The *Ciona intestinalis* branchial sac and its microbiota

H. Rex Gaskins, Joel E. Thurmond, Gerardo M. Nava & Roderick I. Mackie
University of Illinois at Urbana-Champaign, Urbana, IL 61801

Ascidians, larvaceans, and thaliceans form a discrete group of advanced invertebrate species, the urochordates, in the phylum Chordata (Fig. 1). *Ciona intestinalis* is a member of the class Ascidacea, commonly known as tunicates because of their flexible, tough outer covering or "tunic". Although sessile adult tunicates bear little resemblance to typical chordates, their larvae exhibit the four fundamental characteristics of this phylum: i) a dorsal tubular nerve cord, ii) a notochord, iii) pharyngeal gill slits, and iv) a postnatal tail.

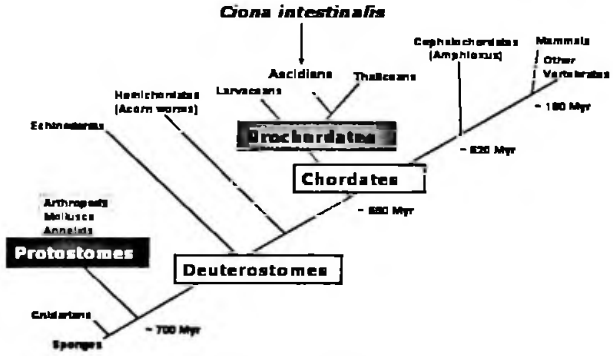

Fig. 1. Phylogeny of the urochoradate *C. intestinalis*, a member of the class Ascidiacea.

Their critical evolutionary position as basal chordates and the simplicity of their embryogenesis have attracted developmental and evolutionary biologists since the turn of the 20th century[14]. Because of this, the genomes of *C. intestinalis* as well as its close relative *C. savignyi* have been fully sequenced recently[2]. The availability of whole genome sequence enables the power of a comparative genomic approach to pursue our long-term goal of determining whether the support by *C. intestinalis* of a bacterial metagenome that contributes to detoxification has influenced, over evolutionary time, its complement of detoxification genes.

These animals are filter feeders and live attached to submerged substrates in the littoral zone of marine waters where they encounter high concentrations of complex polyphenols, halogenated aromatics, methylated sulfides, and some heavy metals. Little is known about the mechanisms by which tunicates detoxify or otherwise tolerate these natural toxins in the marine environment. We are addressing the hypothesis that filter-feeding tunicates rely on bacterial symbionts associated with the branchial sac for this purpose.

The branchial sac precedes the intestine and is an enlarged pharynx whose wall is perforated by numerous tiny gill slits (Fig. 2). It is both a respiratory organ and filter-feeding device. Water flows into the incurrent siphon at approximately twenty milliliters per minute and enters together with food particles the branchial sac. The water passes through the gill slits and then out the excurrent siphon while food remains in the gut and passes posteriorly to be digested. Waste products are secreted from the anus and out the excurrent siphon. The present report describes studies that used molecular ecological as well as conventional cultivation-based microbiological approaches to characterize the predominant microbes associated with the branchial sac of *C. intestinalis*.

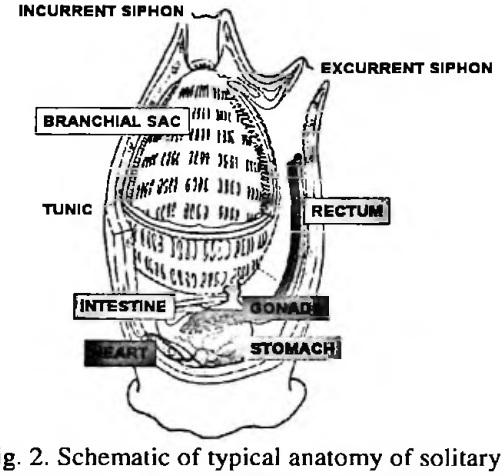

Fig. 2. Schematic of typical anatomy of solitary tunicates. The large branchial sac or pharynx that precedes the intestine is both a filter feeding and respiratory organ.

The branchial sac was dissected from replicate specimens of *C. intestinalis* that colonize sediment trays preceding the running seawater system at the University of Maine Darling Marine Center (http://server.dmc.maine.edu/) or from specimens collected from Cobscook Bay by Gulf of Maine

Marine Life Supply Company (http://www.gulfofme.com) staff and shipped to MDIBL. Animals were maintained in running sea water tanks at MDIBL prior to dissection. DNA was isolated from finely minced tissue using an UltraClean™ Soil DNA Kit (Mo Bio Laboratories; Carlsbad, CA; http://www.mobio.com). 16S-V3 rDNA PCR-DGGE and community structure analyses, and the cloning and sequencing of individual amplicons were performed as described previously[9,10]

Individual 16S-V3 rDNA amplicons were excised from DGGE gels and cloned and sequenced to phylogenetically identify common branchial sac bacteria of *C. intestinalis*. Amplicons found in a minimum of 3 animals were selected for sequencing. Bacteria from 5 major clades were identified (Table 1). The closest matches for the two branchial sac alphaproteobacteria (class in phylum Proteobacteria) sequences were each putative symbionts, one of sponge[5] and the other from a deep sea vent gutless annelid that depends on its endosymbionts for sulfide detoxification[3].

Table 1. 16S-V3 rDNA sequences cloned from the branchial sac microbiota of *C. intestinalis*

Closest Taxon[A]	Taxonomy (Class)	Identities[B]	Functional genes or notable phenotype[C]
Uncultured bacterium TK97	Unclassified Alphaproteobacteria; marine isolate from sponge	164/170 (96.5%)	P450 & GST genes
*Olavius losiae*endosymbiont 3	Unclassified Alphaproteobacteria; from gutless marine oligochete (Annelida)	161/169 (95%)	P450 & GST genes
Shewanella sp. HAW-EB5	Gammaproteobacteria; from marine sediment	189/194 (97%)	P450 & GST genes
Tenacibaculum maritimum	Flavobacteria	176/189 (93%)	P450 genes • pigmented
Reichenbachia agariperforans	Sphingobacteria	174/188 (92%)	P450 genes • pigmented
Psychrobacter glacincola strain ANT9276b	Gammaproteobacteria; from Artic sea ice	193/194 (99%)	P450 & GST genes
Verrucomicrobia sp.	Uncultured bacterium clone VERRUCO1from Salmonid gill	173/182 (95%)	*Verrucomicrobia* endo- (nematode) & ecto- (marine ciliate) symbionts described
Verrucomicrobia sp.	Uncultured *Verrucomicrobia* bacterium clone PI_4z12f;from Plum Island Sound estuary	186/196 (94%)	

[A]Taxon and corresponding accession number of the closest 16S-V3 rDNA sequence match to the cloned amplicons (bands) as determined via a BLAST search of the Entrez Nucleotides database (http://www.ncbi.nlm.nih.gov/ BLAST/).
[B]Percent similarity of sequenced 16S-V3 PCR-DGGE amplicons to sequence of closest taxon.
[C]Some of the 16S rDNA sequences belong to a restricted group of bacterial taxa that possess GST and P450 genes, which are poorly characterized in prokaryotes but are key biotransformation enzymes in eukaryotes. Others belong to bacterial taxa for which marine members possess carotenoid pigments.

In fact, numerous alphaproteobacterial endosymbionts have been described; classic examples being the nitrogen-fixing rhizobia of plant legumes[12] and the ultimate prokaryote endosymbiont, the mitochondrian of eukaryotic cells whose genome is thought to be an alphaproteobacterial descendent[7]. Two additional sequences were most closely related to uncultured *Verrucomicrobia* species. Two exceptionally intriguing cases of symbioses have been described for members of this division, one being an ectosymbiont of marine hypotrich ciliates (genus *Euplotidium*)[13] and another in which obligate intracellular *Verrucomicrobia* species are specifically associated with ovary wall and gut epithelia of *Xiphinema* nematodes[15]. One of the 16S-V3 rDNA sequences exhibited 97% similarity with *Shewanella* sp. HAW-EB5. *Shewanella* are gammaproteobacteria noted for their ability to enzymatically reduce and thereby precipitate metals such as uranium, technetium and chromium, leading to considerable interest in its potential for bioremediation of subsurface sediments and groundwater contaminated with heavy metals and radionuclides[11]. Finally, two of the bacterial 16S-V3 rDNA sequences associated with the *C. intestinalis* branchial sac belong to classes whose marine members are characterized, in part, by the carotenoid pigments they produce. Orange pigmentation is a

phenotypic characteristic of the *Ciona* branchial sac, and we've observed animal-to-animal variation in this trait, consistent with the possibility that the branchial sac pigmentation is bacterial in nature.

With this working hypothesis in mind, we isolated bacteria from branchial sac enrichment cultures with pigmentation as a primary selection criterion. This led to the isolation in pure culture of a pinkish red bacterium that was identified by cloning and sequencing of full length 16S rDNA as a *Methylobacterium* species (Fig 3). The genus *Methylobacterium* is comprised of a group of strictly aerobic, facultatively methylotrophic, gram-negative, rod-shaped bacteria noted, in part, for their production of pink to bright orange-red carotenoid pigments[4].

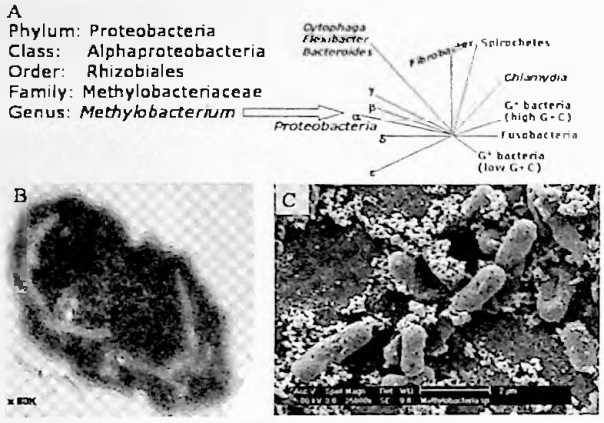

Fig. 3. Phylogeny (A), TEM (B), and SEM (C) photomicrographs of the *Methylobacterium* isolate from *C. intestinalis* branchial sac. The full length 16S rRNA gene shares 99% sequence identity with the uncultured Methylobacteriaceae clone 10-3Ba02, a molecular species derived from a soil library[18].

Methylotrophs are also unique in their ability to grow on one-carbon compounds more reduced than carbon dioxide as sole carbon and energy sources, including methanethiol and dimethylsulfide (DMS), which are important intermediates in the biogeochemical sulfur cycle[4,16]. Important sources of DMS in marine environments include phytoplankton, macroalgae, and coastal vascular plants, which use dimethyl sulphoniopropionate (DMSP), a precursor of DMS, as an osmolyte[17]. Interestingly, these organic sulfur compounds are typically present in high concentrations in shallow marine waters of the littoral zone where tunicates thrive[6].

Transmission electron microscopy was performed[1] to further examine possible niches occupied by the putative branchial sac symbionts.

The TEM photomicrographs revealed sheath-like structures in the stromal region underlying the branchial sac epithelium containing structures of a similar size and morphologically resembling bacteria (Fig. 4). These sheaths appeared to be adherent to the epithelial basement membrane but lacked host cell nuclei. The positive identification of the content of these sheath-like structures requires additional investigation. Efforts are now focused on determining: i) with domain and group specific fluorescence in situ hybridization (FISH) probes whether or not Bacteria and specifically Methylobacteria are found in the stromal region of the *C. intestinalis* branchial sac, and ii) if the *Methylobacterium* isolate can grow on DMS and particularly its precursor, the algal osmolyte DMSP.

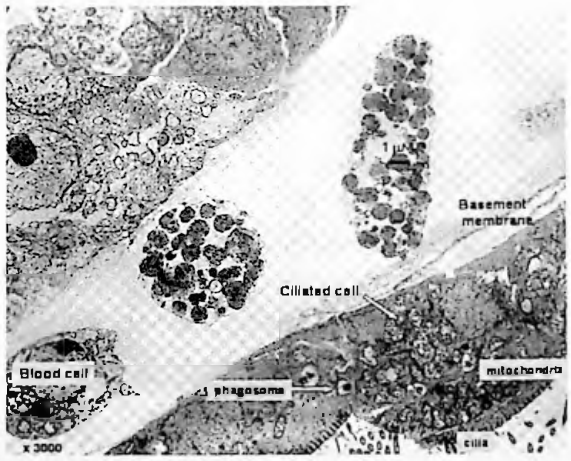

Fig. 4. TEM photomicrograph of *C. intestinalis* branchial sac reveling sheath-like structures possibly containing bacteria in the stromal region underlying the epithelium.

These data will further guide efforts to determine if bacterial detoxification of the methylated sulfides serves as a basis for stable association of Methylobacteria with the *Ciona* branchial sac and if and how this has impacted host genes involved in sulfur metabolism and detoxification.

Supported by a New Investigator Award (HRG) with funds from MDIBL's NIEHS Center for Membrane Toxicity Studies (ES03828-19). Michelle Diamond (University of Illinois) is acknowledged for her assistance with 16S rDNA cloning as are staff of the W. M. Keck Center for Comparative and Functional Genomics (University of Illinois) for sequence analysis. Thanks to Gary King, University of Maine Darling Marine Center, for providing *Ciona* specimens and for useful discussions regarding microbial biogeochemistry.

1. **Amann RI, Binder BJ, Olson RJ, Chrisholm SW, Devereux R, Stahl DA**. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 56: 1919-1925, 1990.
2. **Dehal P, Satou Y, Campbell RK**, et al. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. *Science* 298: 2157-2167, 2002.
3. **Dubilier N, Amann R, Erseus C, Muyzer G, Park SY, Giere,O and Cavanaugh CM**. Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochete Olavius loisae (Annelida). *Mar Ecol Prog Ser* 178: 271-280. 1999.
4. **Green PN**. Methylobacterium. In: *The Prokaryotes:an Evolving Electronic Resource for the Microbiological Community*, 3rd edn. release 3.5. edited by M. Dworkin, S. Fallow, E. Rosenberg, K-H. Schleifer and E. Stackebrandt. New York, USA: Springer-Verlag, 2001 [WWW document] URL http://link.springer-ny.com/link/service/books/10125/.
5. **Hentschel U, Hopke J, Horn M, Friedrich AB, Wagner M, Hacker J and Moore BS**. Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microbiol* 68: 4431-4440, 2002.
6. **Iverson RL, Nearhoof FL and Andreae MO**. Production of dimethylsulfonium proprionate and dimethylsulfide by phytoplankton in estuarine and coastal water. *Limnol Oceanogr* 34: 53067, 1989.
7. **Kurland CG and Andersson SG**. Origin and evolution of the mitochondrial proteome. *Microbiol Mol Biol Rev* 64: 786-820, 2000.
8. **Lueders T, Wagner B, Claus P and Friedrich MW**. Stable isotope probing of rRNA and DNA reveals a dynamic methylotroph community and trophic interactions with fungi and protozoa in oxic rice field soil. *Environ Microbiol* 6: 60-72, 2004.
9. **McCracken VJ, Simpson JM, Mackie RI, and Gaskins HR**. Molecular ecological analysis of dietary and antibiotic-induced alterations of the mouse intestinal microbiota. *J Nutr* 131: 1862-1870, 2001.
10. **Muyzer G, Brinkhoff T, Nubel U, Santegoeds C, Schafer H and Wawer C**. Denaturant gradient gel electrophoresis in microbial ecology. In: *Molecular Microbial Ecology Manual*, Vol. 3.4.4, edited by A. Akkermans, J.D. van Elsas and F. de Bruijn. Boston, MA: Kluwer Academic Publishers, 1998, pp. 1-27.
11. **Nealson KH, Belz A and McKee B**. Breathing metals as a way of life: geobiology in action. *Antonie Van Leeuwenhoek* 81: 215-222, 2002.
12. **Patriarca EJ, Rosarita Tate R, and Iaccarino M**. Key role of bacteral NH metabolism in Rhizobium-plant symbiosis. *Microbiol Mol Biol Rev* 66: 203-222), 2002.
13. **Petroni G, Spring S, Schleifer KH, Verni F, Rosati G**. Defensive extrusive ectosymbionts of *Euplotidium* (Ciliophora) that contain microtubule-like structures are bacteria related to *Verrucomicrobia*. *Proc Natl Acad Sci USA* 97: 1813–1817, 2000.
14. **Satoh N, Satou Y, Davidson B, and Levine M**. *Ciona intestinalis*: an emerging model for whole-genome analyses. *Trends Genet* 19: 376-381, 2003.
15. **Vandekerckhove TT, Coomans A, Cornelis K, Baert P and Gillis M**. Use of the Verrucomicrobia-specific probe EUB338-III and fluorescent in situ hybridization for detection of "Candidatus Xiphinematobacter" cells in nematode hosts. *Appl Environ Microbiol* 68: 3121-3125, 2002.
16. **Visscher PT, Kiene RP and Taylor BF**. Demethylation and cleavage of demethylsulfoniopropionate in marine intertidal sediments. *FEMS Microbiol Ecol* 14:179–190, 1994.
17. **Yoch DC**. Dimethylsulfoniopropionate: its sources, role in the marine food web, and biological degradation to dimethylsulfide. *Appl Environ Microbiol* 68: 5804-5815, 2002.