Gut community diversity of *Geukensia demissa* in response to tidal fluctuations

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Abstract

The ribbed mussel, *Geukensia demissa*, is adapted to survive daily fluctuations in environmental conditions, filtering food and oxygen while submerged in water for only 25% of each day. It is exposed to air during the remainder of the time. How does this drastic shift in environmental conditions affect its ability to utilize food? This study used culture dependent and independent methods to characterize the membership and structure of the gut community of *G demissa* at low and high tide, to determine how diversity changes in response to tidal change. While the sample size was found to be suboptimal, the clone library pointed to *Mycoplasma* sp as a dominant member of the gut community, comprising more than 50% of the sequences. T-RFLP profiles suggested a difference in community structure between the two tidal conditions. Further evidence from phenotypic assays suggested metabolic shifts as well, correlated to tidal change, as the high tide samples were able to use six times more substrate types. Further work is needed to identify key players in the gut community of *G demissa*, and specific roles and interactions that enable the mussel to survive in a fluctuating environment.

Introduction

The ribbed mussel, *Geukensia demissa*, is commonly found in the mid to high littoral zones of estuaries along the eastern United States and the Gulf of Mexico where it lives partially submerged in the peat sediment among marsh grasses such as *Spartina*. It feeds by filtering water of particles as small as 0.2 µm, with a clearance rate of 6.15L/hr.g (Wright, 1982, Riisgard, 1988), consuming phytoplankton, detrital cellulose, diatoms, and bacteria when seasonally available (Kreeger, 2001).

It has been shown that populations of *G. demissa* that spend a greater amount of time emersed between tidal cycles have a greater capacity to digest refractory lignocellulosic carbon sources (Charles, 1997), and observations of an aerial breathing behavior known as 'air-gapping' suggest that these mussels actively modulate aerobic metabolism (McMahon, 1988). What is unclear is the effect of tidal cycles on the membership and structure of the mussel's gut community, whether and how the community shifts between tides, and how its members help the ribbed mussel to make a living as a filter feeder.

While estuarian mussels are generally not commercially valuable, their ecological role as water filters have lead to their use as bioindicators to assess the effects of marine pollution (Culbertson, 2008) and measure the overall health of the ecosystem (Ford, 2005). Understanding the dynamics of the gut community of *G. demissa* and how it

responds to natural perturbations would provide valuable insight into the digestive adaptability of the mussel, and strengthen the predictive capacity of ecological models based on this system.

This study seeks to assess the diversity of the gut community of the ribbed mussel, *G. demissa*, to determine the effect of tidal changes on structure and membership, and to

Methods and Materials

Sample collection:
Ribbed mussels (5-7 cm length) were collected from the *Spartina* shelf at Little Sippiwisset Marsh, Wood's Hole, Ma within an hour of full high or low tide on each day of dissection and extraction (between July 8 and July 24). Stomach and surrounding liver tissue were removed and placed immediately into 1 ml of sterile seawater. Stomach contents were extracted for each analysis by vortexing and pulverizing, and the extracts were kept on ice until used. Blackened mud surrounding the mussels was collected for enrichment using a spatula. Mud extracts were prepared by vortexing 1 g mud in 1 ml of sterile seawater.

Enrichments:
Minimal media was prepared for plates using 7.2 mM of each carbon source (xylan, cellobiose, pectin, or none) separately in 10 ml each of 100X (10%) Seawater base, NH_4Cl, and K_3PO_4, 1mM Na_2SO_4, 1 ml trace minerals, and 15 g of agar per liter. The pH was adjusted to 8.0. Following autoclaving (45 min) and cooling, .1 ml each of vitamin mix and B_{12} was added. Cellulose plates were prepared as above by overlaying a circle of sterile filter paper onto a streaked plate lacking added carbon source. Plates were streaked with three serial dilutions of fresh stomach extract prepared as described above and incubated at 30^{o}C under aerobic conditions, and at room temperature in CO_2/N_2 with added H_2.

Liquid media for additional anaerobic cultures was prepared using the above minimal media recipe, substituting 7.5nM sucrose as a carbon source, and adding a sterile circle of filter paper as a source of cellulose. Diluted mud and stomach extracts were also streaked on LB, SWLYT, and R2A plates and incubated at 30^{o}C under aerobic conditions.

Secondary enrichments of aerobic SWLTY plates provided colonies for PCR. Colonies were collected, suspended and boiled in 20µl of 0.05% NP40 prior to amplification and analysis (as described below).

DNA extraction:
Concentrated stomach extract was prepared by centrifuging 1.5 ml of stomach extract obtained as described above for 10 minutes at 13 rpm, removing and discarding the top 800 µl, then resuspending the pellet in the remaining liquid by vortexing. DNA was extracted from 600-700 µl of the resulting concentrate using MoBio UltraClean Soil DNA Extraction kit (MoBio, Carlsbad, Ca. USA) as directed, using all of the liquid recovered from beadbeating in multiple spin filter steps to maximize DNA recovery.

16S rDNA PCR

The 16S rDNA gene was amplified for a clone library of a sample taken at low tide and isolated colonies by PCR using universal 8f/1492r primers in a 25 µl reaction (12.5µl Promega master mix 2X (Promega, Madison, WI, USA), 2.0 µl (15pmol) 16S_8F, 2.0 µl (15pmol) 16S_1492R, 6.5 µl nuclease-free water, 2.0 µl template). Thermal cycles for each PCR reaction included 5 min of denaturing at 95°C, followed by 30 cycles at 95 °C for 30 s, 46 °C for 30 s, and 72 °C for 90 s, with a final 5 min extension at 72 °C. PCR products were evaluated by ethidium bromide stained gels.

16S Clone libraries

PCR products amplified as described above were cloned into chemically competent cells using the pCR4-TOPO vector as directed in the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Transformed colonies were grown overnight on LB/KAN (50mg/ml) plates (150 µl and 75µl), picked, added to 1.5 ml superbroth liquid media, and incubated overnight. Reactions were cleaned using isopropanol precipitation (100%), and submitted for sequencing.

16S Analysis

Sequence data files were imported into FinchTV (FinchTV 1.4.0 (Geospiza, Inc. Seattle, WA, USA) for initial trimming and conversion to FASTA format. Clone library sequences were aligned and distance matrices were created using MEGA (Tamura, 2007), and imported into DOTUR for OTU assignment, calculation of diversity indices (Ace, Chao1, Simpson, and Shannon), and construction of rarefaction curves to assess sampling efficacy. Neighbor-joining phylogenetic trees of clone library and colony sequences were constructed using TreeBuilder in RDP, and relative abundances of each group were visualized using VAMPS (Visualization and Analysis of Microbial Population Structures http://vamps.mbl.edu).

T-RFLP

Triplicate PCR reactions were performed using DNA extracted as described from stomach extracts from 4 mussels each collected at high and low tides using 8f-FAM/ 1492r primers. Amplification was verified by ethidium bromide stained gels. PCR cleanup was done using QuickStep2 PCR Purification Kit (Edge Biosystems, Gaithersburg, MD, USA), and products were quantified by nanodrop spectroscopy (Thermo Scientific, Wilmington, DE, USA). Enzymes for restriction digest (Hha (GCG^C and SAU961 G^GNCC) were chosen based on comparison of fragments anticipated using NEB Base cutter (http://tools.neb.com/NEBcutter2/index.php) and a restriction enzyme software program shared by Cadillo-Quiroz (unpublished). Restriction digest reactions were set up to use 300 ng of DNA, and the recommended ratios of enzyme, buffer, and water for volumes of 40 µl, and incubated at 37 °C for 3 hours, followed by denaturing at 80 °C for 20 min, and storage at 4 °C. Restriction digest products were cleaned by isopropanol precipitation, then resuspended in HiDi formamide and LIS500 marker. Plate was heated to 60 °C for 5 minutes prior to sequencing.

T-RFLP Analysis

Sequence files were imported into GeneMarker (http://www.softgenetics.com) to obtain electropherograms, and GeneMapper (Applied Biosystems) to prepare data for export into T-Rex (http://trex.biohpc.org) for statistical analysis. Following noise filtering and aligning, frequency of each T-RF, average T-RF richness and sample heterogeneity was calculated from the distance matrices for low and high tide samples as well as for the aggregate for each enzyme.

Phenotypic Assays

GEN III phenotypic assay plates (Biolog, Hayward, CA, USA) were inoculated with 100 µl aliquots from 1 ml of 10^{-4} dilutions of stomach extract from mussels collected at high and low tides added to entire tube of IF-A. This dilution had an average OD of .20, much lower than the recommended OD of .90-.98. Plates were incubated at 30 ^{o}C, and measured with the plate reader every 6-8 hours. Final readings were taken at 39 hours and used to describe results.

Results and Discussion

Enrichments:

Two colonies from the stomach extract and one colony from the mud were isolated from SWLYT media. These isolates are commonly found in marine sediment samples.

MDPlate8G12: *Acinetobacter junii*, strain ACI289 (99% Max Id): Gut
Proteobacteria: Gammaproteobacteria: Pseudomonadales: Moraxellaceae: *Acinetobacter*

MDPlate8H09: *Pseudoalteromonas* sp. (100% Max Id): Mud
Proteobacteria: Gammaproteobacteria: Alteromonadales: Alteromonadaceae: *Pseudoalteromonas*

MDPlate8G09: Uncultured Firmicutes (88% Max Id): Gut
Firmicutes? Clustering with Gammaproteobacteria?

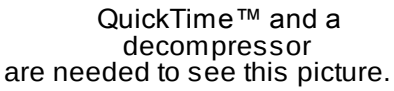


Figure 1: Phylogenetic relationships and identification of three colony isolates obtained from mussel gut extract and mud samples.

There was very little growth in the two-week incubation time on any of the minimal media plates grown aerobically or anaerobically, however, the anaerobic enrichments yielded turbidity in as few as five days. Microscopic observation revealed several morphologies, including long and short rods, tear dropped cells, and cocci. Further enrichment would be necessary to identify these organisms, but this preliminary data

suggests that the communities of microbes found within the stomach of *G. demissa* are morphologically and metabolically diverse.

16S Clone library

A total of 92 sequences were obtained in the clone library from a sample obtained at low tide with a total of 43 unique OTUs, and 17 OTUs at a distance of 0.03 (determined by DOTUR). The rarefaction curves (Figure 2), predict that it is likely that this library does not represent the diversity of the sample, and points to the need for a larger library containing more sequences from more samples.

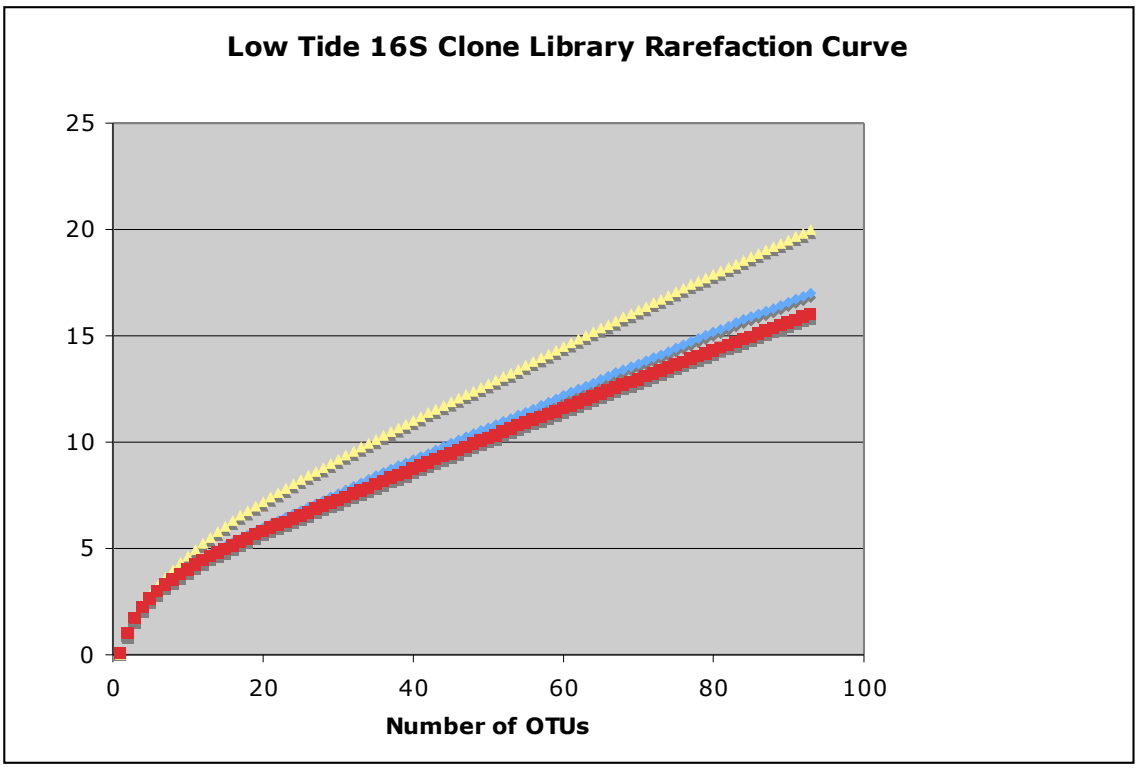
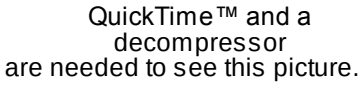


Figure 2: Number of shared OTUs at distances of .01 (yellow), .03 (blue), and .06 (red). Curves appear to be leveling beyond the number of sequences sampled, indicating that more samples would be required to reach optimal sample size.

Analysis of the membership of the clone library and phylogenetic tree of unique OTUs revealed the predominance of *Mycoplasma sp*, (Figure 3) Greater than half of the sequences matched this group with greater than 80% maximum identity. *Mycoplasma* sp have been suggested (Azevedo, 1993) to cause disease in the gills of bivalves such as cockles, but have been also shown (Boyle, 1987) to associate with invertebrates in nondisease causing ways. Loggans (2007) identified as many as 38% of clones as *Mycoplasma* sp in libraries from samples extracted from *G. demissa* intestine over a period of 10 months, suggesting that these could be resident gut species, but their role within the gut has not been established.

A:



B:

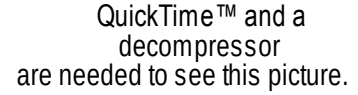


Figure 3: A. VAMPS analysis showing relative abundance of groups classified from the low tide mussel gut 16S clone library. B..Neighbor-joining tree showing clustering of unique OTUs in four distinct groups, predominated by *Mycoplasma* and related sp.

T-RFLP Analysis

A difference in community structure of the gut at high and low tide can be seen in Figure 4, comparing the frequency of TRF by base pair between low, high, and the

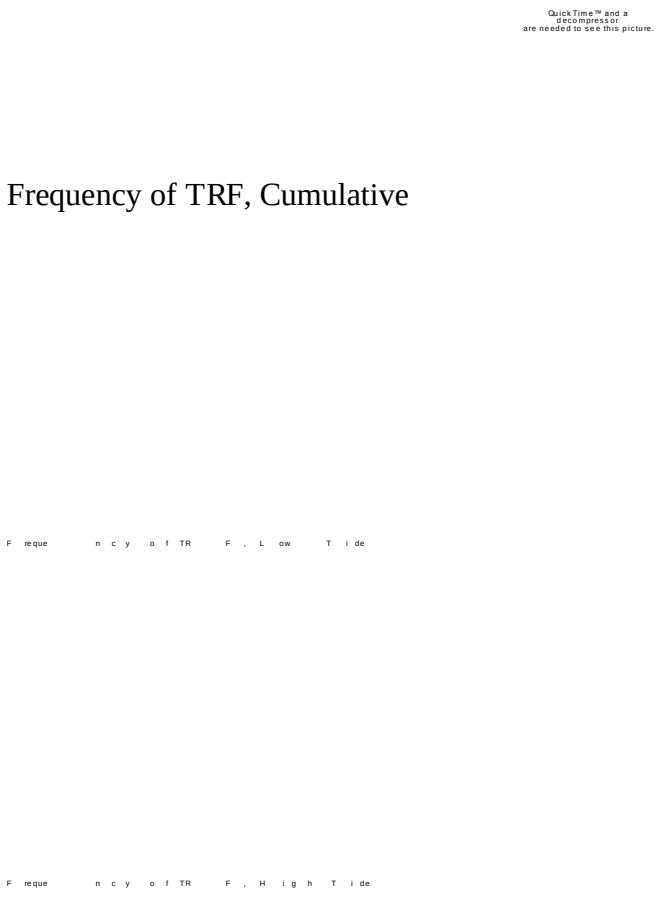


Figure 4: Comparison of the frequency of each peak after noise filtering for the entire community (top), Low Tide (middle) and High Tide (bottom).

cumulative sample. Low tide profiles showed fewer peaks in general, but differences in greater average richness and sample heterogeneity were dependent on the enzyme used (Figure 5), indicating a need for deeper sampling and utilizing more enzymes. It was not possible with the sequences from the clone library to match anticipated peaks *(in silico* digestion) with those that were present in the profiles, but this would help to further differentiate the populations that could be fluctuating. Based on these results, it seems clear that the communities sampled at each tide are more similar to each other than to the other tide, suggesting a community shift as the tides change.

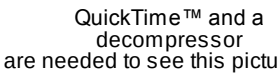
	Ave T-RF richness	Sample heterogeneity
Hha, By sample	80.38	2.52
LT only	89.25	1.53
HT only	75.25	1.72
Sau, By sample	91.88	2.08
LT only	91.25	1.44
HT only	92.5	1.22

Figure 5: Average richness and heterogeneity (beta-diversity) of low and high tide samples

Phenotypic Assays

The results of the phenotypic assays suggested a much greater metabolic diversity than might be expected from the T-RFLP profiles. There is a dramatic shift in the range of carbon sources utilized resulting in 30 unique positive results at high tide (Figure 6).

A



= Unique to Low Tide

= Unique to High Tide

= Common to Both

B

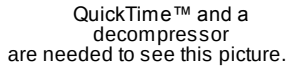


Figure 6: Results of Biolog phenotypic assay using GENIII plates. A: Shows a comparison of positive results between high tide and low tide

Explanations regarding why specific substrates might be utilized, and which organisms might be responsible are beyond the scope of this study, however it seems reasonable that the influx of particles during feeding at high tide would trigger an increase in digestive activity within the gut community, which is suppressed during low tide when fresh food is not available, and accumulated nutrients are being used up. Whether the metabolic shift represents changes in community structure of transients and residents or adaptive responses is unclear, but warrants further study.

Conclusion:

While the small sample size used in this study limited its ability to quantify exact differences between high and low tide gut communities, or to correlate changes in T-RFLP peaks and phenotypic assay results with specific organisms, the data does point to differences between the gut communities at low and high tide. The high tide communities showed greater diversity in population and phenotypic activity. This would

be reasonable to expect given the active feeding and subsequent collection of bacteria and nutrients that is happening during high tide.

This study raises many questions, possibilities for further research, and opportunities to refine techniques including the assessment of functional genes within the community by designing targeted primers for T-RFLP, expansion of clone libraries to include replicates and representatives from high and low tide, standardization of cell counts used in the phenotypic assays, as well as generation of culture recipes and directly inoculating from the plates to enrich specific organisms.

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