydaburg, AK; and various locations in the Philippines from

ecosystems supplied with wood from coniferous forests to mangroves. See Table 3.1 and Figure 3.1 for more details. Shipworms were obtained either by setting baits or collecting found wood in the targeted area that showed evidence of shipworm inhabitation, either holes or calcareous tubes. Shipworm baits consisted of either one piece or many pieces of wood attached to rope and were left out in the water for a period of 6-12 months.

In Yaquina Bay, OR, traps containing a total of 5 or 6 boards, either untreated pine 2x4 boards or driftwood collected from the area, were tied onto the sedentary *WetLabs* research vessel docked in the harbor in Newport, OR. Access to this location was authorized by Alex Derr of Satlantic/*Wetlabs* in Corvallis, OR. In Hydaburg, AK shipworm traps were deployed by students from Hydaburg elementary and high schools under the guidance of collaborator Wendy Smythe. These traps contained 6 pieces of either untreated red cedar 2x4 boards or pieces of driftwood collected in the area and were attached to docks in the Hydaburg Harbor. Traps remained in the water for 9-12 months. Following retrieval of traps, boards containing shipworms were transported to the lab wrapped in damp paper towels on ice. In the lab shipworms were maintained in an aerated aquarium at 10°C until they were sacrificed.

In the Philippines traps were deployed in mangrove and reef ecosystems by local fisherman Joe Arbasto and Romell Seronay with guidance and assistance from Dr. Dan Distel and a team of our collaborators from the University of the Philippines-Diliman, Marine Science University. These traps remained in the water for an average of 10 months and were processed as soon as possible after retrieval. Shipworm inhabited wood from the mangroves was also collected.

Shipworms used in this study were also provided by Ocean Genome Legacy and were collected by Tim Nesseth in Puget Sound, WA.

Isolation of bacterial strains from shipworm tissues.

Extraction of shipworms from wood involved the use of common tools including pliers, axes, and chisels with enough force to break up the wood, but caution to avoid damaging the specimen. During this process shipworms were kept cool by frequent bathing in cold, sterile seawater. Once the specimen was freed from the wood it was rinsed 3 times in sterile seawater and kept on ice until dissection. Small specimens were dissected under a dissecting microscope with fine forceps. First the pallets and valves were removed. The shipworm mantle was then opened using the forceps to tear the tissue from posterior end (near the siphons) to the anterior end on its ventral side to expose organs of interest (caecum and gill), which were then removed and rinsed at least 3 times in cold, sterile, seawater. The tissue was then homogenized in homogenization buffer (See Appendix B for experiments regarding this) using a Dounce homogenizer, diluted in homogenization buffer and 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3} diluents spread onto agar plates.

Shipworm Basal Medium (SBM) 1% agar plates described by Distel et al. (Distel 2002b, Waterbury 1983) with variations was used as growth medium. Usually, the plates contained 0.2% (w/v) Sigmacel 101 (Sigma Aldrich) cellulose as the sole carbon source, but isolation attempts included birch xylan (0.2 % w/v) and a mixture of sucrose, cellobiose, glucose and cellulose with a total concentration of 0.5% (w/v). The standard pH of SBM used was 8.0, but SBM plates with pH ranging from 7.0-8.5 were tested in isolations. Although *Teredinibacter turnerae* possesses the ability to fix atmospheric N_2, 5 mM ammonium chloride was added as a source of combined

nitrogen to facilitate growth of possible non-N_2 fixing endosymbionts. Sterile SBM without agar or carbon source was used as the homogenization buffer during homogenization of tissues. The dissection and plating of all Philippine samples discussed here were carried out in a temporary laboratory at the collection sites in the Philippines by Dr. Daniel Distel, Dr. Abigail Fusaro, and the MSI microbiology team (Malem Flores, Clarisse Quimio, Renan Pamisan, Meriliza Tare, Jem Lapitan, Gwen Limbaco).

Following spreading of tissue homogenate, mother plates were incubated at 10°C, 18°C, room temperature (20-25°C), or 30°C. Once colonies began to appear (1 week to ~4 weeks for slow growing *B. setacea* isolates), individual colonies were picked and re-streaked for isolation on fresh SBM plates. Individual colonies were picked and re-streaked until the strain was pure, approximately 3 times. Purity was determined by observation of colonies with a dissecting microscope and culture wet mounts with an optical light microscope. When an axenic culture was obtained it was transferred to liquid SBM with 0.2% cellulose and ammonium chloride in 15 mL glass test tubes and incubated with shaking (125 RPM) in an orbital shaker at 30°C for preservation in glycerol stocks.

In one attempt to cultivate novel shipworm symbiont strains, *T. turnerae* T7901 culture spent media (cells were spun down and supernatant removed) was spotted onto paper diffusion discs placed on top of the plate post inoculation with gill tissue homogenate in an attempt to stimulate growth of the previously un-cultivated symbionts. Sterile SBM was added to one disc per plate to act as a control.

Growth Curves of Gill Isolates

During my visit to Ocean Genome Legacy Marvin Altamia and I created growth curves for 34 gill isolates. Cultures were grown in liquid SBM as described above and optical density at 600 nm wavelength (OD 600) was measured every 12 hours. Information gathered during this experiment regarding growth rate and physical appearance of culture during exponential growth was applied to the growth of isolates for freezer stock preparation. When broth cultures of strains for preservation began to transition from log growth phase to stationary phase, freezer stocks were created by the addition of 1 mL culture to 250 µL sterile 50% glycerol with subsequent freezing at -80°C. All strains discussed in this thesis have been stored in the Haygood-Tebo strain collection at OHSU and strains originating from Philippine specimens are also stored in the strain collection at Ocean Genome Legacy.

Cellulose degradation capacity in isolates

In this study preliminary screening for cellulase activity in novel shipworm isolates was carried out. Although all strains were isolated from a growth medium containing cellulose as the sole carbon source, it could not be assumed that isolates were growing on cellulose. Other possible sources of nutrition include agar or biomass transferred during inoculation of the plates. Test tubes containing liquid SBM with insoluble cellulose, Sigmacel 101 (Sigma Aldrich), were inoculated with strains and a change in the morphology of the cellulose from a fine powder to a clumpy mat and turbidity in the liquid indicated growth of a strain.

Cellulose is insoluble and remains suspended as particulates in the agar, thus making the medium cloudy. As the cellulose is degraded a zone of clearing can be

detected surrounding the colony. The degree of cellulose clearing on agar plates, as well as the rate at which this appeared were factors considered in determining the potential for cellulose degradation in isolates. This provided for qualitative, but not quantitative results. More extensive characterization is a goal of the MSI microbiology team as a part of the PMS-ICBG project.

Assessment of N_2 fixation capability in novel isolates

To test for the ability of cultivated strains to fix atmospheric N_2 an individual colony was transferred to SBM soft agar tubes (Distel et al. 2002b, Waterbury et al. 1983), which contained SBM plus 0.2% cellulose as the sole carbon source, 0.2% agar, and no added combined nitrogen source. Strains were also transferred to soft agar tubes containing ammonium chloride to act as a control in this experiment. After inoculation cultures were incubated at room temperature until a disc of cellulose clearing (denoted in Figure 3.7) was detected, indicating growth on atmospheric nitrogen (Waterbury et al. 1983). Transfer of mixed colonies to soft agar tubes minus ammonium chloride with subsequent streaking onto agar plates also proved useful in purification of some N_2 fixing isolates.

DNA isolation, amplification, and sequencing for phylogenetic analysis

Amplification of DNA directly from individual colonies on agar plates containing pure cultures was used for all isolates. Phire Animal Tissue Direct PCR Kit (Thermo Scientific, Finnzymes) was used for direct amplification of shipworm gill isolate colonies, which commonly growth beneath the surface of the agar. A 5 µL pipette tip was used to remove an individual colony, including some agar, and place it in a tube containing 20 µL Phire Dilution Buffer plus 0.5 µL DNA Release Additive. Samples were vortexed, incubated 5 minutes at room temperature, incubated at 98°C

for 2 minutes, and then placed on ice. This mixture acted as the template in subsequent PCRs. 16S rRNA genes were amplified by PCR in 50 µL reactions containing 15 µL ddH_2O, 25 µL 2x Phire Buffer, 1 µL Phire Polymerase, 5 µL DNA template and 2 µL each bacterial-domain-specific primers 27f (AGAGTTTGATCATGGCTCAG) and 1492r (GGTTACCTTGTTACGACTT) (Lane, 1991). Post amplification samples were purified using the Qiagen PCR Purification Kit according to the manufacturer's protocol and concentration determined using the NanoDrop ND-1000 (Thermo Scientific). Sequencing of 16S rRNA genes with 27f and 1492r was performed at the OHSU Oregon National Primate Research Center Molecular Biology Sequencing Facility on an ABI3700XL sequencer (Applied Biosystems) or at Beckman Coulter Genomics Inc., Danvers, MA on a Sanger sequencing platform.

Phylogenetic Analysis of gill isolates

All sequence alignment and phylogenetic analyses were carried out using Geneious software (Biomatters Ltd., Drummond et al. 2010). Forward and reverse sequence data in ABI chromatogram format was imported, inspected for quality by eye, and contigs were created using the Geneious assembler tool with default settings, including the exclusion of quality-trimmed ends. Geneious global alignment tool with default settings was used to align novel gill isolate sequences to publicly available gill endosymbiont 16S rRNA gene sequences and various publicly available gamma-proteobacterial strain 16S rRNA gene sequences. Post-assembly and post-alignment of the resulting contigs and alignments were visually inspected and edited to ensure proper alignment.

Several different methods for phylogenetic inference exist, including those that use a distance matrix to estimate evolutionary distance (Neighbor Joining) and those that use discrete character states (Maximum Likelihood) and trees are generated randomly and searched based on an optimality criterion to come up with the most likely phylogenetic representation for the sequences (Holder and Lewis 2003). The divergence of sequences can be inferred by counting the number of dissimilarities between them, but this does not account for multiple substitutions at the same site or sequence reversals. To overcome this, nucleotide substitution models are used to infer actual genetic differences between sequences. The Jukes Cantor genetic distance model assumes the rate of nucleotide substitution and nucleotide frequency is constant, the Kimura model accounts for different rates of transitions and transversions, and the Hasegawa Kishino and Yano (HKY) model accounts for both the different rates of transitions and transversions and different base frequencies (Swofford and Sullivan 2003).

In this study, Geneious Tree Builder Neighbor Joining tree build method with the Jukes-Cantor genetic distance model was used most commonly in the analyses presented here. In addition to phylogenetic inference with the Neighbor Joining tree build method with the Jukes-Cantor genetic distance model, a single multiple sequence alignment containing shipworm isolate sequences and publicly available reference sequences, listed in Table 3.3, was subjected to the Maximum Likelihood tree building method utilizing the Jukes Cantor, HKY, or Kimura genetic distance models performed by the PHYML (Guindon & Gascuel, 2003) plugin in Geneious (Holder and Lewis, 2003). The resulting phylogenetic trees were compared to determine whether major differences in clustering resulted from the different phylogenetic inference methods.

Bootstrapping, performed in Geneious, was used as an assessment of support for the phylogenetic relationships represented on trees (Felsenstein 1985).

3.3 Results and Discussion

Collection of shipworms

In baits placed in both Yaquina Bay, OR and Hydaburg, AK shipworm inhabitation was detected after 9 months, but it is likely this length of time was not necessary. Due to the distance from the lab, trips to the collecting site were not frequent enough to determine an exact time period for shipworm settlement on traps. We also had success scavenging wood below the average low tide mark during an extreme low tide in June 2009, although this method is not as reliable as baits. In the Philippines, both baits and scavenging of mangrove wood have provided many shipworms. During the January 2011 PMS-ICBG collection trip effort was made to correlate wood type with diversity of specimens collected, but information regarding the source of wood was not recorded for specimens discussed here. This information will be valuable when planning future collecting trips and assessing the difference in symbiont communities resulting from diets of various wood types.

Cultivation strategies

These results show that through brute force efforts previously un-cultivated shipworm gill strains can be isolated using the standard shipworm symbiont cultivation procedure described above (Distel 2002b, Waterbury 1983), but that the current isolation methods select for *T. turnerae* strains in warm water shipworm species of the Philippines. Although it is known that *T. turnerae* only makes up approximately 10% of the shipworm gill symbiotic community in *Lyrodus pedicellatus* (Luyten et al. 2006), 90% of strains were *Teredinibacter turnerae*. The relative

abundance of *T. turnerae* in other shipworm species is currently unknown and could be a potential explanation for this discrepancy. The fact that novel strains were isolated only from mother plates where *T. turnerae* was non-existent lead to the hypothesis that *T. turnerae* could be inhibiting growth of other strains during the isolation process.

Results of the isolation in which *T. turnerae* T7901 culture supernatant was spotted onto mother plates showed zones of clearing of colonies surrounding the discs containing the T7901 supernatant. This is the opposite of the result I was expecting and supports the hypothesis that *T. turnerae* strains are inhibiting growth of other strains during the cultivation process. In the control growth extended to the diffusion discs containing sterile SBM. See results in Figure 3.8.

In the case of cultivation of *B. setacea* isolates, decreasing the incubation temperature (18 or 10°C from 30°C) was fruitful in increasing isolate diversity as it yielded five novel isolates and provided cultured representatives of two additional clades. This is not surprising given the average temperature in the host's natural environment of 10-20°C. SBM agar plates at pH 7 and 8 were used in one isolation and resulted in the cultivation of 3 strains each, thus varying the pH over this range does not have an effect on growth of *Bankia setacea* gill endosymbionts. As these strains grow at a very slow rate, a lot of patience was another key factor in their isolation. The addition of multiple carbon sources to SBM growth media resulted in an abundance of growth on the plates. This made it very difficult to pick individual colonies and those that were isolated showed 16S rRNA gene sequence of high similarity (>99%) to common marine bacteria, and therefore were not shipworm symbionts.

Novel Isolates

The PMS-ICBG collaboration has obtained (as of April 2011) 425 shipworm gill and digestive tract isolates on Shipworm Basal Medium (SBM) and a total of 866 total shipworm isolates on a variety of media originating from at least 7 Philippine shipworm species. Of these, 78 isolates originating from Philippine shipworm gill tissue were further analyzed in this study. In addition, a total of 24 isolates were obtained from the cold-water shipworm, *Bankia setacea*, collected in Puget Sound, WA and Hydaburg, AK (## isolated and characterized by Marvin Altamia). Partial (1022 nucleotides) 16S rRNA gene sequences from the 78 isolates from Philippine shipworm gill tissue and 24 *B. setacea* gill isolates were used in phylogenetic analysis.

Phylogenetic analysis shows a close evolutionary relationship of new isolates to shipworm gill clone sequences (Distel et al. 2002a, Luyten et al. 2006, Sipe et al. 2000, Fung and Distel unpublished). We now have cultured representatives covering 5 clades, which is a great improvement over the previous cultivated strains, all of which have been identified as belonging to the single *T. turnerae* clade (Distel 2002b). Phylogenetic relationships are shown in Figures 3.2-3.5. These strains will serve as useful tools for the further exploration of the role of symbionts in maintaining the shipworm symbiosis.

The majority (90%) of isolates from Philippine collected specimens cluster with the *T. turnerae* strains (Clade 1) with greater than 99% sequence similarity within the cluster. Four strains (PMS 1162T), all originating from the same specimen un-identified at the species level, fall into a group with previously un-cultivated *Lyrodus pedicellatus* gill clones, Clade 2 (Luyten et al. 2006, Distel et al. 2002). Although the *L. pedicellatus* clone sequences show the closest similarity to the PMS

1162T strain sequences, sequence similarity is only 96% over 1286 nucleotides. Three of four PMS 1162T strains contain identical 16S sequences and the fourth differs at only two nucleotide positions. Two additional strains (PMS 486K.S.0.12 and PMS 1120W.S.0.4, sequenced by M. Altamia) do not group with any of the main clades, but fell basal to the *T. turnerae* clade. Interestingly, no strains belonging to the *T. turnerae* group were isolated from the mother plates of which the 4 PMS 1162T strains arose.

This analysis showed that the strains tended to group according to host species. While the majority of warm-water symbionts grouped closely with the *T. turnerae* strains in Clade 1 (shown in Figure 3.3) or *L. pedicellatus clone* sequences in Clade 2 (shown in Figure 3.4), none of the isolates obtained from *B. setacea* specimens grouped with *T. turnerae*, but instead fall into 3 distinct clusters (Clades 3, 4, and 5) and are shown in Figure 3.5. Two clusters each contain one of the two dominant ribotypes (Bs 6G and Bs7G) from the extensive *B. setacea* clone library analysis performed by collaborator Jennifer Fung (unpublished) and the others are closely related to the clone sequence published by Sipe et al. (2000). PMS 486K.S.0.12 was isolated from a *Dicyathifer manni* host. This shipworm contains the anatomy in which cellulosic substrate is accumulated in an enlarged anal canal rather than the caecum making it unique in comparison to hosts of other isolates included in this analysis.

It is possible that the difference in habitats between the Pacific Northwest and the Philippines influences symbiont type. The exact mechanism of symbiont transmission is not clear and could offer an explanation for the difference in symbiont communities across shipworm species.

Colony Morphology

Colony morphology for all isolates can be described as predominantly sub-surface and white or off-white in color. Young colonies are almost translucent and can be very difficult to detect on white Sigmacell containing plates. Many colonies have the appearance of an iceberg with most of the colony below the surface and a tip extending above the surface. During extensive laboratory cultivation, surface growth of isolates that originally grew only subsurface became more common. Colonies produce a ring of cellulose clearing when grown on Sigmacell containing agar plates. Upon first glance all shipworm symbiont isolates appear similar in colony morphology, but with closer analysis differences can be detected. See Figure 3.6. Clade 1 (*T. turnerae*) colonies have been termed "flaky" and are characterized by rough outer edges and tend to produce yellow or brownish pigments as they age. Isolates in Clade 2 are white in color and have the appearance of a halo, with a whiter center, under the microscope. *B. setacea* strains in Clade 3 are more similar to Clade 2 and Clades 4 and 5 have a flaky morphology similar to Clade 1. Clade 4 does not produce pigment and Clade 5 produces a purple pigment when it ages (Marvin Altamia, unpublished). Individual cells of PMS 1162T (Clade 2) strains are cocci and Clade 1 cells are rod shaped.

The *T. turnerae* strains exhibit a wide range of production of yellow pigments during growth. Figure 3.7 demonstrates the differences in pigment production during growth of strains in SBM-N+Sig 0.2% agar tubes. The variety in physical characteristics of Clade 1 strains demonstrates that the phenotypic characteristics within the clade are more diverse than phylogenetic analysis of 16S rRNA genes predicts and is consistent with results of a Multi-Locus Sequence Analysis (MLSA) of *T.*

turnerae strains (Marvin Altamia, Nicole Wood, Dan Distel, unpublished) which shows diversity within the *Teredinibacter turnerae* clade. This can be used as motivation for additional characterization and bio-prospecting within the *Teredinibacter turnerae* group.

Metabolic variation

Cellulolytic activity

A difference in the ability to degrade cellulose amongst representatives of the clades exists. Members of Clade 1 (*T. turnerae* strains) possess the most voracious appetite for cellulose, creating large clearings of Sigmacell in agar plates. Members of Clades 2, 3, and 4 are all capable of cellulose degradation, as they do grow with Sigmacell as a sole source of carbon and also create cellulose clearings on agar plates, but these isolates do so at a notably slower rate than the members of Clade 1. Note that these results are qualitative and further quantitative analysis is important. The endosymbionts proposed to produce cellulases responsible for lignocellulose degradation in the digestive tract and represented by strains discussed here reside in gill tissue, physically removed from the source of cellulosic substrate. Thus, in their natural environment it is unlikely that these strains utilize cellulose in their nutrition and rather use a simple sugar, such as glucose provided by the host. Micro-array experiments performed by Brian Fishman (data unpublished) on *Teredinibacter turnerae* T7901 show little differential expression of cellulase-encoding genes in this symbiont when sucrose or cellulose is the provided carbon source. Symbionts may be more efficient producers of cellulases when they are provided with a simple sugar, a carbon source more similar to that of their natural environment, rather than solely cellulose and could be an explanation for the decreased amounts of Sigmacell clearing

by Clade 2, 3, and 4 strains under the culture conditions used here. Altering growth temperature could also affect the degree of cellulose degradation by these strains.

The complete degradation of crystalline cellulose is a very complex process, requiring a suite of enzymes acting synergistically: endo-1,4-β glucanases break β(1->4)-glucosidic linkages, exo-1,4-β-glucanases (cellobiohydrolase) cleave cellobiose or glucose from the non-reducing end, and 1,4-β-glucosidases hydrolyze cellobiose to glucose (Eriksson et al. 1990, Zhang et al. 2006). Both enzymes CelA, purified from *Psiloteredo healdi* symbiont *T. turnerae* T8201 and CelAB, purified from *Lyrodus pedicellatus* symbiont *T. turnerae* T7902, show potential for combined endoglucanase and cellobiohydrolase activity, but many cellulases perform only endoglucanase, cellobiohydrolase, or β-glucosidase activity (Ekborg et al. 2007, Lynd et al. 2002). While some members of the shipworm endosymbiont community may possess the ability to completely break down crystalline cellulose, it is possible that other community members are specialized in only one step of the overall cellulosic breakdown process. This is a potential explanation for the decreased degree of clearing of Sigmacell by strains from Clades 2, 3, and4. Cellulase assays for each enzyme class and genomic analyses are essential in determining the cellulolytic capacity of symbiont types.

N_2 Fixation

Interestingly, unlike *Teredinibacter turnerae,* not all cultivated strains contain the ability to fix atmospheric N_2. Neither the cultivated strains in Clade 2 (PMS 1162T strains) nor the *B. setacea* strains in Clade 3 are able to grow in SBM soft agar tubes

without a combined nitrogen source, thus do not contain the ability to fix atmospheric nitrogen into usable forms. All cultivated members of the *Teredinibacter turnerae* group, Clade 1, and the *B. setacea* strains in Clade 4 have demonstrated the ability to grow utilizing atmospheric N_2 as a sole source of nitrogen. Lechene et al. (2007) applied multi-isotope imaging mass spectrometry (MIMS) with stable isotope N^{15} to show nitrogen fixation by shipworm endosymbionts and transfer to the host, but also found differences in the amount of nitrogen fixation by endosymbionts residing in different gill bacteriocytes in *Lyrodus pedicellatus*. Distel et al. (2002a) showed that endosymbionts segregate themselves into different bacteriocytes in *L. pedicellatus* gill tissue according to phylogenetic identity. This in combination with results of the analysis of N_2 fixation ability in gill endosymbionts in culture showing that only some symbionts are able to fix nitrogen offers an explanation for the variation in N_2 fixation across bacteriocytes observed in MIMS results (Lechene et al. 2007).

The proposed roles of the shipworm gill endosymbionts are in facilitation of digestion of ingested lignocellulosic substrate and in supplementing this nitrogen-deficient diet by providing the host with usable forms of nitrogen. Previously and recently cultivated and characterized *Teredinibacter turnerae* strains show proficient aptitude in both cellulose degradation and nitrogen fixation. In contrast, cultivated shipworm endosymbionts grouping outside the *T. turnerae* clade have displayed lesser degrees of cellulose degradation and lack the ability to transform atmospheric N_2 to forms usable by the host. It has been hypothesized that *Bankia setacea*, not thought to harbor *Teredinibacter turnerae*, contains a symbiont of physiological abilities similar to *T. turnerae*, yet none of the strains assessed here have shown the same potential for degradation of crystalline cellulose. This could be due to culturing conditions and

should be further analyzed. These differences in metabolic characteristics are suggestive of a division of labor amongst gill endosymbionts, in which symbionts play distinct roles in the overall collaborative contribution to host metabolism. This is not unheard of in symbiotic systems, for example methanotrophic and thiotrophic symbionts co-exist in the gills of deep-sea hydrothermal vent mussels (Distel et al. 1995) and the marine gutless oligochaete, *Olavius algarvensis*, contains both sulfate reducing and sulfide oxidizing endosymbionts (Dubilier et al. 2001).

In summary, previously uncultivated bacterial strains closely related to known shipworm symbionts have been isolated from the gills of shipworms from the Philippines and the U.S. Pacific Northwest. We now have strains representative of 5 shipworm symbiont clades. All strains have the demonstrated ability to degrade cellulose, but not all are able to fix N_2 under the growth conditions tested.

Work presented in this chapter provides rationale for further efforts in cultivation of shipworm endosymbiont strains from additional shipworm species, including more specimens with the anatomy characterized by an enlarged anal canal. In this study, physiological characteristics of uncultivated strains detected solely through molecular methods have been inferred from characteristics exhibited by cultivated strains within each phylogenetic clade. The cultivation and phenotypic analysis of additional symbiont strains will provide a more comprehensive understanding of the role of symbionts in this symbiosis. Genomic analysis of novel isolates and metagenomic analysis of gill communities from additional shipworm hosts will provide information useful in analyzing the role of symbionts in the shipworm symbiosis.

Chapter 4. Endosymbiont inhabitation patterns in shipworm gills

4.1 Introduction

The shipworm gill (ctenidium) functions in both respiration and feeding, as is typical of most bivalves. While shipworms receive most attention due to their xylophagous lifestyle, most species also possess the ability to filter feed (Turner, 1966). Gill structure is eulamellibranch, consisting of long, folded filaments with cilia at the end used to draw in water to obtain oxygen and food particles (Distel et al 1991, Turner 1966). See diagram of gill anatomy in Figure 4.1. A food groove present on the gill is used in transporting food particles collected in the gill to the mouth. As depicted in Figure 1.2, the shipworm gill extends a large percentage of its body length. The gill extends posteriorly to the siphons and depending upon the species, reaches to the anterior portion of the visceral mass or just posterior to the caecum (Turner 1966).

Shipworms harbor an extensive community of closely related γ-proteobacterial endosymbionts within specialized cells, bacteriocytes, in their gills (Distel et al. 1991, Distel et al. 2002, Luyten et al. 2006). Anatomically, this is not unique amongst marine mollusks. For example, lucinid clams (Gros et al. 2000, Lechaire et al. 2008) and deep-sea hydrothermal vent mussels (Distel et al. 1995) contain nutritionally important endosymbionts in bacteriocytes within their gills. The *Lyrodus pedicellatus* gill endosymbiotic community consists of at least four distinct ribotypes (Luyten et al. 2006, Distel et al. 2002a) and these endosymbionts have been localized to gill tissue and shown to segregate themselves into bacteriocytes according to phylogeny (Distel et al. 1991, Distel et al. 2002a).

Bacterial localization studies undertaken thus far have involved colony-reared *Lyrodus pedicellatus* (Distel et al. 1991 and Distel et al. 2002a), yet evidence of variations in the composition of shipworm gill communities across species is surfacing, most notably in *Bankia setacea* (Fung and Distel, unpublished). The goal of the work presented in this chapter was to survey gill tissue of additional shipworm species, as well as wild *Lyrodus pedicellatus* for bacterial localization. Detection of newly isolated strains designated PMS 1162T discussed in the previous chapter in shipworm gills was also addressed here.

4.2 Materials and Methods

Specimen collection and preparation for *in situ* hybridizations

Specimens were collected as described in Chapter 3. All specimens with the exception of one very large *B. setacea* (Bs4) were fixed whole. Bs 4 gill tissue was dissected from the rest of the specimen and fixed individually. See Table 4.1 for specimens used. All samples were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, 10 mM Na_2HPO_4, 2.7 mM KCl, 140 mM NaCl, pH 7.4) at 4°C overnight. Samples were transferred to 70% ethanol and stored at -20°C for storage. Prior to paraffin embedding and sectioning it was necessary to remove the calcareous parts (valves and pallets). For specimens large enough, pallets and valves were removed using fine forceps with care not to damage the tissue. In most cases, physical removal of pallets and valves was not possible without damaging tissues, therefore it was necessary to dissolve the pallets and valves in 5% acetic acid.

For preparation of tissue sections suitable for fluorescence *in situ* hybridizations, each tissue sample (whole animal or individual tissue) was embedded in paraffin, sectioned to either 5 or 10 μm, and placed on glass slides (Superfrost Plus,

Fisher Scientific) at the OHSU Oregon National Primate Research Center Histology Core facility. A subset of sections from each sample was stained with haematoxylin and eosin to facilitate identification of tissues of interest. To provide for the most complete analysis of specimens cross-sections and sagittal sections were included in this study.

Probe design

ARB software (Ludwig et al. 2004) was used to design a probe complementary to all publicly available shipworm endosymbiont 16S rRNA sequences for application in a survey of bacterial localization in multiple shipworm species. Geneious software (Biomatters Ltd., Drummond et al. 2010) was used to design a probe suitable for Fluorescence *in situ* Hybridizations (FISH) specific to the 16S rRNA gene sequences of 4 PMS 1162T strains for detection of this newly cultivated symbiont group. The probe, 1162T (T_m =58°C), contains at least 3 centralized base pair mismatches to all known shipworm symbiont 16S rRNA sequences and is not complementary to any non-target sequences in the Ribosomal Database Project (RDP) database. The corresponding negative control probe, 1162T NON, contains two internal base pair mismatches to the 1162T sequence. The probe LP4 sequence (Distel et al. 2002a) was checked against newly obtained 16S sequences using Geneious to ensure that its published specificity for the LP4 group containing the *Teredinibacter turnerae* strains and equivalent to Clade 1 discussed in this work was still true. The LP4 and 1162T probes are complementary to the *E. coli* reference sequence at nucleotide positions 637-662 and 637-665 respectively.

Localization of bacteria by fluorescence *in situ* hybridization

Prior to application on shipworm tissues, hybridization of each probe to its target strains and non-target strains was examined and hybridization conditions were optimized. Axenic cultures of PMS 1162T.S.0a.2, PMS 1162T.S.0a.5, PMS 1178H.S.0a.5, and PMS 926K.S.0a.3 were used to test the specificity and optimize hybridization conditions for 1162T (specific to PMS 1162T strains), 1162T Non, LP4 (specific to Clade 1), and LP4 Non probes. A *Teredinibacter turnerae* T7901 culture was used to test the specificity and optimization of the ShipSymb1273 and ShipSymbNon probes. Probe optimization included a formamide concentration gradient from 30%-50% in the hybridization buffer and hybridization times of 2, 3, and 4 hours. A general bacterial probe, Eub338 (Amman et al. 1990), was used to survey the tissues for all bacteria. ShipSymb1273 was used to survey shipworm species not previously analyzed with FISH for known shipworm endosymbionts. LP4 (Distel 2002a) and 1162T probes were used to detect symbionts of Clades 1 and 2 respectively. Probes Symb a and Symb b (Jennifer Fung, unpublished) were applied to detect *Bankia setacea* symbionts of Clade 3 and 4 respectively. All probes were labeled with fluorescein isothiocyanate (FITC), cyanine 3 (Cy3), or cyanine 5 (Cy5) fluorophores. See Table 4.1 and Figure 4.4a for details of probe specificity.

Cells from cultures were applied to black polycarbonate filters and filters were set on clean slides for hybridization. Shipworm tissue sections were hybridized directly on the slides. Prior to hybridization paraffin was removed from sections with xylenes (5 minute incubation in xylenes, slow rinsing in 95% ethanol and MilliQ water). FISH was performed in a hybridization buffer containing in all cases 0.9 M NaCl, 20 mM Tris-HCl [pH 7.4], 0.01% sodium dodecyl sulfate (SDS) and 35% formamide for all probes except 1162T and 1162T Non, which gave the best results with

45% formamide. All probes were at a final concentration of 5 ng/µL. Hybridization was at 46°C for 2 hours or 4 hours when 1162T was used. Post hybridization, the slides were incubated at 48°C in wash buffer (0.7 M NaCl or 0.35M with 35% or 45% formamide as appropriate, 20 mM Tris-HCl [pH 7.4], 50 mM EDTA, 0.01% sodium dodecyl sulfate) for 20 minutes or 40 when hybridization time was 4 hours. After washing, slides or filters were rinsed in MilliQ water to remove wash buffer and air-dried. Prior to imaging samples were mounted in a 4:1 Citifluor (Citifluor Ltd. London, UK): Vectashield (Vector Labs, Burlingame, CA, USA) mounting medium.

A Zeiss Axio Imager.M1 laser scanning confocal microscope with probe-appropriate excitation wavelengths and LSM 5 Pascal Version 4.0 imaging software (Carl Zeiss, Oberkerchen, Germany) were used for visualization and image capture. Confocal microscopy allows for focusing at a very fine plane (on a nanometer scale) with the exclusion of out of focus fluorescence and optical sectioning through multiple millimeter-thick specimens. A mixed krypton/argon laser is used to excite at wavelengths of 488, 568, and 647 nm. Different emission wavelengths are then detected through appropriate filters for image capture. After image capture, false colors, selected to accommodate most common color vision deficiencies, were artificially assigned to the detected signals. All imaging settings were held constant during capture of positive and negative control samples to ensure signal detected was indeed a result of bound probe.

Fifty images of gill tissue from 7 specimens covering 5 shipworm species were chosen at random and used to quantify results. The number of bacteriocytes stained with symbiont targeted probes (ShipSymb1273, LP4, or 1162T) were counted and

divided by the number of bacteriocytes stained with the universal bacterial probe Eub338 in each image.

4.3 Results and Discussion

Localization of known symbionts in multiple shipworm species

Fluorescence *in situ* hybridizations of shipworm gill tissue from wild *Lyrodus pedicellatus* (collected in Bohol, the Philippines, not from the OGL colony)*, Lyrodus massa,* an unidentified *Lyrodus species* from the Bohol Sea, the Philippines, *Teredo aff. triangularis,* and *Bankia setacea* with the shipworm symbiont specific probe ShipSymb1273 revealed the presence of known shipworm symbionts in each of the species analyzed. Although known shipworm symbionts were detected in each species analyzed, bacteriocytes containing bacteria (detected by the universal bacterial probe) were also present in each species. The ShipSymb1273 probe hybridized to an average of 60% of all bacteria containing bacteriocytes detected with the bacterial probe Eub338. The ShipSymb1273 probe hybridized to very few or no bacteriocytes in the anterior and posterior reaches of gill tissue. See Figure 4.3.

At the time of the design of ShipSymb 1273 it was complementary to all publicly available shipworm endosymbiont 16S rRNA gene sequences, but since then additional sequences that are not complementary to the probe have been obtained. This probe covers approximately 30% of sequences present in an extensive clone library from *Bankia setacea* gill (Fung and Distel, unpublished) and does not bind the four PMS 1162T strains described in the previous chapter, thus we now know the probe is not comprehensive of all shipworm endosymbionts.

The shipworm symbiont directed probe, ShipSymb1273, does not bind all bacteriocytes that hybridize the universal bacterial probe, Eub338, within gill tissue. The bacteria residing in bacteriocytes that only bind the Eub338 probe likely contain a 16S rRNA gene sequence different from the known symbionts at the ShipSymb1273 binding site. This suggests that symbionts that have not been previously detected exist in gill tissue and that symbionts organize themselves into bacteriocytes according to their phylogenetic identity (16S rRNA gene sequence). This pattern of segregation was detected in 7 specimens covering 5 shipworm species (listed above). Multiple symbiont ribotypes have been detected within a single *L. pedicellatus* specimen (Distel et al. 2002a, Luyten et al. 2006) and FISH localization showed symbiont segregation according to phylogeny (Distel et al. 2002a) in colony-reared *L. pedicellatus* (Distel et al. 2002a). Results presented here are consistent with this study and confirm that this pattern of symbiont residence is not an anomaly in colony-reared *L. pedicellatus*, but is a regular trend across at least five wild species.

Localization of 1162T strains in gill tissue

The Ribosomal Database Project (RDP) Probe match tool was used to predict the probe binding specificity of the 1162T probe applied in this study to detect newly isolated PMS 1162T strains assumed to be shipworm gill endosymbionts. RDP probe match did not reveal any sequences complementary to the probe sequence, thus it was assumed that this probe would not bind to non-target bacteria. The 1162T probe contains at least 3 centralized base pair mismatches to other shipworm symbiont sequences. Results of hybridizations with the 1162T probe and cultures of two strains (PMS 926K.S.0a.3 and PMS 1178H.S.0a.5) from the *Teredinibacter turnerae* clade

(Clade 1) and PMS 1162T.S.0a.2 and PMS 1162T.S.0a.5 confirm that this probe only binds the 1162T strains and does not bind the Clade 1 strains.

The 1162T strains were isolated from a host of a currently un-identified species and although tissue from this specimen or another of the same species was not available for FISH, *in situ* hybridizations with the 1162T probe revealed the presence of this symbiont type in 3 of 3 shipworm species analyzed, wild *Lyrodus pedicellatus* (4 different specimens)*, Lyrodus massa,* and *Teredo aff. triangularis*, all of which, including the source of the isolates, were collected in the Philippines. This suggests that these strains are common shipworm endosymbionts, at least amongst Philippine shipworm species.

The 1162T strains show a close phylogenetic relationship to previously detected *Lyrodus pedicellatus* 16S rRNA sequences, but show only 96% sequence identity to their closest relatives, clone sequences from *Lyrodus pedicellatus* (Luyten et al. 2006). Little is known about the shipworm endosymbiont communities in species other than *Lyrodus pedicellatus*. Within *Lyrodus pedicellatus* variation in gill community composition exists. While some symbiont ribotypes were detected by quantitative PCR and constant denaturant capillary electrophoresis in up to 90% of specimens, other ribotypes were only present in less than 50% of specimens (Luyten et al. 2006). In addition, one of the consensus sequences (Lp2) detected by Distel et al. (2002a) was not detected in the clone library analysis of Luyten et al. (2006). Recent clone library analysis of the gill community of *Bankia setacea* by collaborators at Ocean Genome Legacy is showing support for variation in community composition across shipworm species (Fung and Distel unpublished). Given this evidence of community variation across and within species and the fact that the community of

many shipworm species has not received thorough examination, it seems likely that the new shipworm symbiont strains remain unknown.

Localization of the PMS 1162T group in shipworm tissues revealed that these strains were commonly found residing in bacteriocytes characterized by an oblong shape (Figure 4.4 b,c and Figure 4.5 a-c) in contrast to the more common squarish bacteriocytes. This was consistent across all specimens analyzed (3 *L. pedicellatus*, 1 *T. triangularis*, 1 *L. massa*, and 1 *Lyrodus* species). Bacteriocytes binding the 1162T probe were only detected in the middle region of the gill length and made up an average of 53% of total bacteria containing bacteriocytes in regions where they were detected. Bacteriocytes stained with 1162T were rarely found in the portion of the gill lamellum close to gill filaments and were more abundant in the dorsal regions of gill lamellae.

Segregation of cultivated symbiont strains in bacteriocytes

The Clade 1 (*T. turnerae)* strains and Clade 2 (PMS 1162T) strains show varying metabolic characteristics in culture, thus the LP4 (Distel et al. 2002a) probe specific to Clade 1 and the 1162T probe were applied in FISH to determine whether these symbiont groups showed co-habitation in bacteriocytes. Control hybridizations with the LP4 probe and cultures of Clade 1 strains (PMS 926K.S.0a.3 and PMS 1178H.S.0a.5) and Clade 2 strains (PMS 1162T.S.0a.2 and PMS 1162T.S.0a.5) resulted in the LP4 probe binding the Clade 1, but not Clade 2 strains. See Figure 4.2. Application of LP4 and 1162T probes in dual hybridizations with shipworm tissues (the same specimens as described above) resulted in each probe binding to bacteria in separate bacteriocytes. There was no co-localization of both probes to any bacteriocytes, indicating that bacteria targeted by these two probes are mutually

exclusive, separating themselves into bacteriocytes according to their phylogenetic identity. See Figure 4.4a and b.

Bacteriocytes stained with the LP4 probe were consistently detected in the middle to anterior portion of the gill and 1162T stained bacteriocytes were consistently detected only in the middle region of gill tissue in 3 *L. pedicellatus*, 1 *T. triangularis*, 1 *L. massa*, and 1 *Lyrodus* species specimens. Bacteriocytes binding the LP4 probes were found closer to the central axis, with 1162T binding lateral bacteriocytes in the *Teredo aff. triangularis* sections where the central axis and both right and left gill demibranchs were detectable. This distribution for 1162T positive bacteriocytes is shown in Figure 4.5b. In transverse (sagittal) sections from 3 *L. pedicellatus*, 1 *L. massa*, and 1 *L. species* bacteriocytes that bound LP4 were detected in the ventral extent of gill lamellae closest to the ciliated zone with 1162T binding bacteriocytes in the more dorsal regions of gill filaments. In areas where 1162T did not bind bacteriocytes, LP4 hybridized bacteriocytes extending the length of the gill filament. Distel et al. (2002a) reported LP4-labeled bacteriocytes located in the more dorsal and medial regions of the gill, closely adjoining the central axis. This is consistent with results of this study in *T. aff. triangularis,* but not in transverse sections of *Lyrodus massa* and *Lyrodus pedicellatus* specimens.

Probes created by Jennifer Fung (unpublished) to target *B. setacea* ribotypes, Symb a (specific to Clades 4 and 5) and Symb b (specific to Clade 3) and cover the two dominant ribotypes in an extensive *B. setacea* gill clone library, were hybridized to *B. setacea* tissues to assess co-habitation of these strains. Results of hybridizations 1 *B. setacea* showed that these probes did not bind to the same bacteriocytes and indicate that these strains do not cohabitate in bacteriocytes. Figure 4.4c.

Distel et al. (2002a) examined patterns of co-habitation of four shipworm gill endosymbiont phylotypes in colony-reared *L. pedicellatus*. Co-habitation of two different pairs of phylotypes (LP1 and LP3, LP4 and LP2) within a single bacteriocyte was detected, but LP1 and LP3 were never detected in the same bacteriocyte as LP4 or LP2. Similarly, in this study the two groups detected in warm water shipworms, LP4 and 1162T, were never found in co-habitation within a single bacteriocyte. The same is true in the cold water Pacific Northwest shipworm, *B. setacea*, where the symbiont types targeted by the Symb a and Symb b probes were mutually exclusive of each other in bacteriocytes.

A possible explanation for this segregation is division of labor amongst the symbionts. In nature, complete degradation of lignocellulose is a very complex process, usually involving a consortium of microbes (Distel et al. 2002a, Lynd et al. 2002). It is possible that the different symbiont types are specialists, contributing enzymes for a specific step in the complex cellulosic breakdown process. Cultivated representatives of the symbiont types detected here demonstrate differing abilities to breakdown crystalline cellulose in culture. Clade 1 (*T. turnerae*) strains demonstrate the highest ability to degrade crystalline cellulose in comparison to the other cultivated representatives of Clades 2-4. See Chapter 3. The ability to fix atmospheric nitrogen to supplement the nitrogen-deficient xylophagous diet of the host is an important role of symbionts, but is not consistent amongst cultivated strains. Neither the four PMS 1162T strains nor the Clade 3 *B. setacea* strains fix nitrogen in culture. Localization shows that the nitrogen fixing symbionts do not share bacteriocytes with the non-nitrogen fixing symbionts and supports the division of labor hypothesis.

An alternative explanation is that symbiont types vying for the same resources do not co-exist in the same bacteriocytes. Duperron et al. (2007) detected four symbionts in the gills of *Bathymodiolus heckerae* including two thiotrophs, one methanotroph, and one methylotroph and found that the two sulfide-oxidizing symbionts were mutually exclusive of each other, but co-existed in bacteriocytes with the other symbiont types. This is suggestive of segregation due to competition for substrates. In contrast, six different symbiont types were detected in the gills of cold seep *Idas* sp. mussels and included close relatives of the four symbiont types detected in *B. heckerae* plus an additional gamma-proteobacterial symbiont and a *Bacteroides* symbiont (Duperron et al. 2008). In this case, all symbiont types, including the two thiotrophic symbionts, were detected in co-habitation within bacteriocytes thus complicating conclusions describing the rationale for mutual exclusion of symbionts in bacteriocytes.

Further studies incorporating metabolic characteristics of cultivated strains with localization of symbiont types within gill tissue will help answer questions regarding the role of the gill symbionts in the shipworm system. Does the maintenance of multiple symbiont types allow shipworms to adapt to different wood types? How does the symbiont community change with wood source? Are some symbiont types more important during periods of filter feeding by the host? Do the multiple symbiont types play a role in the adaptation of the host to new environments?

Among symbioses containing greater than one symbiont type, this segregation of symbionts to individual host bacteriocytes is rare. More commonly symbionts have been localized to the same bacteriocytes (Distel et al. 1995, Duperron et al. 2006 and

2008, McKinness et al. 2005). An example is the cold seep mussel *Idas sp.*, in which Duperron et al. (2008) detected a remarkable 6 different symbiont types including a methanotroph, thiotrophs, suspected methylotrophs, and a member of the *Bacteroidetes* group residing together in gill bacteriocytes. The mechanisms responsible for the maintenance of the segregation of symbiont types deserve further exploration and will be addressed in the next chapter.

Chapter 5. Interactions between gill endosymbionts

5.1 Introduction

Results of FISH with probes complementary to multiple symbiont groups has revealed an interesting spatial segregation of symbionts to individual bacteriocytes. Differences in metabolic characteristics of cultivated representatives of different shipworm endosymbiont types have also been observed. The genome of the most extensively studied endosymbiont (*Teredinibacter turnerae* T7901) has revealed a substantial potential for the production of bioactive metabolites, with 7.1% of all genes dedicated to secondary metabolite biosynthesis (Yang et al. 2009). This is comparable to known bioactive compound producing actinomycetes, for example *Streptomyces coelicolor* and *Streptomyces avermitilis* contain 5% and 6.6% respectively of their genomes dedicated to secondary metabolite biosynthesis. *T. turnerae* has demonstrated the ability to inhibit growth of *Bacillus subtilis* and *Vibrio anguillarum* in culture (Elshahawi and Haygood, unpublished).

Do the various shipworm symbiont types compete with each other, thus leading to their segregation in bacteriocytes? This is the aim of the work presented in this chapter. To address this, co-plating experiments with cultivated representatives of each symbiont clade were undertaken.

5.2 Materials and Methods

Eight PMS-ICBG strains and *Teredinibacter turnerae* T7901 from Clade 1, two (of four) cultivated strains from Clade 2, Bs02 plus 10 strains from a *B. setacea* collected in Alaska from Clade 3, and Bs08 from Clade 4 were chosen for use in competition experiments. Strains chosen to represent Clade 1 span the morphological diversity observed and host diversity. No cultivated representatives of Clade 5 were

available in our lab during the course of this study and so were not assessed in competition experiments. The *Teredinibacter turnerae* mutant AH03, in which the NRPS (non-ribosomal polyketide synthesis) of the siderophore, common bioactive metabolites, biosynthetic gene cluster is inactivated by plasmid insertion gene disruption, thus making this strain unable to produce siderophores, (Han and Haygood, unpublished) was also incorporated in competition experiments to begin to determine the compound responsible for inhibition.

To test for inhibition of one strain by another, one strain was streaked from an individual colony onto a fresh plate in a single, continuous streak. A second strain from an individual colony of similar age to the first was then streaked perpendicular to the first strain from the outer edge of the plate right up to the first streak in two different places along the first streak. See Figure 5.1 for details. SBM with ammonium chloride and Sigmacell as described in Chapter 3 was the growth medium in all co-plating experiments. After streaking of strains, plates were incubated at 30°C or room temperature and growth was monitored. Strain Bs08 grows much more slowly than the other strains tested and only at room temperature or at colder temperatures. To account for this, Bs08 was streaked onto plates and incubated 5-7 days or until faint growth was detectable prior to adding the competitor strain to the plate. Plates containing Bs08 were always incubated at room temperature or 10° C. When a significant amount of growth was detected plates were photographed.

5.3 Results and Discussion

Cross-streaking experiments involving Clade 1 (*T. turnerae*) strains resulted in zones of clearing of growth of the competitor strain where it came in close proximity

with the Clade 1 strain. Zones of inhibition were not detected in any combinations of strains not involving a Clade 1 strain. Clade 1 strains did not show inhibition of growth of other Clade 1 strains. See Figure 5.2 for photographs of results and Table 3.2 for a summary of results. The Clade 1 strain PMS 936K.S.0a.3 not only inhibited growth of the competitor strain, but grew over the top of it in 90% of cross streaking experiments involving this strain. This is depicted in Figure 5.3.

These results lead to the conclusion that *Teredinibacter turnerae*-like strains are the strongest competitors, inhibiting growth of any symbiont from another clade tested so far under these conditions. None of the Clade 2-4 symbionts tested showed the ability to inhibit growth of any other symbiont strains. Altering growth conditions, including incubation temperature and carbon source may produce different results and should be tested. The T7901 siderophore mutant AH03 did not show any significant differences in its ability to inhibit growth of non-Clade 1 strains and leads to the conclusion that the siderophore is not a major factor in inhibition of symbionts by *Teredinibacter turnerae*.

These results demonstrate the ability of *T. turnerae* strains to inhibit growth of symbionts from other clades. It is possible that this inhibition leads to the segregation of symbionts presented in Chapter 4. The rationale for this could be a division of labor amongst symbionts or due to competition for resources with the strongest symbionts eliminating competitors from the most ideal bacteriocytes.

Distel et al. (2002) showed cohabitation of symbiont phylotype LP 2 and *Teredinibacter turnerae* within bacteriocytes. Currently the closest cultivated representative of the LP 2 phylotype is the Bs02 strain, which contains 94% 16S rRNA gene sequence identity to LP 2. Cultivation of strains more adequately representative

of LP 2 and application in competition experiments will provide valuable insight into the mechanism of and rationale for inhibition of closely related symbionts by *T. turnerae*.

The production of bioactive compounds by symbionts for the benefit of the overall system is quite common, for example beewolf wasps add *Streptomyces* actinobacteria to the cocoons of their offspring, where they produce antimicrobial compounds for defense from pathogens (Kroiss et al. 2010) and *Pseudonocardia* symbionts of attine ants produce antifungal compounds to protect the host's fungal food source from pathogenic fungi (Poulsen and Currie 2010). The production of compounds active against other symbionts proposed to fulfill a similar role in the symbiosis is not well documented. Thus, the phenomenon observed here in which one symbiont is inhibiting growth of another symbiont is unique and deserves further characterization.

Results of experiments presented here provide evidence of the ability of *T. turnerae* strains to inhibit growth of closely related symbionts, but the functionality in the symbiosis is yet to be determined. The current state of knowledge is that *Teredinibacter turnerae* does not exist in the *Bankia setacea* gill symbiont community. Is there a symbiont analogous to *T. turnerae* in *B. setacea* that shows potential for producing bioactive compounds? If non-*T. turnerae* symbionts are not able to fix nitrogen (Clades 2 and 3) is it possible that *T. turnerae* wards them off to protect combined nitrogen that it has fixed from other symbionts, saving it for the host?

Chapter 6. Digestive tract-associated bacteria

6.1 Introduction

The majority of animals that ingest wood or woody plant materials (xylotrophs) harbor microbes in their digestive systems that are thought to aid in the decomposition of recalcitrant plant wall materials (lignocellulose) and in the supplementation of their nutritionally imbalanced diets (Distel 2003). These include termites and related insects, ruminants, and a variety of mammals (Brune and Stingl, 2005, Ohkuma 2003 Hess et al. 2011, Wallace 1994). In contrast, a physical separation between the site of wood accumulation in the caecum and cellulase production by gill endosymbionts has been proposed in the wood degrading shipworm. A rich community of bacterial endosymbionts has been described in the shipworm gill (Distel et al. 2002a, Luyten et al. 2006) and it has been reported that shipworm digestive systems contain few microorganisms (Greenfield and Lane 1953). However, to date, few micrographic and molecular studies of shipworm gut microbiota appear in the literature.

By comparison to many other lignocellulose-consuming animals, shipworms display a relatively simple digestive anatomy. Although many shipworms are capable of filter feeding (Turner 1966, Gallagher et al. 1981) the comparatively small size of the labial palps has been interpreted as evidence of reduced reliance on this feeding method (Turner 1966). Most shipworm species however ingest large quantities of particulate wood (Dean 1978, Gallagher et al. 1981). Microscopic teeth on the surfaces of the valves (shells) are used to burrow in woody substrates producing micron scale particles that are subsequently ingested through the mouth and

delivered to the stomach (Morton 1970). Variation in shipworm anatomy exists across species.

While all species contain a caecum, in some species (hindgut wood accumulators) it is diminished in size and the anal canal is well developed and is thought to be important in wood accumulation (Distel et al. 2011). This study applies only to the group of shipworms characterized by a well-developed caecum, foregut wood accumulators.

After ingestion, wood particles pass through the esophagus and stomach (Morton 1970), which are proportionally small by comparison to other bivalves (Turner 1966). In foregut wood accumulators wood particles then pass into the caecum or appendix, a large blind sac appended to the stomach. This conspicuous organ is often orders of magnitude larger in volume than the stomach and may comprise up to 60% of the body length (Turner 1966). The caecum is elaborated by a large typhlosole, a y-shaped fold of the internal wall surface extending the full length of the caecum. The inner surface of the caecum, including the typhlosole, is lined by a brush border microvillar epithelium (Bazylinski and Rosenberg 1983). The resulting large internal surface area of the caecum and a significant caecal artery passing directly from the heart to the caecum (Lazier et al. 1924) has been interpreted as evidence that the caecum is a site of lignocellulose degradation and is specialized for absorption of lignocellulose hydrolysis products (Bazylinski and Rosenberg 1983). After passage through the caecum, wood particles are returned to the stomach and passed to a relatively short, simple s-shaped tube that constitutes the intestine (Lazier 1924).

Liu and Walden (1970) demonstrated the presence of 23 enzymes associated with breakdown and synthesis of glucose in *Bankia setacea* caecal extract. Reducing sugar assays of pre-caecal and caecal material from a shipworm of the genus *Teredo* show evidence of conversion of cellulosic material to reducing sugars in caecal content, but not pre-caecal material (Greenfield and Lane 1953). This offers further support for the role of the caecum in digestion.

Here we employ Fluorescence *in situ* hybridization (FISH) using fluorescently-labeled, rRNA targeted phylogenetic-group-specific oligonucleotide probes to visualize and enumerate microbial cells within the caecum and intestine of four shipworm species, with an emphasis on *Lyrodus pedicellatus,* a small to medium sized cosmopolitan *species* and *Bankia setacea,* a large temperate Northeast Pacific species.

6.2 Materials and Methods

Fluorescence *in situ* Hybridizations

Shipworm specimens were collected as described in Chapter 3 and the same specimens used for FISH in Chapter 4 were also used in FISH experiments described here. Specimen preservation, sectioning, and removal of xylenes were according to the methods described in Chapter 4. Hybridizations and washing followed the procedure detailed in Chapter 4 for 35% formamide with a 2 hour incubation time. A bacterial probe, Eub338 (Amman et al. 1990), was used to survey the tissues for all bacteria. Eub338 is not completely comprehensive of all bacteria, therefore universal probe U1390 (coverage of all domains) was also employed. The ShipSymb1273 and BsSymb a and b probes were used to detect shipworm symbionts in the digestive tract. The general archaeal probe Arch 915 (Stahl and Amann 1991) was implemented to detect the presence of archaea. Due to their ubiquity in marine environments,

common associations with digestive tracts, and presence in the *B. setacea* gut clone library, probes specific to the *Cytophaga-Flavobacter-Bacteroidetes* group, CFB, (CFB 560 O Sullivan et al. 2001 and CFB 319a Manz et al. 1996) were also applied. Image capture was the same as described in Chapter 4.

To quantify the results, images of sectioned caecum and intestine from 4 specimens (2 *L. pedicellatus,* 1 *L. massa*, and 1 *Lyrodus species*) that had been treated with the bacteria-targeted probe Eub338-cy5 were chosen at random. Fluorescent particles were counted manually for 10 fields of 100 μm x 100 μm in each specimen. Fluorescent particles were similarly counted for 40 fields of 100 μm x 100 μm in paired adjacent sections that had been treated with a negative control probe EubNon-cy5. The mean number of fluorescent particles per field observed in the caecum was subtracted from values observed using the experimental probe in the caecum and the mean number of fluorescent particles per field observed in the intestine using the negative control probe was subtracted from values observed using the experimental probe in the intestine.

6.3 Results and Discussion

Localization of caecum and intestinal microbes

An investigation including 141 tissue sections of 11 shipworm specimens covering 5 shipworm species was completed. Our efforts included the shipworm species *Bankia setacea,* from Oregon and Washington state, and *Lyrodus pedicellatus,* both from culture and wild specimens from Bohol Island, the Philippines with an analysis of 4 and 5 specimens respectively. To extend our study to additional shipworm species we included one specimen each of *Lyrodus massa*, *Teredo aff.*

triangularis, and *Lyrodus* sp. from Bohol Island, the Philippines. These specimens originated from a diverse range of habitats and locations including, cold waters of the United States Pacific Northwest, warm equatorial mangrove and reef environments, and a captive population maintained in culture at Ocean Genome Legacy (Table 4.2).

Although the specimens observed covered a diverse selection of shipworm species and habitats, results of this examination were remarkably consistent across all samples. The caecae of 11 shipworms contained an abundance of woody material indicating active wood consumption (Bazylinski and Rosenberg 1983) by the animal at the time of sacrifice. This study focused on shipworms actively consuming wood, thus any specimens showing signs of decreased wood consumption (empty caecum or burrow sealed at the excavation face by a calcareous lining) were excluded.

In contrast to shipworm gills (Distel et al. 2002), very few microbes were detected in the caecum, Figure 6.1. In approximately 60% of specimens a few bacteria, usually present in clusters of 3-6 cells, were detected among the cellulosic substrate. In approximately 70% of the cases in which bacteria were detected in the caecum, the bacterial cells were located in the anterior region of the caecum near the entrance to the stomach. No additional microbes were detected through hybridization with universal probe U1390, which targets bacteria, eukaryotes, and archaea. Dual probe hybridizations revealed that the few bacterial cells located in the caecum did not hybridize with the shipworm symbiont targeted probe designed for this study (ShipSymb 1273), thus supporting previous results (Distel et al. 2002) in which gill endosymbionts were detected solely in gill tissue.

While the microbial density in the caecum was notably low, a morphologically diverse community of bacteria was observed in the intestines, Figure 6.2. Spiral

shaped bacteria were detected in the intestine of greater than half of all specimens analyzed. See Figure 6.2b. Approximately 90% of spiral shaped bacteria detected were located on the outer surface of the fecal pellets, along the intestinal walls, and in the spaces between. Within the fecal pellets a dense community of cocci and rod-shaped bacteria was observed. The presence of a substantial microbial population was consistent across specimens, but varied along the length of the intestine with no significant difference between the anterior and posterior portions of the intestine. Hybridization with the shipworm symbiont targeted probe (SS 1273) was only detected within the gills (Figure 6.3) and hybridization with the archaea targeted probe (Arch 915) was not detected in any sections examined.

The localization study presented here shows evidence of a caecum devoid of a substantial microbial community, a morphologically varied population of bacteria within intestines, and no gill symbionts in the digestive tract. This is a stark contrast to the gill, densely inhabited by cellulase producing symbionts. Cell counts, with background subtracted, in 40 100 μm x 100 μm fields in caecum and intestine in 4 specimens revealed an average of 0.88 cells/field with a range of 0-14 cells/field and standard deviation of 2.8 cells/field in the caecum and an average of 83.8 cells/field with a range of 0-224 cells/field and standard deviation of 61.3 cells/field in the intestine. Results are shown in Figure 6.4.

The physical separation of cellulase producing endosymbionts in the gill and the site of wood digestion in the caecum in shipworms is intriguing and unique in comparison to other wood degrading symbioses.

Comparison with other cellulose consuming systems

Other well-characterized xylophagous symbioses consist of a complex microbial community at the site of wood digestion. In lower termites protozoan flagellates are responsible for lignocellulose degradation, which results in the release of acetate, CO_2, and H_2. This is followed by fermentation of by-products by bacterial methanogens and acetogens (Brune and Stingl 2005, Ohkuma 2003). Similarly, in the cow rumen a consortium of bacteria, protozoans, and fungi digest lignocellulose, providing acids, sugars, and gases to a fermentative bacterial community. In both of these systems the host receives volatile fatty acids, end products of microbial symbiont metabolism (Wallace 1994, Brune and Stingl 2005). As in the shipworm, termite hindgut and rumen prokaryotes are responsible for fixing atmospheric nitrogen to forms usable by the host. In the shipworm it has been hypothesized that the cellulolytic symbionts reside in the gill, thus transporting cellulases to the caecum where these enzymes digest lignocellulose to simple sugars, which are taken up by the host. Because of the physical separation of symbionts and glucose release, there is no direct competition between host and symbiont for these simple sugars. To maintain this system, it is necessary to guard simple sugars produced in the caecum from scavenger bacteria, those in incoming food and those observed in the intestines.

Maintenance of microbial scarcity in the caecum

The presence of a plethora of bacteria within the intestines and microbial scarcity in the caecum described in this study suggests effort is put forth by either host or gill endosymbionts to keep microbial competitors out of the caecum. Invertebrate host defense systems have commonly adapted to allow for the colonization of specific tissues by selected microbes (McFall-Ngai et al., 2010, Troll et al 2010). Invertebrates

utilize an innate immune system, and unlike vertebrates do not employ adaptive immunity. Host pattern response regulators (PRRs) recognize unique microbe-associated molecular patterns (MAMPs), which trigger signal transduction pathways. Depending on the MAMP presented, production of antimicrobial compounds will be induced (Gross et al. 2009, McFall-Ngai et al. 2010). As well as defending against pathogenic microbes, this system has also been shown to be useful in host colonization by symbiotic bacteria (McFall-Ngai et al. 2010). Similar to vertebrates, immunity in invertebrates also relies on the action of phagocytic cells, reactive oxygen species, and lysozyme (Altura et al. 2011, Gross et al. 2009).

Commonly, symbiotic systems in which a host gains nutrition from the maintenance of a microbial symbiont involve the use of bacterially produced bioactive compounds to defend against invaders (McFall-Ngai 2008). Aphids contain the nutritional symbiont, *Buchnera,* to supplement its sap diet and have recently been shown to contain secondary symbionts of the *Enterobacteriaceae* to defend aphid eggs against killing wasps during incubation in the host body cavity (Oliver et al. 2006). Although the exact purpose is unknown, bioactive compounds patellamides are produced by the nutritionally important symbiont *Prochloron didemni* of *Lissoclinum patella* (Schmidt et al. 2005). Leaf cutter ants cultivate fungi as a source of food and also maintain *Pseudonocardia* bacterial symbionts, which produce anti-fungal compounds, for the purpose of defending the nutritionally valuable fungus from pathogenic fungi (Poulsen and Currie 2010).

The genome of shipworm gill endosybiont *Teredinibacter turnerae* shows not only support for its role in host nutrition, but also high potential for the production of bioactive compounds. Seven percent of the genome is dedicated to secondary

metabolite production and is comparable to *Streptomyces sp.*, known for their production of bioactive compounds (Yang et al. 2009). It is quite possible that *Teredinibacter turnerae* and the other gill endosymbionts not only play an important role in host nutrition, but are also responsible for defending valuable sources of energy from competitor microbes. A summary of the proposed model is illustrated in Figure 6.3.

Genome encoded cellulases

Enzymes encoded by the host nuclear genome may also contribute to lignocellulose degradation. For example, host derived enzymes, including cellulases, xylanases and cellobiosidases have been identified in termites, wood eating roaches, arthropods, mollusks, and nematodes (Wilson 2011, Watanabe and Tokuda 2009, Watanabe and Tokuda 2001). Higher termites utilize both microbially produced cellulases and enzymes produced by the host in lignocellulose degradation (Ohkuma 2003, Warnecke et al. 2007). In the case of the wood boring marine isopod *Limnoria quadripunctata* the digestive system appears to lack a significant microbial community and is thought to rely on host-encoded cellulases for lignocellulose digestion (King et al. 2010).

The near absence of microbial prokaryotes and eukaryotes from the caecum, the largest part of the shipworm digestive system, suggests that contact dependent microbial lignocellulose degrading activity and microbial fermentative metabolism may play a comparatively small role in this symbiosis. If this is the case, the cellulolytic activity observed in shipworm caecum may be due to endogenous cellulases encoded in the nuclear genome of the host. Alternatively, cellulolytic enzymes produced remotely by gill endosymbionts could contribute to this hydrolytic

activity, although no mechanism has been identified and no precedent exists for such a mechanism in intracellular endosymbionts. In either case, investigation of the origin, nature and locations of cellulase enzymes in the shipworm system will be important.

Results of this study confirm the absence of a substantial microbial community in the caecum, an important organ in the xylophagous lifestyle of shipworms and reveal an as yet un-characterized morphologically diverse microbial community in the intestines. This provides rationale for further exploration of symbiont defense mechanisms and presents further knowledge regarding the basic understanding of xylotrophy in this unique and biotechnologically relevant symbiosis.

Chapter 7. Conclusions

At least 3 novel shipworm endosymbiont strains have been isolated by the efforts in cultivation discussed here, one from a warm-water Philippine shipworm species and two from the cold water shipworm *Bankia setacea.* Fluorescence *in situ* hybridizations (FISH) with probes complementary to 16S rRNA gene sequences of the novel isolates in shipworm tissues shows that these isolates are indeed residents of shipworm gill bacteriocytes. The successful isolation of previously un-cultivated symbionts provides motivation for continued effort in cultivation and the opportunity for analysis of physiological characteristics of additional endosymbionts, thus leading to a better understanding of their role in the symbiosis.

FISH in gill tissue of multiple shipworm species has revealed that the pattern of endosymbiont segregation in bacteriocytes according to phylogeny described previously in colony reared *L. pedicellatus* (Distel et al. 2002) is common among shipworms. Preliminary analysis of metabolic characteristics shows that only some symbionts are capable of fixing atmospheric N_2 to forms usable by the host and that the ability to degrade crystalline cellulose varies across symbiont types. Competition experiments demonstrated the ability of *T. turnerae* strains to inhibit growth of strains representative of three other symbiont types, none of which inhibited growth of other strains. Localization of these symbiont types in gill tissue did not show cohabitation of cultivated symbiont strains. Results of localization of symbionts in gill bacteriocytes in conjunction with the ability of Clade 1 strains to inhibit growth of other symbionts suggest that Clade 1 symbionts maintain a mechanism to deter other symbiont types from residing in common bacteriocytes. Potential reasons for this segregation are a division of labor amongst symbionts, with symbiont types segregated based on their

role in the overall symbiosis, or due to competition for resources, with the stronger symbionts banishing the others to less ideal locations in the gill.

The proposed model of lignocellulosic degradation in the shipworm has described a physical separation between the site of production of enzymes important in lignocellulose degradation by gill symbionts and the site of accumulation of cellulosic substrate in the caecum. FISH micrographic examination of the shipworm digestive tract revealed very few microbes in the caecum, but a rich microbial community in the intestine. The microbiota of the intestine may play a role in lignocellulose degradation in the shipworm and deserves further characterization. These results support the current shipworm model and lay the groundwork for a new hypothesis in which gill endosymbionts transport bioactive compounds to the caecum as a defense mechanism for transient competitors. Collectively, results of the study presented here provide rationale for continued study of the shipworm symbiosis for discovery of technologically relevant compounds, important in both cellulosic biofuels production and in medicine.

Appendix A. Cultivation of digestive tract bacteria

A.1 Introduction

While attempts at cultivation have been made (Greenfield and Lane 1953), shipworm digestive tract bacteria have not yet been cultivated. Recent 16S rRNA clone library analysis of *B. setacea* digestive tract (caecum and intestine combined) shows an extensive microbial community and is dominated by *Bacteroidetes* (Fung and Distel, unpublished). Whether transient environmental bacteria or long time residents, digestive tract bacteria are likely competitors for cellulosic substrate and are therefore prime targets for testing bioactive compounds produced by shipfworm gill symbionts. Metabolic analysis of these strains will also provide insight into the role of any bacteria present in the shipworm digestive tract and provide for a further understanding of the symbiosis as a whole.

A.2 Materials and Methods

Attempt 1. Isolation of bacteria from the digestive tract was carried out anaerobically. The isolation medium used was derived from the Hungate anaerobic isolation medium and adjusted to more closely resemble the shipworm intestinal environment. Instructions for the preparation of Hungate anaerobic isolation media (Handbook of Microbiological Media, Ronald M. Atlas 1993) were followed with the following exceptions: Commercially available clam juice was used to replace the rumen fluid used by Hungate, 0.1% (w/v) powdered cellulose (Sigmacell 101) was added, and seawater was provided to more closely mimic the marine environment. Plates were poured under aerobic conditions and transferred to the anaerobic chamber immediately after cooling and remained under anaerobic conditions for at

least three days before inoculation. Phosphate buffered saline (PBS) was used as a homogenization buffer and was gassed with nitrogen to remove all oxygen and transferred to the anaerobic chamber prior to use.

During extraction of the shipworm specimen it was discovered that the calcareous tube was sealed, terminating access to wood and indicating that this animal was no longer actively consuming wood. I proceeded with dissection and found that the caecum was very reduced in size. Instead of using the caecum for isolation of bacteria I used the anterior visceral mass and intestine for isolation. These tissues were rinsed three times in sterile seawater and then transferred to the anaerobic glove box (Vinyl Anaerobic chamber, Model A, Coy Laboratory Products Inc.). The tissues were homogenized, diluted, and spread onto plates under anaerobic conditions. Plates were incubated at room temperature in the anaerobic chamber.

Attempt 2. Two different types of media were used in this attempt at cultivating digestive tract bacteria, both specific for *Bacteroidetes* due to its abundance in a *B. setacea* digestive tract 16S rRNA clone library (Fung and Distel, unpublished). *Bacteroides* minimal media (BMM) was prepared as described in Bacic and Smith (2008). *Bacteroides* Bile Esculin Agar (BBE) contains esculin, a glycoside compound extracted from the horsechestnut tree. Members of the *Bacteroides fragilis* group are unique in their ability to degrade this compound therefore, it acts as an indicator in this medium. When esculin is hydrolyzed, the plates will turn a dark brown color likely indicating the growth of a *Bacteroides* strain (Livingston et al. 1978). This medium contains gentamicin to select against facultative anaerobes and most non-*Bacteroides* gram negative anaerobes. BBE was prepared according to the protocol in the Handbook of Microbiological Media (Ronald M. Atlas 1993).

A large *B. setacea* collected in Hydaburg, AK and showing evidence of active wood consumption prior to sacrifice due to the amount of woody frass excreted was the source of tissues used in this cultivation attempt. The caecum was removed and the majority was given to Sherif Elshahawi for use in chemical analysis. The posterior end of the caecum plus intestine and the anterior visceral mass were used for cultivation as described above.

A.3 Results and Discussion

The first cultivation attempt resulted in 12 isolates from the digestive organs of a starving *B. setacea*. All isolates were facultative anaerobes. 16S rRNA gene sequence comparison in BLAST showed 6 with 99% or greater similarity to *Vibrio* strains (*Vibrio splendidus, Vibrio lentus* and *Vibrio tasmaniensis*) and 4 showed 99% similarity to *Thalassosira lucentensis*. The second cultivation attempt resulted in 6 isolates from the *Bacteroides* minimal media and no isolates from the *Bacteroides* bile esculin media. All of these isolates were also able to grow under aerobic conditions therefore they were not *Bacteroides* strains. 16S rRNA gene sequences were not obtained for these strains.

Vibrio spp. are copiotrophs, ubiquitous in marine environments and were detected in the digestive tract 16S rRNA clone library (Fung and Distel, unpublished). *Thalassosira lucentensis* is a marine α-Proteobacterium with the capacity to utilize carbohydrates as a carbon source (Lopez-Lopez et al. 2002) and therefore may be able to survive in the shipworm digestive tract. *T. lucentensis* was described as an obligate aerobe (Lopez-Lopez et al. 2002). This may be an indication that my cultivation procedure was not strictly anaerobic. The anaerobic chamber used for incubation is used by at least three other members of the lab. The oxygen level rises significantly

whenever the glove box is opened. It could be possible that this influx of oxygen was enough to provide oxygen to the isolates. While *Bacteroides* are not able to divide and reproduce in the presence of oxygen, it is not lethal and in some cases *Bacteroides* strains are handled under aerobic conditions with anaerobic incubation (Bacic and Smith 2008).

It is likely that the cultivated strains from the digestive tract are transient environmental bacteria, not long-term residents of the digestive tract. They have high potential as opportunists in the shipworm caecum, utilizing glucose released during cellulose degradation, and therefore are prime targets for testing anti-microbial compounds derived from shipworm gill endosymbionts.

Appendix B. Homogenization Buffer Optimization

B.1 Introduction

Cultivation independent analyses have revealed at least four symbiont types in the gills of shipworms, yet only one of these has been cultivated (*Teredinibacter turnerae*) in the laboratory. An important step in isolation is the homogenization of tissue to release endosymbionts from host cells, the buffer in which this takes place is the homogenization buffer and is the subject of this Appendix. We hypothesized that amending the homogenization buffer to more closely mimic the internal milieu of the shipworm would alleviate stresses, possibly leading to cell death, felt by bacteria during homogenization of tissue prior to inoculation of growth media.

Regulation of cell volume is critical to cell survival and challenging under conditions of changing ambient osmolarity. Osmolytes are solutes utilized in regulation of cell volume with variability in osmotic stress. Typical osmolytes are polar molecules that have high solubilities in water allowing them to accumulate to high concentrations, lack a hydrophobic region, and lack a net charge (Hochachka and Somero 2002). We hypothesized that shipworm symbionts have adapted to rely on osmolytes used by host cells, therefore compounds commonly utilized in marine invertebrate tissues for osmoregulation were added to the homogenization buffer to alleviate osmotic shock to endosymbionts upon release from host tissue during homogenization.

B.2 Materials and Methods

Homogenization Buffer

Osmolytes commonly utilized by marine invertebrates were added to the SBM homogenization buffer (no carbon source) in concentrations similar to those found in marine invertebrate tissues: Trimethylamine-N-Oxide (TMAO, 25 mM), glycine betaine (35 mM), and free amino acids proline (100 mM) and alanine (100 mM) (Hochachka and Somero 2002). The pH was adjusted to 7, 7.5 or 8.

Simulated homogenizations with *Teredinibacter turnerae* T7901 culture

Prior to application of the Osmolyte Addition Homogenization Buffer (Osm buffer) in an isolation from shipworm tissue this buffer was tested in a simulated homogenization of a *T. turnerae* T7901 culture and cell viability was quantified with the BacTiter-Glo Microbial Cell Viability Assay (Promega). This assay uses the luciferase reaction, in which ATP present in the culture leads to oxygenation of luciferin and luminescence. The amount of luminescence detected is directly correlated to the amount of DNA in the culture and thus is representative of viable cells. A T7901 culture was grown in SBM+N+Sigmacell broth for 30 hours for application in the Osm buffer trial. At the T_0 time point 100 µL of culture was tested. Then 2 mL of culture was aliquoted to 12 different epi-tubes and centrifuged to pellet cells. The supernatant was removed and replaced with either SBM or Osm buffer at pH of 7, 7.5, or 8. Cells were resuspended at incubated at room temperature. At each time point the cell viability was measured in 100 µL of sample, with 3 replicates per sample type according to the BacTiter-Glo Microbial Cell Viability Assay manufacturer's protocol. A blank for each buffer type was measured to detect any

background signal and was subtracted from the luminescence reading for each sample.

Osmolyte Addition Homogenization Buffer in Isolation from Shipworm Gill Tissue

The Osm buffer was applied in 2 different isolations from *B. setacea* gill tissue. To account for any variation in endosymbiont abundance along the length of the gill, it was cut into 6 or 8 pieces and alternating pieces were added to either SBM homogenization buffer (pH 8) or Osm homogenization buffer (pH 8) for a total of either 3 or 4 gill pieces covering the length of the gill per homogenization buffer. Homogenization, inoculation, and incubation proceeded as described in Chapter 3. When colonies appeared they were counted.

B.3 Results and Discussion

Osmolyte Homogenization Buffer Simulations with *T. turnerae*

Results of cell viability analysis after incubation of *T. turnerae* cells in SBM homogenization buffer and Osm buffer were not completely conclusive. See Figure B.1. After 11 minutes of incubation more viable cells were detected in the Osm buffer in samples of pH 7, 7.5, and 8. During the first 11 minutes the amount of ATP present decreased, but after this ATP began to increase in all samples. An explanation is that the shock from centrifugation caused the cells to become inactive. As they adjusted and became more active, the ATP in the sample began to increase. During the time directly after the stress felt by cells due to centrifugation and re-suspension the Osm buffer has a higher capacity for alleviation of cell death than the SBM buffer. Results of this assay do not show conclusive evidence that one homogenization buffer is better

able to alleviate cell death during stress, but provide rationale for implementing the Osm buffer in an actual homogenization and isolation from shipworm gill tissue.

Osmolyte Addition Homogenization Buffer in Isolation from Shipworm Gill Tissue

The first isolation from a starving *B. setacea* that the Osm buffer was applied in resulted in a very dense lawn of colonies on the 10^{0} and 10^{-1} dilution plates and no colonies on the 10^{-2} dilution plates. The shipworm used in this isolation was not actively eating wood at the time of extraction and no shipworm endosymbiont isolates were obtained from this specimen.

The second isolation from a *B. setacea* in which a comparison of both homogenization buffers was attempted resulted in more colonies on plates that had been inoculated with tissue homogenized in Osmolyte addition buffer. See Table B.2. In this case a 10^{-3} dilution was added and homogenates were vortexed more vigorously prior to plating to reduce cell clumping. Twelve additional shipworm endosymbiont isolates were obtained from this isolation. These strains are listed in Table 3.2 and contain AK in the strain name. These strains all Cluster in Clade 3. These results indicate that the Osmolyte Addition homogenization buffer has potential to alleviate cell death during tissue homogenization, but repeating this will make this result more convincing.

Appendix C. The quest for the identity of the vermiform organisms in the caecum of a *Bankia setacea* specimen

C.1 Introduction

During fluorescence *in situ* hybridization of cross sections of *B. setacea* caecum tissue, the presence of large (for bacteria) vermiform organisms was revealed not by probe binding, but by autofluorescence. These organisms are 10- 15 μm in length and approximately 1 μm across. In subsequent hybridizations these organisms bound the universal probe, U1390, indicating that they were living organisms.

This shipworm was quite large (~7 in. in length), had a trifurcated, but not sealed burrow indicating that it was running out of wood, and a large, but not inflated caecum with highly developed gonads. The content of the caecum was different in appearance than that of other full caeca examined. Due to the confined living space of the shipworm within its burrow as they become sexually mature there is no room for the growing gonads to expand outward. As the gonads grow, the animal relies more heavily on filter feeding, thus reducing the amount of wood stored in the caecum and allowing the gonads to grow into this space (Turner 1966). This is a possible explanation for the anomalous caecum observed in this specimen. This was the first specimen I analyzed by FISH and at the time did not know this was an anomaly so proceeded in identifying these intriguing vermiform organisms. They have never been seen in any of the other 12 specimens analyzed.

C.2 Materials and Methods

Microscopy

FISH with bacterial probe Eub338, U1390 covering all domains, eukaryotic probe Euk516, archaeal probe Arch915, and CFB560 for the CFB bacterial group were applied in hybridizations according to methods detailed in Chapter 4. EubNon and EukNon negative control probes containing at least one base pair mismatch were also applied. See Table 4.2 for details of probes used. DAPI and Propidium Iodine nucleic acid binding stains were also applied. Slides were treated with RNAse (from a MoBio soil DNA extraction kit sample) followed by staining with DAPI or Propidium Iodide to allow for the visualization of nuclei, if present, and thus identify these cells as eukaryotes. A *Candida albicans* culture was treated in the same way to act as a control for the accuracy of this method.

Laser Capture Microdissection and 16S rRNA gene amplification

Laser capture microdissection (LCM) was used to obtain individual cells of the vermiform caecum organisms for DNA extraction and 16S rRNA gene sequencing to identify these organisms. LCM was performed at the OHSU NPRC Micro-imaging core facility on the PixCell LCM equipment (they have since replaced this system with a newer one). Paraffin was removed as described in Chapter 4 and slides were dehydrated under a vacuum with DriRite desiccant for approximately 30 minutes prior to LCM. Laser power used was 75-85 mW and duration was 900 µs. This was optimized on non-target areas of the tissue.

After LCM the caps containing target cells were incubated overnight at 37°C in 50 µL Tris-HCl pH 8.3 with Proteinase K (200 ng/µL) in inverted epi-tubes so that the cap was immersed in solution. Next, samples were incubated at 98°C for 15 minutes

to inactivate Proteinase K. This solution was used as a template for PCR amplification of 16S rRNA genes. DNA amplification from nucleic acids from formalin fixed paraffin embedded samples tends to be more successful for shorter sequences (Lin et al. 2009, Gilbert et al. 2007) so primers 27f and 68f were used with 533r and 533f and 1390r were used in PCR. The TOPO TA Cloning Kit for Sequencing (Invitrogen) was used to clone PCR products into TOP10 Electrocomp *E. coli* cells according to the manufacturer's protocol. Plasmids were extracted from 24 clones with the Qiagen Mini Prep Plasmid Extraction Kit and sequenced at the ONPRC molecular biology core with M13 (5'-GTAAAACGACGGCCAG) and M13R (5'-CAGGAAACAGCTATGAC) primers specific to the pCR 4-TOPO plasmid were used in sequencing reactions.

C.3 Results and Discussion

Microscopy

The U1390 probe covering all domains was the only probe tested that hybridized to the target organisms. Although the bacterial probe Eub338 does not hybridize to the vermiform organisms, it did reveal cocci-shaped bacteria intermixed with the much larger target organisms. All nucleic acid stains also stained these organisms. Nuclei were not detected following treatment with RNAse in the target organisms, but nuclei were visible in the *Candida albicans* control.

Laser Capture Microdissection

Analysis of the caps used to collect cells during LCM showed that cells of interest were successfully captured, but amplification of DNA proved challenging. The goal was to include the Eub338 region in the amplified sequence so that sequences could be screened for complementarity to that sequence since the probe does not bind the target organisms, but PCR primer pairs 27F/533R and 68F/533R did not result in

amplification. PCR was successful with the 533F/1389R primer pair and allowed me to proceed with cloning and 16S rRNA gene sequencing of 24 clones. Sequences returned were either of poor quality or were chimeras. Therefore, the identity of these vermiform caecum organisms remains elusive.

Tables

Table 3.1 Shipworm specimens used for microbial cultivation resulting in strains discussed in this chapter.

Specimen ID	Sample ID *	Species	Collection Site	Habitat/ Dominant wood type
Bs 2	NA	*Bankia setacea*	From OGL, Puget Sound, WA	pine board
Bs 3	NA	*Bankia setacea*	From OGL, Puget Sound, WA	pine board
OR Bs4	NA	*Bankia setacea*	Yaquina Bay, OR	estuary, pine board
OR Bs6	NA	*Bankia setacea*	Yaquina Bay, OR	estuary, pine board
OGL Bs#2	Dissection/cultivation at OGL	*Bankia setacea*	Puget Sound, WA	pine board
OGL Bs#5	Dissection/cultivation at OGL	*Bankia setacea*	Puget Sound, WA	pine board
AK Bs 1	NA	*Bankia setacea*	Hydaburg, AK	cedar board
PMS 1090H	PMS 1095S	*Bankia* species	Philippines, Bohol, Bilisan	Mangrove
PMS 1128S	PMS 1133Y	Family Teredinidae	Philippines, Bohol, Bilisan	Mangrove
PMS 1157K	PMS 1162T	Family Teredinidae	Philippines, Bohol, Danajon Bank	coral reef, lumun lumun
PMS 1145H	PMS 1178H	Family Teredinidae	Philippines, Bohol, Danajon Bank	coral reef, seagrass
PMS 995P	PMS 1000X	*Lyrodus massa*	Philippines, Bohol, Cataban	Mangrove
PMS 1016M	PMS 1021W	*Lyrodus massa*	Philippines, Bohol, Cataban	Mangrove
PMS 922W	PMS 926K	*Lyrodus massa*	Philippines, Bohol, Cataban	Mangrove
PMS 988W	PMS 991H	*Lyrodus pedicellatus*	Philippines, Bohol, Danao	reef flat
PMS 1165X	PMS 1169L	*Teredo clappi*	Philippines, Bohol, Danajon Bank	coral reef, lumun lumun
PMS 916L	PMS 920T	*Teredo clappi*	Philippines, Bohol, Cataban	Mangrove
PMS 1114L	PMS 1119U	*Teredo fuller*	Philippines, Bohol, Bilisan	Mangrove
PMS 1002H	PMS 1005M	*Teredo mindanensis*	Philippines, Bohol, Cataban	Mangrove

Table 3.2 List of isolates used in this study. MSI- Marine Science Institute, University of the Philippines- Diliman, microbiology team from the Concepcion laboratory, MA- Marvin Altamia (UP- MSI, Concepcion laboratory and Ocean Genome Legacy), MB- Meghan Betcher, AF- Abigail Fusaro (Ocean Genome Legacy).

TaxonTissueType	StrainName	Isolated By	Sequenced By
Lyrodus massa : gill/ctenidium	1000X.S.0a.1	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1000X.S.0a.2	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1000X.S.0a.3	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1000X.S.0a.4	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1000X.S.0a.5	MSI/MA/MB	MB
Teredo mindanensis : gill/ctenidium	1005M.S.0a.1	MSI/MA/MB	MB
Teredo mindanensis : gill/ctenidium	1005M.S.0a.2	MSI/MA/MB	MB
Teredo mindanensis : gill/ctenidium	1005M.S.0a.3	MSI/MA/MB	MB
Teredo mindanensis : gill/ctenidium	1005M.S.0a.4	MSI/MA/MB	MB
Teredo mindanensis : gill/ctenidium	1005M.S.0a.5	MSI/MA/MB	MB
Teredo mindanensis : gill/ctenidium	1005M.S.0a.6	MSI/MA/MB	MB
Teredo mindanensis : gill/ctenidium	1021W.S.0a.1	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1021W.S.0a.2	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1021W.S.0a.3	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1021W.S.0a.4	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1021W.S.0a.5	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	1021W.S.0a.6	MSI/MA/MB	MB
Teredo fulleri Clapp, 1924 : gill/ctenidium	1133Y.S.0a.1	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill/ctenidium	1133Y.S.0a.2	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill/ctenidium	1133Y.S.0a.3	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill/ctenidium	1133Y.S.0a.4	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill/ctenidium	1162T.S.0a.2	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill/ctenidium	1162T.S.0a.3	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill/ctenidium	1162T.S.0a.4	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill/ctenidium	1162T.S.0a.5	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill/ctenidium	1169L.S.0a.1	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	1169L.S.0a.2	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	1169L.S.0a.4	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	1169L.S.0a.5	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	1169L.S.0a.6	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	1169L.S.0a.7	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	1169L.S.0a.8	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	1169L.S.0a.9	MSI/MA/MB	MB

Teredo clappi : gill/ctenidium	1169L.S.0a.10	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	1178H.S.0a.1	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	1178H.S.0a.3	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	1178H.S.0a.4	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	1178H.S.0a.5	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	1178H.S.0a.6	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	1178H.S.0a.7	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	1178H.S.0a.8	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	1178H.S.0a.9	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	1178H.S.0a.10	MSI/MA/MB	MB
Family Teredinidae (Gen. sp. TBD) : gill	920T.S.0a.1	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	920T.S.0a.2	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	920T.S.0a.3	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	920T.S.0a.4	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	920T.S.0a.5	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	920T.S.0a.7	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	920T.S.0a.8	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	920T.S.0a.9	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	920T.S.0a.10	MSI/MA/MB	MB
Teredo clappi : gill/ctenidium	926K.S.0a.1	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	926K.S.0a.2	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	926K.S.0a.3	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	926K.S.0a.5	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	926K.S.0a.6	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	926K.S.0a.7	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	926K.S.0a.8	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	926K.S.0a.9	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	926K.S.0a.10	MSI/MA/MB	MB
Lyrodus massa : gill/ctenidium	991H.S.0a.1	MSI/MA/MB	MB
Lyrodus pedicellatus : gill/ctenidium	991H.S.0a.2	MSI/MA/MB	MB
Lyrodus pedicellatus : gill/ctenidium	991H.S.0a.3	MSI/MA/MB	MB
Lyrodus pedicellatus : gill/ctenidium	991H.S.0a.4	MSI/MA/MB	MB
Lyrodus pedicellatus : gill/ctenidium	991H.S.0a.5	MSI/MA/MB	MB
Lyrodus pedicellatus : gill/ctenidium	991H.S.0a.6	MSI/MA/MB	MB
Lyrodus pedicellatus : gill/ctenidium	991H.S.0a.7	MSI/MA/MB	MB
Lyrodus pedicellatus : gill/ctenidium	991H.S.0a.8	MSI/MA/MB	MB
Lyrodus pedicellatus : gill/ctenidium	991H.S.0a.9	MSI/MA/MB	MB
Dicyathifer manni: gill/ctenidium	486K.S.1a.12	MSI/AF	MA
Teredo fulleri Clapp, 1924 : gill/ctenidium	1120W.S.0a.4	MSI	MA
Bankia setacea: gill/ctenidium	Bs 02	MA/MB	MA
Bankia setacea: gill/ctenidium	Bs 03	MA/MB	MA
Bankia setacea: gill/ctenidium	Bs 04	MA/MB	MA
Bankia setacea: gill/ctenidium	Bs 05	MA/MB	MA
Bankia setacea: gill/ctenidium	Bs 06	MA/MB	MA
Bankia setacea: gill/ctenidium	Bs 08	MA/MB	MA
Bankia setacea: gill/ctenidium	Bs 12	MA	MA

Bankia setacea: gill/ctenidium	AK Bs1	MB	MB
Bankia setacea: gill/ctenidium	AK Bs2	MB	MB
Bankia setacea: gill/ctenidium	AK Bs3	MB	MB
Bankia setacea: gill/ctenidium	AK Bs4	MB	MB
Bankia setacea: gill/ctenidium	AK Bs5	MB	MB
Bankia setacea: gill/ctenidium	AK Bs6	MB	MB
Bankia setacea: gill/ctenidium	AK Bs8	MB	MB
Bankia setacea: gill/ctenidium	AK Bs9	MB	MB
Bankia setacea: gill/ctenidium	AK Bs10	MB	MB
Bankia setacea: gill/ctenidium	AK Bs12	MB	MB
Bankia setacea: gill/ctenidium	AK Bs14	MB	MB
Bankia setacea: gill/ctenidium	AK Bs16	MB	MB

Table 3.3 List of reference sequences used in phylogenetic analyses.

Strain Name	Accession Number	Reference
Hahella chejuensis KCTC 2396	AF195410.1	Lee et al. 2001
Cellvibrio japonicus	NR 028836.1	Nagy et al. 2002
Pseudomonas aeruginosa PAO1	DQ777865.1	Mohana et al. 2007
Pseudomonas putida KT2440	AE015451.1	Nelson et al. 2002
Oceanospirillum multiglobiferum	NR 024652.1	Satomi et al. 1998
Microbulbifer thermotolerans	AB304802.1	Miyazaki et al 2008
Microbulbifer agarilyticus	AB304799.1	Miyazaki et al 2009
Oceanobacter kriegii IFO	NR 024655.1	Satomi et al. 1998
Candidatus Endobugula sertula BnPV	AF006607.1	Haygood and Davidson 1997
Candidatus Endobugula sertula BnSP	AF006606.2	Haygood and Davidson 1998
Saccharophagous degradans 2-40	CP000282.1	Weiner et al. 2008
Teredinibacter turnerae T7902	NR027564.1	Distel et al. 2002b
Teredinibacter turnerae CS30	AY949835.1	Distel et al. 2002b
Teredinibacter turnerae T8602	EU604077.1	Yang et al. unpublished
Teredinibacter turnerae T7901	EU604078.1	Yang et al. unpublished
Teredinibacter turnerae T0609	EU604079.1	Yang et al. unpublished
Teredinibacter turnerae CS32	AY949836.1	Trindade-Silva et al. 2009
RT 18 L. pedicellatus clone	DQ272313	Luyten et al. 2006
RT 20 L. pedicellatus clone	DQ272307	Luyten et al. 2006
RT 19 L. pedicellatus clone	DQ272306	Luyten et al. 2006
RT 1 L. pedicellatus clone	DQ272301.1	Luyten et al. 2006
RT 14 L. pedicellatus clone	DQ272315	Luyten et al. 2006
RT 12 L. pedicellatus clone	DQ272316	Luyten et al. 2006
RT 22 L. pedicellatus clone	DQ272309	Luyten et al. 2006
RT 24 L. pedicellatus clone	DQ272312	Luyten et al. 2006
RT 21 L. pedicellatus clone	DQ272314	Luyten et al. 2006
RT 5 L. pedicellatus clone	DQ272300.1	Luyten et al. 2006
RT 7 L. pedicellatus clone	DQ272310	Luyten et al. 2006
RT 15 L. pedicellatus clone	DQ272311	Luyten et al. 2006
B. setacea clone	AF102866	Sipe et al. 2000
LP2 L. pedicellatus clone	AY150184	Distel et al. 2002a
Bs 7G B. setacea clone		Fung and Distel, unpublished
Bs 5G B. setacea clone		Fung and Distel, unpublished
Bs 2G B. setacea clone		Fung and Distel, unpublished
Bs 6G B. setacea clone		Fung and Distel, unpublished
Bs 3G B. setacea clone		Fung and Distel, unpublished
Bs 1G B. setacea clone		Fung and Distel, unpublished

Table 3.4. Summary of characteristics of strains representative of each Clade. The Inhibition of Other Strains column is addressed in Chapter 5.

Clade	Number of Isolates	N_2 Fixation	Cellulose Clearing	Growth Temperature	Colony Morphology	Inhibition of Other Strains
1	72	Yes	++	RT or 30°C	Flaky + yellow pigment	Yes
2	4	No	+	RT or 30°C	Halo	No
3	19	No	+	RT or 30°C	Halo	No
4	3	Yes	+	RT, 10°C, or 18°C	Flaky	No
5	2	No	+	RT, 10°C, or 18°C	Flaky + purple pigment	No

Table 4.1 Probes applied in Fluorescence *in situ* hybridizations of gill tissues and their specificity for shipworm endosymbiont clades. Eub338 probe is described in Amann et al. 1990 and LP4 is described by Distel et al. 2002a. All other probes designed for this study.

Probe	Sequence	Reference	Probe complementary to:				
			Clade 1	Clade 2	Clade 3	Clade 4	Clade 5
Eub338	GCTGCCTCCCGTAGGAGT	Amann et al. 1990	X	X	X	X	X
ShipSymb 1273	ACTGTTTTATGGGATTAGCTC	This study	X	X (except 1162T)		X	X
1162T	TCTTCTGTACTCTAGCAATCCAGTTCTG	This study		X 1162T only			
BsSymb 3a	CTGTACTCTAGTTACCCAGTTCTA	Fung, unpublished				X	X
BsSymb 3b	CTGTACTCTAGCTACACAGTTCTA	Fung, unpublished			X		
LP4	GCTGTACTCAAGTTACCCAGTTCTA	Distel et al. 2002a	X				
EubNon	ACTCCTACGGGAGGCAGC	Amann et al. 1990					
ShipSymb Non	ACTGTTTTATGTGATTAGCTC	This study					
1162T Non	TCTTCTGTACTAGAGCAATCCAGTTCTG	This study					
LP4 Non	GCTGTACTCAACTTACCCACTTCTA	Distel et al. 2002a					
BsSymb aN	CTGTACTCTACTTACCCAGATCTA	Fung, unpublished					
BsSymb bN	CTGTACTCTACCTACACAGATCTA	Fung, unpublished					

Table 4.2 Specimens applied in fluorescence *in situ* hybridization bacterial localization studies. The PMS 1593W sample includes 3 *Lyrodus pedicellatus* specimens. All 3 were paraffin embedded and sectioned together.

Specimen ID	**Sample ID ***	**Species**	**Collection Site**	**Habitat/ Dominant wood type**
OR Bs4	NA	*Bankia setacea*	Yaquina Bay, OR	estuary, pine bait
OR Bs5	NA	*Bankia setacea*	Yaquina Bay, OR	estuary, scavenged wood
OGL Bs1	NA	*Bankia setacea*	from OGL, Puget Sound, WA	pine board
OGL Bs2	NA	*Bankia setacea*	from OGL, Puget Sound, WA	pine board
OGL Lp2	NA	*Lyrodus pedicellatus*	OGL Colony	pine board
PMS 1140T	PMS 1140U	*Lyrodus pedicellatus*	Philippines, Bohol, Bil-isan	Mangrove
PMS 1592U	PMS 1593W	*Lyrodus pedicellatus*	Philippines, Bohol, Balicasag	coral reef, pine bait
PMS 785M	PMS 1180L	*Lyrodus massa*	Philippines, Bohol, Danajon Bank	coral reef, lumun lumun
PMS 1173T	PMS 1171P	*Lyrodus* species	Philippines, Bohol, Danajon Bank	coral reef, lumun lumun
PMS 1035H	PMS 1038M	*Teredo* aff. *triangularis*	Philippines, Bohol, Danao	mangrove, pine bait

Table 6.1 Probes applied in FISH bacterial localization studies in digestive tract tissues.

Probe	Sequence	Target	Reference
Eub338	GCTGCCTCCCGTAGGAGT	Universal bacteria	Amann et al. 1990
EubNon	ACTCCTACGGGAGGCAGC	NA	Manz et al. 1992
ShipSymb1273	ACTGTTTTATGGGATTAGCTC	Shipworm symbionts	This study
ShipSymbNon	ACTGTTTTATGTGATTAGCTC	NA	This study
BsSymb a	CTGTACTCTATTACCCAGTTCTA	*B. setacea* symbionts	J. Fung (unpublished)
BsSymb aN	CTGTACTCTACTTACCCAGATCTA	NA	J. Fung (unpublished)
BsSymb b	CTGTACTCTAGCTACACAGTTCTA	*B. setacea* symbionts	J. Fung (unpublished)
BsSymb bN	CTGTACTCTACCTACACAGATCTA	NA	J. Fung (unpublished)
CFB560	WCCCTTTAAACCCART	Cytophaga-Flavobacter-Bacteroides	O' Sullivan et al. 2001
CFB319a	TGGTCCGTGTCTCAGTAC	Cytophaga-Flavobacter-Bacteroides	Manz et al. 1996
Arch915	GTGCTCCCCCGCCAATTCCT	Universal archaea	Stahl and Amann 1991
U1390	GACGGGCGGTGTGTACAA	All domains	Zheng et al. 1996

Table B.1 Number of colonies obtained on SBM+N+sig plates after homogenization of *B. setacea* gill tissue in either Osmolyte Addition Homogenization Buffer or SBM homogenization buffer. Each number is an average of 2 replicate plates. TMTC= too many to count, a very dense lawn of bacteria.

Dilution	Osmolyte Addition Homogenization Buffer	SBM Homogenization Buffer
10^{0}	TMTC	TMTC
10^{-1}	TMTC	TMTC
10^{-2}	210	2.5
10^{-3}	25	0

Figures

Figure 1.1 Photographs of 2 shipworm specimens. A. *Lyrodus pedicellatus* specimen PMS 549X. Photograph courtesy Marvin Altamia and the PMS-ICBG project. **B.** *Bankia setacea* specimen during extraction from wood.

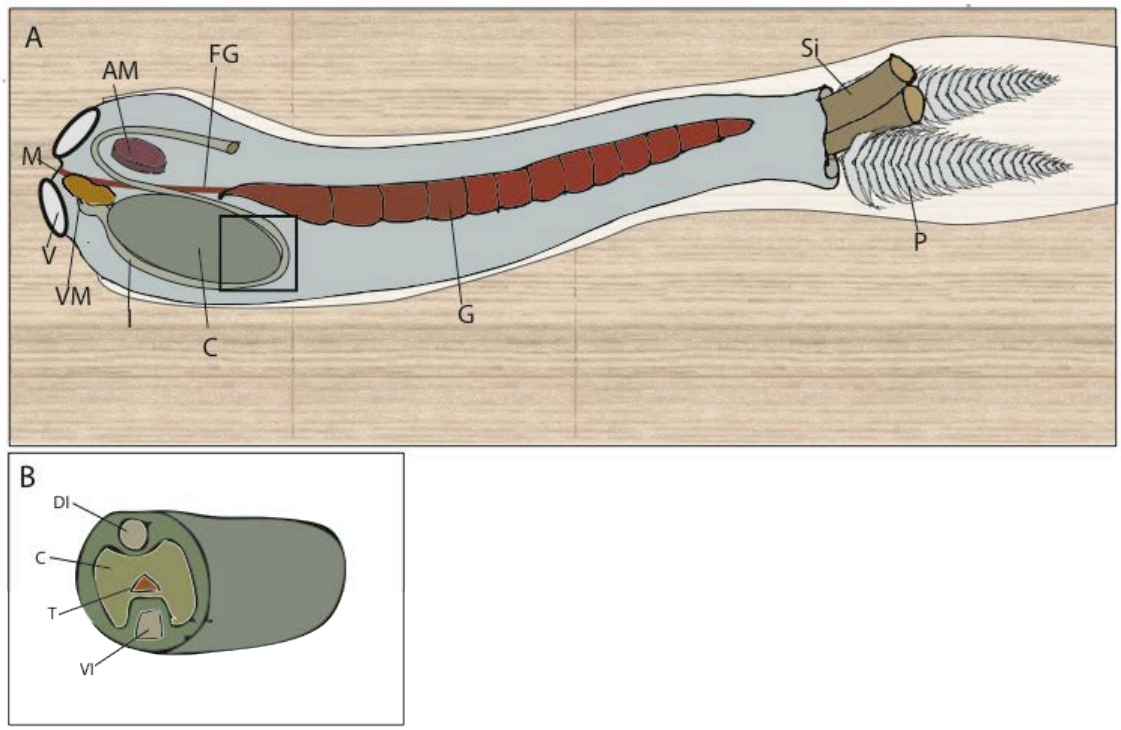


Figure 1.2 Diagram depicting basic shipworm anatomy. A. AM- Adductor muscle, M- Mouth, V- Valve, VM- Visceral Mass (includes stomach and digestive glands), I- Intestine, C- Caecum, FG- Food Groove, G- Gill (Ctenidium), P- Pallet, Si- Siphon (Based on Turner 1966) **B.** Drawing of a cross-section of the area within the black box in A. DI- Dorsal Intestine, C- Caecum, T- Typhlosole, VI- Ventral Intestine.

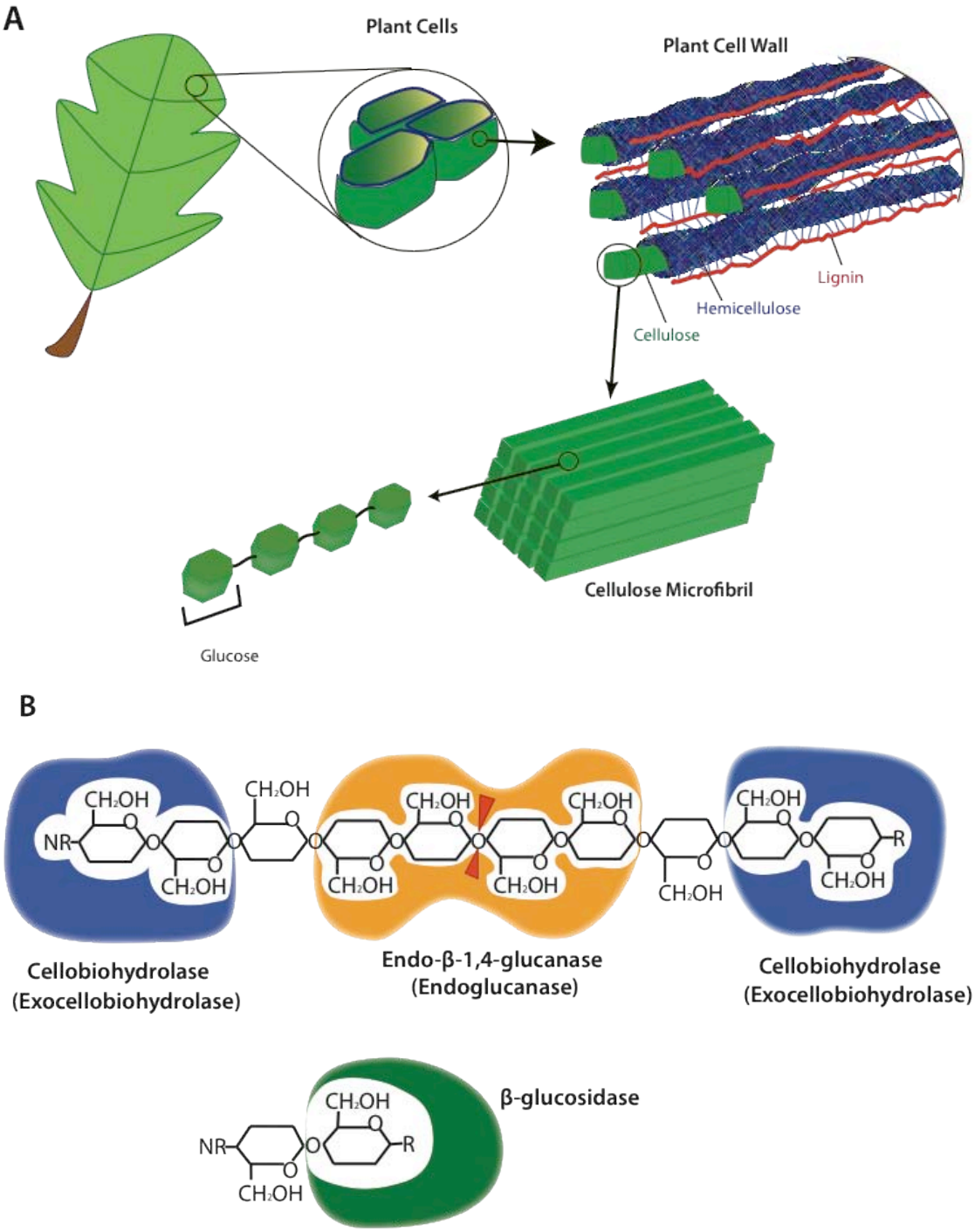


Figure 1.3 Figures demonstrating the complexity of lignocellulose degradation.
A. Diagram illustrating the cell wall components comprising lignocellulose.
B. Schematic of cellulosic breakdown to glucose by synergistic activity of endoglucanases (endo-β-1,4-glucanase), exoglucanases (cellobiohydrolase), and β-glucosidases.

	Teredinibacter turnerae	*Saccharophagus degradans*	*Cellvibrio japonicus*	*Nausitermes* Community
Glycoside Hydrolase Domains	101	130	122	704

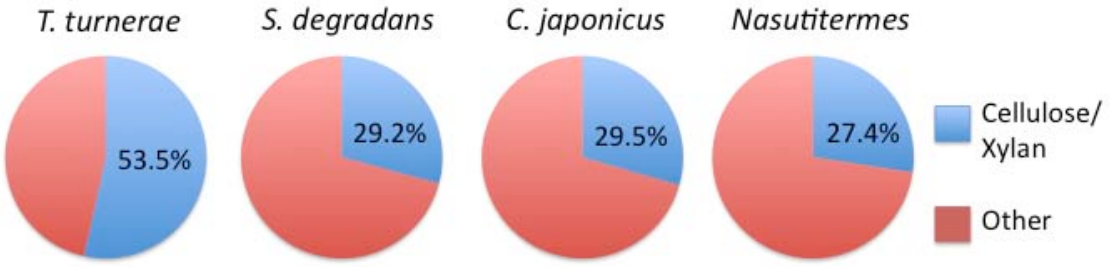


Figure 1.4 Glycoside hydrolase domain substrate specificity in *T. turnerae* T7901. While the number of GH domains in *T. turnerae* is not outstanding (top table), *T. turnerae* contains a large proportion of GH domains dedicated to cellulosic substrates (in blue on pie charts). *T. turnerae* is highly specialized for woody substrates. (Data from Yang et al. 2009)

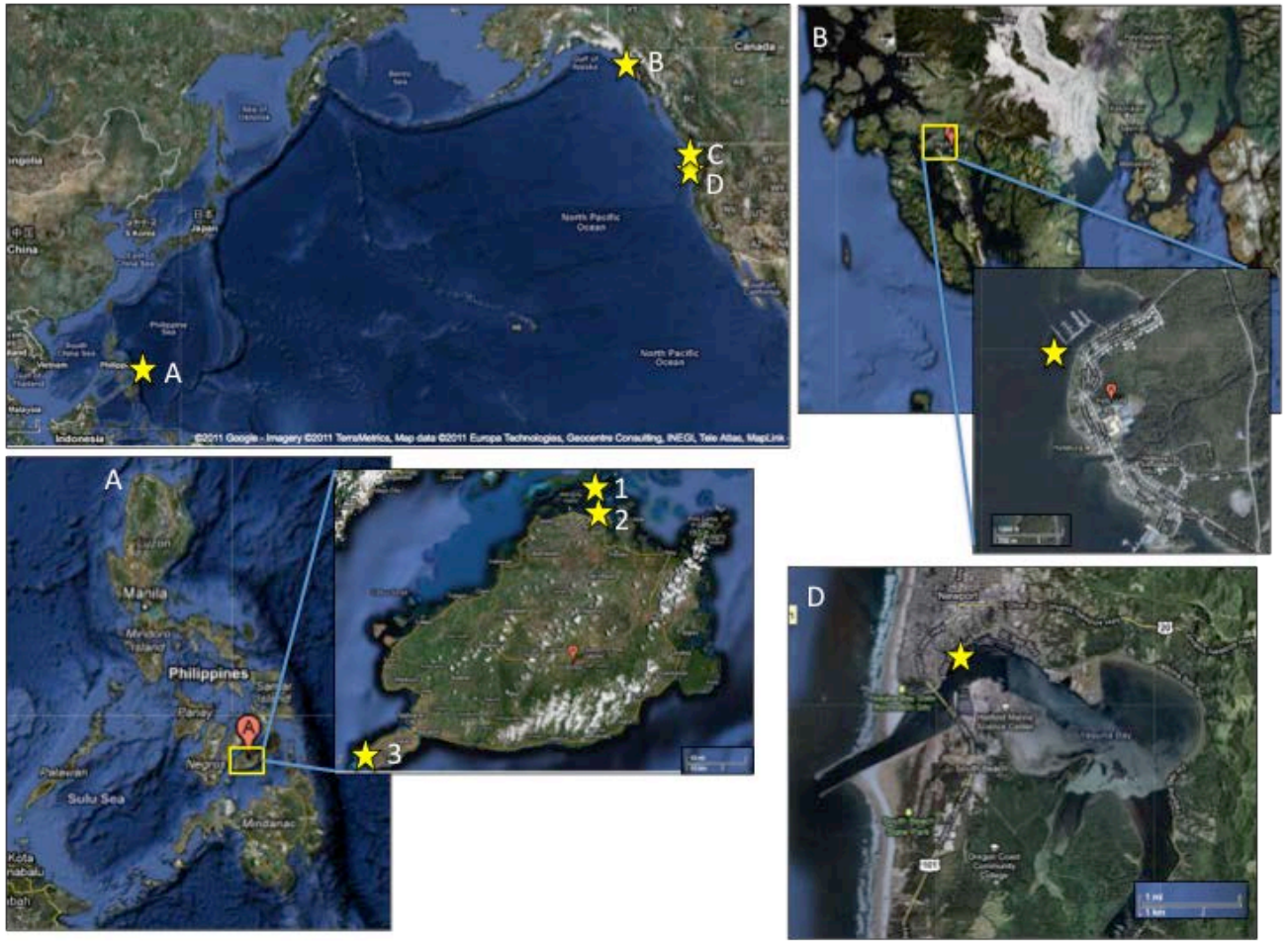


Figure 3.1 Collection sites spanning the northern Pacific Ocean. **A.** Philippines. All collections in the Philippines were a part of the PMS-ICBG collaborative project. 1. Cataban, Bohol 2. Danajon Bank, Bohol 3. Bil-isan, Bohol. **B.** Hydaburg, Alaska. All collections here involved the students from Hydaburg Public Schools under the direction of Wendy Smythe as a part of the NSF Geoscience Education program. **C.** Puget Sound, WA. (Close up not shown.) Collections through Ocean Genome Legacy. **D.** Yaquina Bay, OR. Traps were attached to the Satlantic WetLabs stationary research vessel in the Newport Harbor.

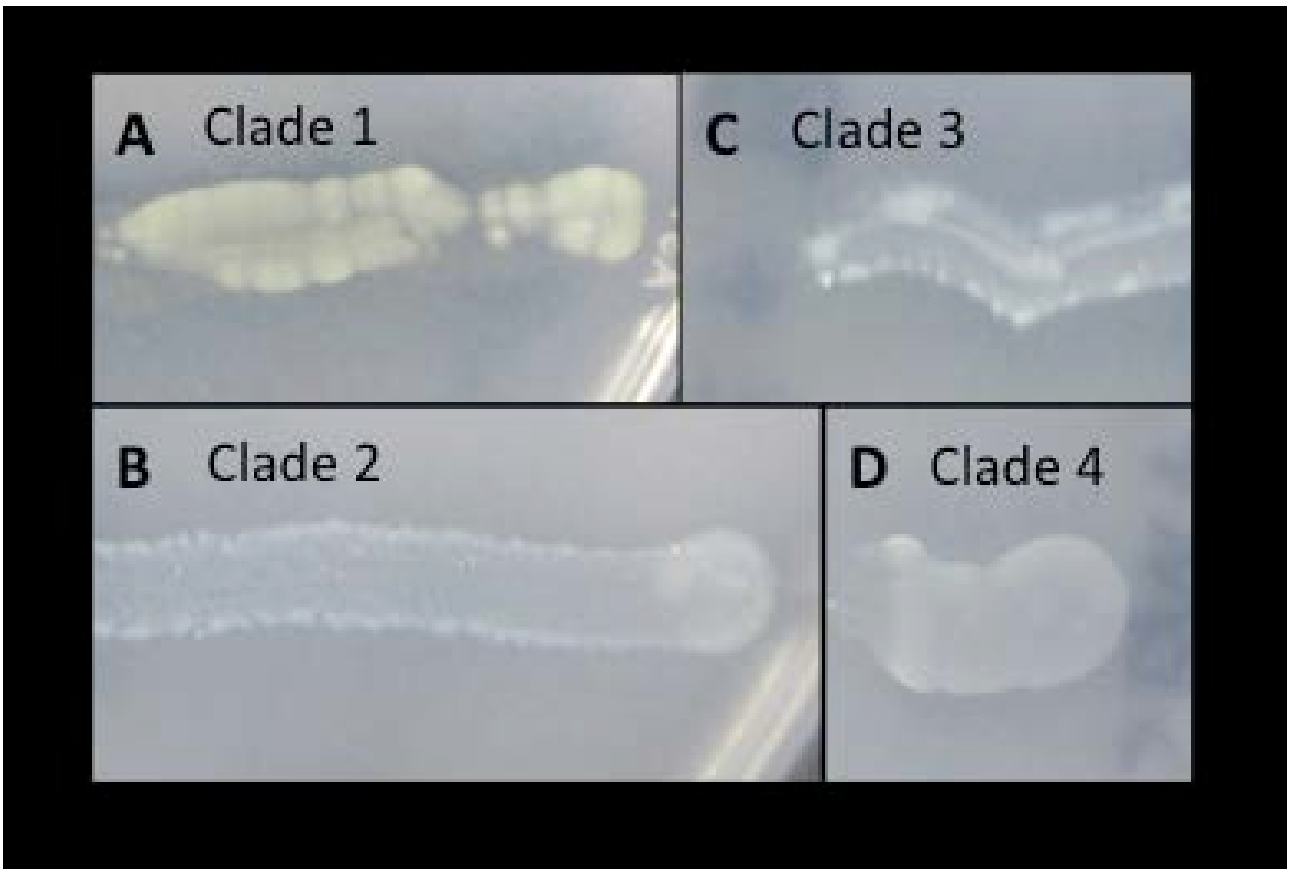


Figure 3.2 Photos of growth characteristics of strains representative of each shipworm symbiont clade. A. PMS 1178H.S.0a.5, Clade 1. **B.** PMS 1162T.S.0a.5, Clade 2. **C.** Bs02, Clade 3. **D.** Bs08, Clade 4. Note the morphological similarity in A and D and B and C. All cultures are grown on SBM+N+Sigmacell agar plates.

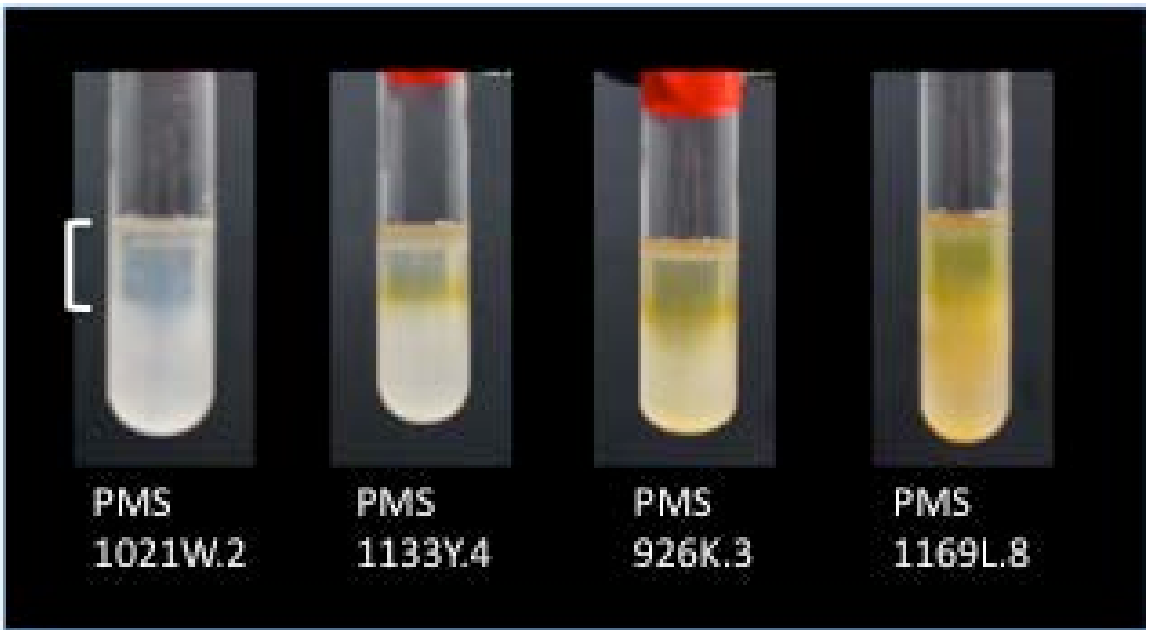


Figure 3.3 Photographs of 4 different Clade 1 PMS strains after 15 days growth in 0.2% agar SBM-N+Sigmacell containing test tubes. Note the difference in production of yellow pigment by these closely related (99% sequence similarity) strains. The white bracket highlights the disc of clearing of cellulose indicative of growth under N_2-fixing conditions. The clearing is quite large in these photographs due to the age of the cultures. Isolate IDs are truncated versions of complete PMS-ICBG strain names. Full names are: 1021W.S.0a.2, 1133Y.S.0a.4, 926K.S.0a.3, 1169L.S.0a.8.

Figure 3.4. Neighbor-joining phylogram of partial 16S rRNA gene sequences showing the identity of recently cultivated shipworm symbiont strains. All shipworm symbiont strains shown in bold, clone reference sequences are in plain text. * indicates strains whose genomes have been sequenced. * indicates strains approved for genome sequencing at the Department of Energy Joint Genome Institute. Bootstrap support based on 1000 replicates.

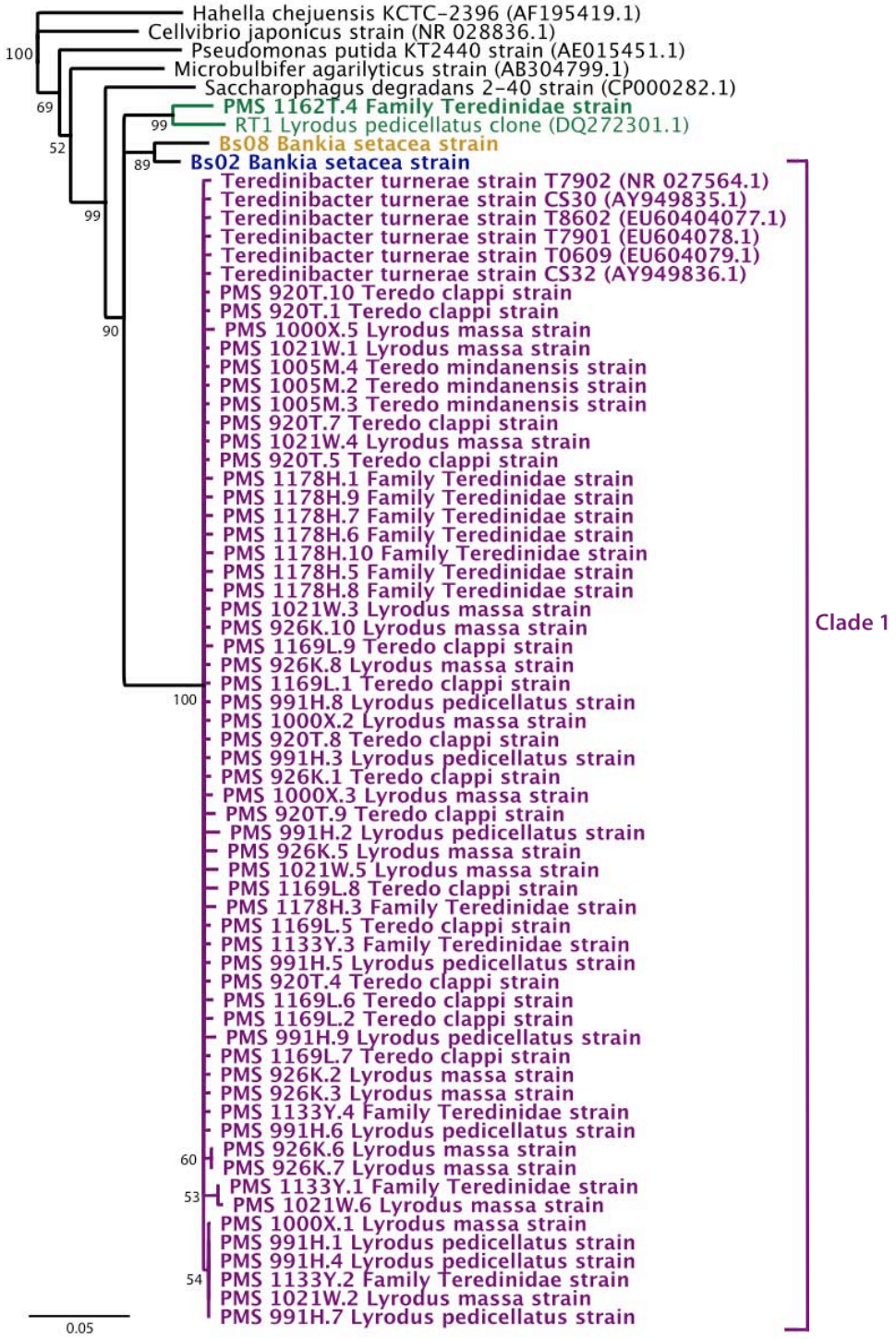


Figure 3.5 Neighbor-joining phylogram of partial 16S rRNA gene sequences showing the identity of recently cultivated shipworm symbiont strains with focus on Clade 1. All shipworm symbiont strains shown in bold, clone reference sequences are in plain text. Bootstrap support based on 1000 replicates.

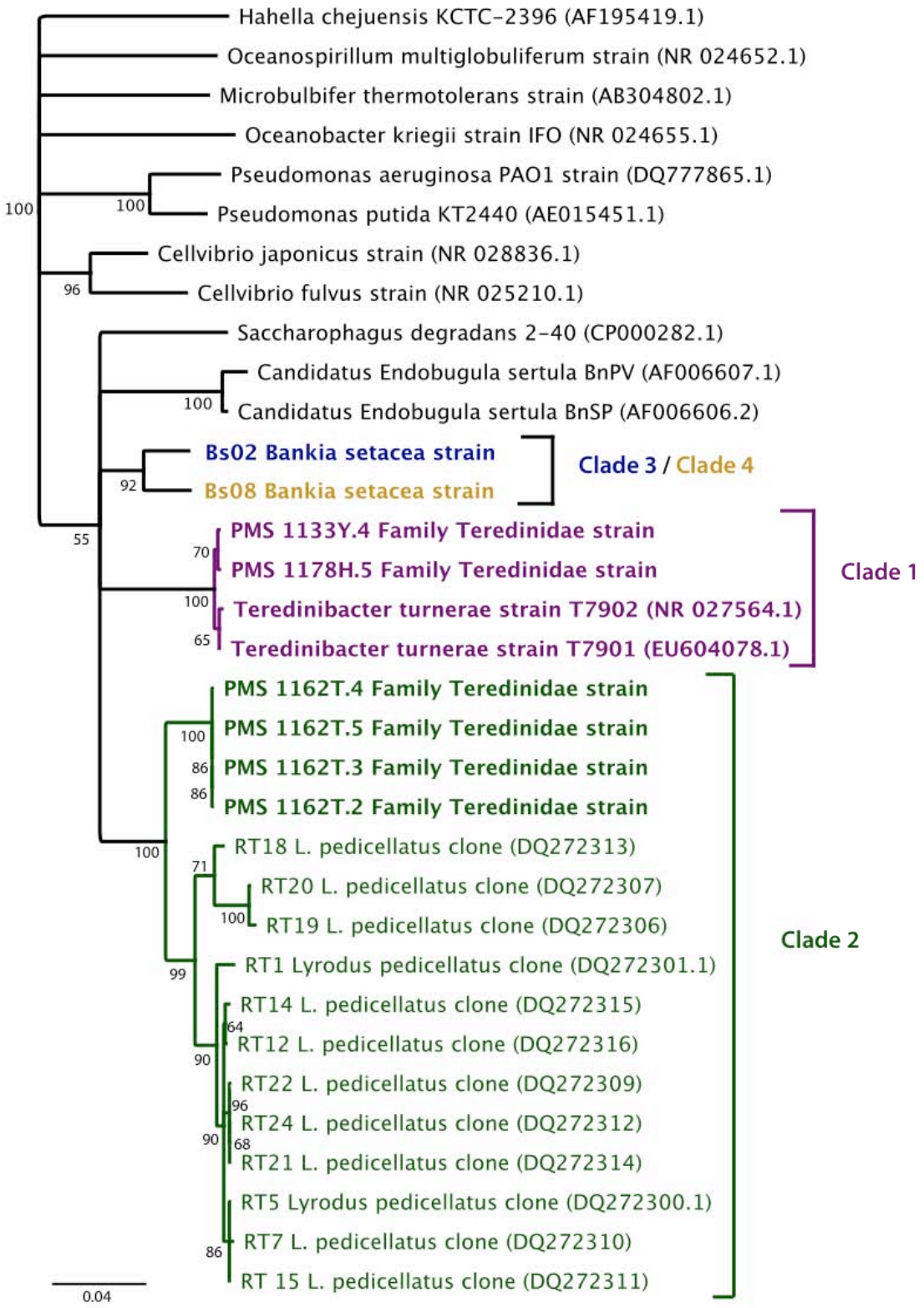


Figure 3.6 Neighbor-joining phylogram of partial 16S rRNA gene sequences showing the identity of recently cultivated shipworm symbiont strains with focus on Clade 2. All shipworm symbiont strains shown in bold, clone reference sequences are in plain text. Bootstrap support based on 1000 replicates.

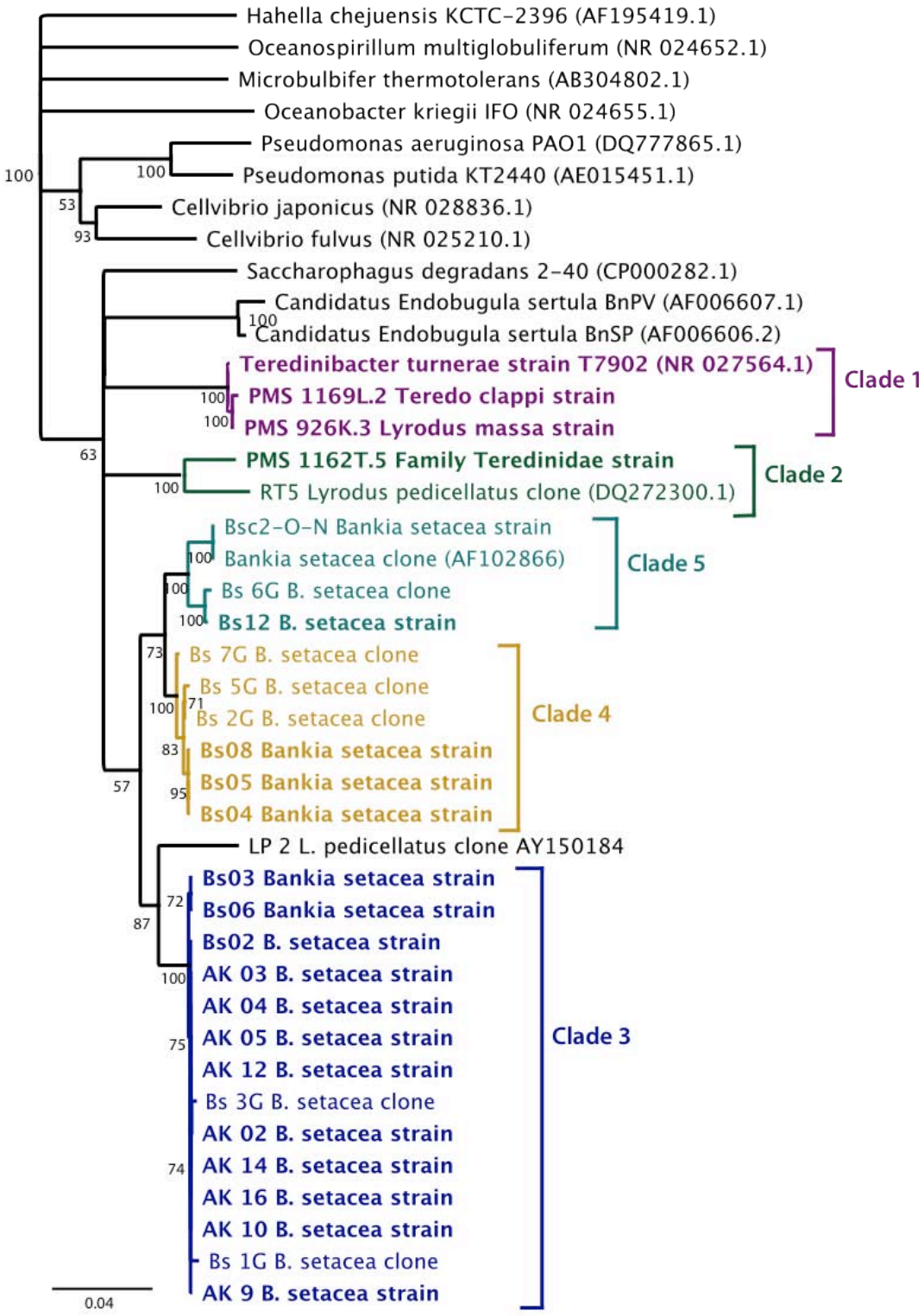


Figure 3.7 Neighbor-joining phylogram of partial 16S rRNA gene sequences showing the identity of recently cultivated shipworm symbiont strains with focus on Clades 3 and 4. All shipworm symbiont strains shown in bold, clone reference sequences are in plain text. Bootstrap support based on 1000 replicates.

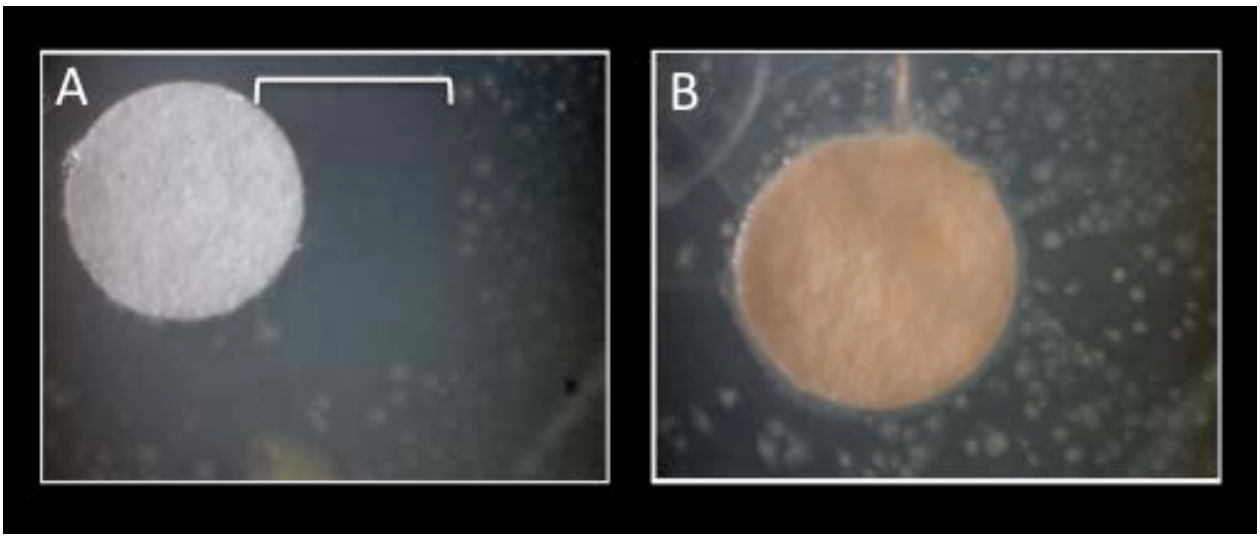


Figure 3.8 *T. turnerae* spent media inhibits growth of bacteria. A. *T. turnerae* spent media was added to the white paper disc in an attempt to stimulate growth of other shipworm symbionts, but instead it inhibited growth of bacteria, denoted by the white bracket. **B**. Control, sterile SBM was added to the disc in this case. The disc turned pink and colonies appeared close to the disc. Both discs were placed on the same SBM+N+Sigmacell agar plate.

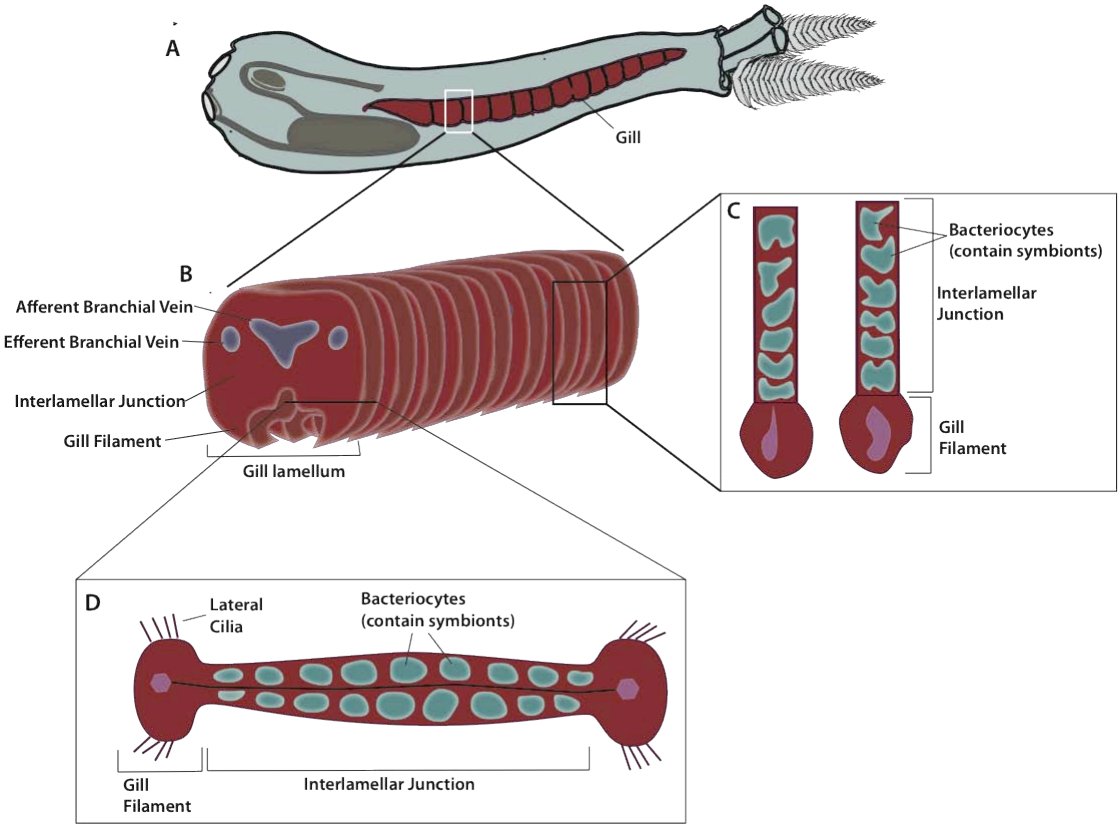


Figure 4.1 Representation of gill anatomy indicating the location of bacteriocytes. **A.** Depicts a diagram showing the position of the gill in the shipworm. **B.** Shows a close up of a region of gill. The individual gill lamellae, which are tightly packed like the pages of a bound book, are visible in this view. **C** and **D** illustrate the location of symbiont containing-bacteriocytes within the interlamellar junctions of gill lamellae.

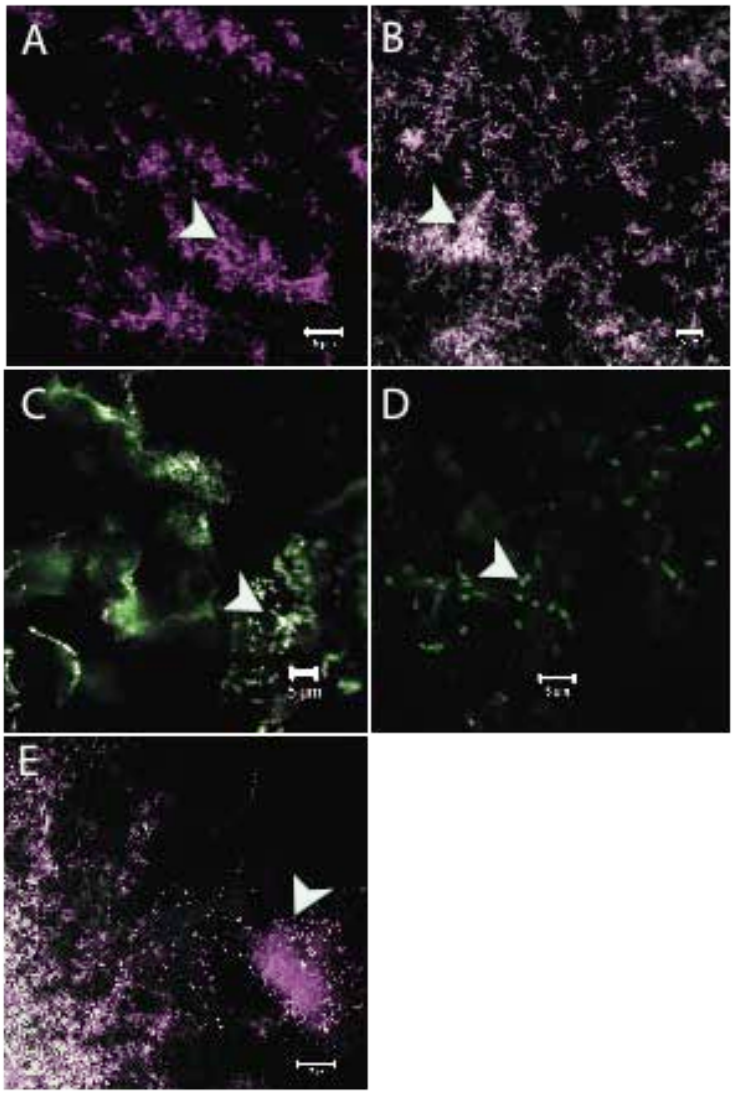


Figure 4.2 Dual probe *in situ* hybridizations with bacterial probe Eub338 and LP4, 1162T, or ShipSymb symbiont specific probes to confirm probe specificity. **A.** PMS 1178H.S.0a.5 (Clade 1) pure culture probed with Eub338 (magenta) and 1162T (green). The 1162T probe does not hybridize to this strain. **B.** PMS 1178H.S.0a.5 pure culture probed with Eub338 (magenta) and LP4 (green), co-localization of both probes is shown in white. **C.** PMS 1162T.S.0a.5 pure culture probed with Eub338 (green) and 1162T (magenta), co-localization is shown in white. Green in the background is autofluorescence of residual cellulose from the growth medium. **D.** PMS 1162T.S.0a.5 pure culture probed with Eub338 (green) and LP4 (magenta). LP4 does not hybridize to this strain. **E.** *Teredinibacter turnerae* T7901 pure culture probed with Eub338 (green) and ShipSymb 1273 (magenta). Co-localization of both probes shown in white. Note the similarity in cell morphology, curved rods, in the PMS 1178H.S.0a.5 and T7901 cultures and the coccus morphology in the PMS 1162T.S.0a.5 culture. Scale bars in A-D are 5 μm and 10 μm in E.

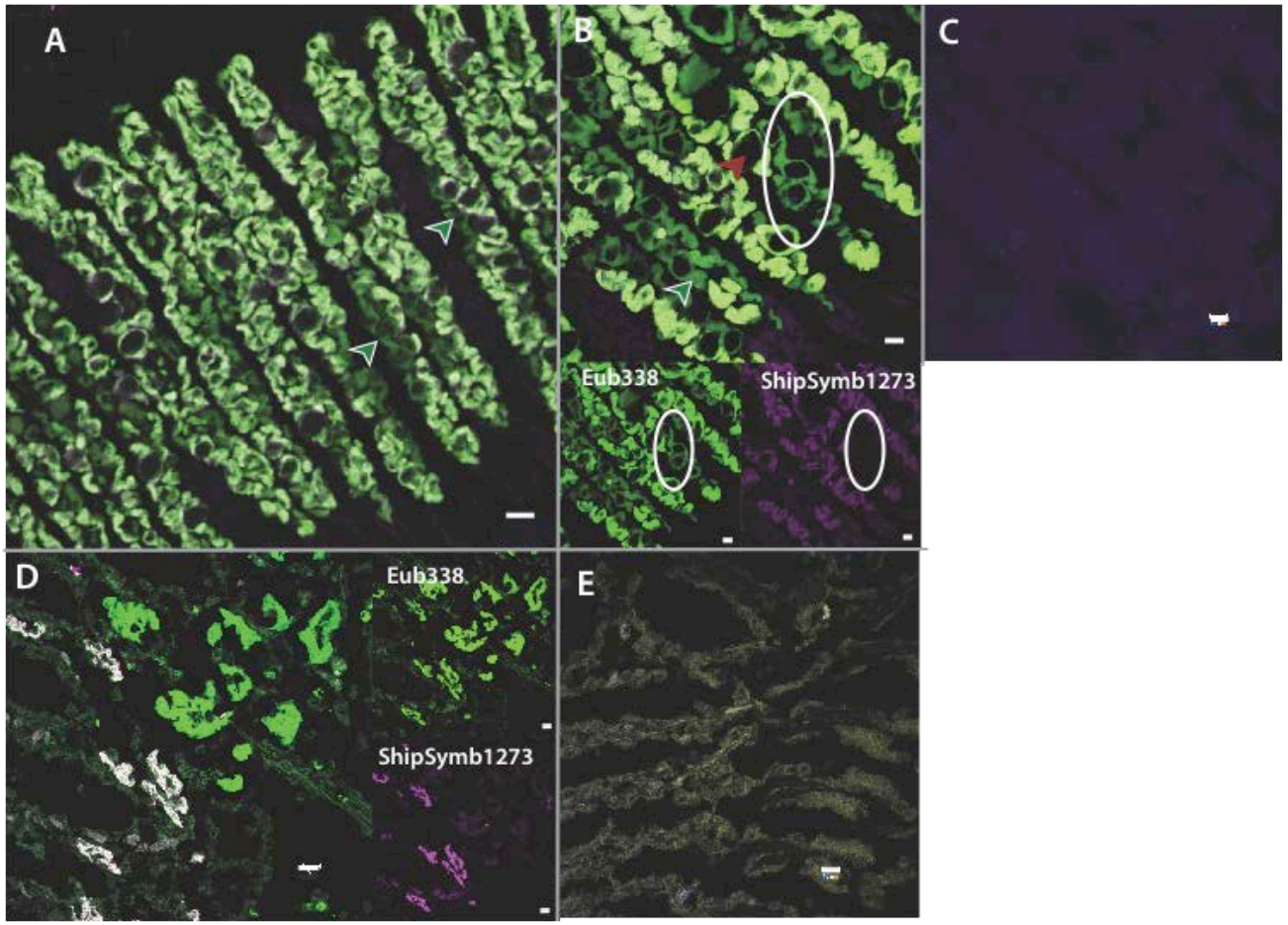


Figure 4.3 Shipworm symbiont-targeted probe, ShipSymb1273, does not hybridize to all gill bacteriocytes. A, B, and D show tissue probed with Eub338-cy5 bacterial probe (green) and ShipSymb 1273-cy3 (magenta). Green arrows point out bacteriocytes containing symbiont types with 16S rRNA gene sequences that are not complementary to the ShipSymb 1273 probe. Insets in B and D show probes individually, Eub338 in green and ShipSymb1273 in magenta. **A.** *Lyrodus massa.* Scale bar = 20 µm. **B.** *L. massa.* The red arrow points out a host cell nucleus. Circles contain bacteriocytes housing bacteria that do not bind ShipSymb1273. **D.** *Bankia setacea.* **C and E.** Region of gill tissue similar to the one shown in B and D respectively probed with EubNon-cy5 and ShipSymbNon-cy3 negative control probes. B-E Scale bar = 10 µm.

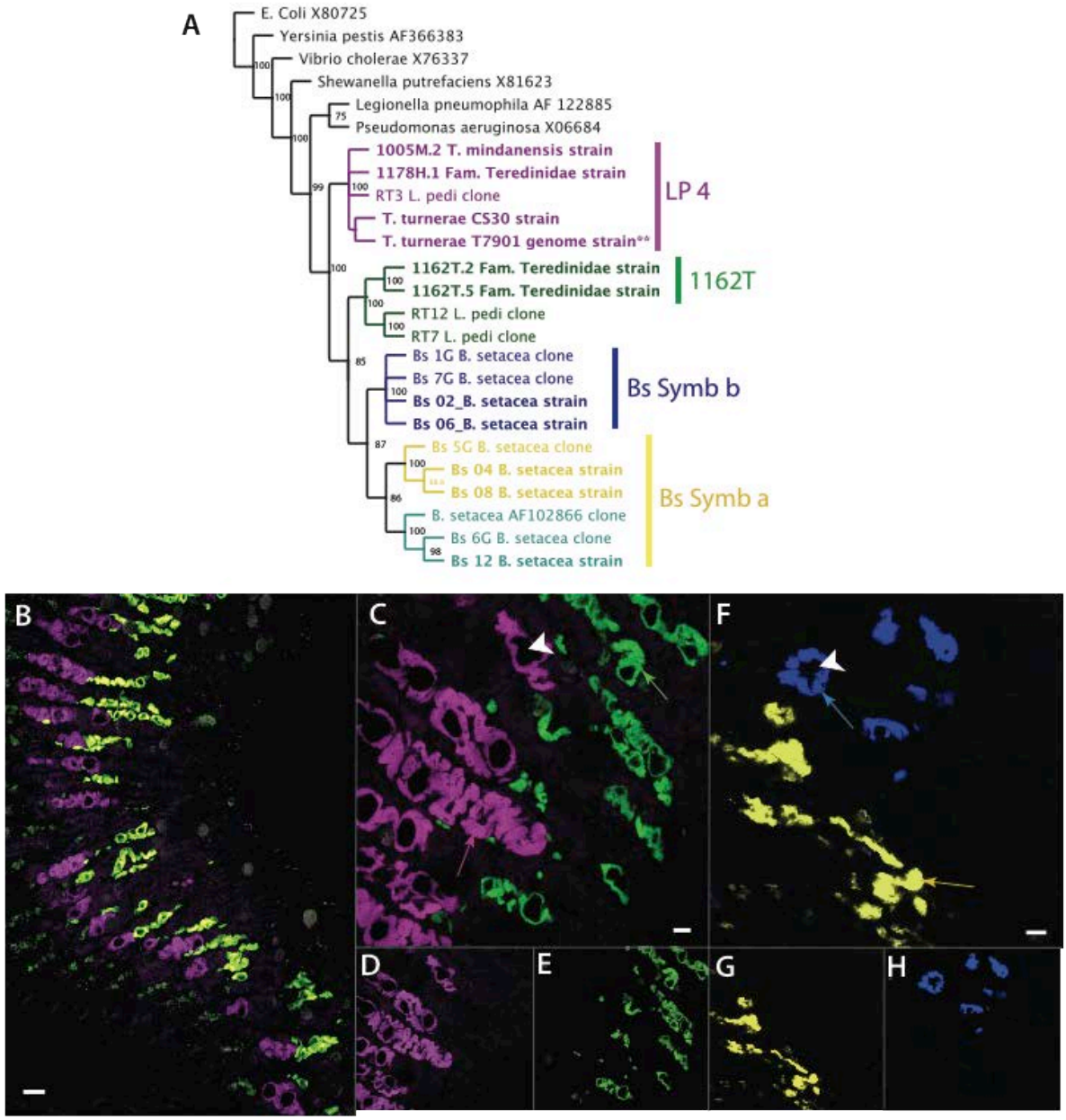


Figure 4.4. Symbiont types are segregated in gill bacteriocytes. A. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences of representative shipworm gill symbionts illustrating the specificity of symbiont type-specific probes applied in FISH with gill tissue. **B and C.** *Lyrodus massa* gill tissue probed with 1162T-cy5 (green) and LP4-cy3 (magenta) probes. **D and E** show probes LP4-cy3 and 1162T-cy5 individually from the same image as in B. **F.** *Bankia setacea* gill tissue probed with Bs Symb a-cy3 (yellow) and Bs Symb b-cy5 (blue). **G and H** show probes Bs Symb a-cy3 and Bs Symb b-cy5 individually from the same image as in F. Scale bar = 10 μm in C-H and 50 μm in A. White arrows point to host cell nuclei.

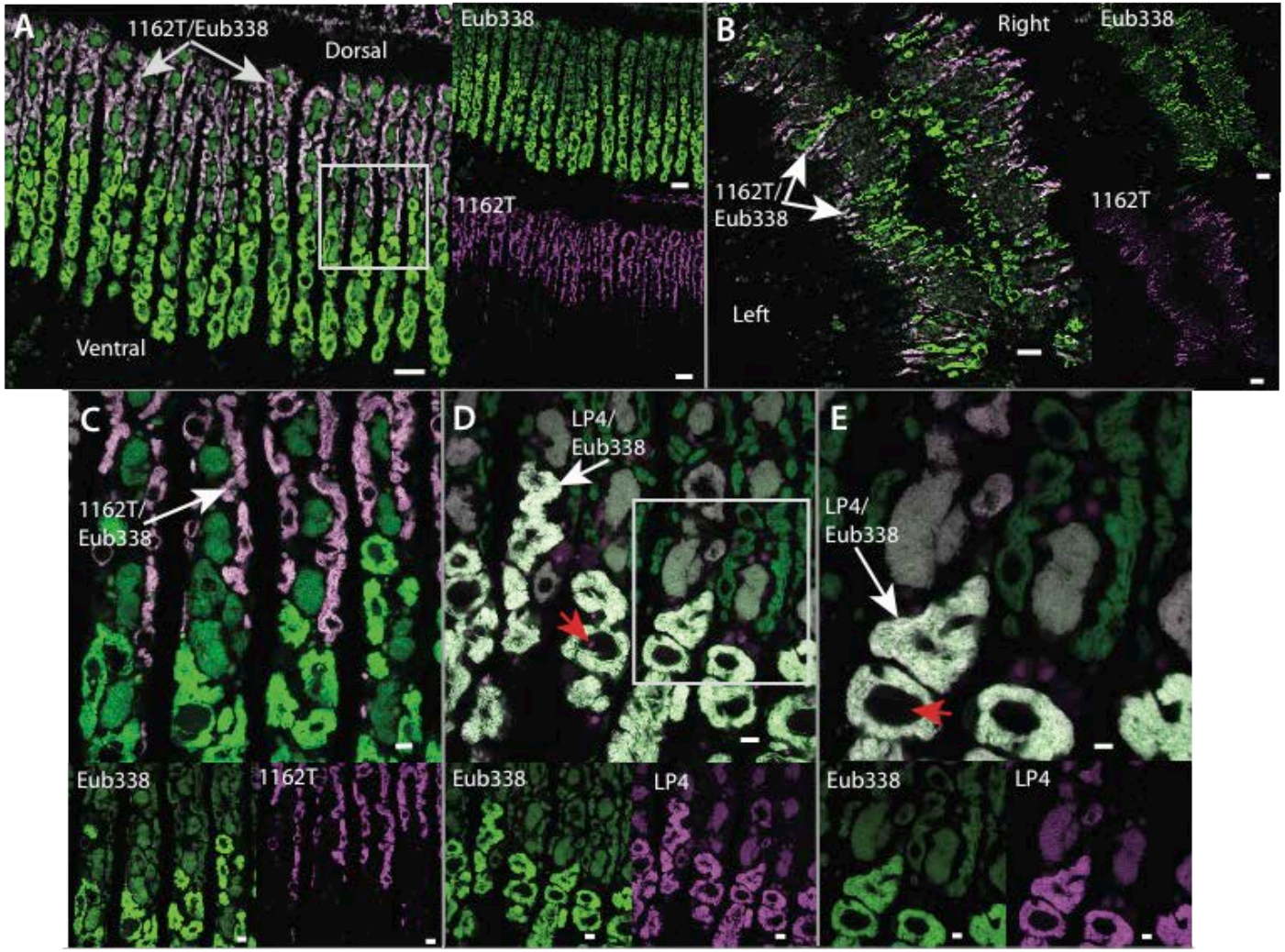


Figure 4.5 Distribution of bacteriocytes containing 1162T and LP4 complementary bacteria. A-C LSCM micrographs of tissue sections hybridized with Eub338-cy3 (green) and 1162T-cy5 (magenta) probes. D and E hybridized with Eub338-cy5 (green) and LP4-cy3 (magenta) probes. Co-localization of both probes appears white. Probe colors were falsely assigned during image capture. **A.** Sagittal section (similar to Figure 4.1c) of *Lyrodus pedicellatus* gill tissue. **B.** Lateral section of *Teredo aff. triangularis* gill tissue showing the left and right demibranchs. The central axis runs approximately from top left to bottom right of this image. **C.** *Lyrodus pedicellatus.* Close up of a region enclosed by the box in A. **D.** *Lyrodus pedicellatus.* Next consecutive section to the one shown in A and C probed with LP4 (magenta) and Eub338 (green). **E.** *Lyrodus pedicellatus.* Close up of a region enclosed by the box in D. Scale bar A and B=50 µm, C and D=10 µm, E=5 µm. All images show the middle (lengthwise) region of the gill. All insets show probes individually.

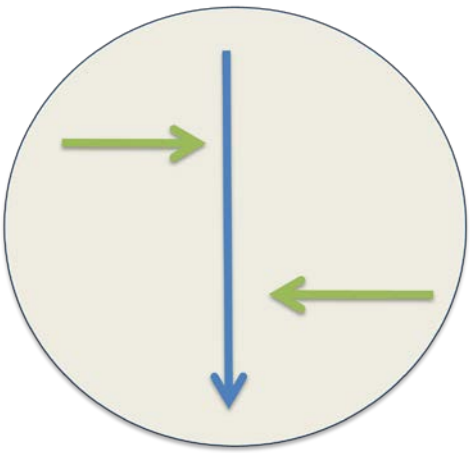


Figure 5.1 Diagram of the co-plating assay used to test competition between gill symbiont types. One strain is streaked vertically onto the plate (green arrow) and a second strain is streaked perpendicularly to the first (green arrows). The second strain streak ends very close to, but not touching the first strain.

Figure 5.2. Results of cross-streaking experiments with shipworm symbiont strains. Growth medium in all photos is SBM+N+Sigmacell. Red arrows denote regions of clearing of growth, an indicator of inhibition of one strain by the other.

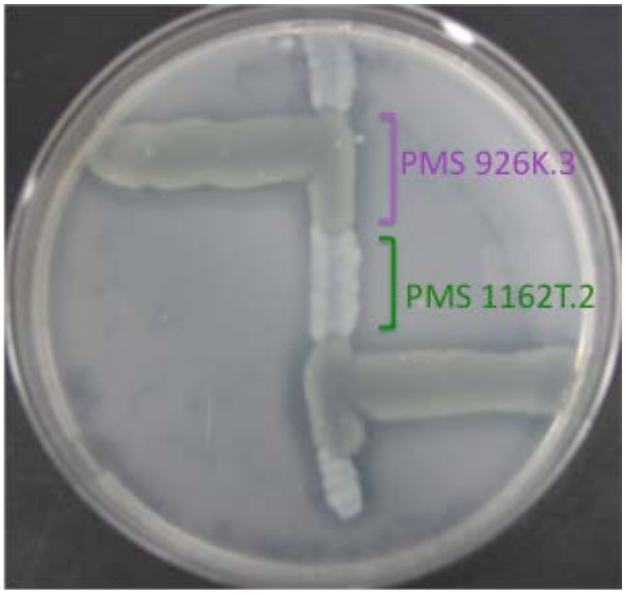


Figure 5.3 Photograph depicting the typical result of co-plating with PMS 926K.S.0a.3 with a strain of Clades 2-4. This strain did not just inhibit growth of the competitor, but extended growth into the region previously occupied by the other strain.

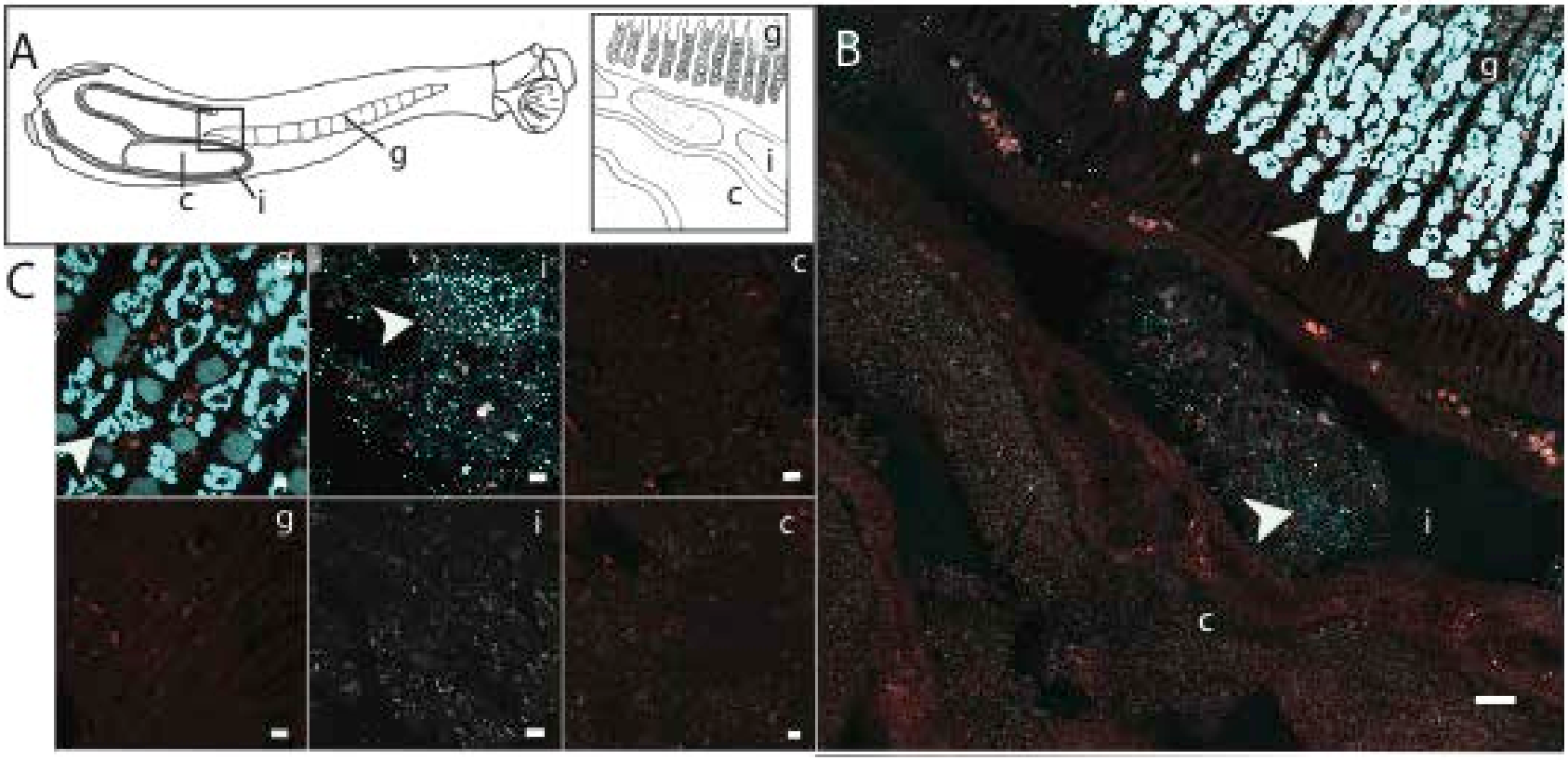


Figure 6.1 Overview of fluorescence *in situ* hybridization localization of bacteria in a *Lyrodus pedicellatus* shipworm specimen. g- gill, i- intestine, c-caecum. **A.** Drawing adapted from Turner 1966 showing shipworm anatomy. Inset shows a close up of the region within the box and is representative of the micrograph in B. **B.** LSCM micrograph of a tissue section showing gill, intestine, and caecum. Eub338-cy5 universal bacterial probe is shown in turquoise and is representative of bacteria. Autofluorescence from tissue and lignocellulose within the caecum and intestinal content is in red. Arrows point out locations where bacteria was detected. Scale bar=50 µm. **C.** Top row- Close up images corresponding to tissues annotated in B. Eub338-cy5 universal bacterial probe is shown in turquoise and autofluorescence is in red. Arrows point to: gill- bacteria densely packed in bacteriocytes, Intestine- Numerous bacteria within the fecal bolus. Bottom row- negative control images corresponding to images in column B. Scale bars=10 µm.

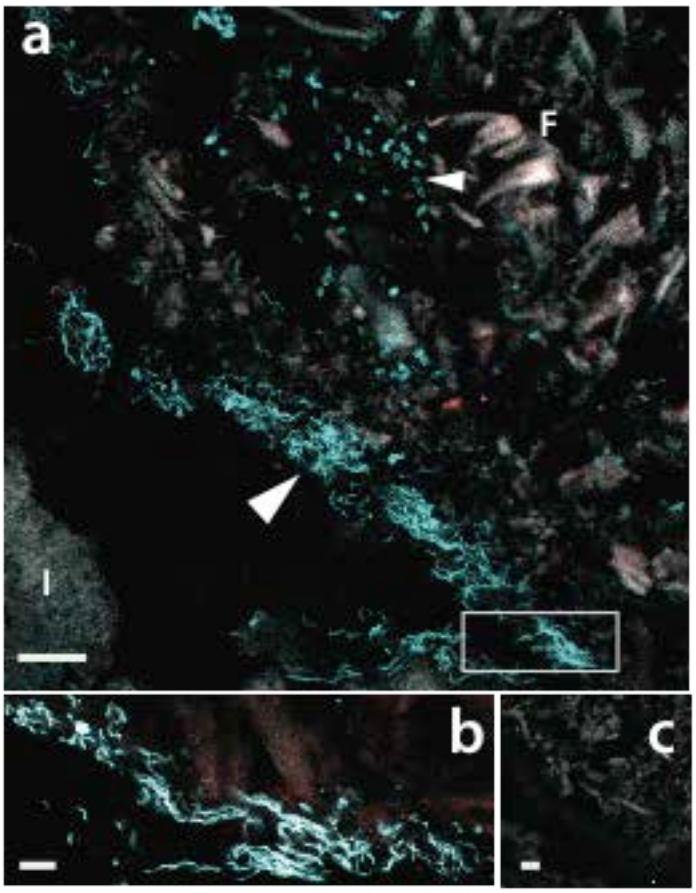
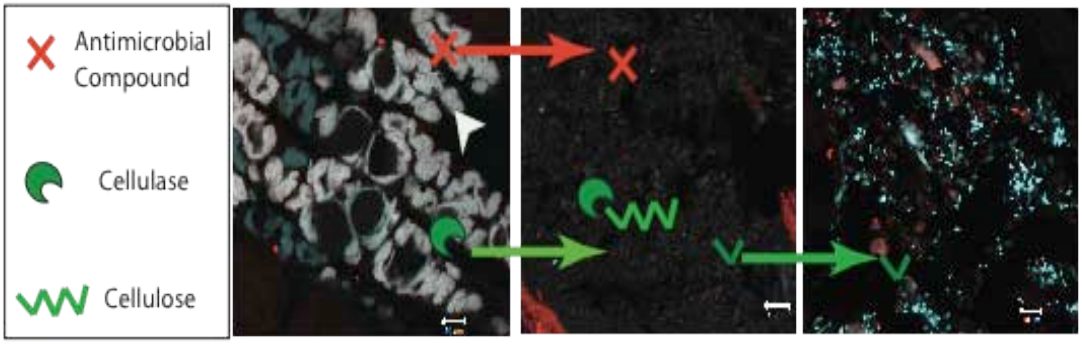


Figure 6.2 Bacterial morphotypes in the intestine of *Bankia setacea.* Confocal micrographs depicting hybridization of a bacteria-domain specific 16S rRNA directed oligonucleotide probe (Eub338) and a negative control probe (EubNon) to sectioned tissues of *Bankia setacea*. Both probes are labeled with the fluorochrome cy5 shown false colored in turquoise in images shown. **a.** Tissue section showing the fecal pellet (F) and the intestinal wall (I) hybridized with Eub338-cy5. Note the presence of multiple cell morphologies, spiral (large arrow) and rod or cocci (small arrow). **b.** Enlarged view of the region in the box in a. **c.** Tissue section adjacent to that shown in (a) hybridized with EubNon-cy5. Scale bars are 10 μm in (a) and (c) and 5 μm in (b).



	Gill (Ctenidium)	Caecum (Appendix)	Intestine
Symbionts or Bacteria present?	Symbionts	Very few Bacteria	Bacteria
Cellulose Accumulation?	No	Yes	Not determined

Figure 6.3 Proposed model of the shipworm symbiosis as supported by results of this study. FISH micrographs illustrate a summary of results showing that cellulase-producing symbionts are confined to the gills where lignocellulose degradation does not occur. Micrographs- FISH images of *Lyrodus massa* tissue sections (Left panel-gill, Middle panel- caecum, Right panel-intestine) representative of the average result for each tissue. Bacteria hybridized with the Eub338-cy5 probe shown in turquoise, co-localization with endosymbiont specific ShipSymb1273-cy3 probe in white. Gill contains densely packed endosymbionts, hypothesized to produce and transport enzymes for digestion of lignocellulose and bioactive compounds to the caecum. The caecum contains very few bacteria, but is the site of accumulation of cellulosic substrate. The intestine contains numerous bacteria, potential targets of symbiont-produced anti-microbial compounds and degraders of by-products of lignocellulosic digestion.

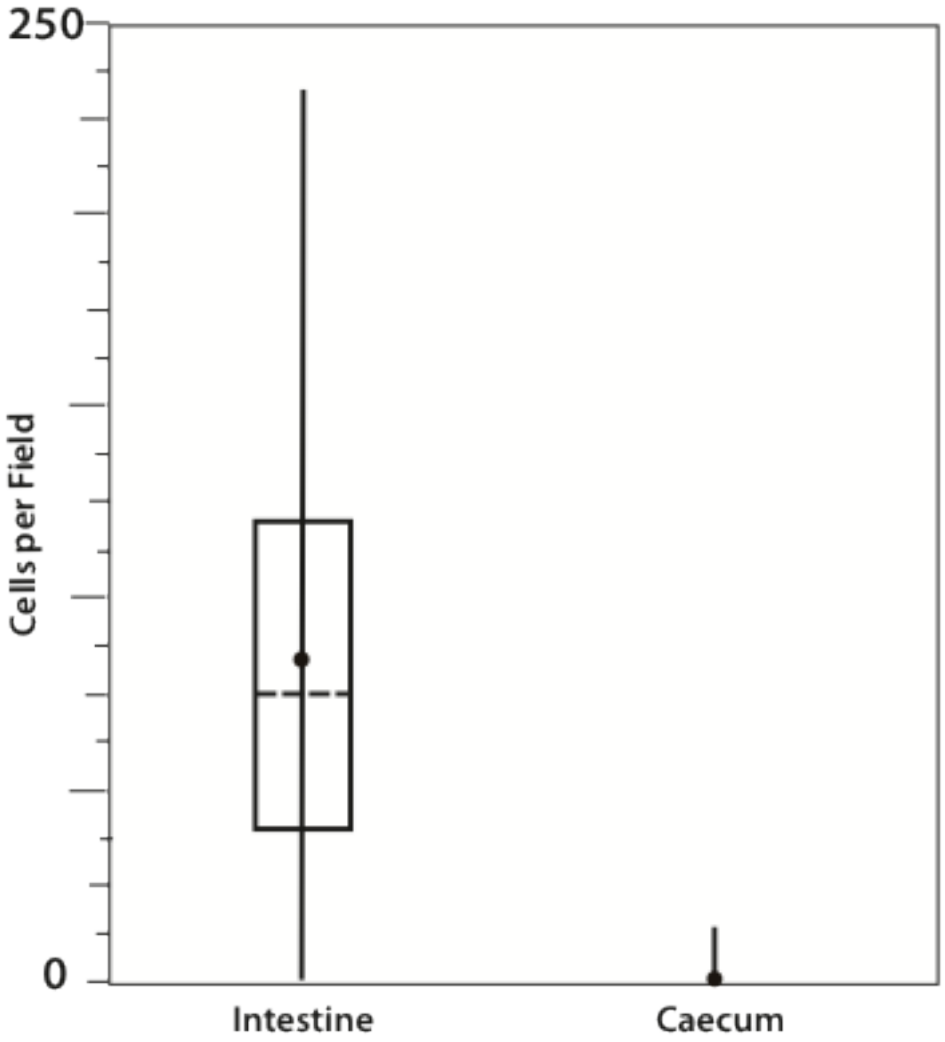


Figure 6.4. Frequency of bacteria observed in contents of intestine and caecum. Box plot showing frequency of bacteria observed per field in intestine and caecum of four shipworm species. Forty randomly selected fields (100 µm x 100 µm) were examined for each tissue by laser scanning confocal microscopy using the bacteria-targeted probe Eub338 cy5 or the negative control probe EubNon cy5. Vertical lines indicate range of values, horizontal dashed lines and solid circles indicate median and mean values respectively, and open boxes indicate upper (top line) and lower (bottom line) quartiles respectively. Note upper and lower quartiles and median in caecum have zero values and have been omitted for clarity.

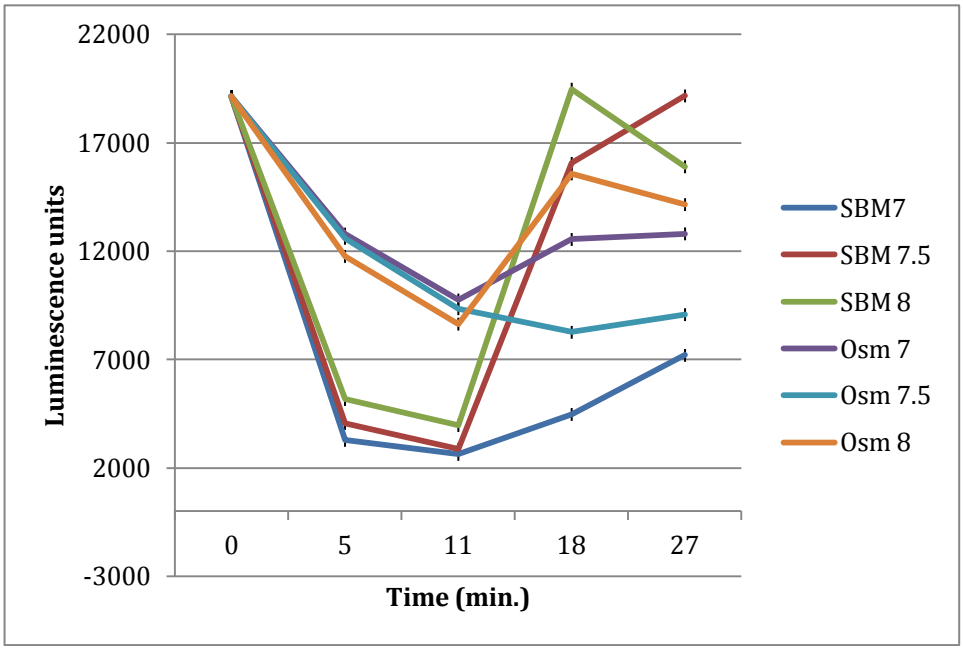


Figure B.1 Cell viability as represented by luminescence released from the reaction of luciferin with ATP in the presence of luciferase over time. Error bars indicate the average error of results obtained by this assay. Error was calculated for replicates of each sample at each time point and the average of these values is represented in error bars.

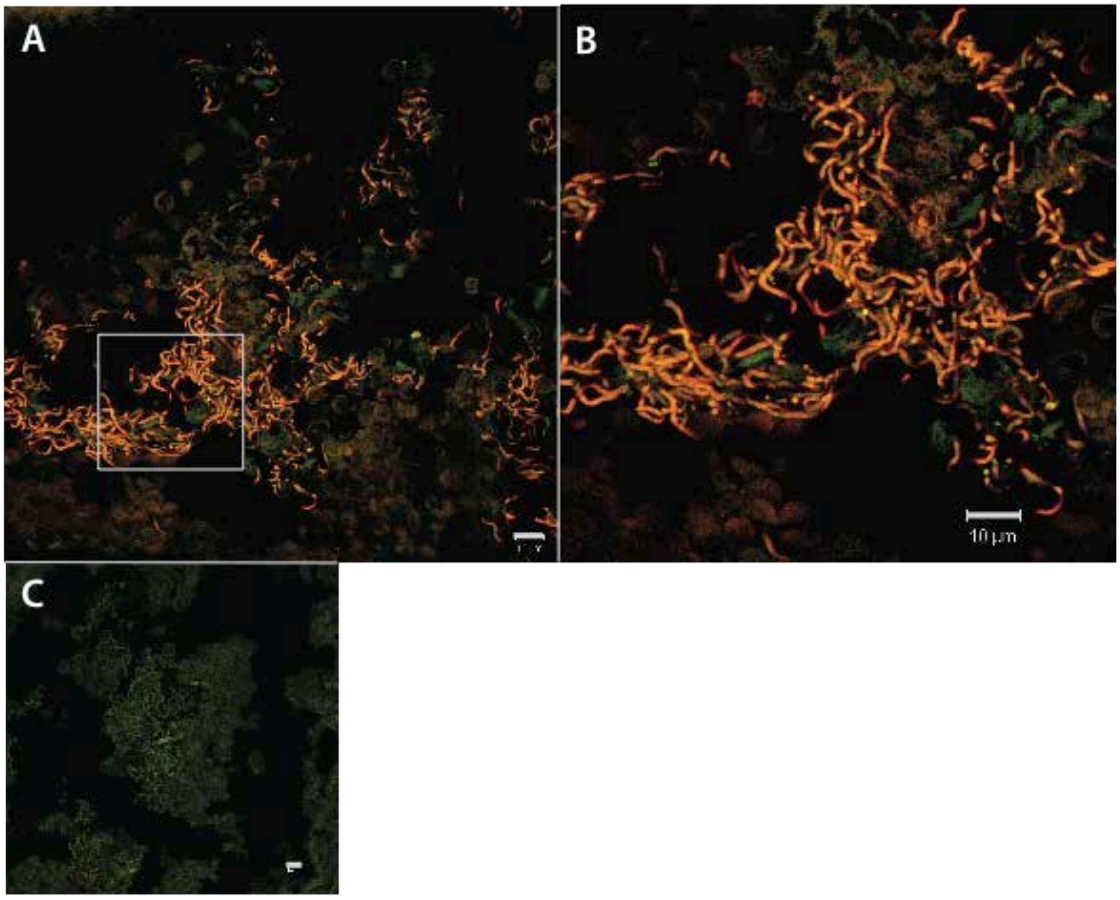


Figure C.1 A. FISH micrographs illustrating the vermiform caecum organisms in a *Bankia setacea* caecum. A. Caecum tissue hybridized with U1390-cy3 probe (red) and Eub338-cy5 (green). **B**. Close up of the region inside the box in A. **C.** Neighboring section to that shown in A hybridized with EubNon-cy5 (green). Scale bar in each image = 10 µm.

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Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Environ Microbiol* **56**: 1919-1925.

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Biographical Sketch

Meghan Betcher was born in Choteau, Montana on August 26, 1981. She received a B.S. in Microbiology, Microbial Ecology track from the University of Montana in 2004. During her undergraduate study Meghan performed undergraduate research in the lab of Dr. William Holben and then continued on as a research assistant during the rest of her school and for a year following graduation studying the impacts of forest fires on soil microbial communities and the impacts of noxious weeds on soil microbial communities.

Meghan then decided to experience life outside of the lab and travelled in Argentina where she worked on a farm, spent time working as a fire lookout in the Bob Marshall Wilderness in Montana, and worked in the cellar learning the art of wine making at two different wineries in Washington and Oregon. Meghan then moved to Portland and worked with the Bicycle Transportation Alliance before enrolling at OHSU in the Environmental Science and Engineering, joining the Haygood lab, and developing a keen interest in shipworms and their symbionts.