Marine Ecology. ISSN 0173-9565

ORIGINAL ARTICLE

Characterization of bacterial community structure in vestimentiferan tubeworm *Ridgeia piscesae* trophosomes

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Keywords
Hydrothermal vent; *Ridgeia piscesae*; T-RFLP; trophosome; vestimentiferan endosymbionts.

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Accepted: 21 December 2006

doi:10.1111/j.1439-0485.2007.00151.x

Abstract

Terminal-restriction fragment length polymorphisms (T-RFLPs) were used to address the question whether there are multiple phylotypes of bacteria within the trophosome of the vestimentiferan tubeworm *Ridgeia piscesae* in addition to the known endosymbiont. Clone libraries were constructed to aid in the identification of the additional bacterial phylotypes. Individual *R. piscesae* specimens were collected from Juan de Fuca Ridge, Axial Caldera and Explorer Ridge, Magic Mountain. Clone library analyses revealed only one bacterial phylotype (expected *R. piscesae* endosymbiont) within the Explorer Ridge trophosomes. However, the Axial Caldera clone library revealed five operational taxonomic units (OTUs). Three of the resulting phylotypes detected (designated RAE) were putative thioautotrophic symbionts within the *γ-Proteobacteria* which belonged to the anticipated endosymbiont group (RAE OTU 1), the *Maorithyas hadalis* symbiont II group (RAE OTU 3), and the *Halothiobacillus* group (RAE OTU 4). The remaining two phylotypes were most likely opportunistically derived. RAE OTU 5 was an *α-Proteobacterium* within the *Roseobacter* group and RAE OTU 2 was within the *Cytophaga–Flavobacterium–Bacteroidetes*. T-RFLP analyses revealed that all 15 trophosomes evaluated contained at least RAE OTUs 1 and 4. Five of the 15 trophosomes contained at least RAE OTUs 1, 3, and 4 and two of the trophosomes contained at least five RAE OTUs. Cluster analysis of the T-RFLP data revealed three distinct clusters. The number of taxa within the trophosome may be an indication of the general health of the tubeworm. This study strongly suggests that we have discovered and identified more than one bacterial phylotype within the trophosome of vestimentiferan *R. piscesae*.

Problem

Ridgeia piscesae is a vestimentiferan tubeworm found at hydrothermal vents along Explorer, Juan de Fuca, and Gorda Ridges in the northeastern Pacific Ocean (Jones 1985; Black *et al.* 1998). Vestimentiferan tubeworms, in general, can also be found at sulfide-rich coldwater seeps and hydrocarbon seeps (Cavanaugh *et al.* 1981; Felbeck 1981; Fisher *et al.* 1997). *Ridgeia piscesae* is found only at ridge axis locations and can exhibit significant phenotypic plasticity ranging from long and skinny (*c.* 250 mm long and *c.* 5 mm wide) to short and fat (*c.* 150 mm long and *c.* 19 mm wide) morphotypes (Southward *et al.* 1995). The overall length of tubes potentially ranges from *c.* 18 mm to *c.* 1.5 m (Jones 1985) and can be gold, brown, grey, translucent white, or black in color (Southward *et al.* 1995).

Ridgeia piscesae were once hypothesized to be several different species based on their highly variable morphology. At least three distinct morphospecies (*R. piscesae*, *R. phaeophiale*, and the 'Ring' species) were described that were found along at the Juan de Fuca, Gorda, and

Explorer Ridges (Jones 1985; Tunnicliffe *et al.* 1985, 1986; Tunnicliffe 1988; deBurgh *et al.* 1989). Despite distinct differences in morphology, molecular phylogeny has shown that all the morphotypes originally thought to be different species are in fact the same species exhibiting a high degree of gene flow (Black *et al.* 1997).

There are two possible modes in which vestimentiferans acquire their endosymbionts: (1) horizontally or *de novo* from established vestimentiferan populations or (2) vertically or from parent to progeny (Jones & Gardiner 1988, 1989). Multiple studies have shown that vestimentiferan tubeworms acquire their endosymbionts horizontally by either ingestion during larval recruitment or by infection during larval stages (Edwards & Nelson 1991; Cary *et al.* 1993; Laue & Nelson 1997). More recently, there is evidence that transfer occurs post-larval recruitment, through a process of infection and migration to the dorsal mesentery followed by proliferation of the trophosome with simultaneous apoptosis of surrounding tissue (Nussbaumer *et al.* 2006). The endosymbionts shared among all host species of vestimentiferans found at hydrothermal vents (*e.g. Ridgeia*, *Riftia*, *Tevnia* and *Oasisia*) are virtually identical (Feldman *et al.* 1997; DiMeo *et al.* 2000). This lack of variability of endosymbionts across multiple host species of vestimentiferans supports the horizontal acquisition of endosymbiont hypothesis. In contrast, if endosymbionts were transferred vertically (*i.e.* directly from parent to offspring), there would be significant genetic variability among endosymbionts across host species due to a lack of gene flow (Distel *et al.* 1988; Edwards & Nelson 1991; Feldman *et al.* 1997; Laue & Nelson 1997; Nelson & Fisher 2000). Furthermore, microscopic observations reveal that *Riftia pachyptila* larvae possess a ciliated duct that connects the trophosome to the surrounding seawater which acts as a digestive tract (Jones & Gardiner 1988, 1989). Larval vestimentiferans are dispersed and undergo a free-feeding trochophore stage (Jones & Gardiner 1988, 1989; Young *et al.* 1996; Southward 1999). After the larvae settles, its digestive tract closes and the trophosomes enlarge (Jones & Gardiner 1988, 1989). Additionally, bacterial symbionts have not been found in either vestimentiferan eggs or sperm (Cavanaugh *et al.* 1981; Cavanaugh 1985). These studies offer strong support that vestimentiferan larvae are being infected from the same pool of bacteria in the surrounding seawater and that endosymbionts are transferred horizontally most probably by the infection of mesodermal tissue (Bright & Sorgo 2003; Nussbaumer *et al.* 2006).

The known endosymbionts of *R. piscesae* and other vestimentiferans found at hydrothermal vents are within the subdivision *γ-Proteobacteria* (Stahl *et al.* 1984; Lane *et al.* 1985; Distel *et al.* 1988; Feldman *et al.* 1997). These endosymbionts encompass 0.2% divergence based on SSU rRNA gene comparisons across the East Pacific Rise and Northeast Pacific phylotypes (Nelson & Fisher 2000). Other phylotypes associated with the chitineous tube surrounding *R. pachyptila* have been found to include mostly *ε-Proteobacteria* along with several *α-*, *δ-* and *γ-Proteobacteria*. However, the endosymbiont of *R. pachyptila* was not detected within the tube bacterial assemblage (López-García *et al.* 2002).

Several studies that provide strong evidence against the 'single taxon of endosymbiont within the trophosome' hypothesis have been conducted (deBurgh *et al.* 1989; Naganuma *et al.* 1997a,b); however, this hypothesis is still widely accepted. One study using transmission electron microscopy methods found two morphologically distinct bacterial endosymbionts within *R. piscesae* (deBurgh *et al.* 1989). Other microscopic studies have also revealed more than one morphotype of endosymbiont within vestimentiferan tubeworms (Cavanaugh *et al.* 1981; Cavanaugh 1985; Fisher 1990) and that bacterial cells are markedly larger towards the center of the trophosome (Gardiner & Jones 1993; Bright *et al.* 2000). Studies by Naganuma *et al.* (1997a,b) discussed a second phylotype belonging to the *ε-Proteobacteria* detected within trophosomes of *Lamellibranchia* sp. found inhabiting cold methane seep communities using both microscopy and *in situ* hybridization. Although these studies contradict the accepted hypothesis, more than a single bacterial phylotype within trophosomes has not been found in vestimentiferans collected from hydrothermal vents.

Previous methods used to characterize the populations within the vestimentiferan trophosome community may not have been sensitive enough to resolve minor populations, which may be present at relatively low numbers. Endosymbionts of *R. pachyptila* exist at an extremely high density of up to 3.7×10^9 cells·g^{-1} of trophosome (Cavanaugh *et al.* 1981; Powell & Somero 1986). The high density of a single majority taxon within the trophosome may act to mask detection of minor taxa within the trophosome using traditional molecular techniques. There are also biases in traditional PCR methods towards amplifying the phylotype which makes up the majority of the population. During the exponential phase of PCR, the major phylotypes in the population may become saturated more quickly so that other minor populations may remain undetected using PCR-based clone library methods alone (Polz & Cavanaugh 1998).

Terminal-restriction fragment length polymorphism (T-RFLP) detects multiple taxa within a community (Liu *et al.* 1997). T-RFLP is a sensitive genotyping technique that can detect all ribotypes within a community containing low to intermediate richness (Engebretson & Moyer 2003). During amplification, the 5′-end of the forward

primer is labeled with a florescent dye. The amplified DNA fragments are digested with a suite of restriction enzymes and the terminal 5′ fragments are accurately sized using capillary electrophoresis. Each phylotype resolves to a unique series of fragments which can be detected as a distinctive fingerprint within a given trophosome community. SSU rDNAs (*i.e.* SSU rRNA genes) are widely used because these genes contain both conserved and variable regions and are universally conserved within the Domain *Bacteria*.

The primary goal of this study was to determine whether *R. piscesae* found at different hydrothermal vents contain more than one taxon of endosymbiont and to find other potential endosymbionts using culture-independent molecular methods. T-RFLP techniques were used in conjunction with traditional clone library and sequencing techniques to determine the community structure within several trophosome samples collected from the Magic Mountain area on Explorer Ridge and at Axial Seamount on Juan de Fuca Ridge.

Material and Methods

Sample collection

Samples containing multiple specimens (*c.* 20–30 individuals) of *R. piscesae* were collected at Tubeworm Vent, which were a cluster of hydrothermal vents at the Magic Mountain area of the Explorer Ridge, during two separate dives on July 28, 2002 (dive no. R666) and August 1, 2002 (dive no. R670). Multiple specimens of *R. piscesae* were also collected at Casper Vents (dive no. R661) within the caldera of Axial Volcano along the Juan de Fuca Ridge, on July 18, 2002. Specimens were removed from their habitat using the ROPOS mechanical arm and carried to the surface in a sealed biobox to prevent contamination from ambient seawater. Tubeworms were immediately dissected and trophosomes were aseptically removed. Trophosomes, including outermost membrane, were repeatedly rinsed in cold sterile 1X PBS and stored at −80 °C until DNA extraction. A total of 15 individual tubeworm trophosomes were analyzed in this study (n = 12 from Magic Mountain area, Tubeworm Vent and n = 3 from Axial Caldera area, Casper Vent).

Genomic DNA extraction and purification

Genomic DNA (gDNA) was extracted using either QIAamp Tissue Kit (Qiagen, Valencia, CA) or DNeasy Tissue Kit (Qiagen) according to manufacturer's protocol with the following modifications. A higher concentration of proteinase K and longer incubation times were needed in order to lyse bacteriocytes and bacterial cell walls completely. When the QIAamp Tissue Kit was used, trophsomes were defrosted on ice and cut into 25–100 mg pieces with a sterile surgical knife. Trophosomes were cut from different locations (*i.e.* middle and end) when extractions were done in duplicate. Cells were lysed by adding 22 μl of proteinase K (20 mg·ml^{-1}) and then incubated at 55 °C for 16 h. Genomic DNA was eluted twice with 100 μl Buffer AE. When the DNeasy Tissue Kit was used, trophsomes were defrosted on ice and rinsed in cold sterile 1X PBS. Trophosomes were cut into 25–75 mg pieces using sterile stainless steel scissors. Cells were lysed by adding 250 μl buffer ATL and 28 μl proteinase K (20 mg·ml^{-1}) and incubated at 55 °C for 20 h. Samples were then centrifuged at 13,800 × *g* for 5 min to separate the sulfide residues in sample. The supernatant (210 μl) was then transferred to the column provided. Genomic DNA was eluted twice with 100 μl Buffer AE.

Desalting and concentrating gDNA

The product from the gDNA extraction was added to a Montáge PCR Centrifugal Filter Device (Millipore, Bedford, MA). Samples were centrifuged at 735 × g for approximately 15 min or until the filters were slightly moist. The filtrate was removed and 300 μl of molecular biology grade water was added and centrifuged to rinse the sample. This step was repeated. Samples were eluted twice with 100 μl molecular grade water. Genomic DNA yields were quantified using a NanoDrop ND-1000 (NanoDrop, Wilmington, DE) and diluted to 10 ng·μl^{-1} using filter sterilized 10 mM Tris, pH 8.0.

Amplification of SSU rDNA for T-RFLP

Bacterial gDNAs from trophosome tissues were amplified using the forward primer (5′ – TNA NAC ATG CAA GTC GRR CG) which corresponds to positions 49–68 of *Escherichia coli* SSU rRNA and the reverse primer (5′ – RGY TAC CTT GTT ACG ACT T) which hybridizes to the 1510–1492 positions (Brosius *et al.* 1978; Moyer *et al.* 1994). R corresponds to the purine analog dK, Y corresponds to the pyrimidine analog dP, and N is an equal mixture of both analogs at a single position (Glen Research, Sterling, VA). The forward primer was also labeled with 6-FAM on the 5′- end (Applied Biosystems, Foster City, CA). PCR reactions were performed using 50 ng of gDNA, 5 U *Taq* DNA polymerase (Promega Biosciences, San Luis Obispo, CA), 1X PCR buffer (Promega Biosciences), 2.5 mM $MgCl_2$, 200 μM each dNTP, 10 μg BSA, 1 μM forward primer, 1 μM reverse primer, and molecular biology grade water to a final volume of 50 μl. The reaction mixtures, excluding the BSA and *Taq* polymerase, were heated to 95 °C for 2 min and allowed to cool to 4 °C. The *Taq* and BSA mixture was immediately

added and sample tubes were placed in a GeneAmp 9700 thermocycler (Applied Biosystems). The PCR reactions were cycled 30 times under the following conditions: denaturation of DNA at 94 °C for 1 min, hybridization at 56 °C for 90 s, elongation of DNA at 72 °C for 3 min, and a final elongation step for 7 min at the end of the 30th cycle. PCR products were tested via gel electrophoresis. Five microliters of the PCR product was added to 3 μl of 10X loading buffer, and 3 μl of 1 KB ladder (Promega Biosciences) was mixed with 2 μl 10X loading buffer. The samples were then added to polymerized 1% Seakem agar (FMC Bioproducts, Rockland, ME) with *c.* 0.5 $\mu g \cdot ml^{-1}$ ethidium bromide in 1X TAE and run in 1X TAE at *c.* 80 V for *c.* 45 min. Every gel included a negative and a positive control. If the positive control failed to amplify or there was amplification in the negative control, the PCR reaction series was discarded and the PCR reactions were run again.

T-RFLP preparation

Two to three PCRs were pooled and their products were desalted using the Montáge PCR Centrifugal Filter Device (Millipore) with a 100 μl final elution volume using molecular biology grade water. PCR products were then digested with eight restriction endonucleases (New England Biolabs, Ipswich, MA) with the following 5′ to 3′ recognition sites: *Alu*I (AG▼CT), *Bst*UI (CG▼CG), *Hae*III (GG▼CC), *Hha*I (GCG▼C), *Hinf*I (G▼ANTC), *Mbo*I (▼GATC), *Msp*I (C▼CGG), and *Rsa*I (GT▼AC). Each reaction included the following: 5 U of restriction endonucleases (New England Biolabs), 1X NEB 2 Buffer (New England Biolabs), molecular biology grade water, and 15 μl PCR product for a total of 30 μl per reaction. Each reaction was incubated at 37 °C overnight (*c.* 16 h) with the exception of samples digested with *Bst*UI which were incubated at 60 °C. Digests were desalted with Sephadex superfine G-75 (Amersham Biosciences, Piscataway, NJ). Sephadex G-75 was weighed (20 g), added to 400 ml molecular biology grade water, and autoclaved to hydrate Sephadex beads. After allowing slurry to cool and allowing the beads to settle at the bottom of the flask, the top 90% of water above the beads was decanted. The Sephadex slurry was added (800 μl) to Whatman, Unifilter 96 well 25 μm polypropylene mesh 800 μl capacity plates with associated Uniplate (Whatman, Floraham Park, NJ) and centrifuged to form columns at *c.* $750 \times g$ on a swinging bucket centrifuge at 4 °C (Sorvall, Asheville, NC) for 5 min. Entire restriction digests were then added to Sephadex columns and centrifuged at *c.* $750 \times g$ for 8–10 min at 4 °C into a clean semi-skirt 96-well PCR plate. Samples were then dried down at 60 °C using a vacuum centrifuge (Eppendorf, Westbury, NY). After samples were thoroughly dried, 15 μl of formamide and 0.4 μl of ROX-Genescan-500 internal dye standard (Applied Biosystems) were added. The samples were then denatured at 95 °C in a thermocycler for 5 min and cooled to 4 °C. Samples were then separated with an ABI 3100 Genetic Analyzer using a 50 cm capillary array (Applied Biosystems).

T-RFLP analysis and normalization

Terminal-restriction fragment length polymorphism chromatograms were analyzed using GeneMapper v.3.7 (Applied Biosystems). Peaks <50 bp were not included in the analysis to eliminate primer dimers and other small charged molecules. Peaks >500 bp were also excluded due to limitations of the size standard and capillary electrophoresis. Peaks over 6000 relative fluorescence units (RFUs) were diluted with formamide and ROX-500 and rerun, whereas if the main peak(s) was under 1000 RFU then the sample was reamplified and rerun. The chromatograms were imported into BioNumerics (Applied Maths, Saint-Martens-Latem, Belgium). The terminal-restriction fragments (T-RFs) were then normalized to the internal size standard. Cluster analysis was then performed using unweighted pair group method with arithmetic mean (UPGMA) and Pearson product–moment correlation (Häne *et al.* 1993). The cophenetic correlation coefficient was calculated to assess the robustness of the assigned clusters.

Clone library construction

Genomic DNA was amplified with the same parameters as described above without 5′-FAM label and both primers had 5′- phospholinks added to facilitate ligation reactions. PCR reactions were checked by gel electrophoresis and products were desalted and quantified. The PCR products were then ligated using the pCR 2.1 vector kit (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's directions. To determine whether clones contained the proper size insert, PCR products were generated using putative positive screening PCR methods (Moyer 2001). The PCR products were assayed for size using gel electrophoresis against a 1 kb ladder. Putative positive clones were each inoculated in 5 ml of LB + Amp broth and incubated for 16 h at 37 °C in a shaking incubator at 220 rpm. One milliliter of the culture was added to 600 μl of 80% glycerol and immediately flash frozen in liquid nitrogen. The remaining 4 ml of culture was centrifuged at *c.* $750 \times g$ for 8 min. Plasmids were extracted according to QIAprep Spin Miniprep Kit protocol (Qiagen). Plasmid concentrations were quantified and then the 5′- end of each insert was partially sequenced.

OTU determination

Sequences from the clone library analysis were imported and edited using the program Bioedit (Hall 1999). Sequences were trimmed; starting with the primer and approximately 350–400 bp were used in the analysis. The number of unique operational taxonomic units (OTUs) found within *R. piscesae* samples from Axial Caldera and Explorer Ridge (RAE) was determined using DOTUR (Schloss & Handelsman 2005) as well as results from *in silico* cuts from T-RFLP data. A unique OTU is defined as an operational taxonomic unit or group that demonstrates ≤97% similarity to the known *R. piscesae* endosymbiont and occurs more than once within the clone library. The SSU rDNA clone partial sequences were compared with sequences found in the Ribosomal Data Project using the sequence match function (Maidak *et al.* 2000).

Full SSU rDNA sequencing

Plasmid DNA was amplified using the same parameters as stated above except, M13F and M13R primers were used and 0.5 ng of plasmid DNA was added to the reaction. PCR product subsamples (*c.* 10%) were run on a 1% agarose gel to test PCR efficiency and then the remaining PCR products were cleaned and used as templates for sequencing. Full sequences of SSU rDNAs were contiguously assembled using BioNumerics (Applied Maths).

In silico restriction digests and data evaluation

Predicted fragment sizes were determined *in silico* for the eight restriction enzymes used in this study and compared with the peaks found in the T-RFLP data. *In silico* cuts are computer simulated restriction digests where the size of the restriction fragments are predicted. *In silico* determination of T-RF lengths was performed using the program Bioedit for both partial and full sequences of the SSU rRNA gene. The fragment length of the terminal end of the sequence was recorded and compared with the fragments found in the T-RFLP chromatograms.

Phylogenetic analysis

Full sequences were compared to those in the GenBank database using BLASTn (Altschul *et al.* 1990) and the percent similarities were recorded. The most similar sequences to the RAE OTUs were downloaded and imported into the ARB software environment. These sequences were then aligned to the SSU_Jan04 database using the ARB fast aligner (Ludwig *et al.* 2004) and additional sequences were selected and exported into Bioedit alignment tool for further editing. Phylogenetic comparisons were restricted to the moderately to highly conserved nucleotide positions: 101–182, 221–451, 480–838, 848–997, 1036–1133, 1139–1283, and 1296–1448 (*E. coli* numbering system). Phylogenetic placements were calculated with fastDNAml ver. 1.2.2 (Olsen *et al.* 1994) using the general two-parameter model of evolution (Kishino & Hasegawa 1989) and allowing for the global swapping of branches. The search for an optimal tree was repeated, using these parameters, until the best log likelihood tree was calculated in at least three independent tree calculations. Each tree was bootstrapped 100 times allowing global swapping of branches. The search for each bootstrap was repeated until the best log likelihood score was calculated for at least two independent bootstrap calculations.

Nucleotide sequence accession numbers

The SSU rRNA gene sequences representing the OTUs for *R. piscesae* from Explorer and Axial (RAE OTUs) used in this study have been deposited in GenBank and assigned accession numbers DQ660820 through DQ660827.

Results

Initial trophosome screening

Initial T-RFLP results revealed the presence of multiple peaks in many trophosome samples (*e.g.* Fig. 1). Samples with multiple peaks were hypothesized to contain multiple bacterial taxa. Representative trophosomes with the most diverse community structure from Axial Caldera, Juan de Fuca Ridge and Magic Mountain, Explorer Ridge were selected to construct clone libraries. PCRs using archaeal primers were also attempted, however, amplification did not occur indicating no *Archaea* were detected (data not shown).

Clone library analysis

Three SSU rDNA clone libraries were constructed each from trophosome tissue originating from an individual *Ridgeia piscesae* tubeworm collected either from Explorer Ridge or from Axial Caldera (RAE) (Table 1). One *R. piscesae* bacterial symbiont clone library with 52 clones was constructed using SSU rDNA from a trophosome collected from Axial Caldera (R661A). Two clone libraries, R666A (28 clones) and R670D (84 clones), were constructed from Explorer Ridge *R. piscesae* trophosomes. RDP Sequence Match searches of the partial sequences revealed an initial estimate of the richness of bacterial groups represented within the three trophosome clone libraries. Trophosomes from Explorer Ridge exhibited a single

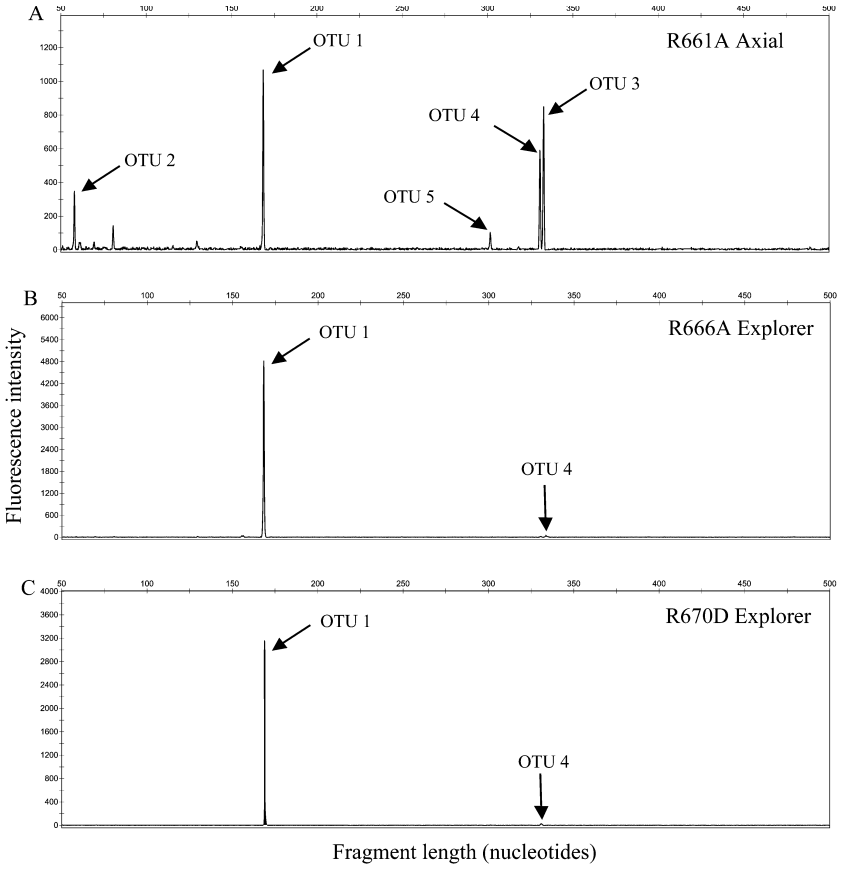

Fig. 1. *Ridgeia piscesae* trophosome bacterial community structure as determined by terminal-restriction fragment length polymorphism analysis using the restriction enzyme *Hha*I. The sample from chromotogram A was collected Axial Caldera, Juan de Fuca Ridge. Samples from chromotograms B and C were collected from Magic Mountain, Explorer Ridge. OTU 1 was the expected endosymbiont peak.

Table 1. Clone library partial sequence analysis summary.

trophosome sample I.D.	collection location	total number of clones evaluated (N)	total number of groups identified[a]
R666A	Explorer Ridge, Magic Mountain, Tubeworm Vent	28	1
R670D	Explorer Ridge, Magic Mountain, Tubeworm Vent	84	1
R661A	Juan de Fuca Ridge, Axial Caldera, Casper Vent	52	5

[a]A group must contain two or more clones to constitute an operational taxonomic unit or OTU.

group or OTU of bacterial clones. Analysis of the trophosome clone library from Axial Caldera detected five groups of clones or OTUs, four of which were distinct from the two Explorer Ridge clone libraries. The most frequent group found within the trophosomes examined was the anticipated endosymbiont of *R. piscesae*. Four additional OTUs were also detected from the following taxonomic groups, *Bacteroidetes*, *α-Proteobacteria*, and two *γ-Proteobacteria*. Several single-occurrence (*i.e.* singleton) phylotypes were also found, but were not evaluated further. Based on the parameters of ≥97% similarity and more than two clones per group, five OTUs were classified using the program DOTUR (Schloss & Handelsman 2005). Representative clones from each OTU were fully sequenced.

Full sequence and phylogenetic analysis

Each representative SSU rDNA sequence extracted from individual *R. piscesae* trophosomes that was fully sequenced is listed in Table 2. Some of the OTUs were fully sequenced in duplicate or triplicate (*i.e.* two or three clones were analyzed) to ensure reproducibility and to reaffirm that phylotypes were robust and not chimeras. *R. piscesae* endosymbionts from Axial Caldera, Juan de Fuca Ridge and Magic Mountain, Explorer Ridge (RAE) OTUs 1, 3, and 4 cluster within the *γ-Proteobacteria* class (Fig. 2). RAE OTU 1 is the *γ-Proteobacteria R. piscesae* endosymbiont and belongs to the vestimentiferan tubeworm thioautotrophic symbiont group (Feldman *et al.* 1997; Nelson & Fisher 2000). The closest relative to RAE

Table 2. Full SSU rRNA sequence analysis. Closest relatives and similarity of bacterial RAE OTUs found within trophosomes.

trophosome sample I.D.	clone no.	OTU[a]	closest relative[b]	similarity (%)
R670D	80	RAE OTU 1	*R. piscesae* endosymbiont (U77480)	99
R670D	32	RAE OTU 1	*R. piscesae* endosymbiont (U77480)	99
R670D	54	RAE OTU 1	*R. piscesae* endosymbiont (U77480)	99
R661A	44	RAE OTU 2	Uncultured bacterium clone CH2b108 (DQ228645)	96
R661A	36	RAE OTU 2	Uncultured bacterium clone CH2b108 (DQ228645)	96
R661A	8	RAE OTU 3	*Maorithyas hadalis* gill thioautotrophic symbiont II (AB042414)	91
R661A	20	RAE OTU 3	*Maorithyas hadalis* gill thioautotrophic symbiont II (AB042414)	91
R661A	1	RAE OTU 4	Uncultured bacterium clone AT-s2-59 (AY225636)	92
R661A	15	RAE OTU 5	*Roseobacter* sp. GAI-109 (AF098494)	96

[a]RAE or *Ridgeia piscesae* collected from Axial Caldera, Juan de Fuca Ridge and Magic Mountain, Explorer Ridge.
[b]As determined by BLASTn.

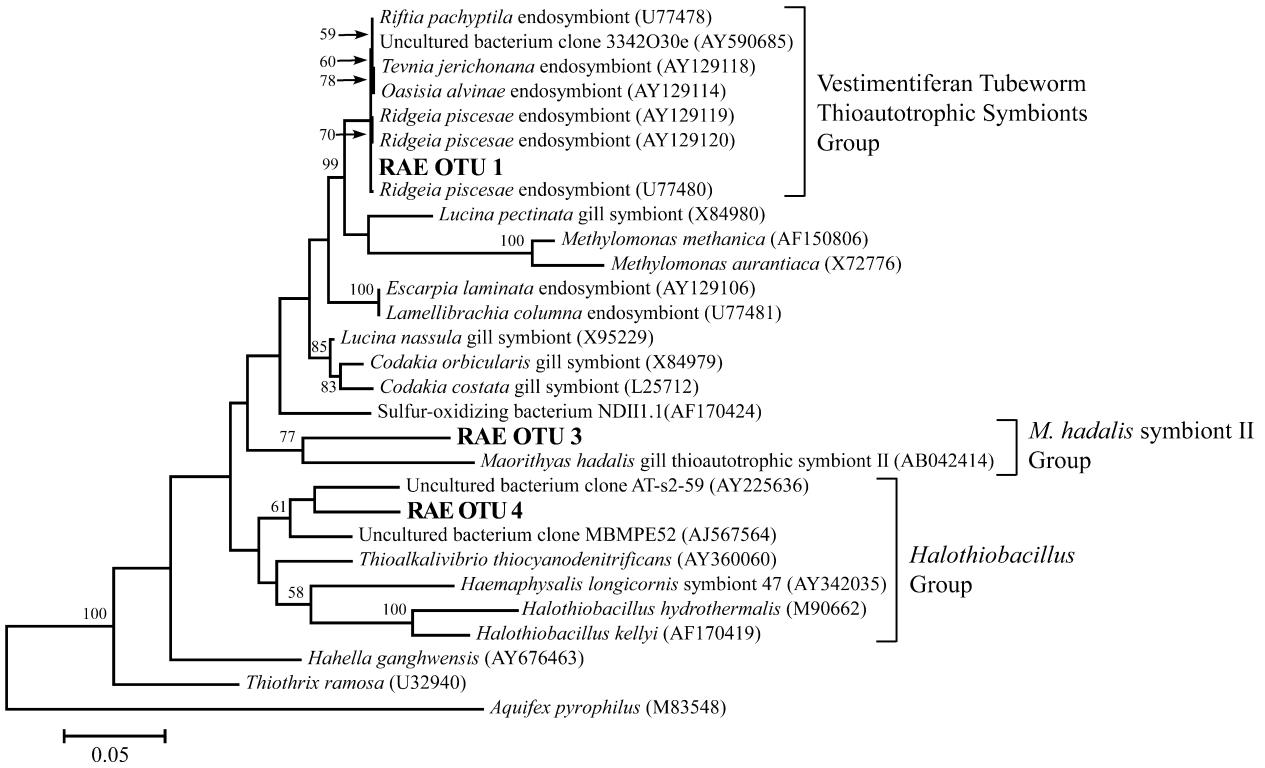

Fig. 2. *γ-Proteobacteria* phylogenetic tree. Maximum-likelihood phylogenetic tree showing the position of RAE OTUs 1, 3, and 4. This includes different bacterial taxa including the closest BLAST matches and representative neighbors. Only bootstrap values above 50 are shown. Scale bar represents five substitutions per 100 positions.

OTU 3 is *Maorithyas hadalis* gill thioautotrophic symbiont II (Fujiwara *et al.* 2001). RAE OTU 4 clusters within the *γ-Proteobacteria* class and is most closely related to uncultured bacterium clone AT-s2-59 collected from a hydrothermal vent on the Mid Atlantic Ridge (López-García *et al.* 2003) and uncultured bacterium clone MBMPE52 sampled from the Pacific Nodule Province A station (Xu *et al.* 2005). RAE OTU 5 clusters within the *Roseobacter* group (Fig. 3) and is most closely related to the organic sulfur metabolizing *Roseobacter* sp. (González *et al.* 1999) and uncultured *Roseobacter* NAC11-6 collected from a North Atlantic Algal Bloom, 400 km south of Iceland (González *et al.* 2000). RAE OTU 2 clusters within the *Cytophaga–Flavobacterium–Bacteroidetes* or CFBs (Fig. 4). RAE OTU 2 is most closely related to uncultured bacterium clone CH2b108 collected from Mothra Hydrothermal Vent Field, Endeavour Segment of the Juan de Fuca Ridge (Kelley *et al.* 2004).

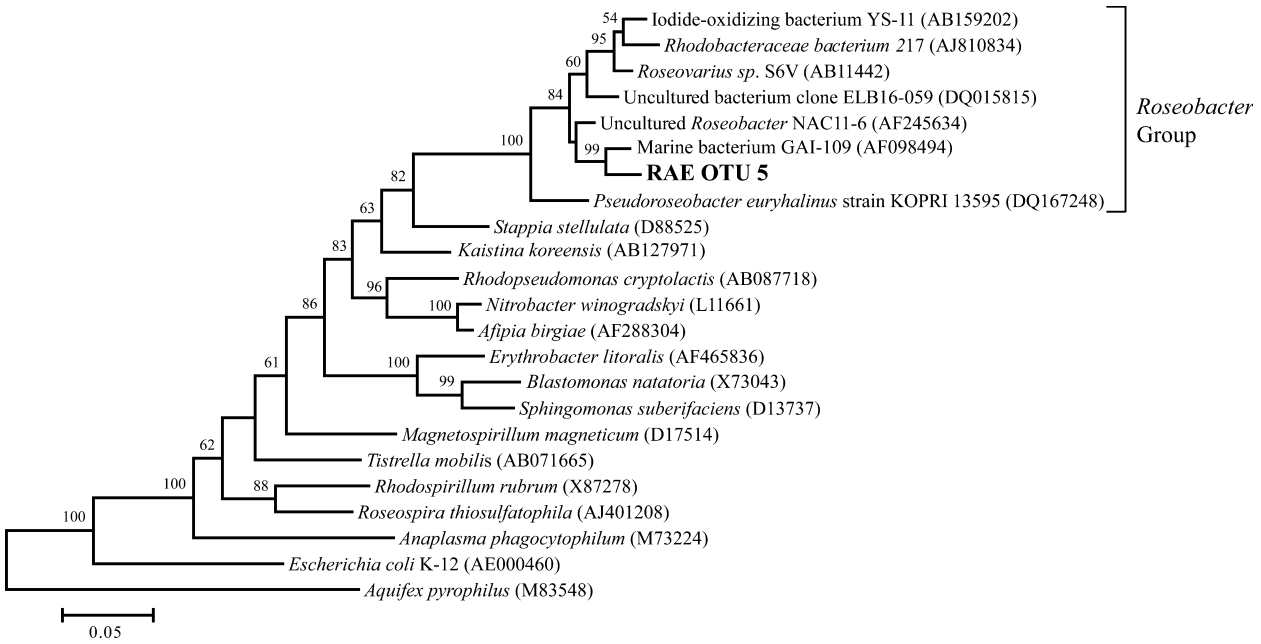

Fig. 3. *α-Proteobacteria* phylogenetic tree. Maximum-likelihood phylogenetic tree showing the position of RAE OTU 5. This includes different bacterial taxa including the closest BLAST matches and representative neighbors. Only bootstrap values above 50 are shown. Scale bar represents five substitutions per 100 positions.

Confirming community structure using *in silico* T-RFLP

Full sequences were cut *in silico* with each of the eight restriction enzymes (*Alu*I, *Bst*UI, *Hae*III, *Hha*I, *Hinf*I, *Mbo*I, *Msp*I, and *Rsa*I) to determine the T-RF size (bp) for each of the five RAE OTUs. The detection limits for T-RFLP are optimal between 50 and 500 bp (Engebretson & Moyer 2003). The majority of T-RF fragments were within the optimal detectable range (data not shown). RAE OTUs were considered present if they were found in at least three actual T-RFLP digests. All T-RFLP chromatogram data confirm that RAE OTU 1 was present in all samples. As an example of this comparison process, the bacterial community structure within three trophosomes collected from Axial Caldera and Explorer Ridge, as determined by treatment with *Hha*I, revealed T-RF fragments within a detectable range for all five RAE OTUs (Fig. 1). *Ridgeia piscesae* trophosomes collected from Axial contained RAE OTUs 1 through 5 (Fig. 1A) and those from Explorer Ridge contained RAE OTUs 1 and 4 (Fig. 1B and C). RAE OTU 4 was ≤1% of the trophosome communities from Explorer Ridge (Fig. 1B and C) in this example.

T-RFLP of multiple trophosome samples

Replicate trophosome samples were analyzed via T-RFLP with the exception of tubeworms 13 and 14 (samples R661A and B), which did contain sufficient biomass. To summarize these findings, one representative T-RFLP fingerprint from each trophosome was chosen for cluster analysis. The RAE OTUs present in each trophosome sample are listed in Table 3. RAE OTUs 1 and 4 were found in all 15 trophosomes analyzed. RAE OTUs 2 and 5 were only found within trophosome samples 13 and 14, which were sampled from Axial. RAE OTU 3 was found within trophosome samples 3 and 5 sampled from Explorer and trophosomes 13, 14, and 15 sampled from Axial. All 5 OTUs were present in T-RFLP data within trophosomes 13 and 14 sampled from Axial (consistent with clone library-derived data). Three clusters resulted from UPGMA/product–moment cluster analysis of 15 T-RFLP bacterial community fingerprints from *R. piscesae* trophosomes using 8 restriction digest treatments (Fig. 5). *Ridgeia piscesae* trophosome bacterial community Cluster 1 consists of three trophosome samples: R666B, R670G, and R666A, which were all collected from Explorer Ridge, Magic Mountain. Cluster 2 includes both trophosomes collected from Juan de Fuca, Axial Caldera and Explorer Ridge, Magic Mountain. This cluster was by far the largest and includes: R666C, R666D, R670A, R670B, R670C, R670D, R670E, R670F, R670H, and R661C. Cluster 3 consists of trophosomes collected exclusively from Axial Caldera (R661A and R661B).

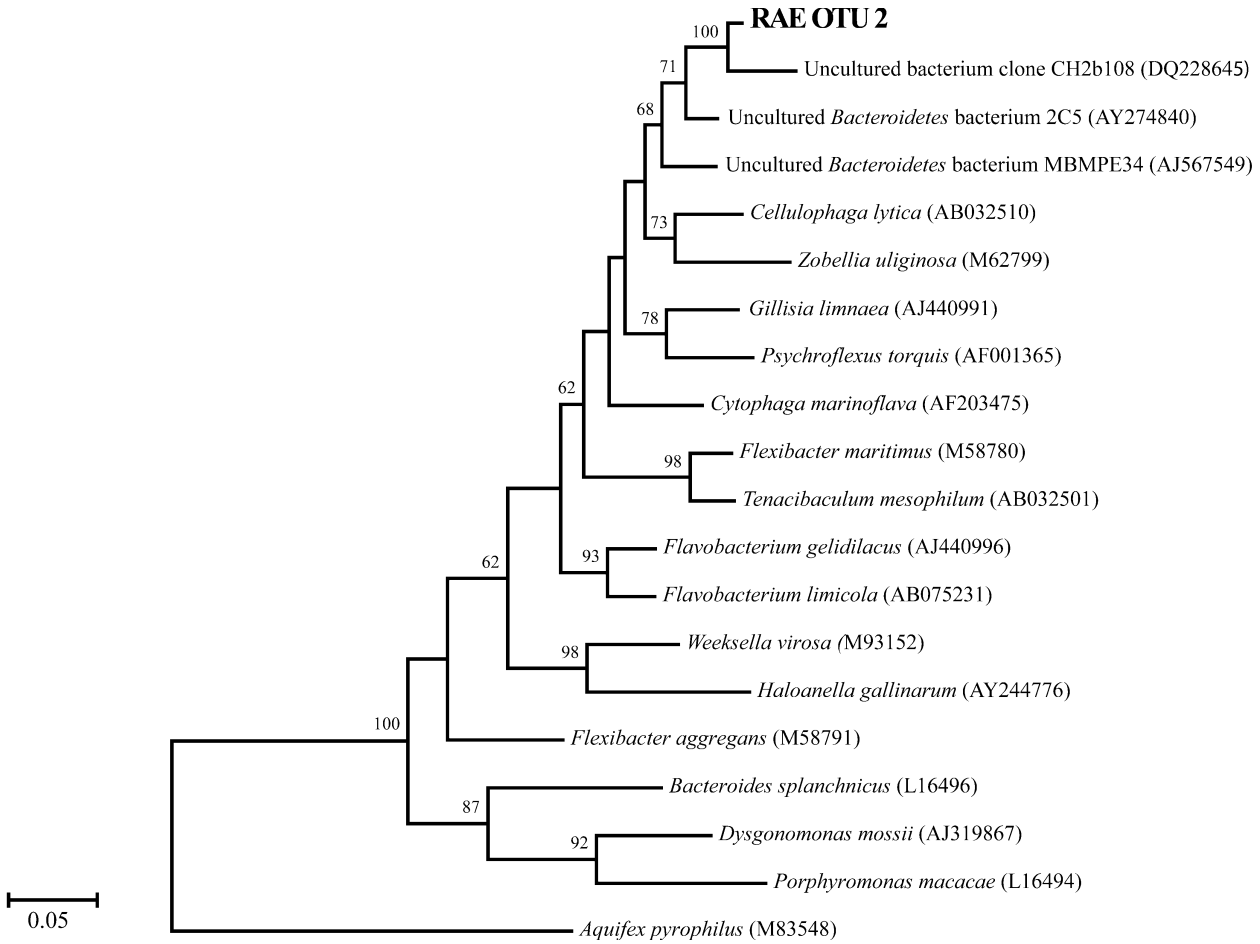

Fig. 4. *Cytophaga–Flavobacterium–Bacteroidetes* (CFBs) phylogenetic tree. Maximum-likelihood phylogenetic tree showing the position of RAE OTU 2. This includes different bacterial taxa including the closest BLAST matches and representative neighbors. Only bootstrap values above 50 are shown. Scale bar represents five substitutions per 100 positions.

Table 3. Terminal-restriction fragment length polymorphism analysis summary of multiple trophosome samples.

trophosome sample I.D.	collection location	RAE OTU 1	RAE OTU 2	RAE OTU 3	RAE OTU 4	RAE OTU 5	total number of RAE OTUs detected
R666A	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R666B	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R666C	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	3
R666D	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R670A	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	+	+	–	3
R670B	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R670C	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R670D	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R670E	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R670F	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R670G	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R670H	Explorer Ridge, Magic Mountain, Tubeworm Vent	+	–	–	+	–	2
R661A	Juan de Fuca Ridge, Axial Caldera, Casper Vent	+	+	+	+	+	5
R661B	Juan de Fuca Ridge, Axial Caldera, Casper Vent	+	+	+	+	+	5
R661C	Juan de Fuca Ridge, Axial Caldera, Casper Vent	+	–	+	+	–	3

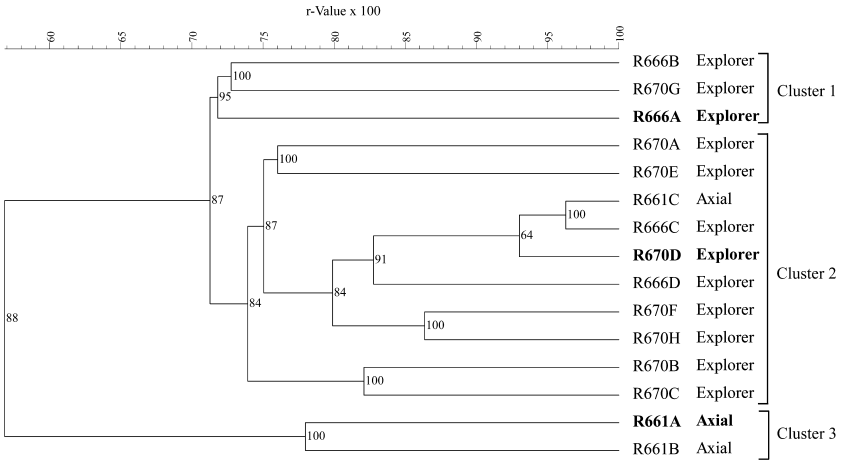

Fig. 5. UPGMA/Pearson product–moment cluster analysis of 15 terminal-restriction fragment length polymorphism bacterial community fingerprints from *Ridgeia piscesae* trophosomes using eight restriction digest treatments on each sample. Three *R. piscesae* trophosome bacterial community clusters were identified and designated Clusters 1, 2, and 3. The numbers at the nodes represent the cophenetic correlation values. Clone libraries were constructed from the trophosome samples in bold type.

Discussion

Ridgeia piscesae and all other vestimentiferan tubeworms from hydrothermal vents are thought to have a single bacterial endosymbiont within their trophosomes (Stahl *et al.* 1984; Lane *et al.* 1985; Distel *et al.* 1988; Feldman *et al.* 1997; Nelson & Fisher 2000). The goal of this study was first, to determine whether *R. piscesae* found at different hydrothermal vents contained more than one bacterial phylotype within the trophosome using T-RFLP techniques. The second goal was to identify any additional phylotypes within the trophosome community using DNA cloning and sequencing methods. This study is the first to provide evidence for the existence of multiple taxa of bacterial endosymbionts within vestimentiferan trophosomes collected from hydrothermal vents using molecular methods.

The results from the clone library analyses revealed five RAE OTUs within the trophosome collected from Axial Caldera, but only one RAE OTU was detected within Explorer Ridge, Magic Mountain trophosomes. All five RAE OTUs found within trophosomes collected from Axial Caldera were also found in T-RFLP chromatograms (*e.g.* Fig. 1). Clone library analyses were unable to detect more than one taxon of bacterial endosymbiont from trophosomes collected from Explorer Ridge. However, T-RFLP data show that all trophosomes from both Axial and Explorer contain RAE OTUs 1 and 4 (Fig. 1 and Table 3). T-RFLP chromatograms of bacterial endosymbionts collected from Explorer Ridge show RAE OTU 4 to be ≤1% of the community. A plausible explanation for why RAE OTU 4 was not found in Explorer Ridge clone libraries was that it was improbable to find any of these clones within the two libraries (84 clones from R670 and 20 clones from R666). For example, if RAE OTU 4 was ∼1% of the community in R670D from Explorer Ridge. This means that at least 100 clones would need to be evaluated to find a single RAE OTU 4 clone. As RAE OTUs were assigned to groups containing two or more clones, approximately 200 clones would need to be evaluated to find RAE OTU 4 within the R670D clone library.

Terminal-restriction fragment length polymorphism chromatograms revealed that additional taxa were present within both Explorer and Axial trophosomes, but these were not found within any of the clone libraries analyzed. T-RFLP is therefore considered more sensitive than clone library analyses because this technique can detect more taxa within a community containing low to intermediate richness, such as those found within *R. piscesae* trophosomes. Each population results in a specific array of T-RFs which, in turn, generates a composite fingerprint of the trophosome community. From the T-RFLP data for R661A and R661B trophosomes, more than five RAE OTUs were detected and these data were considered in the cluster analysis. Many more clones would be needed to potentially identify these additional phylotypes, which were found using T-RFLP; however, this was not the goal of this study.

RAE OTU 1 is the *R. piscesae* endosymbiont (similarity 99%) which is in the *γ-Proteobacteria* (Feldman *et al.* 1997; Nelson & Fisher 2000). This clade forms a group called the vestimentiferan tubeworm thioautotrophic symbionts. Phylogenic analysis (Fig. 2) supports the hypothesis that endosymbionts across vestimentiferan host species found at hydrothermal vents are virtually identical. RAE OTU 3 is most closely related to the *M. hadalis* gill thioautotrophic symbiont type II (similarity 91%), which belongs to the *γ-Proteobacteria* (Fig. 2). Symbiont type I is associated with thioautotrophic symbionts of vesicomyid clams as well as deep-sea and cold seep mussels and is found in the outer region of the bacteriocyte. Symbiont type II, in contrast, is distantly related to the free-living chemoautotrophic bacteria *Thiomicrospira* and

Hydrogenovibrio and is very distant to other known bacterial symbionts in the *γ-Proteobacteria*. Type II endosymbionts are found within the inner region of the *M. hadalis* bacteriocyte, and are sulfur-oxidizing chemoautotrophs. *Maorithyas hadalis* gill thioautotrophic symbiont types I and II were the first to show spatial partitioning within the bacteriocyte among intracellular endosymbionts in marine invertebrates using molecular techniques (Fujiwara *et al.* 2001). RAE OTU 4 is most closely related to an uncultured bacterial clone ATs-s2-59 (similarity 92%), which is in the *Halothiobacillus* group within the *γ-Proteobacteria*. This clone was collected in a sediment core from the Rainbow site, a hydrothermal vent area on the Mid-Atlantic Ridge, and is a putative sulfur-oxidizing chemoautotroph (López-García *et al.* 2003). Another close relative to RAE OTU 4 was collected from deep-sea sediments from the Pacific Nodule A Province off of China (Xu *et al.* 2005).

RAE OTU 5 is most closely related to a *Roseobacter* sp. (similarity 96%), which is a marine heterotrophic *α-Proteobacteria* (Fig. 3). This isolate was collected from coastal Georgia seawater. These bacteria degrade the sulfur-containing osmolyte dimethyl sulfoniopropionate and produce dimethyl sulfide (DMS). This bacterium can also reduce dimethyl sulfoxide to DMS and degrade DMS into methanethiol. This was the first known marine bacteria that possess both the demethylation and cleavage pathways (González *et al.* 1999). Another near relative was collected from an algal bloom located 400 km south of Iceland (González *et al.* 2000).

RAE OTU 2 belongs to the CFBs (Fig. 4). All cultured CFBs are heterotrophic and are known to be abundant in aquatic ecosystems, primarily oxic surface waters of lakes, rivers and oceans. Members of the CFBs are involved with cycling organic carbon in aquatic environments (González & Moran 1997; Glöckner *et al.* 1999). The most closely related bacterium (similarity 96%) to RAE OTU 2 was recovered from an *in situ* incubator that was deployed in the wall of an active black smoker located at the Mothra Hydrothermal Field within the Endeavour Segment of the Juan de Fuca Ridge. This bacterium may be associated with microbial mats covering the outer chimney walls (Kelley *et al.* 2004). Another close relative was found at the Delaware Estuary 10 km from Cape Henlopen, which contains water from the Delaware River and Delaware Bay. These bacteria may dominate fluxes of dissolved organic matter (DOM) and other aspects of carbon cycling within aquatic ecosystems (Kirchman *et al.* 2003).

RAE OTUs 2, 3, 4, and 5 are the first known occurrences of multiple bacterial phylotypes within the trophosome of a vestimentiferan found at hydrothermal vents. Our initial hypothesis was there would be at least one additional bacterial phylotype inhabiting the trophosome; similar to what was observed in previous studies using both hybridization techniques (Naganuma *et al.* 1997a,b) and electron microscopy (deBurgh *et al.* 1989). One explanation for our observation of an unexpectedly diverse trophosome community (*e.g.* trophosome samples R661A and R661B; Table 3) was contamination from bacteria associated with the tube or bacterial contaminants associated with nearby microbial mats, vent fluids, or ambient seawater. Bacterial clone libraries constructed from *R. pachyptila* outer tubes were mainly composed of *ε-Proteobacteria* (68%) as well as *δ-* (8%), *α-* (3%), and *γ-Proteobacteria* (1%) (López-García *et al.* 2002). In addition, the bacterial phylotypes found associated with tubes were most closely related to those found within deep-sea sediments and hydrothermal vents (López-García *et al.* 2002), which again may indicate horizontal transmission through infection from the environment. Sulfur-dominated hydrothermal vents at Axial Seamount were found to be dominated by *ε-Proteobacteria* within vent fluids (Huber *et al.* 2003) and microbial mats (Lynch 2000) and both studies included background seawater controls. None of the RAE OTUs we detected belonged to the *ε-Proteobacteria*, therefore, we consider it unlikely that the trophosomes were contaminated with bacteria associated with the tube, vent fluids, or microbial mats which are all known to be dominated by *ε-Proteobacteria* at Axial Seamount.

Another possible explanation for the unexpected taxa richness detected in the R661A and R661B trophosome samples is that they may have been experiencing less than optimal growth conditions (*i.e.* were in poor health or stressed) at the time of collection. All other tubeworms appeared healthy, around 0.20–0.65 m in length, and *c.* 7 mm wide with oxidized sulfur-compound residues within bright red trophosomes. The R661A and R661B trophosomes came from tubeworms that were short (*c.* 0.1 m long) and shriveled without any visible oxidized sulfur residues. These trophosomes were brown in color and appeared to be unhealthy, but in both cases the tubeworm was alive at the time of collection. It is possible that when a tubeworm is unhealthy, conditions become more favorable for the minor taxa to infect or invade the trophosome. For example, RAE OTU 2 is a potential heterotroph, which may be utilizing the DOM within the trophosome. In the R661A clone library, RAE OTU 2 was also dominant within the community (data not shown). All trophosomes examined by T-RFLP contained RAE OTUs 1 and 4, which are both putative sulfur-oxidizing chemoautotrophs (Table 3). The morphotypes that deBurgh *et al.* (1989) identified could have been RAE OTUs 1 and 4 as T-RFLP analyses revealed that all trophosomes contained these OTUs. Additionally, R666C, R670A, R661A, R661B, and R661C trophosomes contained RAE

OTU 3, which is also a putative sulfur-oxidizing chemoautotroph (Table 3). As these OTUs all have similar metabolisms, which can potentially benefit the tubeworm, it is feasible that at least one additional taxon of sulfur-oxidizing bacterium may be functioning as an additional endosymbiont within *R. piscesae* trophosomes. The metabolisms of these bacteria have not been formally described because they have not been isolated in pure culture. It must also be recognized that similar phylotypes do not necessarily account for similar metabolisms. Additional phylotypes within the trophosome with similar metabolisms may benefit the worm by providing sugar or amino acids that the primary endosymbiont does not synthesize. Another plausible explanation for the occurrence of multiple thioautotrophic symbionts is that additional phylotypes may be supporting the tubeworm when H_2S is not present at optimal concentrations. It is also possible that these other thioautotrophs are oxidizing other sulfur redox states, such as elemental sulfur or thiosulfate. These other thioautotrophic RAE OTUs may always be present in small numbers or they may be opportunistically derived from the environment. Only the potentially unhealthy or stressed R661A and R661B trophosomes contained RAE OTUs 2 and 5 in high numbers, which are putative heterotrophs. These heterotrophs may be compromising or even decomposing tubeworm tissues (such as the outermost trophosome peritoneal cells *sensu* Gardiner & Jones 1993), using the trophosome as a carbon source.

Three distinct trophosome community clusters were formed in the cluster analysis of T-RFLP data (Fig. 5). Community Cluster 1 and 2 were more similar to each other than Cluster 3. Cluster 3 was the most diverse group with at least five RAE OTUs. Cluster 2 had at least two or three OTUs present and Cluster 1 only contained two RAE OTUs. This pattern of clusters fits with our relative health hypothesis, described previously, where Cluster 1 could have potentially encountered optimal growth conditions (*i.e.* most healthy) and was composed of the known endosymbiont along with RAE OTU 4 in low numbers. The largest cluster (Cluster 2) could have been exposed to more general growth conditions (*i.e.* fair health) and was composed mainly of the known endosymbiont and may have contained greater numbers of RAE OTU 4. This cluster may also have contained RAE OTU 3 as well as other taxa. Cluster 3 contained at least five RAE OTUs and was the only cluster with trophosomes that contained RAE OTUs 2 and 5 (both putative heterotrophs). The clustering of trophosomes is based on both the number and abundance of OTUs, thereby indicating that Cluster 3 trophosomes were possibly exhibiting a poorer health state than any of the other trophosomes sampled. In the future, we hope to use FISH analysis to support our relative health hypothesis, as this efficacious method will provide both location and distribution evidence for these taxa.

In this study, T-RFLP as well as traditional clone library and sequencing methods were used in conjunction to determine the community structure within individual *R. piscesae* trophosomes collected from Juan de Fuca Ridge, Axial Caldera and Explorer Ridge, Magic Mountain. T-RFLP analyses showed that all trophosomes examined contain RAE OTU 1, which was the expected *R. piscesae* endosymbiont. Also, to a lesser extent, RAE OTU 4 was present in all T-RFLP chromatograms, which was a member of the *Halothiobacillus* group within the *γ-Proteobacteria*. Only RAE OTUs from the *γ-Proteobacteria* were found within trophosomes sampled from Explorer Ridge (OTUs 1, 3, and 4), whereas additional putative heterotrophs (OTUs 2 and 5) were detected in trophosomes R661A and R661B from Axial Caldera. This study strongly suggests the existence of multiple phylotypes of bacterial endosymbionts within the *R. piscesae* trophosome. In addition, we demonstrate that T-RFLP is a sensitive molecular fingerprinting method for determining the bacterial community structure, with enhanced sensitivity over clone library and sequencing analyses alone.

Acknowledgements

We gratefully acknowledge the captain and crew of the R/V *Thomas G. Thompson* and the ROPOS operations team as well as the participants of the 2002 Submarine Ring of Fire expedition to the Explorer Ridge, with special thanks to chief scientist Robert Embley, Verena Tunnicliffe and Kim Juniper. We also thank Ray Lee and our anonymous reviewers for their help with revisions and Andrea Curtis for help with initial gDNA extraction methods. This project was funded in part by a grant from NOAA's Office of Ocean Exploration and by funding through the Office of Research and Sponsored Programs at Western Washington University.

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