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SHORT COMMUNICATION

Bacterial Diversity and Abundance in Shell Biofilms from the Freshwater Snail *Pleurocera canaliculatum* (Cerithioidea: *Pleuroceridae*)

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Abstract

Mollusk shells provide a hard substrate for aquatic biofilm colonization. While most work has focused on bivalve shells and grazing, little work has focused on gastropod shells and the microbes growing on them. We sampled biofilms from 14 *Pleuroceracanaliculatum* and analyzed them using a metagenomic approach. Microbial diversity varied between individuals, and rarefaction suggested that 63 snails would need to be sampled to capture all of the estimated genus-level diversity. *Cyanobacteria* and species of *Novosphingobium* and *Methylosoma* were the most abundant taxa across all shells.

Key words: bacterial diversity on snail shell, biofilms on gastropod shells, metagenomics approach

Mollusk shells can make up a significant portion of the available hard substrate in aquatic systems (Gutiérrez *et al*., 2003). Shells can harbor a diversity of prokaryotic and eukaryotic organisms enclosed in an extra-cellular matrix growing in multicellular biofilms (Lopez *et al*., 2010). Biofilms often promote both species abundance and richness locally, and impose benefits and costs to shells carrying epibionts. In terms of biofilms, research on snail interactions with freshwater biofilms generally focuses on grazing (*e.g.* Sheldon and Walker, 1997; Lopez-Doval *et al*., 2010; Hladyz *et al*., 2011; Lundqvist *et al*., 2012), including snails grazing on other snails' shells (Abbott and Bergey, 2007). Work on biofilms growing on mollusk shells has focused on bivalves (Gillanand De Ridder, 1997; Gillan *et al*., 1998; Ivanov *et al*., 2006; Bischoff and Wetmore, 2009). For snails, Abbott and Bergey (2007) reported that freshwater snail shells are generally free of algal coverings and may harbor diatoms, but no mention of prokaryotic biofilm diversity on snail shells was given in this or any other reference.

Given the importance of both snails (Johnson, 2009) and biofilms (Besemer *et al*., 2012) in the ecology of freshwater systems, we aimed to examine the diversity of bacteria in biofilms growing on snail shells. We sampled snails and biofilms from Bayou Bartholomew, a slow-moving eutrophic waterway in Arkansas and Louisiana. The bayou has a long history of habitat degradation, from bank erosion due to agriculture, to heavy metal and other contaminant inputs from industry (Layher, 2005). We focused on the siltyhorn snail, *Pleurocera canaliculatum* (Pleuroceridae), an oviparous algal grazer that can be found on soft and hard substrates (Dillon, 2000) and that is common in Bayou Bartholomew (Minton *et al*., 2008).

Fourteen *P. canaliculatum* (Fig. 1) were collected by hand from Bayou Bartholomew in Bastrop, Louisiana (32.8017°N, 91.9495°W) in September 2013. Shells were covered in dark oxides along with visible biofilms, and snails were crawling on either the bayou bottom or submerged hard surfaces (*e.g.* bridge pilings, woody debris). Nitrile gloves were worn to avoid human contamination. Biofilms were scraped from individual shells using sterile razor blades and placed directly into bead-beating tubes. For comparison, we collected two sediment samples from the site. DNA was extracted from biofilms using the Power Soil DNA isolation kit (Mo Bio Laboratories), according to manufacturer's

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Fig. 1. Photograph of *Pleurocera canaliculatum* shell

directions. For metagenomic analysis, DNA samples were sent to MrDNALab (Shallowater, Texas). The *16S rDNA* gene variable region V4 was amplified from the bacterial DNA samples using the 515/806 primer pair (Caporaso *et al*., 2011). A single-step, 30 cycle PCR using HotStarTaq Master Mix kit (Qiagen, USA) was run (94°C for three minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for one minute, followed by a final elongation step at 72°C for five minutes). Sequencing was performed on an Ion Torrent PGM following the manufacturer's guidelines. Sequence data were processed using MrDNALab's proprietary analysis pipeline for depletion of barcodes and primers, followed by removal of sequences less than 150 bp, sequences with ambiguous base calls, and homopolymer runs exceeding 6 bp. Sequences were de-noised, chimeras were removed, and operational taxonomic units (OTUs) defined by clustering at 97% similarity. Final OTUs were taxonomically classified using nBLAST against a curated GreenGenes database (DeSantis *et al*., 2006).

Using the species-level counts, we performed sample-based rarefaction with extrapolation in EstimateS 9 (Colwell, 2013) to determine if our sample size was large enough to capture the bacterial diversity on the shells. We used EstimateS to calculate Chao's abundance-based Jaccard indices between snails and sediment samples to determine how similar the bacterial communities were between shells and the environment. Chao-Jaccard indices are based on the probability that two randomly chosen individuals, one from each of two samples both belong to species shared by both samples. This approach reduces the negative bias that undermines the usefulness of traditional similarity indices, especially when incompletely sampling rich communities (Chao *et al*., 2005). Finally, we used principal coordinates analysis of genus-level counts in STAMP (Parks *et al*., 2014) to visualize differences in diversity between snails and the sediment.

Metagenomic analysis yielded an average of over 68,500 sequences per snail shell that could be matched to 1,258 bacterial genera in 64 phyla. Proteobacteria and Cyanobacteria were the two most abundant phyla on *P. canaliculatum* shells. The most abundant genus on shells was *Novosphingobium*, followed by *Leptolyngbya* and *Methylosoma*. Sample-based rarefaction indicated that 14 shells sufficiently captured phylum-level bacterial diversity but were insufficient to capture the genus-level diversity on them. Rarefaction extrapolation suggested that sampling 63 shells would be needed to accurately describe bacterial communities at the genus level, estimated to be 2,014 genera. Chao-Jaccard indices for genus-level pairwise comparisons of shells to shells and sediment to sediment ranged from 0.873 to 0.987, where a value of 1.0 indicated identical samples. Chao-Jaccard indices comparing shells to sediment were much lower, ranging from 0.656 to 0.707. Principal coordinates analysis suggested that the shell and sediment bacterial communities possessed a high degree of overlap (Fig. 2).

Our data suggest that individual snails have diverse bacterial communities growing on their shells, and that these communities differ between snails. The most abundant bacteria found on *P. canaliculatum* shells reflected the biology of the bayou and the life history of the snail. Species in the genus *Novosphingobium* are gram-negative bacteria that break down aromatic compounds including phenol, nitrobenzene, and carbofuran. They are frequently isolated from aquatic environments exposed to high anthropogenic activities (Gan *et al*., 2013). The bayou watershed regularly receives agricultural, residential, and industrial inputs that likely include the aromatics used by *Novosphingobium* (Kresseand Fazio, 2002). *Leptolyngbya* species are cyanobacteria commonly found in soils and in periphyton and metaphyton of freshwater and marine systems. *Leptolyngbya* also live epiphytically in the extra-cellular matrix produced by other organisms, explaining their presence in the biofilm (Komárek and Hauer, 2013). Cyanobacteria are also common in eutrophic systems like the bayou, and the shells of *P. canaliculatum* remain exposed in the water column. Other cyanobacteria occurring in high abundance were *Chamaesiphon*, *Phormidium*, and *Pleurocapsa*. Finally, species of *Methylosoma* are aerobic methanotrophs, active in sediments where methane and oxygen meet (Rahalkar*et al*., 2007). *Pleurocera* spend time crawling on the benthos, likely bringing them in proximity to *Methylosoma*.

McClean (1983) showed the importance of snail shells in shaping benthic community structure. Our

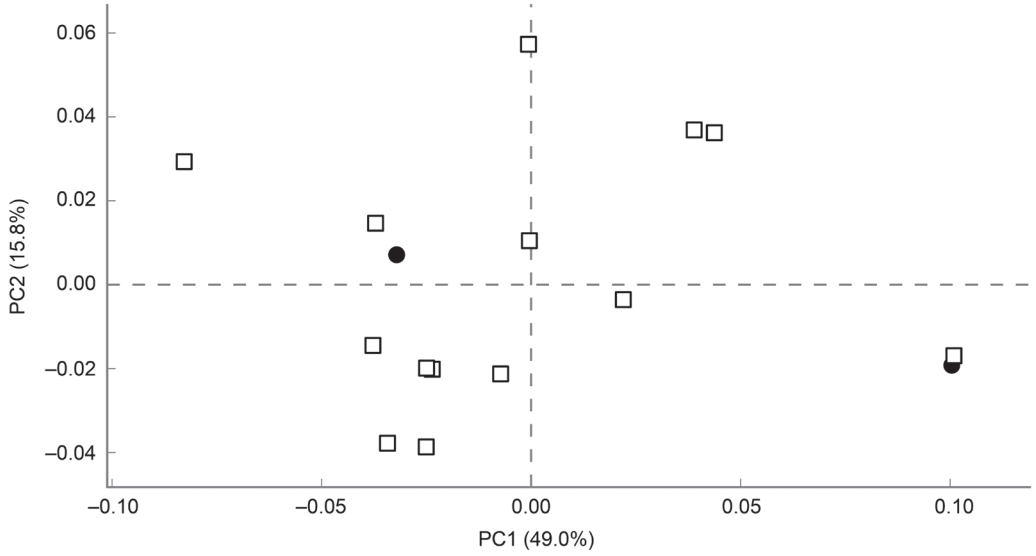

Fig. 2. Principal coordinates analysis of genus-level diversity counts showing variation among bacterial communities on snail shells (open squares) and in the sediment (filled circles). Data for the first two coordinates and the percent variation explained by each are shown

findings represent the first study of snail shell microbes and we suggest that snail shells play a role influencing microbial diversity and abundance in freshwater systems. Aquatic biofilms show seasonal variation (Olapade and Leff, 2005) and succession (Tien and Chen, 2013). We hope our data can serve as a baseline data for other shell biofilm studies and represent a starting point for future research on how shell biofilms change temporally.

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