Comparison of bacterial communities among geographically distinct populations of the benthic dinoflagellate *Prorocentrum lima*

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Abstract

Members of the *Prorocentrum lima* species complex (PLSC) are most frequently found in high cell abundance in the tropics and sub-tropics and are almost invariably toxigenic. Epiphytic and planktonic bacteria are known to co-occur with such toxigenic *Prorocentrum* but reciprocal allelochemical interactions with the dinoflagellate microbiome have been under-investigated and are poorly understood. The aim of the present study was to identify and compare the bacterial community cultured together with isolates of the PLSC from geographically distant coasts of Mexico. Profiles of bacterial communities associated with dinoflagellate clones were obtained by high-throughput sequencing of 16S V3-V4 amplicons. Our results show that the microbiome of *P. lima* is associated with the location of origin. A similar bacterial diversity was found free-living in culture medium or closely associated with the host dinoflagellate cells. The microbiome comprised a total of fourteen bacterial classes where alpha- and gamma-proteobacteria and Bacteroidia dominated the respective communities. Specifically, members of genera *Labrenzia*, *Roseitalea, Cohaesibacter, Marivita, Muricauda, Marinobacter* and *Masilia* were most abundant. Based on known interactions of these genera with other microeukaryotes, they may sustain symbiotic relationships with *P. lima* populations via a variety of allelochemicals and reciprocal nutrient enrichment mechanisms within the phycosphere. Bacterial members identified will improve the study of the toxigenicity, ecology, and biotechnological potential of this benthic dinoflagellate group.

Keywords: Dinoflagellate, *Prorocentrum lima*, microbiome, xenic culture, allelochemicals

Introduction

Marine benthic species of the dinoflagellate genus *Prorocentrum* (Ehrenberg) are found around the globe in shallow waters living mainly attached to macroalgae, seagrasses or other benthic substrates (Durán-Riveroll *et al*., 2019). Epibenthic dinoflagellates are exposed to a microbiome comprising free-living and substrate-attached bacteria and microeukaryotes. Chemical interactions can occur in the extracellular space surrounding the cell, denominated as the phycosphere (Seymour *et al*., 2017) into which chemicals can diffuse and be exchanged. Chemical interactions within the phycosphere can be either beneficial or detrimental to the epibenthic dinoflagellate, or perhaps mutually beneficial for the microbiome. In the antagonistic case, allelochemicals generated by the dinoflagellate may limit parasitism or reduce competition for inorganic nutrients from the bacterial component, whereas certain bacteria may inhibit growth or survival of the dinoflagellate. In the mutually beneficial case, dissolved or particulate organic matter may be produced by the dinoflagellate, in reciprocal exchange for vitamins or organic macronutrients generated by the bacteria.

The strategic importance of the microbiome in dinoflagellate toxin production and regulation in benthic dinoflagellates is unclear. Associated bacteria within the phycosphere of the benthic dinoflagellate *P. lima* (Ehrenberg) F. Stein influence growth rates and likely also toxin production rate and cell toxin content (Tarazona-Janampa *et al*., 2020). Elucidation of the microbiome composition is therefore the first step in defining these interactions.

The genus *Prorocentrum* contains more than seventy species, with almost all of the known toxigenic species considered primarily benthic or epibenthic. Some *Prorocentrum* species have not been fully classified and many morphotypes remain provisionally unresolved within various species complexes. Within the *P. lima* species complex (PLSC), molecular methods directed to target sequences of the rRNA gene are required for reliable species classification (Cembella *et al*., 2021).

We analyzed the microbiome associated with cultures of taxonomically confirmed PLSC isolates from two geographically distant sites on Mexican coasts to evaluate the dependence of the origin on the microbiome structure. The microbiome components were further analyzed to compare the similarity between bacterial communities found free-living in the culture medium and those associated directly to the dinoflagellate cells.

Material and Methods

Prorocentrum lima *isolation, culture, and harvest*

Prorocentrum lima cells were isolated from macroalgae and seagrasses from Bahía de La Paz, Baja California Sur (Gulf of California, n = 6), and from the Veracruz Reef System (Gulf of Mexico, n = 5). Microscopic examination was performed under a stereo-dissecting microscope (Discovery.V8, Zeiss). *Prorocentrum lima* single cells were isolated by micropipette into 96-well microplates containing 100 µL of 50%-strength GSe growth medium modified without soil extract prepared from filtered and autoclaved

seawater at salinity 36. Clonal cultures were maintained on a 12:12 h light:dark cycle at 80 µmol photons m^{-2} s^{-1} illumination. Where multiple cell divisions were observed, well contents were transferred to 24-well plates with 2 mL of nutrient medium. After observed growth, cells were transferred to 60 x 15 mm Petri plates with modified 50% GSe medium and later maintained as reference cultures at the Department of Marine Biotechnology, CICESE, Ensenada, Mexico.

Dinoflagellate-associated bacterial biomass was generated in 250 mL Erlenmeyer flasks of isolates maintained as xenic reference cultures for approximately two years with monthly transfers (~24 transfers). In late exponential growth phase, dinoflagellate cells and bacteria in the growth medium were separated using UV-sterilized 20 µm mesh sieves under a laminar flow hood. *Prorocentrum lima* cells with their attached or mucus-associated microbiome and free-living bacteria from the growth medium were recovered separately in sterile microtubes by centrifugation (6000×*g* for 3 min). The supernatant was discarded and the cell pellets were frozen at -20 °C until DNA extraction and amplification.

Microbiome sequencing and data analysis
Samples were homogenized in a FastPrep-24 5G instrument (MP Biomedicals) using three cycles of 30 s at 6.0 m s^{-1} with resting incubations of 5 min on ice. Total DNA was extracted with DNeasy Power soil Kit (QIAGEN) according to manufacturer's instructions. The V3-V4 region of the 16S rRNA gene (Klindworth *et al*., 2013) was amplified to generate Illumina sequencing libraries following the 16S metagenomic sequencing library preparation B protocol. Amplification reactions used KAPA HiFi HotStart DNA Polymerase (KAPA Biosystems). Amplification products were cleaned with Agencourt AMPure XP beads. Dual indexing was performed with Nextera XT index kit according to the manufacturer's instructions. Libraries were quantified by fluorometry with a Qubit 3.0 (Life Technologies) and sequenced as 250 bp paired-end reads on a MiSeq machine (Illumina).

Sequencing reads were processed in R (v.3.6) with the dada2 v.1.14 general workflow with proper parameter adjustments. Denoised reads were used to form Amplicon Sequence Variants (ASVs) and finally remove chimeras. Taxonomic assignments in dada2 used the SILVA 138.1 database with a confidence threshold of 80. Further analysis of the sequence-count per sample table was conducted in R with functions for phylogenetic and ecological analysis of microbiome data available in the DECIPHER, phangorn, microbiome, phyloseq, and vegan packages. A maximum likelihood tree of ASV sequences was inferred using a GTR+I+G model. Sequence counts per sample were transformed to relative abundances and summarized by respective taxonomic assignment to the class level for visualization of the bacterial community composition. The structure of bacterial communities in the two locations of origin was evaluated by a PERMANOVA analysis (499 permutations) of UniFrac distance matrices, validated by analysis of variance of the groups' dispersions. The first two components of a Principal Coordinates Analysis (PCoA) were used for visualization of the categorical groups.

Results and Discussion

The microbiome of *P. lima* isolates from the Gulf of California was substantially different from that of isolates from the Gulf of Mexico (Fig. 1). A total of 648 ASVs were detected; on average, the samples contained 63 ASVs (min. 45, max. 86). The dinoflagellate cell host- and culture medium-associated bacterial communities of isolates from the two locations of origin were significantly different regarding both UniFrac metrics. This result is likely evidence of the initial differences in the bacteria present at these two locations and thus available for interaction with the dinoflagellate. Once a xenic culture has been established, a diverse microbiome can be sustained through dozens of host-cell generations, an effect also observed for other microalgal species (Jackrel *et al*., 2020). Nevertheless, the probability of clonal selection in long-term xenic cultures cannot be excluded here.

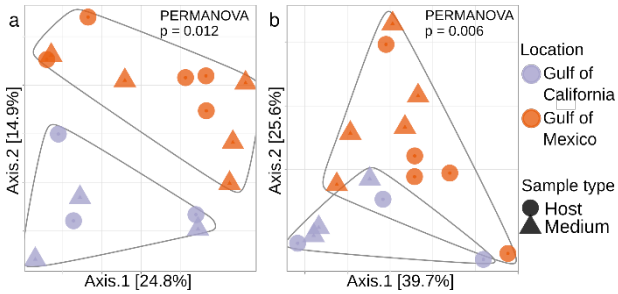

Fig. 1. Bacterial community structure in *Prorocentrum lima* cultures. Principal coordinates analyses of UniFrac distances (a, unweighted; b, weighted) is shown for microbiomes obtained by sequencing of 16S rRNA amplicons of bacteria directly associated with host or free-living in culture medium. Grouping by location of origin was evaluated by PERMANOVA.

The microbiome structure of bacterial samples taken as free-living from the culture medium versus associated to the host dinoflagellate cells did not cluster separately in the ordination. Hence, they cannot be considered as separate groups. One possible explanation for this overlap is that culture conditions – confinement within a small, enclosed, and stable environment – create an artificially expanded "phycosphere" that allows a relatively easy and generalized access to nutrients and potential allelochemicals as secondary metabolites exchanged by molecular diffusion. In natural benthic environments, the phycosphere is more susceptible to disruptive wave action, tidal flux, and wind-driven turbulence that favor molecular dispersion. Under these circumstances, the microbiome must be retained, e.g. within mucus aggregates or biofilms, close to the dinoflagellate cells to facilitate effective interactions for metabolite exchange.

The second objective of this study was to determine the composition of the bacterial community within the microbiome. Similarities at the class level, between paired samples of bacteria from culture medium and host-cells were clear (Fig. 2). At the highly diverse class level, fourteen classes were identified among all sequenced samples. Alphaproteobacteria was the dominant class, occupying > 50%, and up to a maximum 95%, of the ASV composition in each sample. This class has been found commonly associated to planktonic eukaryotic microalgae, such as natural populations of diatoms and other dinoflagellates (Lawson *et al*., 2018; Maire *et al*., 2021). Other abundant and widely distributed bacteria belonged to classes Bacteroidia, Gammaproteobacteria, and

Plancto-mycetes. Classes with substantial ASV abundance (> 1%) but present only in some samples were Phycisphaerae and Kapabacteria, mainly in cultured isolates from the Gulf of California (Fig. 2).

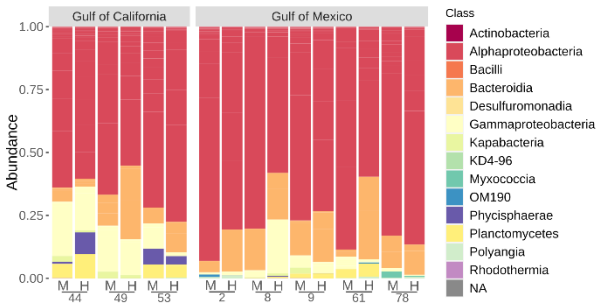

Fig. 2. Bacterial community profiles of P. lima xenic cultures based on 16S rRNA gene sequence amplicons. Relative abundances at the class level are shown for bacteria associated directly to host dinoflagellate cells (H) cells versus free-living in culture medium (M).

A previous attempt to culture bacteria associated with *P. lima* yielded isolation of bacteria from three classes: Actinobacteria, Alphaproteobacteria and Gammaproteobacteria (Tarazona-Janampa *et al*., 2020). Our current results confirm the presence of these three classes but add eleven more classes to the diversity profile. The maintenance of such huge bacterial diversity sustained over years in xenic dinoflagellate cultures may assist in designing optimal culturing conditions to target the other members of the *P. lima* microbiome community for more detailed study.

A closer examination of the members of Alphaproteobacteria reveals the dominance of order Rhizobiales, represented by genus *Labrenzia* as the member present in all isolates with at least 1% abundance. Heterotrophic species in this genus may play a role in the dinoflagellate hosts-stress tolerance due to dimethylsulfoniopropionate (DMSP) production (Lawson *et al*., 2018). Other genera identified from Rhizobiales were *Roseitalea* and *Cohaesibacter*, both previously found as core members of endosymbiotic dinoflagellates of Symbiodinaceae (Lawson *et al*., 2018; Maire *et al*., 2021). This congruence is rather remarkable given the striking differences in the phycosphere for free-living epibenthic dinoflagellate such as *P. lima*. Finally, the genus *Marivita* (family Rhodobacteraceae) may provide vitamins to the dinoflagellate host contributing to its growth (Park *et al*., 2017). Representing the Bacteroidia, the genus *Muricauda* (family Flavobacteriaceae) may also help its host by scavenging reactive oxygen species through carotenoids (Maire *et al*., 2021). *Marinobacter* and *Massilia* represent highly abundant ASVs of Gammaproteobacteria. Some members in these genera can render nutrients such as iron and phosphate biologically accessible for microeukaryotes and presumably other bacteria. In *Massilia*, this bioavailability function was only described in a terrestrial member but this genus has been associated with diatoms in the marine environment (Lupette *et al*., 2016).

In conclusion, our results show a complex microbiome and suggest potential allelochemical interactions can regulate respective growth and perhaps survival of dinoflagellates and their microbiome members. Despite substantial differences in microbiome structure depending upon the origin of the dinoflagellates, common members of the consortium were found. The specific identity of these bacteria varies by location, but the roles and functions may fill the same specific niches of symbiotic or antagonistic interactions. This study provides

an initial template for elucidation of the role of the microbiome in dinoflagellate toxigenicity and their ecological interactions in natural benthic populations.

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