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Bacterial diversity of siliciclastic sediments in a *Thalassia testudinum* meadow and the implications for *Lucinisca nassula* chemosymbiosis

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ABSTRACT

Despite the ecological and economic importance of *Thalassia testudinum* (turtle grass) meadows along the Caribbean and Gulf of Mexico coasts, and recognition that microbial activities are critical to plant growth and health, the bacterial diversity of these habitats has been poorly studied. Based on comparative analyses of 16S rRNA gene sequences from sediments in a *T. testudinum* meadow, 25 major taxonomic groups (excluding candidate divisions) were retrieved, including *Alpha-* *Delta-*, and *Gammaproteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Acidobacteria*, *Spirochaetes*, and *Firmicutes*. The distribution of bacterial groups was linked to a strongly hypoxic and sulfidic redox gradient. The diversity is potentially novel because phylogenetic affinities of sediment sequences compared to contextually annotated environmental clones from different habitats or to cultured representatives indicated approximately 41% were more closely related to each other than to sequences retrieved from these other habitats. Of all the relationships, very few (2.4%) were to cultured organisms, but 27% were to environmental clones retrieved from shallow marine shelf and coastal sediments or from mangroves, estuarine, or wetland sediments. Rare sequences were closely related to endosymbiont groups of *Lucinisca nassula* (Lucinidea: Bivalvia) hosts collected from the same meadow, which may indicate that the sediment is a potential reservoir for free-living symbionts. This study provides insight into the ecological and evolutionary relationships of the *Thalassia*–lucinid–bacteria system in tropical to sub-tropical regions.

1. Introduction

Thalassia spp. (turtle grass) along the Caribbean and Gulf of Mexico coasts are considered some of the most ecologically and economically important seagrasses due to abundant biomass and high rates of primary productivity (den Hartog, 1970; Dawes et al., 1985; Byron and Heck, 2006; Larkum et al., 2006). In addition to being integral to many different biogeochemical cycles in marine sediments, *Thalassia*, like other seagrasses, also influence coastal sedimentation because aboveground biomass baffles and detains sediment particles and the rhizome and root systems stabilize sediments and prevent particle resuspension (e.g., Gacia and Duarte, 2001; Orth et al., 2006). The diversity of epi- and in-fauna associated with *Thalassia* meadows is generally higher than from unvegetated habitats, with animals including crustaceans, echinoderms, molluscs, and numerous others (e.g., Heck and Wetstone, 1977; Hemminga and Duarte, 2000; Peterson and Heck, 2001). The Lucinidae, which comprise several distinct clades, including the *Myrtea* and *Anodontia* clades and the *Fimbria* and *Phacoides* lineages (Williams et al., 2004), are among some of the most prevalent infaunal taxa associated with shallow suboxic, calcareous and siliciclastic sandy sediments in *Thalassia* meadows (Jackson, 1972, 1973; Barnes and Hickman, 1999; Taylor and Glover, 2006).

Modern lucinids are found in diverse marine habitats in addition to seagrass meadows, including fjords, mangrove swamps, cold seeps, and hydrothermal vents (Wiley and Felbeck, 1995; Gros et al., 1996; Imhoff et al., 2003; Duperron et al., 2005; Taylor and Glover, 2006), as long as there are strong redox gradients due to the presence of hydrogen sulfide, which supports chemosymbiotic associations with sulfur-oxidizing (thiotrophic), endosymbiotic bacteria. In general, seagrass beds are particularly suitable habitats for lucinids (e.g., Barnes and Hickman, 1999; Peterson and Heck, 2001; Hebert et al., 2007), likely because the dense rhizome network offers refuge from predation and can help to establish and maintain the redox gradient in the soft, usually homogeneous sediments. The burrowing bivalves also impact sediment biogeochemistry by affecting pore water composition through sulfide

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oxidation, oxygen introduction, and nutrient suspension (e.g., Peterson and Heck, 2001; Reynolds et al., 2007). But, even with evidence from the fossil record that this plant–lucinid association also persisted in the geologic past (e.g., Jackson, 1972, 1973), a complete understanding of the interactions within the *Thalassia*–lucinid–bacteria system is lacking, not only because the bacterial diversity of *Thalassia* sediments has not been fully documented, but because knowledge about processes affecting lucinid chemosymbiosis in tropical to sub-tropical seagrass meadows remains poorly studied. As such, it has been difficult to know how the microbial diversity and ecology of any lucinid habitat, in particular seagrass meadows, might affect lucinid life-cycles, symbiont distribution and diversity, and biogeochemical cycling within the habitat.

Here, we describe the bacterial diversity of siliciclastic sediments in a *Thalassia testudinum* meadow from Cedar Key, on the Gulf of Mexico side of Florida (USA). We compare the 16S rRNA gene taxonomic diversity to previously retrieved environmental sequences that were differentiated into one terrestrial and seven marine habitat types. Because seagrass meadows are susceptible to changing environmental conditions (e.g., Duffy and Emmett, 2006; Orth et al., 2006), processes affecting the distribution of microbes within the *Thalassia*–lucinid–bacteria system are considered. The results also provide insight into the potential distribution of free-living forms of lucinid symbionts. Some sediment sequences were closely related to endosymbionts from multiple *Lucinisca nassula* (formerly *Lucina nassula*; Taylor and Glover, 2000) hosts from the same *Thalassia* meadow. Although free-living symbionts are known for non-bivalve host taxa (e.g., Harmer et al., 2008), the lucinid–bacteria association is somewhat unique among marine bivalves. To date, free-living bacterial symbionts are not well documented for non-lucinid marine bivalves (Imhoff et al., 2003; Dubilier *et al.*, 2008), although recent molecular evidence suggests both thiotrophic and methanotrophic endosymbionts for *Bathymodiolus* spp. mussels are present in their habitats (Won et al., 2003; Crépeau et al., 2011). In contrast, free-living cells phylogenetically affiliated with lucinid symbionts have been identified from marine sediments (e.g., Weidner et al., 2000; Ravenschlag et al., 2001), and specifically from *T. testudinum* seagrass bed sediments (e.g., Gros et al., 1996, 2003a). Furthermore, aposymbiotic (symbiont-free) lucinid juveniles have been successfully infected with symbionts purified from different lucinid hosts (Gros et al., 2003a), and starved aposymbiotic adults reintroduced into the environment took up new symbionts (Gros et al., 2012). Consequently, studying the bacterial diversity of various lucinid habitats may potentially reveal more information about the ecological and evolutionary relationships between lucinids and their environments through time.

2. Materials and methods

2.1. Sample collection

Sediment and water samples were collected off of the unpopulated Dog Island at Cedar Key from Levy County, Florida (29° 7.457′ N; 83° 18.483′ W) in June 2005, before the leaf biomass seasonal maximum (Iverson and Bittaker, 1986). The shallow (0.5–2 m depth) water supports dense *T. testudinum* meadows and is reported to host a variety of lucinid bivalves (http://www.jaxshells.org/cedarkey.htm). Taxonomic names used herein are consistent with Taylor and Glover (2000). A 1 m by 1 m sampling grid was established in a *T. testudinum* meadow to retrieve 30 cm-long by 4 cm-diameter cores and collect lucinid individuals. Two cores were taken 1 m apart, at the NW and SE corners of the grid. Cores were placed on ice for transport and then stored at −20 °C. Within the sampling grid, only *L. nassula* were encountered, and ten individuals (size range: 0.9–1.9 cm) were collected manually by sand-sieving. Almost all (8 of 10) of the lucinids were situated below the sediment surface to ~20 cm depth; the remaining two were in the top 10 cm. Lucinids were forced open, fixed immediately in 95% ethanol, transported on ice, and stored at 4 °C until dissection.

2.2. Sediment geochemistry and mineralogy

Sediment pore fluid geochemistry and sediment mineralogy were evaluated to provide context for possible changes in microbial community composition with depth. Pore water was sampled from seagrass sediments in the sampling grid, from a depth of between 20 and 30 cm, by low-flow pumping through a mini-piezometer. Physiochemical parameters were determined on site immediately from the pumped fluids using standard electrode methods (APHA, 1998), including temperature and pH with a double junction electrode, total dissolved solids (TDS), and oxidation–reduction potential. Dissolved sulfide and trace-level dissolved oxygen (DO) were measured using CHEMetrics (Calverton, VA) colorimetric methods on a portable V-2000 multi-analyte photometer (APHA, 1998). Marine water was collected from within 10 cm of the top of the seagrass meadows. All water was pumped through Whatman glass microfiber filters and 0.2 μm PVDF filters, and filtered water was collected in separate HDPE bottles for anion and acid-preserved cation measurements. Concentrations of major ions were determined by ion chromatography (Dionex, USA) and inductively coupled plasma mass spectrometry.

Sediment water and organic carbon content were estimated from 3 to 5 g of sediment subjected to the loss-on-ignition (gravimetric, dry oxidation) method, whereby hydrated sediments were dried overnight at 65° and weighed for water content. The dehydrated material was combusted at 550 °C for 2 h to obtain the organic carbon content, following a measurement after heating to 360 °C for 2 h and homogenization to ensure even combustion. The 360 °C step provided clay and oxide structural water content. All samples were cooled in a desiccator to ensure no atmospheric water was absorbed prior to weighing. Core sediments were analyzed for textural properties, basic grain size, and mineralogy. Mineralogy was determined from 2 g of material at each core depth, following homogenization, separation by centrifugation, drying, and grinding to a fine powder. Powder mounts were analyzed with a BRÜKER Siemens D-5000 XRD using the operating setting of 40 kV and 30 milliamps. X-ray diffractograms of 27 common minerals were compared using JADE pattern processing software version 6.1 (MDI, USA). Minerals were identified from diffraction peak position and visual confirmation.

2.3. Sediment nucleic acid extraction

Each of the 4-cm diameter cores was aseptically divided into ~10 cm depth intervals while frozen. After thawing, triplicate aliquots of ~3 g wet sediment were taken from the center of the cores at each depth for total environmental DNA extraction. For each, sediments were suspended in the lysis buffer provided by the DNeasy extraction kit (Qiagen Inc., USA) and shaken for 30 min to 2 h with a Burrell wrist action shaker to disaggregate the material. After shaking, 20 μl of proteinase K were added and the samples continued to shake at room temperature for up to 2 h. Large particles and root pieces were allowed to settle for 10 min, then ~3 ml of cloudy supernatant was taken and nucleic acids were extracted, following manufacturer instructions with additional modifications: 5 ml additional fresh extraction buffer and 40 μl additional proteinase K were added to the supernatant; suspension rotational shaking was done at 225 rpm overnight at 55 °C; an additional separation of 750 μl supernatant was done prior to