ORIGINAL ARTICLE

Bacterial diversity associated with the tunic of the model chordate *Ciona intestinalis*

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The sea squirt *Ciona intestinalis* is a well-studied model organism in developmental biology, yet little is known about its associated bacterial community. In this study, a combination of 454 pyrosequencing of 16S ribosomal RNA genes, catalyzed reporter deposition-fluorescence *in situ* hybridization and bacterial culture were used to characterize the bacteria living inside and on the exterior coating, or tunic, of *C. intestinalis* adults. The 454 sequencing data set demonstrated that the tunic bacterial community structure is different from that of the surrounding seawater. The observed tunic bacterial consortium contained a shared community of <10 abundant bacterial phylotypes across three individuals. Culture experiments yielded four bacterial strains that were also dominant groups in the 454 sequencing data set, including novel representatives of the classes *Alphaproteobacteria* and *Flavobacteria*. The relatively simple bacterial community and availability of dominant community members in culture make *C. intestinalis* a promising system in which to investigate functional interactions between host-associated microbiota and the development of host innate immunity.

Introduction

The tunicate *Ciona intestinalis* has been studied as a model organism in developmental biology since the late 1800s, and has recently regained popularity as an experimental model because of advances in sequencing and molecular biology. Tunicates are named for their protective outer coating or tunic, which in *C. intestinalis* consists of a layer of cellulose and mucopolysaccharide exterior to the epidermis (De Leo *et al.*, 1981). Several studies have documented abundant bacteria both in and on the tunic of *Ciona* (De Leo and Patricolo, 1980; De Leo *et al.*, 1981; Di Bella *et al.*, 2011). However, the phylogeny and ecological role of the tunic bacteria remain completely unknown. This host-associated bacterial community is of special interest given the tractability of *Ciona* as an experimental system.

Several factors have contributed to the resurgence of *Ciona* as a model organism, not least the availability of both the *C. intestinalis* and the related

C. saviygni genome sequences (Dehal *et al.*, 2002; Small *et al.*, 2007). The *Ciona* genomes are the smallest sequenced chordate genomes at 150–170 Mb in length and encode ∼16 000 genes. In addition, molecular and genetic methods are well established in *Ciona,* including DNA electroporation, mRNA microinjection and whole-mount fluorescence *in situ* hybridization (FISH) of embryos (Christiaen *et al.*, 2009a, b, c). The phylogenetic position of tunicates as our closest invertebrate relatives has also renewed interest in *Ciona* as a model organism (Delsuc *et al.*, 2006). *Ciona* is a basal invertebrate chordate (phylum: Chordata, subphylum: Tunicata, class: Ascidiacea). Like all invertebrates, tunicates lack adaptive immune systems, and thus present the opportunity to study the innate immune system in isolation.

The *Ciona* tunic is a jelly-like coating that surrounds the animal mantle epithelium (Figure 1; De Leo *et al.*, 1981, 1997). The thin, outermost layer of the tunic is a tough, fibrous cuticle composed mainly of cellulose. The interior of the tunic, often referred to as the tunic matrix or 'ground substance', is a thick, amorphous layer of cellulose fibrils and polysaccharides. Small tunicate cells of a variety of types are scattered throughout the tunic matrix. The *Ciona* tunic functions as an intermediary zone between the interior and exterior of the animal,

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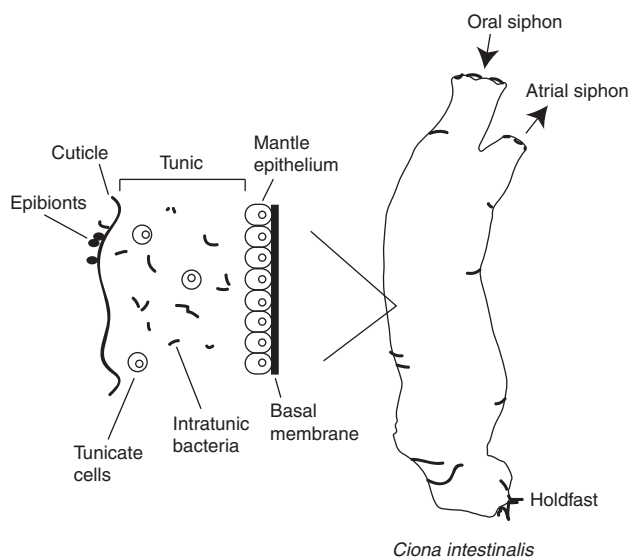
Figure 1 Schematic of *C. intestinalis* adult and a detail showing a cross-section of the tunic (redrawn after De Leo *et al.*, 1997). The width of the tunic from mantle epithelium to cuticle is ~200–400 μm thick (De Leo *et al.*, 1981).

similar to mucus layers in humans. As such, it serves as a site of self/non-self recognition and a barrier to infection (Di Bella and De Leo, 2000), making it attractive as a model for the role of innate immunity in establishment and maintenance of a surface-associated microbiome.

Tunicate bacterial communities are also of interest for their potential as sources of new natural products. The competitive and complex environment of sessile marine invertebrates may select for bacteria that can produce bioactive compounds. On average, 35 new natural products have been discovered from tunicates in each year since 2007 (Blunt *et al.*, 2011), and many of these compounds are likely produced by microbial symbionts rather than the animal host (Schmidt and Donia, 2010). The uncultivated cyanobacterium *Prochloron didemni* has been recently shown to produce a suite of bioactive peptides including the patellamides and cyanobactins, which were originally isolated from the host tunicate *Lissoclinum patella* (Schmidt *et al.*, 2005). In another example, the intracellular symbiont *Candidatus Endoecteinascidia frumentensis* has been implicated in production of the alkaloid ecteinascidin-743 from the tunicate *Ecteinascidia turbinata* (Rath *et al.*, 2011). Even the model tunicate *Ciona* has yielded bioactive bacteria. The bacterium *Pseudoalteromonas tunicata* D2 was originally isolated from the surface of *C. intestinalis* (Holmstrom *et al.*, 1998) and has been shown to produce a variety of bioactive molecules including the antifungal compound tambjamine, the purple pigment violacein and 3-formyl-tyrosine metabolites (Franks *et al.*, 2005, 2006; Blasiak and Clardy, 2010).

This study aimed to characterize the tunic microbiome of adult *C. intestinalis* from Woods Hole, MA, USA, to determine if there is a specific, tunic-associated community, and to identify the abundant phylotypes in and on the tunic. In order to provide a comprehensive analysis of the tunic bacterial community, we used a variety of techniques including 454 16S ribosomal RNA (rRNA) gene amplicon sequencing, microscopy and bacterial culture. We report the presence of several shared and dominant phylotypes across three *C. intestinalis* individuals. In addition, we succeeded in isolating four novel strains of tunic bacteria, all of which were abundant in the amplicon sequencing analysis. This is in contrast to many other environments in which cultured bacterial community members are rare within the total sequenced community (Shade *et al.*, 2012). The combination of 16S rRNA gene amplicon-based bacterial community analysis and available cultured isolates will provide a basis for future investigation of the ecological function of the tunic bacterial community.

Materials and Methods

C. intestinalis *collection, dissection and DNA extraction*

Initial microscopic observations of *C. intestinalis* tunic were performed on three adult individuals obtained from Woods Hole Marine Biological Laboratory (MBL) Marine Resource Center (MRC) aquaculture facility in July 2011. All further microscopy, 454 sequencing and bacterial isolation were performed on 15 wild-caught *C. intestinalis* adults directly collected from Eel Pond (41.526, −70.670) by the MBL MRC in Woods Hole in December 2011. The water temperature at the collection site was 7 °C. Three 50 ml Falcon tubes of seawater were collected at the site of the *C. intestinalis* collection. Specimens were shipped overnight to the Institute of Marine and Environmental Technology (IMET) in a large bag of seawater on ice and processed immediately on arrival. The seawater samples were syringe filtered with Sterivex—GV 0.22 μm filter units (Millipore, Billerica, MA, USA). The filter units were opened and the filters were removed with sterile scalpels and stored at −20 °C until DNA extraction. Three additional 50 ml water samples were taken from the collection bag containing the shipment of *Ciona* and were processed as described.

Multiple ~0.5 cm³ tunic samples for DNA extraction and fixation were sterilely dissected from three *C. intestinalis* individuals. For tunic and cuticle samples (1–3TC), each section was rinsed 3× in sterile artificial seawater (ASW) and then processed for DNA extraction or fixation. For tunic interior samples (1–3T), the exterior cuticle was removed from the section with tweezers before rinsing 3× in sterile ASW. DNA was extracted from tunic samples or water filters using the Mo Bio PowerSoil DNA Isolation kit (Mo Bio, Carlsbad, CA, USA). The tunicate COI gene was sequenced from two

individuals from Woods Hole, and these tunicates were confirmed to belong to *C. intestinalis* cryptic sp. B (Caputi *et al.*, 2007; Supplementary Figure S1).

Tunic fixation and catalyzed reporter deposition (CARD)-FISH
Tunic samples were fixed overnight at 4 °C in 4% formaldehyde solution prepared from 16% metha-nol-free formaldehyde (Thermo Scientific, Rockford, IL, USA) in ASW. Fixed samples were washed in ASW and incubated in 15% and then 30% sucrose in ASW for 1 h each at room temperature. Tunic samples were embedded in Tissue Tek OCT com-pound (Sakura, Torrence, CA, USA), flash-frozen in liquid N_2 and sectioned to 8 μm on a Tissue Tek Cryo$_3$ microtome (Sakura). Sections were transferred to poly-L-lysine-coated slides (Electron Microscopy Sciences, Hatfield, PA, USA). CARD-FISH was performed according to established protocols (Pernthaler *et al.*, 2002). Tunic sections were permeabilized for 30 min with 15 mg ml^{-1} lysozyme in 50 mM EDTA, 100 mM Tris pH 8 at 37 °C. Endogenous peroxidases were inactivated with 0.15% H_2O_2 in methanol for 30 min. Horseradish peroxidase-labeled probes were obtained from Bio-mers (Ulm, Germany) and used at a working concentration of 50 ng μl^{-1}. Hybridizations were performed under established stringent hybridization conditions: 46 °C, 3 h hybridization, 35% formamide (EubI-III, Non338, CF319a) or 0% formamide (Euk516, Non338; Amann *et al.*, 1990; Manz *et al.*, 1996; Ishii *et al.*, 2004). Amplification was per-formed using the Tyramide Signal Amplification system (Perkin Elmer, Waltham, MA, USA) with tyramides diluted 1:10 in provided amplification buffer. DNA was counterstained with 4′,6-diami-dino-2-phenylindole (DAPI). The hybridization stringency of the CF319a probe was confirmed by testing against alphaproteobacterial isolate CWH001 (negative control) and flavobacterium *Tenacibacu-lum mesophilum* MBIC 4357 (positive control). Bacterial cell density was calculated by direct counts of DAPI-stained objects in 10 fields of view in 8 μm tunic sections. The percentage of CF319a or EubI-III-labeled cells relative to total DAPI-stained cells was calculated by comparing DAPI and Cy3 counts from 10 fields containing 35–115 cells.

Culture and phylogenetic analysis of tunic-associated bacteria
A ∼1 cm^3 section of tunic from *C. intestinalis* individual 2 was pressed through a sterile 100 μm nylon Cell Strainer (BD Falcon, Bedford, MA, USA) and diluted in 1 ml sterile ASW. This solution was serially diluted in ASW and 100 μl of the 10^3 and 10^5 dilutions were plated in triplicate on Difco Marine Agar 2216 (MA2216) (BD, Sparks, MD, USA) and natural seawater agar (autoclaved natural seawater with 1.5% agar) and observed for 2 weeks.

Four main morphotypes (CWH001, CWH003, CWH007 and CWH016) were subcultured to isolation on MA2216 at 20 °C. For DNA extraction and cryo-preservation, tunic isolates were grown in 5 ml Difco Marine broth 2216. Isolates were cryopreserved in 30% glycerol at −80 °C, but only CWH001 and CWH003 could be revived from the cryopreserved stock. DNA was extracted using the UltraClean Microbial DNA isolation kit (Mo Bio). The 16S rRNA gene was amplified and sequenced using primers 27F 5′-AGAGTTTGATCMTGGCTCAG − 3′ and 1492R 5′-TACGGYTACCTTGTTACGACTT-3′ (Lane, 1991). The 50 μl PCR reactions contained ∼50 ng genomic DNA, 0.5 units Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) in supplied PCR Rxn buffer, 2 mM MgCl$_2$, 0.2 mM dNTP mix (Fermentas, Glen Burnie, MD, USA) and 0.2 μM of each primer. The amplification conditions were as follows: 96 °C for 5 min, followed by 25 cycles of 92 °C for 30 s, 46 °C for 2 min, and 72 °C for 1 min 30 s, with a final extension at 72 °C for 10 min. PCR products were sequenced on an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in IMET's BioAnalytical Services Laboratory.

16S rRNA gene sequences of isolates and their closest cultured and uncultured BLAST hits were aligned using the SILVA Incremental Aligner (SINA) v1.2.11 (Pruesse *et al.*, 2012). Aligned sequences were added to 'The All-Species Living Tree' Project (LTP) database LTPs108 by maximum parsimony in ARB (Ludwig *et al.*, 2004) and the alignment was further refined manually. Type strains representing closely related and representative groups were selected for phylogenetic analysis. A 50% positional base frequency filter was calculated over either all *Alphaproteobacteria* or *Bacteroidetes*. The final alignments comprised 1262 bp and 1211 bp for the *Alphaproteobacteria* and *Flavobacteria* trees, respectively. Alignments were exported and ana-lyzed with Phylip 3.69 (Felsenstein, 2005) to generate neighbor-joining and maximum parsimony consensus trees with 1000 × bootstraps. Maximum-likelihood trees with 1000 × bootstraps were calculated with PhyML 3.0 Online (Guindon *et al.*, 2010) using the HKY85 substitution model with empirical base frequencies and estimated transition/ transversion ratio, invariable sites and gamma distribution parameter. There was good agreement between the neighbor-joining, maximum parsimony and maximum-likelihood trees with well-supported nodes consistent across all three methods.

454 16S rRNA amplicon sequencing
A total of six DNA samples from three *Ciona* individuals were analyzed by 454 sequencing of 16S rRNA amplicons. One DNA sample from tunic interior (T) and one from tunic and cuticle (TC) were sequenced from each individual. One DNA sample for each water type including water from the

collection site (CW) and shipment bag (BW) were also analyzed. DNA concentrations ranged from 5 to $9\,ng\,\mu l^{-1}$ as measured by Nanodrop 2000 spectrophotometer (Thermo Scientific). PCR amplification and 454 barcoded amplicon sequencing were performed by Research and Testing Laboratory (Lubbock, TX, USA) on the 454 Life Sciences GS FLX+ platform (Roche Diagnostics, Branford, CT, USA). The V1–V3 region of the 16S rRNA gene was amplified using primers 28F (barcoded 5′-GAGTTT GATCNTGGCTCAG-3′) and 519R (5′-GTNTTACN GCGGCKGCTG-3′). Sample 1TC was amplified and sequenced twice as the first sequencing run generated <2000 reads. Only the second sequencing run of 1TC was included in the analysis. A complete list of samples and barcoded primers is available in Supplementary Table S1.

Data processing was performed in Mothur v1.28.0 (Schloss et al., 2009) and generally followed the procedures recommended in Schloss et al. (2011). Raw sequencing reads were denoised using the Pyronoise algorithm (Quince et al., 2011) and sequences were required to have exact barcode and primer matches, no ambiguous base calls, and no homopolymers >8 bp. Barcode and primer sequences were removed and then sequences with <250 bp were discarded. Finally, sequences were all trimmed to exactly 250 bp before analysis. Sequences were preclustered using an abundance weighted single-linkage preclustering step at ∼2% difference (maximum 2-bp difference over 250 bp) as originally recommended by Huse et al. (2010). Chimeras were identified and removed using the UChime algorithm (Edgar et al., 2011). Sequences were classified against the Ribosomal Database Project (RDP) database release 9 (Cole et al., 2009) with a bootstrap cutoff of 50% (Wang et al., 2007). Sequences that classified as chloroplasts were removed. A distance matrix was generated using pairwise.seqs in MOTHUR and clustering was performed using the average neighbor method at 3% to generate operational taxonomic units (OTUs). Details of read numbers following each processing step are given in Supplementary Table S2.

Near complete 16S rRNA gene sequence for OTU2 was obtained using the specific primer A1CWF 5′-G GAATCTACCTTATAGTGGGGGATAACTTC-3′ along with 1492R for amplification from sample 2T as described for 27F/1492R but with annealing at 55 °C. Sequencing was performed using specific primers A1CWF and A1CWF_mid 5′-GCTAAACGTCGGGTC TTAGGAT-3′.

Sequence data are available in the GenBank database under accession no. KF494349-55 and the MG-RAST database under project 5387 with metagenome IDs 4530382.3-89.3.

Results

Tunic microbiome analysis
DNA from six water samples and three tunicate individuals was used to create 16S rRNA gene amplicon libraries that were initially screened by denaturing gradient gel electrophoresis (DGGE) to compare the bacterial communities (Supplementary Methods and Supplementary Figure S2). Based on this analysis, two water samples and six tunicate samples were selected for 454 sequencing. Two samples were sequenced from each tunicate individual, one sample of tunic and exterior cuticle (TC), and one sample containing only interior tunic (T). DNA from associated seawater samples was sequenced from both the collection site (CW) and the shipping bag (BW). The 454 amplicon libraries were randomly subsampled to 2500 sequences per sample, which provides nearly complete coverage (97–99% Good's coverage) of tunic libraries at 3% OTU clustering (Supplementary Table S3). The 16S rRNA gene libraries from water samples were more diverse than those of the tunic samples as assayed by both DGGE and 454 sequencing, with an average of 51 OTUs detected in the tunic samples and 526 OTUs and 278 OTUs detected in the collection bag water and seawater respectively (Figure 2, Supplementary Figure S3 and Supplementary Table S3). The number of OTUs detected in tunic samples ranged from 15 to 147. In addition, the tunic and cuticle samples had greater richness than the tunic-only samples from the same individual, with 15–22 OTUs observed for tunic-only samples and 29–147 OTUs observed for tunic and cuticle samples (Supplementary Table S3 and Supplementary Figure S3).

The sequences in the tunic 16S rRNA gene fragment libraries were classified by RDP into three main phyla, Proteobacteria, Bacteroidetes and unclassified Bacteria (Figure 2a). The tunic Proteobacteria sequences belonged mainly to Alphaproteobacteria or could not be further classified (unclassified_Proteobacteria), but all other classes within the Proteobacteria were detected in at least one tunic sample. Actinobacteria, Planctomycetes, Firmicutes, Verrucomicrobia, Chloroflexi and Armatimonadetes were all also present in low abundance in at least one tunicate. Only tunicate individual three (both 3T and 3TC) contained 1–3% of sequences from an OTU categorized as 'unclassified bacteria' by RDP. Alignment of a representative sequence from this OTU to the SILVA SSU reference database indicated that this OTU groups with the candidate division BD15. Sequences from the phylum Cyanobacteria comprised 2% of collection seawater sequences (CW) and 33% of shipping bag seawater sequences (BW) but were not detected in any tunic DNA library. In addition, the bacterial groups TM7, Acidobacteria, Fusobacteria, Spirochaetes, Nitrospira and OD1 were detected in seawater amplicon libraries but not in tunic libraries.

Only nine OTUs contained >1% of the total tunicate sequences and accounted for >90% of the total tunicate sequences (Figure 3). These OTUs were classified as Flavobacteria, unclassified
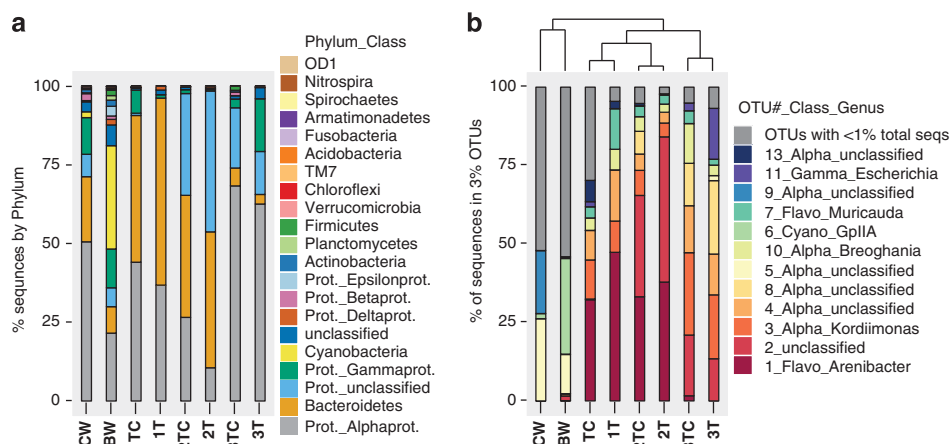
Figure 2 Bacterial community analysis by 454 sequencing of 16S rRNA genes. Sample names are: collection site seawater (CW), shipping bag seawater (BW), *C. intestinalis* individuals 1–3 tunic and cuticle (1–3TC) or tunic only (1–3T). (**a**) Relative abundance of sequences classified to the phylum level by RDP. Proteobacteria are expanded to the class level. (**b**) Relative abundance of sequences grouped into 3% OTUs. A representative sequence from each OTU was used for RDP classification. Rare taxa (OTUs containing <1% of total sequences) are grouped together. Dendrogram at top of figure shows UPGMA clustering of the samples based on the Yue and Clayton estimator of similarity, θ, which compares community structures.

Proteobacteria, *Alphaproteobacteria* and *Gammaproteobacteria* (Table 1). Using specific primers that target the OTU 2 sequence, the second-most abundant group in tunicate samples, we were able to obtain a near complete 16S rRNA gene sequence for this group, and RDP classification indicates that it also belongs to the *Alphaproteobacteria*. The eight most abundant OTUs were detected in all tunicate individuals. Hence, the most abundant OTUs were also the most commonly shared OTUs. Less common tunicate OTUs were only detected in one or two tunicates.

Beta diversity analysis demonstrated that the bacterial community structures of the water and tunicate samples were clearly different (Figure 2b). The water samples consistently grouped separately from the tunicate samples in UPGMA clustering based on the Yue and Clayton θ estimator and Bray Curtis similarity metrics (Schloss *et al.*, 2009). A clustering analysis of DGGE banding patterns also supported separate clustering of the water and tunicate bacterial communities (Supplementary Figure S2). Within the tunicate samples, the bacterial communities clustered by tunicate individual rather than by sample type (interior tunic only vs tunic and cuticle). The bacterial community of tunicate 3 was most different, with fewer reads corresponding to OTU 1 (Flavobacteria_Arenibacter) and more corresponding to proteobacterial OTUs (Figure 3). The clustering of DGGE banding patterns of samples from tunicate 3 provided a similar outcome (Supplementary Figure S2).

Bacterial culture from C. intestinalis *tunic*
Four main bacterial morphotypes were isolated from *C. intestinalis* tunic and 16S rRNA gene sequencing

demonstrated that these four morphotypes each corresponded to an abundant OTU in the 454 data set (Table 1). Three of the isolates also had 100% identity to sequenced bands from the DGGE analysis (Supplementary Figure S2). ML trees showing the position of the four new bacterial isolates in relation to their closest BLAST hits and reference strains are shown in Figure 4. Isolate CWH001, corresponding to the third-most abundant tunicate OTU, clusters within the alphaproteobacterial *Kordiimonas* genus and shares 95% identity with type strain *Kordiimonas gwangyangensis*. RDP classification of isolate CWH003 (OTU 8) supports affiliation within the alphaproteobacterial genus *Kiloniella* (confidence 84%); however, the SINA classifier assigns this sequence to the *Rhodospiralles* order and the *Thalassospira* genus. CWH003 shares only 90% identity with the type strain *Kiloniella laminariae*. The phylogenetic analysis does not give strong support for affiliation with any characterized genera, but CWH003 does group consistently with the *Kiloniella* and *Pelagibius* genera rather than within the *Thalassospira*. The isolate CWH007 has 95% 16S rRNA sequence identity to type strain *Flagellimonas ecklioniae* and 96% identity to type strain *Muricauda olearia* CL-SS4. The RDP classifier assigns CWH007 (OTU 7) to the *Muricauda* genus with 100% confidence. The phylogenetic analysis shows CWH007 clustering consistently within a group of *Flavobacteriaceae* including the *Muricauda, Croceitalea* and *Flagellimonas* genera, but does not support clear affiliation with any genus. Isolate CWH016, corresponding to the most abundant tunicate OTU 1, groups with strong support within the flavobacterial genus *Arenibacter*, and this isolate has 94% 16S rRNA gene sequence identity to the type strain *Arenibacter palladensis*.

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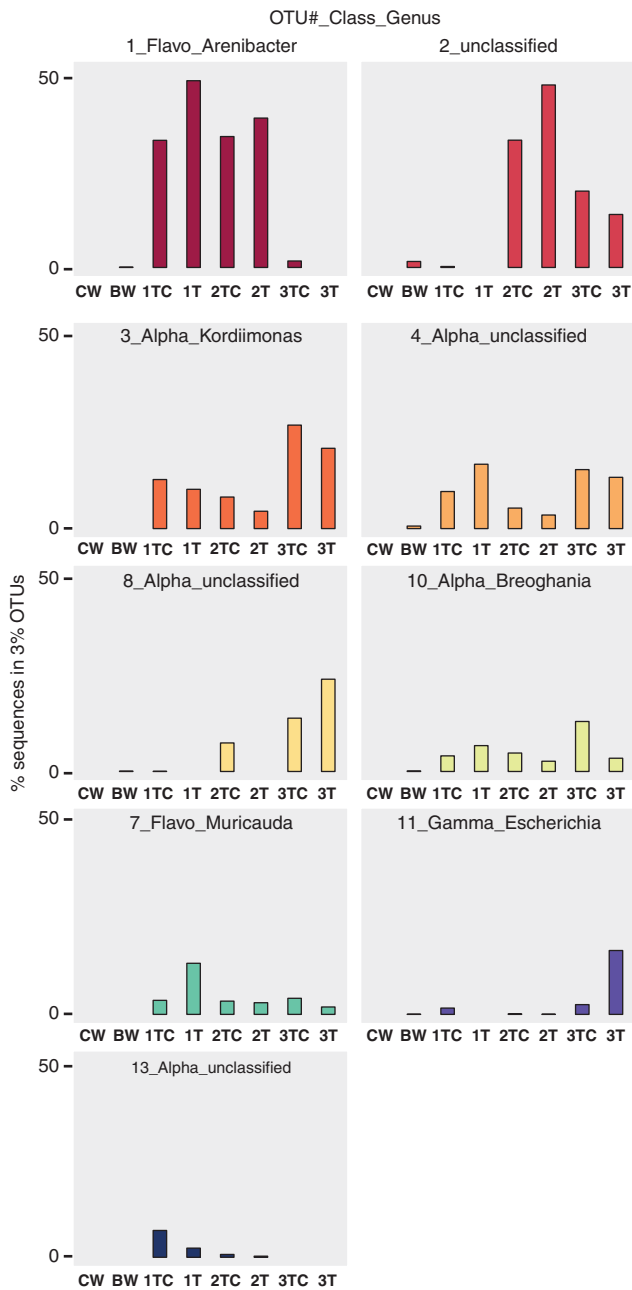
Figure 3 Expanded view of Figure 2b to allow direct comparison across samples. Each panel shows the relative abundance of sequences within an OTU. Only OTUs containing >1% of the total reads from tunicate samples are shown.

CARD-FISH of bacteria in C. intestinalis *tunic*
Microscopy of Gram-stained tunic sections revealed an even, dense population of Gram-negative bacteria embedded throughout the tunic matrix, interspersed with a variety of tunicate cells (Figure 5a). General bacterial and eukaryotic CARD-FISH probes were used to visualize bacteria and tunicate cells within tunic sections (Figures 5b and c). The interior tunic contains 1–2 μm bacteria evenly spaced within the tunic matrix, with few, spherical ~10 μm diameter

tunicate cells. The majority of the interior tunic bacterial population was labeled by the specific CF319a Cytophaga-Flavobacteria probe, which is an exact match to both isolates CWH016 (OTU 1) and CWH007 (OTU 7) (Figure 5d and e). By directly counting DAPI-stained bacterial cells within tunic sections, we estimated $2.6 \pm 0.9 \times 10^9$ cells cm^{-3} for individual 1, $2.8 \pm 0.6 \times 10^9$ cells cm^{-3} for individual 2 and $8.8 \pm 0.2 \times 10^9$ cells cm^{-3} for individual 3 (sample s.d. are indicated). The percentage of cells specifically labeled by CARD-FISH was quantified for individual 2 by comparing Cy3-labeled cells with total DAPI counts. The EubI-III probe detected an average of $78 \pm 10\%$ of the DAPI-stained cells, whereas the specific Cytophaga-Flavobacteria probe CF319a labeled $37 \pm 9\%$ of cells within the tunic.

Discussion

The presence of bacteria within the tunic of *C. intestinalis* was first reported over 30 years ago with electron microscopy images showing bacilli of ~2 μm within tunic sections (De Leo *et al.*, 1981). In a separate study, De Leo and Patricolo (1980) also observed a specific association with a probable cyanobacterial symbiont in *C. intestinalis* from tropical waters. Since these initial reports, only a few studies have remarked on the tunic bacteria (De Leo *et al.*, 1997; Di Bella *et al.*, 2011), and their phylogeny had never been investigated. Here we described the first culture and non-culture-based investigation of the *Ciona* tunic bacterial community.

Analysis of the beta diversity of tunic bacterial communities based on 454 sequencing of 16S rRNA amplicons showed that variation between individuals were greater than the variation between the tunic-only samples (T) and the tunic and exterior cuticle samples (TC). That is, the samples clustered by individual rather than by whether or not they contained exterior cuticle. For example, the bacterial community of 1T was more similar to that of 1TC, than to 2T or 3T. This was surprising, given that we anticipated that different bacterial groups might be important on the interior and exterior of the tunic. We expected that surface-associated epibionts would be found only in the TC samples, while bacteria living within the interior tunic matrix would be found in both T and TC samples. These individual bacterial community differences observed in the sequence data were also supported by 16S rRNA DGGE analysis using a different primer set. One possible explanation for these differences is simply that the dissection and removal of the exterior bacteria from T samples was imperfect, minimizing apparent community differences between the interior and exterior of the tunic. This explanation is unlikely to completely explain the observed differences as the TC samples had consistently higher measures of richness than their T

Table 1 Classification of abundant tunic OTUs

OTU#	Isolate	RDP classification class/genus	Closest uncultured and cultured BLAST hits			
			Acc. #		Source	Max. Id (%)
1*	CWH016	Flavobacteria/Arenibacter	DQ486485	Arenibacter sp. DG1238	Dinoflagellate	95
2*	—	Alphaprot./unclassified1	HQ326311	Uncultured clone SBS-RV-031	Biofilm	99
			DQ167245	Kopriimonas byunsanensis KOPRI 13522	Marine biofilm	88
3*	CWH001	Alphaprot./Kordiimonas	EU287361	Uncultured bacterium clone S26–61	Arctic sediment	99
			GU289640	Kordiimonas sp. MEBiC06554	Seawater	96
4	—	Alphaprot./unclassified2	HQ163356	Uncultured Alphaprot. SHZW738	Seawater	92
			JQ085424	alphaproteobacterium NH6–42	Seawater	85
8*	CWH003	Alphaprot./Kiloniella	EU287341	Uncultured bacterium clone S26–41	Arctic sediment	99
			AB571945	Alphaproteobacterium AKA07–5	—	96
10	—	Alphaprot./Breoghania	FJ543036	Uncultured Alphaprot. B07–10D	Earthworm gut	88
			GQ272328	Alphaproteobacterium UBF-P1	Sand after oil spill	86
7*	CWH007	Flavobacteria/Muricauda	FJ203619	Uncultured bacterium clone SHFH709	Coral	98
			FJ348472	Flavobacteriaceae bacterium A9	Marine sponge	97
11	—	Gammaprot./Escherichia	KC002412	Marine bacterioplankton clone	Seawater	100
			JX120100	P4-3B_16 Bacterium P618/	Swine feces	100
13	—	Alphaprot./unclassified3	JF297198	Uncultured Hyphomicrobiaceae	Lobster shell legion	98
			EF554909	Devosia sp. G-He10	—	87

Abbreviations: OTU, operational taxonomic unit; RDP, Ribosomal Database Project; rRNA, ribosomal RNA.
Top BLAST hits and closest cultured BLAST hits for the nine OTUs with >1% of total 454 16 rRNA gene sequence abundance from tunicate samples. When available, almost complete 16S sequence was used for RDP classification and BLAST searches as indicated by an asterisk. BLAST search was performed on 18 January 2013.

counterparts, indicating that some groups occur only in the exterior samples. Alternatively, most bacterial groups that occur on the tunic exterior may also be present within the tunic interior matrix. Finally, similarity of community structure within individual tunic samples may reflect a true variation of the tunic microbiome among individuals. This is similar to what has been observed in the human microbiome, where each individual has a unique microbiome, and as sequencing effort of individuals increases, the core of shared taxa decreases, although functional traits of the community may be conserved (Turnbaugh et al., 2009; Consortium, 2012).

Although the bacterial communities of each Ciona individual were different, the most abundant amplicons were consistently detected across all three tunicate individuals. The observed tunic bacterial communities contain dominant phylotypes that are shared between individuals and enriched compared with the surrounding seawater. The diversity of the observed C. intestinalis tunic microbiome was much lower than the diversity of the vertebrate-associated microbiome. For example, the microbiome of human gut and skin are expected to contain ∼30 000 and ∼2000 OTUs at 3% clustering (Huse et al., 2012) compared with the 15–150 OTUs observed in Ciona tunic samples. Low diversity has also been observed in other invertebrate microbiomes including those associated with the cnidarian Hydra (Fraune and Bosch, 2007) and the leech (Kikuchi and Graf, 2007).

The observation of shared and abundant phylotypes across these tunicate samples raises questions about whether these resident phylotypes are conserved across time and space. The three tunicate individuals sequenced here were collected from a single site in December 2011, and future study will be required to determine the stability of the tunic bacterial community with respect to different C. intestinalis populations (cryptic subsp. A vs B described here), geography, seasons, environmental conditions and health. In addition, although Ciona is a solitary tunicate, it is often found growing in large clusters of individuals, which could facilitate future investigation of the effect of proximity and genetic diversity on the associated microbiome.

The Ciona tunic has several advantages as a potential model system for extracellular or host surface-associated bacterial communities, including the relative simplicity of the resident community, with <10 abundant phylotypes observed in these individuals, and the ability to culture representatives of at least 4 of these abundant groups. Our initial culture effort was minimal, and we expect that more extensive culturing efforts with more media types and longer incubation times could yield more cultivable diversity. Future experimental work will be required to characterize the ecological role of these isolates within the tunic community and determine if they have functional importance. The isolate CWH001, which corresponds to OTU3, like all of the Kordiimonas spp. that have been described
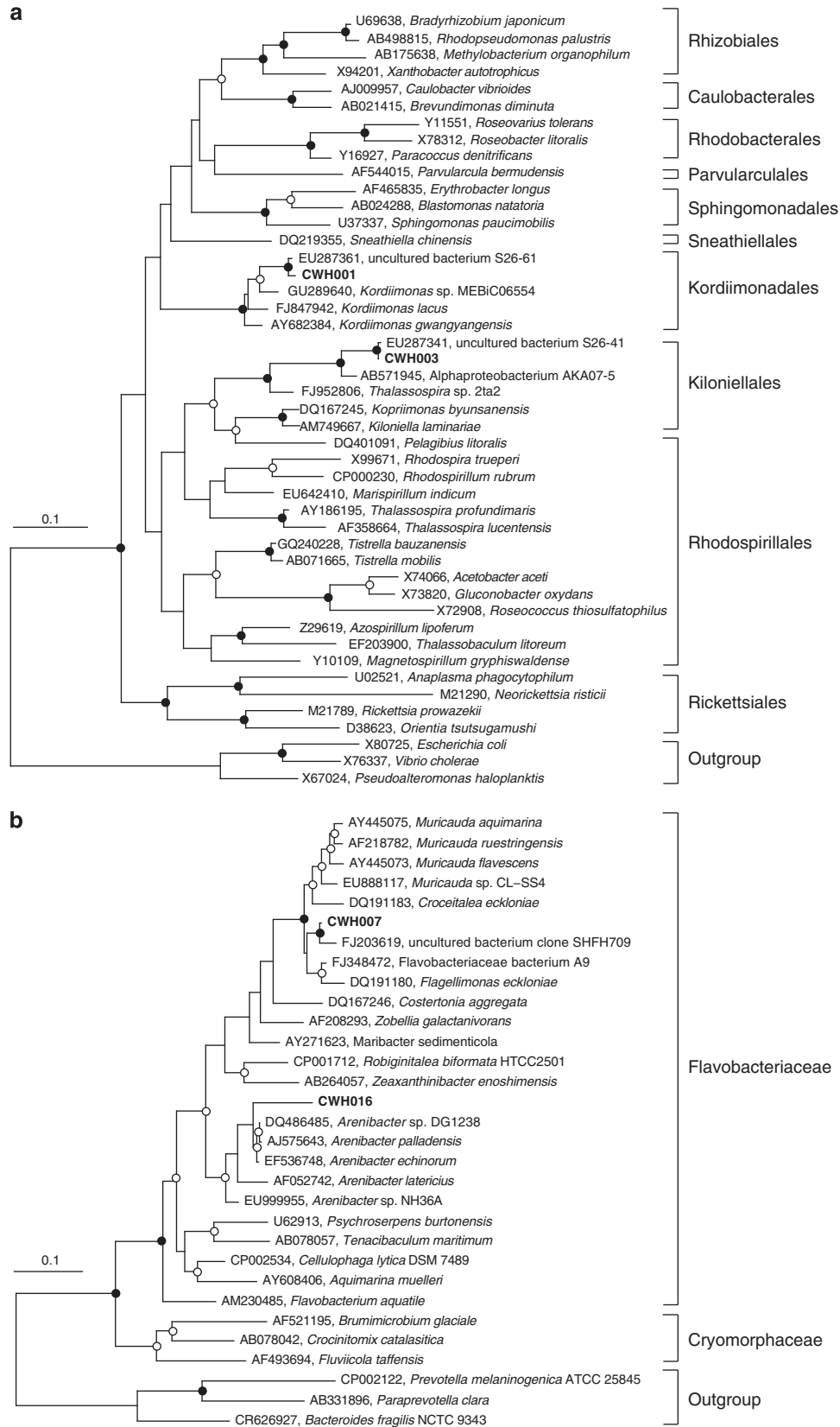
Figure 4 Maximum likelihood trees with 1000× bootstrap resampling of *Alphaproteobacteria* (**a**) and *Flavobacteria* (**b**) isolates from *C. intestinalis* tunic. Sequences from this study are shown in bold. Nodes with >90% bootstrap support are indicated by closed circles and nodes with >50% support by open circles.
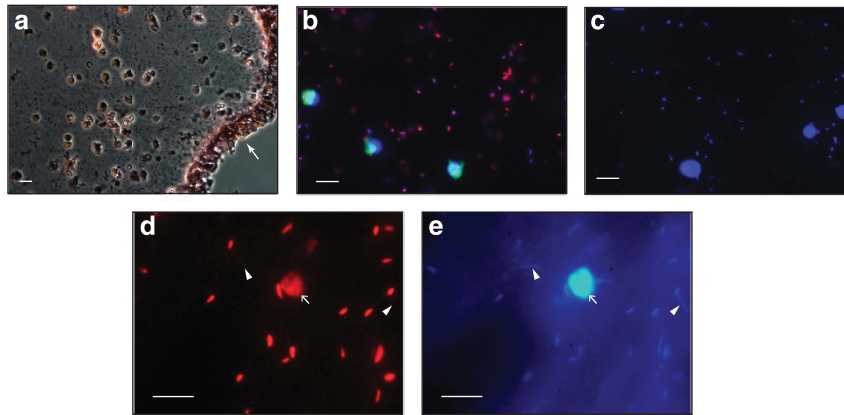
Figure 5 Epifluorescent micrographs of CARD-FISH-labeled bacteria within the *C. intestinalis* tunic. (**a**) Gram-stained tunic and cuticle section. White arrow denotes exterior cuticle. (**b**) *C. intestinalis* individual 5 from Woods Hole July 2011 collection. EubI-III-labeled bacteria (red), Euk516-labeled tunicate cells (green) and DAPI-stained DNA (blue). (**c**) Same tunicate individual as in **a** with double Non338 (red, green) negative control showing no hybridization. DNA is stained with DAPI (blue). (**d**) *C. intestinalis* individual 2 from Woods Hole December 2011 collection. CF319a-labeled Flavobacteria-Cytophaga bacteria (red). Unlabeled bacteria are indicated with white arrowheads. White arrow shows tunicate cell. (**e**) Same image as C showing DAPI-stained DNA (blue). White scale bars are 10 μm.

thus far, is an aerobic, marine bacterium. Members of the *Kordiimonas* genus have also been detected through culture-independent methods in the marine sponge *Axinella corrugata* (GenBank accession EF092170) as well as in the bacteria associated with the accessory nidamental gland of several cephalopod species (Pichon *et al.*, 2005; Collins *et al.*, 2012). These nidamental bacteria are thought to be vertically transmitted to the cephalod eggs or egg sac and to function in some protective capacity. Interestingly, cephalopod nidamental bacteria have also been shown to include flavobacterial groups (Collins *et al.*, 2012), and clone libraries from the squid *Loligo pealei* egg capsule contained several sequences that were closely related to the alphaproteobacterial OTU 8/isolate CWH003 (Barbieri *et al.*, 2001).

Despite mention of the presence of some intra-tunic bacteria in several *C. intestinalis* studies (De Leo *et al.*, 1981, 1997; Di Bella *et al.*, 2011), it was quite surprising to observe such a dense bacterial population within the *C. intestinalis* tunic. Bacteria were observed throughout the tunic in all specimens of the Woods Hole *C. intestinalis* population that we examined (multiple individuals across both summer and winter collections). Similarly dense intratunic bacteria are commonly observed in colonial tuni-cates, including the *Synoicum adareanum* and *Lissoclinum patella* (Riesenfeld *et al.*, 2008; Donia *et al.*, 2011). Although the *C. intestinalis* specimens used in this study appeared to be healthy adults and the bacteria were observed spread evenly through-out the tunic tissue, we cannot completely discount the possibility that the observed tunic bacteria represent a pathogenic infection. Alternatively, the tunic bacteria could function to protect the tunicate against fouling or pathogens through production of

natural products, chemical signals or competition. In future work, investigation of the tunic micro-biome across larger populations, geographic regions and tunicate life stages could begin to provide clues about the stability and ecological roles of these bacterial associations.

The tunic microbiome is of special interest given that *Ciona* is re-emerging model system for the study of innate immunity in a basal chordate. Invertebrate model systems have revealed a surprising specificity and complexity in these host–microbe associations, despite the lack of an adaptive immune system (reviewed in Nyholm and Graf, 2012). For example, study of the *Vibrio fisheri*-squid symbiosis demon-strated that recognition of microbial-associated molecular patterns can mediate symbiosis or immune response depending on the context (Koropatnick *et al.*, 2004). The *C. intestinalis* genome encodes only two Toll-like receptor homo-logs, which both activate the transcription factor nuclear factor-KB in direct response to multiple microbial-associated molecular patterns (Sasaki *et al.*, 2009). *C. intestinalis* has also been shown to express a complement gene, CiC3-1, in hemocytes (Pinto *et al.*, 2003), and opsonins termed variable region-containing chitin binding proteins are expressed in the gut epithelium and hemocytes (Dishaw *et al.*, 2011). *Ciona* mounts an inflamma-tory response when the tunic is injured through injection of seawater, *Escherichia coli* lipopolysac-charide, or sheep erythrocytes (Parrinello *et al.*, 1977, 2007). This response includes a visible encapsulation reaction at the injured site and activation of the prophenoloxidase system and melanization (De Leo *et al.*, 1997; Cammarata *et al.*, 2008). Several antimicrobial peptides are also expressed in tunic granulocytes in as part of the

tunic inflammatory response (Di Bella *et al.*, 2011). The results presented in this study, along with our ability to culture major phylotypes, provide new insights into the *Ciona* tunic bacterial community and lay the foundation to study the interactions between the observed tunic bacteria and their hosts.

Conclusions

The tunic-associated bacterial community of *C. intestinalis* is distinct from that of the surrounding seawater and contains an abundant alphaproteobacterial and flavobacterial phylotypes. A dense population of tunic bacteria living within the tunic interior was observed by CARD-FISH across multiple individuals and collections, and was comprised of mainly flavobacteria. Although *C. intestinalis* is a well-studied model organism, a minimal culturing effort yielded several novel bacterial isolates that will be interesting to investigate for their natural product potential. Unlike in many other systems, these culturable bacteria were also abundant members of the tunic-associated community as assayed by 16S rRNA gene-based methods. The availability of these dominant community members in culture will be a valuable resource for interrogating their role with respect to the tunicate host. The *C. intestinalis* tunic bacterial community represents an exciting model system in which to study the interplay between the host microbiome and innate immunity in development.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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