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Composition and Function of a Novel Consortial Endosymbiosis in the Shipworm Lyrodus pedicellatus

Daniel L. Distel
Principal Investigator; University of Maine, Orono

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Final Report for Period: 03/2000 - 02/2004 **Submitted on:** 12/02/2003
Principal Investigator: Distel, Daniel L. **Award ID:** 9982982
Organization: University of Maine
Title:
Composition and Function of a Novel Consortial Endosymbiosis in the Shipworm Lyrodus pedicellatus.

Project Participants

Senior Personnel

Name: Distel, Daniel
Worked for more than 160 Hours: Yes
Contribution to Project:

Name: Eash, Angela
Worked for more than 160 Hours: No
Contribution to Project:
Undergraduate student involved in conducting experiments and analyzing data; received support from this grant.

Name: Bosse, Benjamin
Worked for more than 160 Hours: No
Contribution to Project:
Undergraduate student involved in conducting experiments and analyzing data; received support from this grant.

Post-doc

Graduate Student

Name: Luyten, Yvette
Worked for more than 160 Hours: Yes
Contribution to Project:
This student performed experiments contributing to the project in partial fulfillment of requirements of a Masters Degree.

Name: Nair, Nitin
Worked for more than 160 Hours: No
Contribution to Project:
Graduate student recently joined the project. Has not yet received any support fromt the grant.

Undergraduate Student

Name: Amin, Mehwish
Worked for more than 160 Hours: Yes
Contribution to Project:
This undergraduate student performed experiments related to this project in partial fulfillment of the research requirements for the degree of Bachelor of Science.

Name: Burgoyne, Adam
Worked for more than 160 Hours: Yes
Contribution to Project:
This student helped to perform experiments in support of this grant while working as a student lab aid. Support was provided by this grant.

Name: Wiley, Jennifer
Worked for more than 160 Hours: Yes
Contribution to Project:

Undergraduate student involved in conducting experiments and analyzing data; received support from this grant.

Name: Chen, Tim

Worked for more than 160 Hours: No

Contribution to Project:

Undergraduate student involved in conducting experiments and analyzing data; received support from this grant.

Technician, Programmer

Name: Morrill, Wendy

Worked for more than 160 Hours: Yes

Contribution to Project:

This technician performed experiments in support of this research with salary partially provided by this grant.

Other Participant

Research Experience for Undergraduates

Organizational Partners

Other Collaborators or Contacts

Dr. Martin Polz, Assistant Professor, Dept. of Civil and Environmental Engineering (MIT) has participated in this research by providing access to, and assistance with, quantitative PCR devices and methods being developed at MIT.

Activities and Findings

Research and Education Activities: (See PDF version submitted by PI at the end of the report)

Please see uploaded combined activities and findings PDF file.

Findings: (See PDF version submitted by PI at the end of the report)

Please see uploaded activities and findings PDF file.

Training and Development:

Education and Training

The awarded funding has helped us to continue our active support for student and teacher education, research and training activities. This project has helped to support several graduate and undergraduate students and a high school teacher summer intern at U. Maine. Students were involved in all levels of research and performed the majority of experimental work. High school, undergraduate and graduate students as well as high school science teachers have completed research projects in both laboratories resulting in numerous authorships, including one coauthorship and a pending first authorship for undergraduate students in the Distel lab. Students have received the following awards for research conducted in the Distel laboratory on projects related to this grant: 1) The Barry M. Goldwater Scholarship for Outstanding Undergraduates in Science and Mathematics, The Pfizer Summer Undergraduate Research Fellowship, The Maine Science and Technology Foundation MERITS Summer Fellowship, The Intel High School Science Talent Search (semifinalist), the Maine Upward Bound Summer Internship SOAR Award and others. All listed awards included cash scholarships or stipends ranging in value from $1000-$7500, demonstrating the value of NSF dollars for leveraging additional student support from other agencies and for boosting the academic careers of students at all levels. We will continue our ongoing efforts to recruit broadly, taking advantage of existing personal and programmatic links to institutions serving underrepresented minorities.

Outreach Activities:

Outreach Activities

In the summer of 2000, large-scale destruction of coastal wooden structures due to shipworm activity was observed for the first time in the Penobscot Bay area. Waters this far north were formerly thought to be too cold to too sustain shipworm activity. The recent expansion of shipworm populations from the south caused the complete destruction of newly a newly erected municipal pier in Belfast, Maine less than a year after its completion.

In response to this local disaster, we have begun sampling larval shipworm populations in Belfast Harbor and elsewhere to help determine the identity of the invading species, the extent of infestation and the relationship of seasonal temperature change to shipworm activity. We have also consulted with local harbormasters and fishermen and conducted a survey to determine the extent of shipworm infestation problems in Maine and to suggest ways to limit future damage. We have also offered interviews to the local press to help disseminate reliable information about the newly arrived pests. In addition we have begun work on a shipworm website to help provide information to the public regarding our work and the biology of shipworms in general, however this will not likely become public until spring or summer.

Journal Publications

Distel, D. L., W. Morrill, N. MacLaren-Toussaint, D. Franks, and J. Waterbury, "Teredinibacter turnerae gen. nov., sp. nov., a dinitrogen-fixing, cellulolytic,
endosymbiotic gamma-proteobacterium isolated from the gills of wood-boring
molluscs (Bivalvia: Teredinidae)", Int J Syst Evol Microbiol, p. 2261, vol. 52, (2002). Published

Distel, D. L., D. J. Beaudoin, and W. Morrill, "Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve Lyrodus pedicellatus (Bivalvia: Teredinidae)", Appl. Environ. Microbiol., p. 6292, vol. 68, (2002). Published

Xu, P.-N., W. Morrill, and D. L. Distel, "Purification and characterization of a cellulase (endo-1, 4-?-D glucanase) from the cellulolytic system of the wood-boring mollusc Lyrodus pedicellatus (Bivalvia: teredinidae).", Marine Biology, p. , vol. , ( ). Accepted

Fu, Y., C. O?Kelly, M. Sieracki, and D. L. Distel., "Protistan grazing analysis by flow cytometry using prey labeled by in vivo expression of fluorescent proteins", Appl. Environ. Microbiol., p. 6848, vol. 69, (2003). Published

Books or Other One-time Publications

Distel, D. L., "The biology of wood-boring bivalves and their bacterial endosymbionts", (2003). Book, Published
Editor(s): T. P. Schulz, B. Goodell, and D. D. Nicholas
Collection: Wood deterioration and preservation: Advances in our changing world.
Bibliography: p. 253-271. American Chemical Society, Washington, DC.

Web/Internet Site

URL(s):

Description:

Other Specific Products

Contributions

Contributions within Discipline:

1. We have demonstrated the existance of symbiotic bacterial consortia in two host species demonstrating that this type of symbiosis, which has not been observed before, is not an aberation limited to a single species. This puts a new spin on the study of bacterial endosymbioses since we must now consider whether other symbioses formerly thought to be monocultures may infact involve multiple related

symbiont species.

2. We have cloned three new cellulases, the first from an intracellular symbiont, and have begun its characterization.

Contributions to Other Disciplines:

We have applied techniques developed by our colleagues ant MIT (Polz Lab) to reduce heteroduplex and chimera formation in 16S rDNA libraries generated from mixed microbial populations and have provided a first test for these methods in parallel with standard PCR cloning methods for a natural mixed bacterial population. These data will be of interest to a broad range of researchers in microbial ecology and other fields in which PCR products from mixed populations must be cloned and analyzed. We have also helped to develop the application of Constant Denaturant Capillary Electrophoresis CDCE for use in microbial ecology applications.

Contributions to Human Resource Development:

We have trained seven undergraduate students to isolate DNA, do PCR, run gels, analyze and edit DNA sequences and other techniques widely used in molecular biology. We have also trained two graduate students in more advanced areas of molecular biology. These skills will be valuable to them when they seek to enter the workforce or pursue graduate education.

Contributions to Resources for Research and Education:

Contributions Beyond Science and Engineering:

Categories for which nothing is reported:

Organizational Partners

Any Product

Contributions: To Any Resources for Research and Education

Contributions: To Any Beyond Science and Engineering

Project: IBN-9982982 ($275,151, 03/15/00-02/28/03, "Composition and function of a novel consortial endosymbiosis in the shipworm *Lyrodus pedicellatus"*)

Participants:
PI: D. L. Distel
Graduate Students: Yutao Fu, Yvette Luyten, Nitin Nair
Undergraduate Students: Adam Burgoyne, Angela Eash, Tim Chen, Benjamin Bosse, Jennifer Wiley, Mehwish Amin
Technician: Wendy Morrill

Organizations involved as partners: None

Other Collaborators: The principal collaborator was Dr. Martin Polz, Assistant Professor, Dept. of Civil and Environmental Engineering (MIT) who participated in this research by providing access to, and assistance with, quantitative PCR devices and methods being developed at MIT.

Major research and education activities of the project.

The work has: a) demonstrated the existence of a consortial polysymbiosis in the shipworm *L. pedicellatus*, containing at least four co-occurring symbiont ribotypes (4), b) characterized and formally named a cultivated symbiont of shipworms, *Teredinibacter turnerae*, gen. nov., sp. nov., (5), c) cloned and partially characterized three new cellulase genes from *T. turnerae*, (2, 8, 9), d) purified and characterized the principal endoglucanse detected in tissues of L. pedicellatus (13), e) isolated two new, as yet un-named, species of the genus *Teredinibacter* from the shipworms *T. navalis* and *L. pedicellatus*, and f) provided evidence for a symbiotic consortium in the shipworm *T. navalis* that is similar to the consortium demonstrated in *L. pedicellatus*. The work has resulted in three publications in print (3-5), one manuscript in press (13), one related published manuscript (6), four manuscripts in preparation (2, 8-10), and numerous abstracts, e.g. (1, 11). Additional publications are planned.

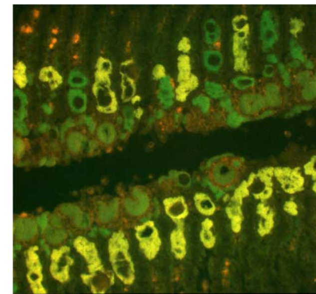

Figure 1. Dual-probe FISH of bacteriocytes showing differential staining of two symbiont ribo-types (arrows) by specific oligo-nucleotide probes. (color figure)

Education and Training

The awarded funding has helped us to continue our active support for student and teacher education, research and training activities. This project has helped to support several graduate and undergraduate students and a high school teacher summer intern at U. Maine. Students were involved in all levels of research and performed the majority of experimental work. High school, undergraduate and graduate students as well as high school science teachers have completed research projects in both laboratories resulting in

numerous authorships, including one coauthorship and a pending first authorship for undergraduate students in the Distel lab. Students have received the following awards for research conducted in the Distel laboratory on projects related to this grant: 1) The Barry M. Goldwater Scholarship for Outstanding Undergraduates in Science and Mathematics, The Pfizer Summer Undergraduate Research Fellowship, The Maine Science and Technology Foundation MERITS Summer Fellowship, The Intel High School Science Talent Search (semifinalist), the Maine Upward Bound Summer Internship SOAR Award and others. All listed awards included cash scholarships or stipends ranging in value from $1000-$7500, demonstrating the value of NSF dollars for leveraging additional student support from other agencies and for boosting the academic careers of students at all levels. We will continue our ongoing efforts to recruit broadly, taking advantage of existing personal and programmatic links to institutions serving underrepresented minorities.

Summary of findings and products of IBN-9982982

Four distinct bacterial ribotypes were detected in a small 16S rDNA clone library (48 clones) constructed from two adult specimens of *L. pedicellatus.* Fluorescent *in situ* hybridization (FISH) was used to localize the four ribotypes to bacteriocytes in sectioned gills of *L. pedicellatus* (Fig. 1). The ribotypes are all closely related to each other and include the cultivated bacterium *T. turnerae*. The distribution of ribotypes is non-random. *T. turnerae* was predominantly observed in bacteriocytes located dorsally and medially nearest the afferent branchial vein and epibranchial chamber.

Four new 16S rDNA clone libraries (~ 96 clones each) have been constructed from individual and pooled specimens of *T. navalis* and *L. pedicellatus*. The libraries were constructed using low numbers of PCR cycles to reduce chimera formation during PCR, a high fidelity polymerase (*pfu*) and a "reconditioning PCR" method (12) to prevent chimeras due to cloning of heteroduplex PCR products with subsequent random excision-repair by the *E. coli* MutHLS system. The sequence diversity in libraries constructed by this method should therefore more accurately reflect the true diversity in the sample population. Among the most significant results observed is that libraries from different individuals of the same species vary significantly in composition, particularly with regard to the numerically predominant ribotypes.

Primers and control DNAs for constant denaturant capillary electrophoresis (CDCE) (7) have been developed and tested for symbiont ribotypes of *L. pedicellatus* and *T. navalis.* Results indicate that these primers can be used to simultaneously and quantitatively amplify all symbiont ribotypes so far identified in *L. pedicellatus*. The identities of four ribotypes and their corresponding CDCE peaks have been confirmed by co-migration with cloned standards to date.

Figure 2. CDCE profiles

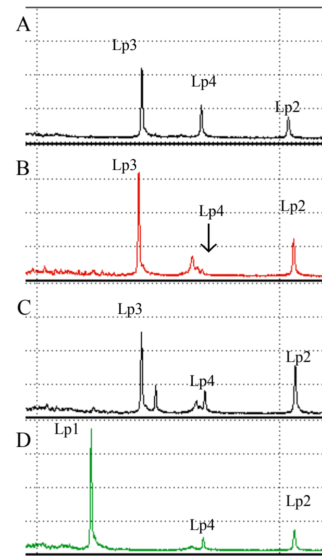

Figure 2 (right) shows CDCE profiles from four specimens of *L. pedicellatus* (A-D) sampled from laboratory cultures over a two year period. Note 1) the numerically dominant ribotype varies dramatically from individual to individual, 2) two ribotypes, Lp1 and Lp3, appear to occur interchangeably as the dominant ribotype, and 3) the relative abundance of the nitrogen-fixing cultivable ribotype Lp4 (*T. turnerae*) varies dramatically among specimens. Unidentified peaks are unlabeled.

A plasmid library was constructed in pBSII using genomic DNA from the cultivated endosymbiont *T. turnerae.* Five thousand clones were screened for potential secreted cellulase activity using the artificial fluorogenic substrate MUC (methlyumbelliferyll cellobioside). Eleven positive clones were detected. One of these (p28) is an endoglucanase whose complete open reading frame contains 1809 bp encoding a 602 amino acid polypeptide of 63 kD (Fig. 3). The sequence encodes a plausible ribosome-binding site, a signal peptide, cellulose-binding domain, linker region and single catalytic domain and has been designated *Tt eglA*. *Tt eglA* has activity against carboxymethylcellulos (CMC) and phosphoric acid swollen cellulose PSC, but not microcrystalline cellulose (Avicel) as expected for an endoglucanase.

A second clone, p47, contains a single ORF encoding a signal sequence and a 340 amino acid poly-peptide of 40 kD with a high degree of sequence similarity to the non-modular endoglucanase Cel A from *Cellvibrio mixtus* (Fig. 3). This clone shows activity against MUC and exonuclease activity against phosphoric acid swollen cellulose however, no hydrolytic activity was detected against carboxymethylcellulose. It has been designated *Tt exoA*.

A lambda library of *T. turnerae* genomic DNA was also screened for hydrolytic activity against carboxymethylcellulose using the Congo Red plaque assay method. A lambda clone (λ2.2) was identified that expresses an unusual bifunctional cellulase of 105.9 kD that contains both endo- and exoglucanase domains (Fig. 5) and expresses activity against both CMC, PSC, and Avicel. The identities of the putative functional domains have been confirmed using transposon mutagenesis.

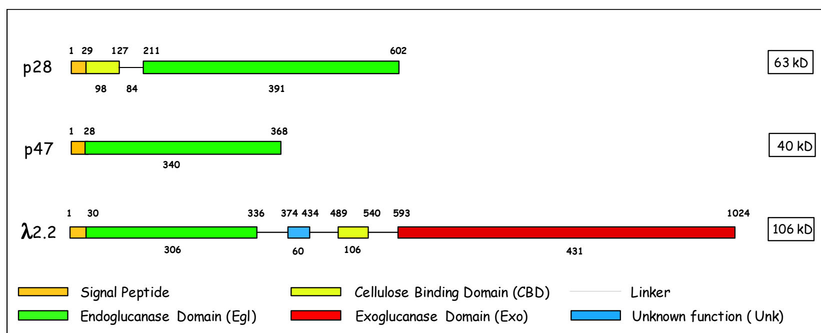

Figure 3. Cellulases of *T. turnerae* (color figure).

An endoglucanase (designated Lp-egl-1, molecular weight about 40 kDa) has been purified to homogeneity from tissues of *L. pedicellatus* (13). Molecular weight, Km, pI, temperature and pH range, substrate specificity, and hydrolysis products have been

determined and shown to differ from the principal endoglucanase secreted by *T. turnerae* in pure culture (Tt-egl-1). Lp-egl-1 also shows no cross reactivity with a polyclonal antibody generated against Tt-egl-1.

Two new putative symbiont species (Tni-1, and Ttw-1) have been isolated in pure culture from gills of *T. navalis*, and *L. pedicellatus*, respectively andutative Ttw strains have been isolated from four additional shipworm species. Tni-1 is identical in 16S rRNA sequence to ribotype Tn3, recovered from *T. navalis* individual and pooled-specimen genomic DNA 16S PCR libraries. Both new strains share about 95% sequence identity with each other and with *T. turnerae* and so likely constitute new species in the genus *Teredinibacter*. Both isolates are microaerophilic, fix dinitrogen, and grow with cellulose as a sole carbon source but differ from *T. turnerae* in colony morphology, and ability to grow on various sugars.

1. **Burgoyne, A., W. Morrill, L. Li, and D. L. Distel.** 2003. Presented at the 103rd General Meeting of the American Society for Microbiology.
2. **Burgoyne, A., W. Morrill, L. Li, and D. L. Distel.** in preparation. Cloning, expression and characterization of a putative bifunctional cellulase from *Teredinibacter turnerae*, endosymbiont of wood-boring mollusks.
3. **Distel, D. L.** 2003. The biology of wood-boring bivalves and their bacterial endosymbionts, p. 253-271. *In* T. P. Schulz, B. Goodell, and D. D. Nicholas (ed.), Wood deterioration and preservation: Advances in our changing world. American Chemical Society, Washington, DC.
4. **Distel, D. L., D. J. Beaudoin, and W. Morrill.** 2002. Coexistence of multiple proteobacterial endosymbionts in the gills of the wood-boring bivalve *Lyrodus pedicellatus* (Bivalvia: Teredinidae). Appl. Environ. Microbiol. **68:**6292-9.
5. **Distel, D. L., W. Morrill, N. MacLaren-Toussaint, D. Franks, and J. Waterbury.** 2002. *Teredinibacter turnerae* gen. nov., sp. nov., a dinitrogen-fixing, cellulolytic, endosymbiotic gamma-proteobacterium isolated from the gills of wood-boring molluscs (Bivalvia: Teredinidae). Int J Syst Evol Microbiol **52:**2261-9.
6. **Fu, Y., C. O'Kelly, M. Sieracki, and D. L. Distel.** 2003. Protistan grazing analysis by flow cytometry using prey labeled by in vivo expression of fluorescent proteins. Appl. Environ. Microbiol. **69:**6848-6855.
7. **Khrapko, K., H. A. Coller, X. C. Li-Sucholeiki, P. C. Andre, and W. G. Thilly.** 2001. High resolution analysis of point mutations by constant denaturant capillary electrophoresis (CDCE). Methods Mol. Biol. **163:**57-72.
8. **Li, L., W. Morrill, and D. L. Distel.** in preparation. Cloning and heterologous expression of a cellulase (endo-1, 4-β-D glucanase) from *Teredinibacter turnerae* (γ–Proteobacteria), an endosymbiont of wood-boring molluscs (Bivalvia: teredinidae).
9. **Li, L., W. Morrill, and D. L. Distel.** in preparation. A nonmodular cellulase from *Teredinibacter turnerae* (g-Proteobacteria) an endosymbiont of wood-boring molluscs (Bivalvia: teredinidae) exhibits exo-1, 4-b-D glucanase activity.
10. **Luyten-Jarvis, Y. A., J. R. Thompson, M. F. Polz, and D. L. Distel.** in preparation. Analysis of the composition of polytypic symbiont populations in

gills of the wood-boring mollusk *Lyrodus pedicellatus* using constant denaturant capilary electrophoresis (CDCE) and quantitative polymerase chain reaction (QPCR).

11. **Luyten-Jarvis, Y. A., J. R. Thompson, M. F. Polz, and D. L. Distel.** 2003. Presented at the 103rd General Meeting of the American Society for Microbiology.
12. **Thompson, J. R., L. A. Marcelino, and M. F. Polz.** 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Res **30:**2083-8.
13. **Xu, P.-N., W. Morrill, and D. L. Distel.** 2003, in press. Purification and characterization of a cellulase (endo-1, 4-β-D glucanase) from the cellulolytic system of the wood-boring mollusc *Lyrodus pedicellatus* (Bivalvia: teredinidae). Mar. Biol.

Findings

Findings are summarized with respect to progress toward testing of four hypotheses posed in the original proposal.

Hypothesis I. Symbiont populations, observed in the gills of shipworms contain several bacterial phylotypes rather than a bacterial monoculture.

a) The presence of four distinct bacterial 16S rRNA ribotypes has been confirmed by in situ hybridization methods in the gills of *L. pedicellatus*. Results confirm that the four ribotypes could represent up to four, but no less than two distinct symbiont phylotypes, i.e., one or more ribotypes may be differentially expressed by a single phylotype. A manuscript describing the evidence for the coexistence of multiple phylotypes is has been submitted.

b) It was reported in 1983 that one of the symbionts of *L. pedicellatus* can be grown in pure culture. This symbiont was never fully characterized nor formally described and named. We have submitted a manuscript describing this bacterium (and 58 similar isolates from shipworm gills) and formally proposed a new genus and species *Teredinibacter turnerae* gen. nov., sp. nov. with strain T7902 from *L. pedicellatus* as the type strain. The manuscript is tentatively accepted pending revision.

c) Graduate student, Yvette Luyten is examining the symbiont populations of a second species of shipworm, *Teredo navalis*, which has invaded the coastal waters of Maine in the past two years. Yvette is currently screening a 16S rDNA library created from genomic DNA extracted from the symbiont-containing gills of an individual specimen and a second library from three pooled specimens of *T. navalis*. To date Yvette has screened a total of 189 clones from the two libraries using RFLP. Six RFLP patterns have been identified and at least three representative clones are being sequenced for each pattern (some patterns may be redundant, representing different orientations of the same insert). So far three distinct 16S rDNA sequences have been identified among the *T. navalis* clones. Although more may be identified with continued screening, the most abundant RFLP patterns have been identified and results from the two libraries are very similar (see Table 1).

Phylogenetic analyses (see Figure 1) reveal that the three sequences identified in *T. navalis* are very similar, but not identical to the sequences observed in gills of *L. pedicellatus.* In fact, the new sequences are more closely related to the *L. pedicellatus* sequence than any other known bacterial 16S rDNAs. Moreover, sequences from the two host species fall into three groups with each host containing at least one sequence from each group.

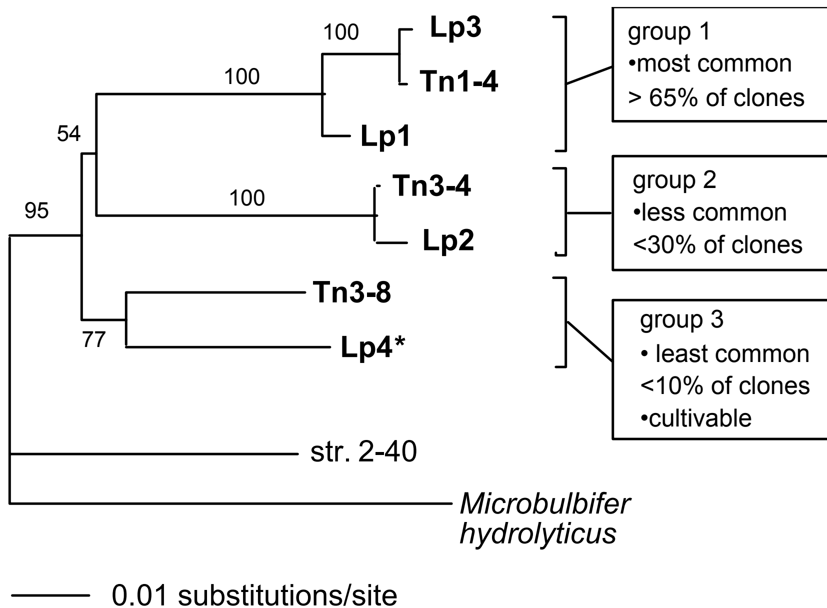

Figure 1. Phylogenetic relationships among symbiont 16S rDNA sequences cloned from host shipworm species *Lyrodus pedicellatus* (Lp) and *Teredo navalis* (Tn). Sequence Lp4 is identical to that of the cultivated symbiont *Teredinibacter turnerae* and sequence Tn3-8 is identical to a newly isolated teredinibacter-like strain from *Teredo navalis.* Evolutionary distance tree with bootstrap proportions for 100 replicates.

Kimura 2-parameter distance matrix

	1	2	3	4	5	6	7	8	9
1 tn1-4	-								
2 tn3-4	0.0775	-							
3 tn3-8	0.0752	0.0665	-						
4 lp3	0.0021	0.0767	0.0744	-					
5 lp1	0.0140	0.0685	0.0669	0.0148	-				
6 lp2	0.0818	0.0043	0.0690	0.0810	0.0743	-			
7 Tt.Lp4	0.0753	0.0689	0.0496	0.0745	0.0670	0.0713	-		
8 str.2-40	0.0881	0.0841	0.0777	0.0864	0.0800	0.0900	0.0833	-	
9 M.hyd	0.1074	0.1123	0.0874	0.1073	0.1011	0.1113	0.1008	0.0947	-

Table 1. Distance matrix comparing diferences among symbiont 16S rDNA sequences cloned from host shipworm species *Lyrodus pedicellatus* (Lp) and *Teredo navalis* (Tn).

d) Yvette has also been attempting to cultivate new symbiont strains from *T. navalis*. Several candidate strains are in various stages of enrichment. One of these (Tn-i1) has been isolated in pure culture and its 16S rDNA sequence has been determined to be identical to that of clone Tn3-8, the most Teredinibacter-like clone from *T. navalis*. This strain may constitute a second species in the new genus Teredinibacter and may represent only the second bacterial symbiont species of bivalves to be grown in pure culture.

Hypothesis II. Symbiont phylotypes observed in shipworms comprise distinct, stable, predictable and well-defined bacterial consortia with non-random spatial distribution in the gill tissue.

a) Phylogenetic analyses suggest that the two host species may each harbor different strains of at least three common symbiont species corresponding to groups 1-3 in figure 1. Moreover, the proportions of each symbiont species appear to be similar in both host species as well as in libraries constructed from a single individual and three pooled individual specimens of *T. navalis*. In situ hybridization experiments are planned to determine the distribution of the three symbiont species in *T. navalis*.

b) We have designed and synthesized two sets of GC clamped fluorescent primers to be used for quantitative PCR (QPCR) employing constant denaturant capillary electrophoresis (CDCE) to determine quantitatively the composition of symbiont populations in gill tissues of *L. pedicellatus*. Internal positive control molecules identical to the target sequence at all but one nucleotide position have also been synthesized. Preliminary tests demonstrate that these primers can be used to successfully amplify all four putative symbiont 16S rDNAs.

Hypothesis III. Cellulolytic enzymes are produced by symbionts in the gills of the shipworm *L. pedicellatus* and are transported to the gut where they aid in wood digestion.

A critical first step toward testing this hypothesis is the cloning and expression of symbiont cellulases. Screening of an initial plasmid library constructed from genomic DNA from the cultivated endosymbiont *Teredinibacter turnerae* revealed several clones with putative cellulase activity as detected with the fluorogenic substrate MUC. Only one of these clones (designated p28b9) expresses demonstrable endoglucanase (carboxymethylcellulase) activity in *E. coli*. Sequence analysis revealed that p28b9 contained a gene fragment composed of a putative endoglycolytic catalytic domain and an unusual serine-rich linker sequence. No cellulose-binding domain was identified on this fragment. To recover the entire gene sequence a second genomic library, composed of larger inserts, was constructed in lambda phage. This library was screened using a probe specific for a portion of the p28b9 putative catalytic domain. A clone containing the complete gene was identified and complete sequence was determined. The complete sequence contains a plausible open reading frame of 1809 bp encoding a 602 amino acid polypeptide of 65.5kd. Included is a plausible signal peptide, cellulose binding domain, linker region, and catalytic domain. The complete gene has been designated Tt EglA.

Previously we reported that we had not yet been successful in expressing a functional full-length gene product from Tt EglA. This remains true. Expression had only been successful for the originally isolated gene fragment containing the linker plus catalytic domain. Subcloning the full-length gene and the catalytic domain alone did not result in measurable expression of carboxymethylcellulase activity in *E. coli,* either in the plasmid pBS II or in the specialized expression vector PET22b.

We have since determined that expression of the p28b9 product in *E. coli* is dependent on the presence of a downstream sequence tentatively identified as a transcriptional terminator. This sequence was removed in all previous clones. Subsequent construction of a subclone of p28b9 that retains the putative termination sequence but eliminates

further downstream sequence retains the ability to be expressed in *E. coli*. We are currently working to construct a full length TtEglA clone that contains the terminator sequence. We are also working on methods to improve purification of the p28b9 protein product for enzymatic characterization.

We have also demonstrated that the p28b9 product cross reacts with a polyclonal antibody raised against the purified major endoglucanase secreted by *T. turnerae* when grown in pure culture. Thus p28b9 appears to be the gene encoding the major secreted endoglucanase of *T. turnerae* when in pure culture.

In attempt to further explore potential cellulase genes present in the *T. turnerae* genome, the lambda library of *T. turnerae* genomic DNA was screened again for cellulase expression using an activity stain that utilizes a more realistic substrate (carboxymethylcellulose). Two additional lambda clones have been identified that express and secrete endoglucanase activity in *E. coli.* PCR amplifications using primers specific for the putative endoglucanases previously identified suggest that at least one of the two lambda clones encodes anendoglucanase that is distinct from the genes previously cloned. The second lambda clone has not yet been examined.

The new lambda clone (currently designated CR2) expresses and secretes endoglucanase activity at a considerably higher level than p28b9. This high level of expression will facilitate purification and characterization of this endoglucanase. All indications suggest that this clone will provide sufficient quantity of purified enzyme for synthesis of specific antibodies. These antibodies will be used for in situ immunohistochemical investigations to be used in testing Hypothesis IV and for experiments aimed toward revealing the mechanism of cellulase transport from the symbiont containing shipworm gill to the gut where cellulose degradation occurs.

Hypothesis IV. Individual symbiont phylotypes produce different enzymes that contribute to a complete cellulolytic system.

The experience that we have gained from cloning cellulolytic enzymes from the cultivated symbiont *T. turnerae* will be invaluable in the more challenging problem of cloning cellulolytic enzymes from non-cultivable symbionts in the gill tissue (activities for years 2-3). These difficulties will be reduced by the development of efficient expression assays. Screening for expression of endoglucanase activity should help circumvent problems due to contamination of the genomic library with eukaryotic DNA. Our success in cultivation of additional putative symbionts may also be valuable in exploring this hypothesis.