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PRESENCE AND ABUNDANCE OF THE TYPE VI SECRETION SYSTEM IN A COASTAL OCEAN ENVIRONMENT

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Table of Contents

List of Figures and Tables

Abstract

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Bacteria-bacteria interactions are critically important to fundamental biological processes in the ocean such as nutrient cycling and the carbon pump. These interactions are often governed by physical and chemical effectors that are poorly understood. The presence and relative abundance of bacterial predators utilizing the type VI secretion system (T6SS) were examined at local and global scales to better understand local bacterial community dynamics, through the use of a two-year time series of weekly samples from Monterey Bay and metagenome data from the TARA Oceans project. We found that relative abundance of bacterial predators in both datasets is negatively correlated with availability of essential nutrients. Statistical analyses suggest that, when abundant, these bacterial predators reduce the abundance of other bacterial species thereby influencing bacterial community composition. Taken together, these data show that predatory bacteria play a role in determining bacterial community structure under given environmental conditions and may influence ecosystem functions performed by the bacterial community.

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Chapter 1: Background and Introduction

1. Bacteria-Bacteria Predation

Marine bacteria constitute the largest portion of biomass in the world's oceans (1, 2), and are responsible for the cycling and regeneration of nutrients throughout the ocean (3). Most regenerated primary production relies on nutrients that pass through the microbial loop, whereby bacteria and other microorganisms metabolize organic compounds and return them to non-organic, bioavailable forms (4). Function and efficiency within the microbial loop are affected by composition and activity of the microbial community. For example, diverse functional groups of bacteria contribute to nutrient cycling, nutrient fixing and ecosystem function (5–7). Fenchel (3) describes functional categories of bacteria acting within the microbial loop in very specific metabolic processes like oxidation of ammonia or C-1 carbon compounds. Shifts in bacterial community composition that also affect functional group representation may change the manner in which the microbial community cycles nutrients and organic carbon and are thus important to understand.

Bacteria in ocean ecosystems are generally found to be concentrated between 10^3 cells/mL - 10^6 cells/mL, which is thought to be much lower than their carrying capacity (8). This difference is in part created by top-down predation pressures. Larger organisms such as protists are well-known predators of bacteria (9, 10), but predation of bacteria is also carried by other bacteria (9, 11). Most of these predatory bacteria are non-obligate predators, which typically take nutrients and carbon from the surrounding environment (12). These predators will use predation to gather nutrients and reduce competition when freely available nutrients are scarce (9, 13). Evidence for bacteria-bacteria predation has been reported in laboratory settings (11, 14, 15) and in terrestrial, fresh-water aquatic, and marine environments (11, 12, 16–18).

One method of bacteria-bacteria predation, and the focus of this study, is through the use of the bacterial Type VI secretion system (T6SS) (19, 20). Bacteria-bacteria predation takes many forms, all involving the killing of neighboring bacterial cells and uptake of lysed nutrients (11). T6SS specifically involves the direct injection of toxic compounds, known as effectors, into a target cell (21, 22). These effectors quickly cause cell lysis, killing the target cell and freeing nutrients for absorption by the predator (23).

Predator-bacteria interactions, which can shape microbial communities, can have an impact in the recycling of nutrients in ocean ecosystems. Knowledge of the role of T6SS as a predatory mechanism between bacteria is currently limited. The following chapter examines predatory bacteria with T6SS, the environmental drivers of their presence and abundance locally and globally, and the putative effects of these predatory bacteria on the wider bacterial community. We present evidence that these bacteria occupy specific habitats within the ecosystem, that their abundance within those habitats are influenced by environmental conditions, and that abundance of predator bacteria may influence bacterial community composition. Bacterial predation pressures are poorly understood but our work indicates that they are important in understanding changes in the bacterial community.

2. Monterey Bay Environment

Santa Cruz Wharf, the study site for time-series bacterial community data in the following study, is located within Monterey Bay. It is therefore important to consider the common patterns and seasonalities within the region, to inform the following discussion of the bacterial community in the area, and the T6SS predatory bacteria within that community. The region is controlled by the California Current system and is subject to a yearly cycle of wind-driven upwelling and downwelling (24–26). In general, flow along this region follows the seasonal winds, with southeastward currents in the spring and summer months promoting upwelling and northwestward currents in the winter months promoting downwelling (25, 26). The spring and summer upwelling driven by these currents occurs outside Monterey Bay

itself, but provides cold, nutrient rich water that enters the bay near Point Año Nuevo (24, 27). This infusion of nutrients into the bay drives spring and summer phytoplankton blooms and leads to a peak in yearly primary productivity (28–31). Upwelling relaxation occurs late in the summer followed by the infusion of warm, high saline equatorial water by the Davidson Current in fall and winter (32). Along with seasonal variability in wind stress and upwelling events, Monterey Bay is also impacted by seasonal patterns in precipitation. The study site for this paper at Santa Cruz Wharf is near the mouth of the San Lorenzo River, and is impacted by runoff during the rainy season, generally between November and March. Bacterial populations shift with these changing environmental conditions, both in response to phytoplankton blooms (33) and in response to changing nutrients and temperatures (33, 34) as is examined here in chapter 2.

Chapter 2: Presence and Abundance of the Type VI Secretion System in a Coastal Ocean Environment

1. Introduction

1.1 T6SS, a mechanism of Bacterial Predation

T6SS is one of the secretion systems used by bacteria to predate on other bacteria (19, 20). It is present in up to 25% of Gram-negative bacteria species by some estimates, mostly within the proteobacteria (22, 35–37). This secretion system is a versatile mechanism that delivers effector proteins directly into target cells, and has been shown to be a factor in pathogen virulence and a mechanism for killing other bacteria in laboratory studies (20, 38). Bacteria with T6SS display contact-dependent anti-microbial properties, and have been shown to use this for predation of other bacteria in culture experiments (19, 20). These experiments suggest that T6SS evolved for use as a competitive and predatory mechanism which was later co-opted as a mechanism for pathogen virulence.

In structure, T6SS is strikingly similar to a bacteriophage tail, with a trans-membrane anchor and contractile sheath which extends to puncture target cells (22, 39). After puncturing a target cell, T6SS delivers effector proteins which are toxic to the target cell. These effectors can attack the cell wall, cell membrane, or nucleic acids depending on the specific protein being delivered, and generally lead to cell lysis (23). The primary extracellular structure of T6SS is comprised of two proteins, valine-glycine repeat G (VgrG) and haemolysin coregulated protein (Hcp), which are therefore used as proxies for T6SS activity (19, 39, 40). VgrG forms the “cap” of the T6SS assembly and is propelled by a sheath formed from the Hcp structural protein; both proteins, as well as several others, are needed for T6SS to function (15, 39, 41). While the structure of T6SS is well conserved among T6SS containing bacteria, the gene corresponding to the structural proteins is not. To date, most T6SS pathways have been identified by protein structure rather than by gene sequence (14, 22, 40, 41).

Bacterial predators are known to be prevalent in natural environments (11), and T6SS is a method of bacterial predation found widely amongst sequenced bacteria (22, 35–37). Information is currently scarce or nonexistent as to when having a T6SS is beneficial for bacteria in natural settings, what environmental or biological conditions might drive its usage, and to explore the effects T6SS predation may have on the rest of the bacterial community. It has been suggested that bacterial predation plays a role in modulating the composition of bacterial communities (11), but this process and its implications are poorly understood compared to predatory pressures in eukaryotic communities.

1.2 Objectives

Here, we address three questions regarding the presence and abundance of bacteria with T6SS genes in the coastal ocean, specifically at our study site at the Santa Cruz Wharf in Monterey Bay, California. First, whether these bacteria are present in the coastal ecosystem and assess their abundance through time. Second, what environmental and biological conditions drive abundance of these T6SS-containing bacterial predators, using correlational analyses to compare relative abundance with environmental metadata. Lastly, what changes these bacteria might cause in the larger bacterial community, by comparing changes in bacterial operational taxonomic unit (OTU) abundance to the abundance of T6SS-containing bacteria. By combining these measurements and analyses, we may begin to understand the impact of this secretion system on the wider bacterial community in the coastal ocean. This will provide insight on the importance of bacteria-bacteria interaction via T6SS in the environment and its potential impact on microbial food webs and biogeochemical cycling.

2. Methods

2.1 Sampling

In order to assess the changes through time in the bacterial community of Monterey Bay, water samples were taken weekly and analyzed through 16S rRNA sequencing.

Sampling times and location corresponded with sampling for SCCOOS HAB database, the source of physical ocean data examined in this study. Water samples were collected at the end of the Santa Cruz Wharf. This location is approximately 700 meters from shore, with a maximum depth of approximately 10 meters.

A Niskin bottle was used to collect 3 water samples of 1.3 liters each at 0, 1.5, and 3 meters below the ocean surface. These samples were thoroughly homogenized, then divided into 3 replicates of 1 liter. Each replicate was filtered first through sterile, prepackaged 3.0 µm Cellulose Nitrate filters and then through sterile, autoclaved 0.2 µm Durapore filters on 47mm Nalgene filter towers. Immediately after filtering, filters were stored at -80C until extraction. These filter sizes are respectively considered to represent the particle-attached bacterial community (3.0 µm) which adheres to particles in the water and are trapped on the larger filter size, and free-living bacteria (0.2 µm) which pass through the larger filter size and are sequentially trapped by the smaller filter size.

2.2 DNA Extraction

Whole community DNA was extracted from both 3.0 µm and 0.2 µm filters using the PowerWater DNA Isolation kit (MO BIO Laboratories, Inc., CA), per manufacturer instructions. Briefly, samples were physically lysed and removed from filters by bead-beating, then chemically lysed using the proprietary chemicals from the PowerWater kit. Following lysis and removal of debris, DNA was captured on the provided filter column, washed and eluted into sterile vials. DNA extract was tested for quality and quantity, and frozen at -80C prior to Next-Generation sequencing.

2.3 Testing Sample Quality

Following extraction, samples were tested for DNA quality through PCR using primers 515F and 806R for bacterial 16S ribosomal subunit V4 region (42). Samples with no amplification were tested again following a 1:10 dilution in nanopure PCR water, after which the remainder of un-amplified samples were removed from this analysis.

DNA extracts of good quality were then quantified using the Quant-iT PicoGreen dsDNA assay. Extract aliquots were diluted 50x in 1x TE buffer and PicoGreen dye. DNA standards were created ranging from 0ng/mL to 2000ng/mL. Fluorescence of both samples and standards was read on a Spectramax plate reader spectrophotometer at Ex=480nm and Em=520nm. Fluorescence readings were converted to extract DNA concentration using the calculated linear standard curve (R^2>.99).

2.4 Next-Generation Sequencing and Sequence Processing

After ensuring that DNA extracts were of good quality and quantifying extract concentrations, an aliquot of each extract was taken for sequencing. These aliquots were normalized to concentrations between 1-10ng DNA/µL by dilution with nanopure PCR water. These samples were plated on 96 well plates, sealed with aluminum sealing tape, and frozen at -80C. When the samples were frozen, the plates were sent to the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory for Illumina MiSeq Next-Generation Sequencing. Sequencing targeted 16S rRNA V4-V5 regions, using primers 515F and 806R (SEQUENCE).

Post-processing of sequencing results was carried out using the Quantitative Insights Into Microbial Ecology (QIIME 1.3) pipeline, as laid out in Sison-Mangus et al., 2014 (43). Briefly, OTUs were picked using Pynast with 97% similarity (44), then identified using RDP classifier 2.2 (45). Singletons and chloroplast sequences were removed prior to data analysis. Raw sequencing reads have been deposited in Genbank.

2.5 Qiime Output Analysis

Following sample analysis through the modified QIIME pipeline, relative abundances were averaged across replicates to recover OTU abundance and standard error by sampling date and limited to the 1200 most abundant OTUs for further analysis. BLASTn searches were run on OTUs that were not already identified to genus level. These OTUs were assigned a genus if BLASTn matched them at a ≥97% similarity threshold to a known genus.

Genera of interest were identified as those known to contain core structural genes of the T6SS pathway (S. 1), with accessions available through NCBI or UNIPROT KB databases. In most cases, genera of interest were represented by multiple OTUs within this data set. The percent abundances of these OTUs were summed to determine the percent abundance of genera of interest within the bacterial community for each sample date. Relative abundance by genus and date and physical ocean conditions by date from SCCOOS data sets were used to statistically assess the relationships between bacteria with T6SS, the rest of the bacterial community, and the ocean environment.

2.6 Metagenome Sequencing

Ten samples corresponding to five sampling dates were chosen from the Santa Cruz Wharf sample set for metagenome sequencing. These samples were selected to represent a range of seasons, environmental conditions and expected T6SS predator abundance. These samples were checked for DNA quality before being processed by GENEWIZ through their metagenome sequencing pipeline. Nucleotide reads were downloaded, then built into BLAST databases using BLAST+ 2.9.0. These databases were then searched for any sequences matching known *hcp*, *vgrg*, *clpV*, *vipA*, and *vipB* sequences belonging to 21 genera of bacterial predators.

2.7 TARA Oceans Database

All nucleotide reads from the TARA Oceans database were analyzed for the presence of bacterial *hcp* and *vgrg* sequences. The complete set of nucleotide reads was downloaded, then built into BLAST databases using BLAST+ 2.9.0. These databases were then searched for any sequences matching known *hcp* and *vgrg* sequences belonging to bacterial genera confirmed to be present in the TARA database. Gene abundance was counted by matching reads per million mapped reads belonging to each genera with representation in the TARA samples. This was done both to confirm the presence of those

genes in the expected bacterial species, and to compare worldwide patterns of abundance to those found in local SC Wharf time series samples.

2.8 Statistical analyses

Genus abundance by size fraction was examined by comparing size fractions across all samples by two-way student's t-test to compare fraction preference (free-living vs particle-attached) of each genus of interest. Each genus was compared pairwise between its abundance in the 3.0 µm filter fraction and the 0.2 µm filter fraction at each time point (Table 1). Genera showing a statistically significant higher abundance in one of the size fractions was labeled as adhering to the corresponding niche.

In order to understand the effects of environment and community composition on bacterial predator abundance, the correlations between bacterial and environmental data were examined. Canonical correspondence analysis (CCA) was used to examine the correlations between the abundance of bacteria genera of interest and physical and biological environmental factors. Abundance data for bacterial genera with T6SS was cross referenced with environmental data using the VEGAN package in R. Adonis was used to calculate statistically significant environmental variables ($p < 0.05$) using 999 permutations. All environmental variables were then passed to the VEGAN cca function and plotted against sample dates and genus abundance (figs. 5-6, code available in supplementary materials). The same analysis was used in analyzing data from TARA Oceans, with bacterial abundance represented by vgrg and hcp gene reads per million mapped reads.

To assess the effects of bacterial predators on the rest of the bacterial community, a cross-correlation time lag analysis was run between predator abundance at the genera level and abundance of each bacterial OTU from local samples. Interactions were determined using a time lag of 1 week. Network diagrams were built to show relationships between predatory and other OTUs bacteria with statistically significant negative cross-correlations (less than -0.5, $p<0.05$), representing relationships that were potentially predatory in nature.

Each of these relationships was plotted as a grey line connecting one predatory genus to a potential prey OTU. These relationships were plotted using Cytoscape 3.7.2 (fig.7).

3. Results

3.1 Presence of T6SS in the coastal ocean.

Metagenome sequencing of samples from Santa Cruz Wharf indicates the presence of core T6SS genes belonging to 21 genera of bacteria across 10 samples from 5 time points (fig. 1). These samples were chosen for their representation of a broad range of expected predator abundance and environmental conditions such as phytoplankton bloom status, and to represent every season. Sequences identified as *vgrG*, *hcp*, *clpV*, *vipA*, and *vipB* (core structural genes of T6SS) (S.1) were found to be present in each of these samples. The presence of core T6SS genes in these samples confirms that these 21 genera are present at Santa Cruz Wharf samples and that they have the genetic capacity to carry out predation using the type VI pathway. These 21 genera, therefore, make up the predatory bacteria population that is tracked through the rest of this study.

Predatory bacteria were consistently present in local time-series coastal samples, when analyzed using Illumina next-generation sequencing of bacterial 16S V4 region. These T6SS-containing bacteria were tracked in 3.0 µm and 0.2 µm filter fractions of water from Santa Cruz Wharf. Some combination of these genera was present in every analyzed sample and at each filter size fraction. Bacteria with the machinery for T6SS-related predation ranged in relative abundance from 1% to 11% of the total bacterial community in these samples, with standard error ranging between 0.01% and 0.88% (fig. 2). Putative predatory bacteria had higher relative abundance at Santa Cruz Wharf in winter months, and lower relative abundance through the summer, with a minimum in late June and early July. This seasonality appeared in data across 2014 and 2015 and in both filter fractions, aside from one high abundance outlier in August of 2014.

T6SS genes may vary greatly in nucleotide and protein sequence, but function of T6SS has been shown to be highly conserved (37). While T6SS is a horizontally transferred system, a large portion of sequenced proteobacteria have been found to possess some configuration of 13 core genes that make up active T6SS (15, 37, 41, 46). References for genes used as markers of T6SS loci in the bacteria discussed herein are compiled in supplementary materials (S1), and 5 of those structural genes have been quantified in SC Wharf samples through metagenome sequencing. Thus, while it cannot be assumed that every species containing T6SS genes uses T6SS machinery, we must for the purposes of this community-level study assume that those bacteria with loci coding for conserved T6SS proteins produce functional T6 systems.

3.2 Different bacterial predators occupy different niches.

Bacterial predator abundances varied significantly between the particle-attached and free-living communities. While total predator abundance varied slightly in these two size fractions with the attached fraction having more predatory bacteria (Two sample T test, $p = 0.0476$, measured difference = 0.61% of total community), composition of each community was widely different (fig. 2, table 1). Six genera of potentially predatory bacteria appear only in the 3.0 µm size fraction (*Planctomyces, Bradyrhizobium, Mesorhizobium, Methylobacterium, Agrobacterium,* and *Serratia*). Of the remaining 15 genera, only 5 do not show a significant difference in relative abundance between filter size fractions.

To understand the niches that each genera of predatory bacteria inhabits, abundances of each genera were compared between size fractions using a two-way Student's t-test (table 1). By this analysis, 4 genera were identified primarily in the free-living fraction (Ruegeria, Acinetobacter, Francisella, and Pseudomonas), while 12 genera were primarily particle-attached (Planctomyces, Bradyrhizobium, Mesorhizobium, Agrobacterium, Rhodobacter, Janthinobacterium, Pseudoalteromonas, Psychromonas, Serratia, Shewanella, Teredinibacter, and Vibrio) ($p < 0.05$). The remaining 5 genera (Mesorhizobium,

Sphingomonas, Arcobacter, Enterobacter, and Halomonas) do not show a statistically significant difference in percent relative abundance between the size fractions. These have been labeled “mixed”. *Ruegeria* and *Acinetobacter* dominated the free-living predator community, while the attached community was primarily composed of *Vibrio*, *Psychromonas*, *Planctomycetes*, and *Shewanella* (fig. 3). *Halomonas* also made up a large percentage of particle-attached predators, but statistically was a member of the “mixed” niche. While this analysis was carried out using 16S sequencing data to provide measures of relative abundance, T6SS genes for each of these bacteria have been confirmed present in SC Wharf samples through metagenome sequencing.

3.3 Presence of T6SS on a global scale

Core T6SS genes were found to be universally present in globally distributed samples. In order to compare local results to patterns on a global scale, metagenome data from the TARA Oceans project was examined. This examination specifically targeted two genes, *vgrg* and *hcp* from 8 bacterial predators known to be present in TARA Oceans samples. BLAST searches of TARA Oceans metagenomes returned reads of *vgrg* and *hcp* from every tested sample, ranging from 0.002 to 1.1 reads per million mapped reads (RPM). RPM at each sampling site can be found in figure 4. T6SS genes had highest relative abundance in samples from the southern Atlantic Ocean gyre and the Indian Ocean gyre and lowest relative abundance in samples taken from the coastal environment, through the Mediterranean Sea and around southern Africa.

3.4 Correlational analyses between bacterial predators and environmental factors.

To better understand the root drivers of bacteria-bacteria predation, canonical correspondence analysis (CCA) was used to assess correlations between T6SS predator abundance and several key environmental parameters (figs. 5, 6). Significance of environmental variables is based on ADONIS analysis of variance at 999 permutations. Arrow length in the ordination plots represents strength of the relationship of each

environmental parameter to composition of the community of bacterial predators. Asterisks by parameter names denote a p-value of $p < 0.05$.

In the Santa Cruz Wharf timeseries samples (fig. 5), environmental conditions considered included toxic *Pseudo-nitzschia* (Toxic_PN) cells/mL, nitrate (NO3), ammonia (NH3), phosphate (PO4), and silicate (Si) concentrations, chlorophyll concentration (chl) as a proxy for autotroph abundance, and water temperature (temp). In the particle-attached fraction (fig. 5A), the first two axes explained 16% of variance in predator population. Three distinct response groups are observed within the predator population. First, there is a group of predators responding positively to silicate ($F=4.45$, $p=0.002$), consisting of *Rhodobacter, Shewanella, Sphingomonas,* and *Methylobacterium*. Second, a group responds positively to temperature ($F=1.96$, $p=0.087$) and negatively to the presence to toxic *Pseudo-nitzschia* ($F=1.82$, $p=0.11$). This group consists of *Agrobacterium, Halomonas, Jathinobacterium, Enterobacter,* and *Shewanella*. Finally, the third response group shows strong negative responses to ammonia ($F=4.1$, $p=0.003$) and phosphate ($F=1.83$, $p=0.10$), and consists of *Francisella, Serratia, Pseudomonas, Ruegeria,* and *Acinetobacter*. Several remaining genera are observed that fall near the origin of this CCA plot, and therefore are not varying in relative abundance with the factors considered in this study. In the free-living fraction (fig. 5B), the first two axes explained 17.4% of variance in predator population. Two distinct response groups are noted among bacterial predators in this filter fraction. As in the particle-attached fraction, there is a group that varies strongly with silicate ($F=3.72$, $p=0.01$), and in this plot is also closely aligned with temperature ($F=2.21$, $p=0.07$). This group consists of *Rhodobacter, Sphingomonas,* and *Shewanella*. The other group of predatory bacteria showing strong responses are varying negatively with toxic *Pseudo-nitzschia* ($F=2.32$, $p=0.059$) and chlorophyll concentration ($F=1.12$, $p=0.35$). This predator group consists of *Jathinobacterium, Vibrio,* and *Pseudoalteromonas*. Finally, there is again a group of predators that do not seem to be responding to the variables considered in this study.

The TARA Oceans metagenomes show similar CCA patterns to the Santa Cruz Wharf samples (fig. 6). For this analysis, read count abundance of T6SS genes within metagenome samples were compared to potential environmental drivers. Depth was the most significant driver of T6SS gene abundance ($f=11.19$, $p=0.006$), and relative abundances of *Rhodobacter* and *Shewanella* were tightly correlated to this factor. While strong relationships with phosphates and ammonia were expected based on observations from local samples, these nutrient concentrations increase with depth which overwhelms latitudinal variation. Most predatory bacteria from these samples varied more directly in a negative correlation with autotroph abundance ($f=3.39$, $p=0.077$), with responses from *Acinetobacter, Pseudoalteromonas, Pseudomonas, Ruegeria, Vibrio,* and the total abundance of T6SS gene reads.

3.5 Bacterial predators may drive successional dynamics in the bacterial community.

T6SS-containing bacteria, when in high abundance, are correlated with a later decrease in specific OTUs. Time-lagged cross correlation analysis showed a significant decrease in relative abundance of many members of the bacterial community ($p<0.05$) following high abundances of bacterial predators (fig. 7A and 7B). The grey lines in this diagram each represent a negative relationship between a specific predatory genus and one OTU from the rest of the bacterial community. Amongst free-living bacteria, there were concentrations of negative interactions between bacterial predators and bacterial OTUs in the Actinobacteria, Bacteroidia, Beta-, Delta-, and Epsilonproteobacteria, and the Thaumarcheota. There was a noticeable lack of negative interactions between these potential predators and the Alphaproteobacteria and Flavobacteria. Within the particle-attached community, potential predation activity was more evenly distributed compared to the free-living community. Within the particle-attached community, negative interactions did not show a noticeable difference in density by OTU class. These network plots demonstrate that T6SS-

containing bacteria potentially cause decreases in relative abundance of specific OTUs within the bacterial community in a manner consistent with predatory activity.

4. Discussion

4.1 Presence and Abundance of Bacterial Predators

T6SS predatory bacteria are omnipresent in both local time-series samples and global ocean samples. These bacteria make up a large segment (up to 11%) of both the attached and free-living bacterial communities in the coastal ocean and are present in every sample and at every time point examined (figs. 1, 2, 4). There is a greater variety of T6SS predators that exist mainly in the particle-attached fraction, including six genera that were found there exclusively (fig. 3, table 1). Only 4 of the 21 genera examined were mainly found in the free-living fraction. These patterns indicate that having a T6SS could be a competitive advantage to predatory bacteria that attach themselves to organic particle hotspots ranging from marine snow to living phytoplankton. Similarly, free-living bacteria are highly dispersed in the water column compared to particle-attached bacteria, and the encounter rate of bacterial prey could be slim, thus having a T6SS as a predatory mechanism could only become advantageous under particular conditions that promote bacterial-bacterial competition. In this study, for instance, bacterial predators in the free-living fraction were putatively predating on members of Beta-proteobacteria (fig. 7B), one of the marine bacterial groups that are commonly found in low abundance in coastal ecosystems (47) but have been observed to increase in abundance and bloom in low biomass winter season (48). Particle-attached predators, on the other hand, must compete on the local scale of the particle to which they are attached. These particle-attached predators have higher encounter rate with other microbes, as particles in the ocean tend to be hotspots of microbial activity (49, 50). Being particle attached, however, limits their selection of prey to their immediate microbe neighbors. For this reason, particle attached predators may prey more generally on competing bacteria with the same particle-attached niche (fig. 7A).

4.2 Changes in predator abundance related to environmental factors

In general, predatory bacteria increase in relative abundance when there are fewer resources available. In such times and locations, it is likely that their ability to kill neighboring bacteria provides a competitive advantage through procuring nutrients and reducing competition for other scarce resources. This pattern is seen through time in the Santa Cruz Wharf samples and spatially across large areas in the TARA Oceans samples. Interestingly, the relative abundance of predatory bacteria in Santa Cruz Wharf samples was positively correlated to silicate concentrations and negatively to ammonia concentrations and autotroph abundance. Predatory bacteria in the free-living fraction also had a negative response to water temperature. Possibly, bacteria with predatory traits are being outnumbered or outcompeted during times when high bacterial production is coupled to high phytoplankton biomass. Ammonia is being consumed at a higher rate by heterotrophic bacteria (51) while phytoplankton preferentially use ammonia as N-substrate for primary production (52, 53). During phytoplankton blooms and nutrient-rich upwelling events, bacteria have access to large pools of organic carbon and other growth factors (54–56). When upwelling and phytoplankton blooms cease, however, bacteria must compete over much more limited particulate and dissolved organic nutrients. SC Wharf time series data indicates that predatory bacteria thrive relative to the rest of the bacterial community during these times.

Global samples reveal similar environmental trends in predator bacterial abundance, given considerations for the differences in sampling methods. The strongest effect seen in TARA Oceans data was of increasing abundance of T6SS genes with depth, followed by decreasing abundance with increased autotroph abundance. The T6SS gene frequency global patterns indicate that relative abundance of bacterial predators increases as resources become scarce. These bacteria become more prevalent in deeper water, as available particulate organic carbon (POC) and dissolved organic carbon (DOC) sources diminish (57, 58). Moreover, predatory bacteria are more prevalent in areas with lower biomass of primary

producers, such as the gyres and non-coastal environments (Fig. 4). Overall, these results agree with the results from the SC Wharf time series and demonstrate that predatory bacteria gain a competitive advantage at times and places where resources are limited.

4.3 Importance of Predatory Bacteria to Ecosystem function

The structuring of bacterial communities is known to be influenced by factors including top-down control such as protist grazers that feeds on heterotrophic bacteria (9, 10, 59), bottom-up control such as the availability of nutrients and organic matter (33, 34), and allelopathic chemicals such as antibiotics (60–62). The impact of predatory bacteria on bacterial assemblages, however, has rarely been considered as a potential factor. Besides known environmental, biological, and chemical factors, this study suggests that bacteria with predatory traits could also be playing a role in structuring bacterial communities in the ocean. The presence of predatory bacteria is strongly correlated with a later reduction in number of many the most common bacterial OTUs in both size fractions (fig. 7a and 7b). While this correlational analysis cannot definitively pinpoint the cause of this relationship, it suggests bacteria-bacteria predation, with predators targeting specific bacterial OTUs, as a possible cause. If this is the case, bacterial predators may not only influence the structure of the bacterial assemblage but may also influence their successional dynamics, and subsequently, impact ocean biogeochemical cycling. By changing the relative proportion of bacterial OTUs, predatory bacteria can change the dynamics within the bacterial community and their collective function in recycling of nutrients (microbial loop) and the microbial transformation of organic carbon from labile to recalcitrant states (microbial carbon pump) (63, 64). Shedding light to some unconsidered factors such as bacteria-bacteria predation is therefore crucial to understanding the mechanisms that control ocean ecosystems health and function.

Figures and Tables

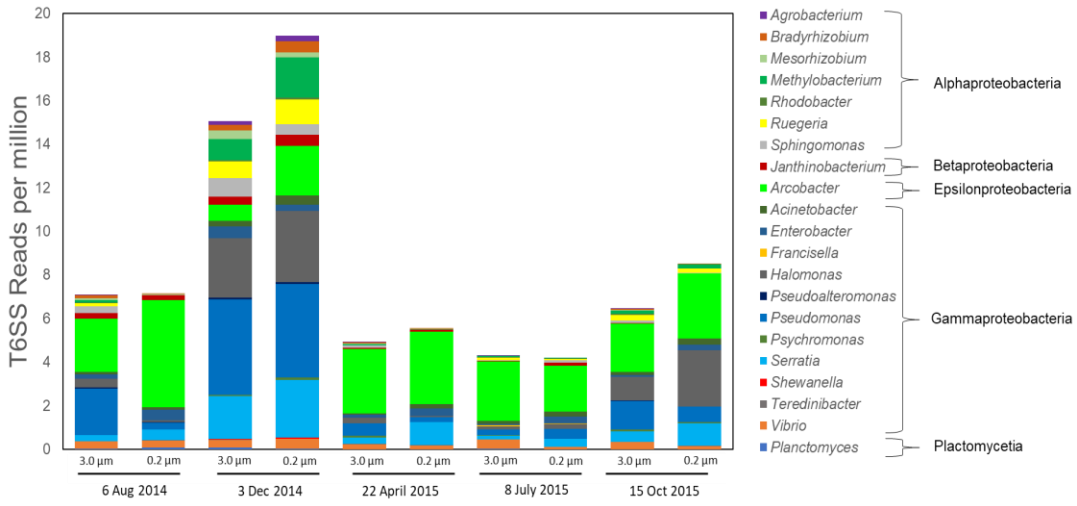

Fig. 1. Measure of metagenome reads belonging to T6SS in RPM across 10 samples from 5 time points. Columns represent a single filter fraction (3.0 or 0.2 µm) and are grouped by sample date. Samples were chosen for range in predator relative abundance via 16S NGS from highest observed (6 August 2014) to lowest observed (8 July 2015). The remaining samples were chosen from samples with medium relative predator abundance to cover all seasons and to include a high concentration bloom of toxic Pseudo-nitzschia (22 April 2015, 1.2x10^5 cells/L). 21 genera of T6SS-containing bacterial predators were present across these samples, identified both by metagenome reads of core T6SS structural genes and by 16S next generation sequencing.

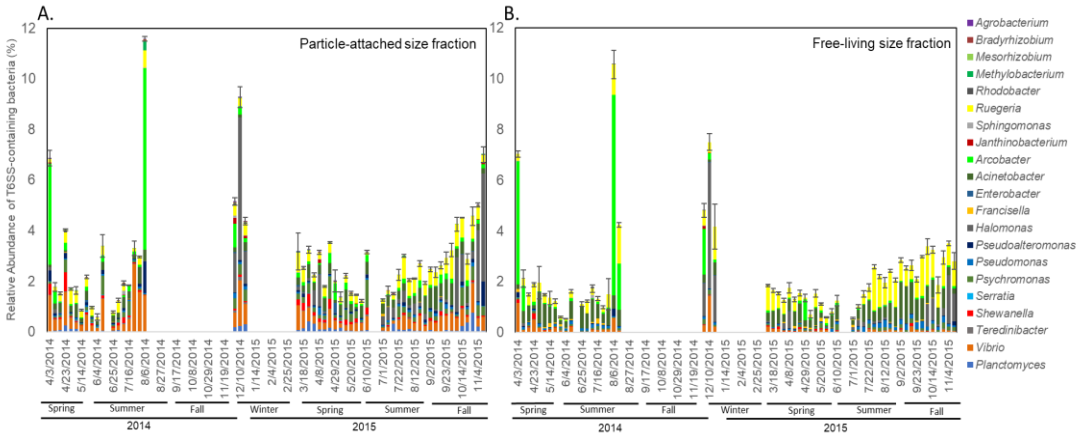

Fig. 2. Relative abundance of bacterial genera with T6SS-encoding genes by filter size fraction 2014-2015. A. particulate-attached 3.0 µm filter size fraction. B. free-living 0.2 µm filter size fraction. Relative abundance (%) is based on the number of reads from 16S amplicon sequencing belonging to each bacterial genus, averaged across triplicate samples. Error bars represent standard error of the mean for the total population of predatory bacteria across those samples.

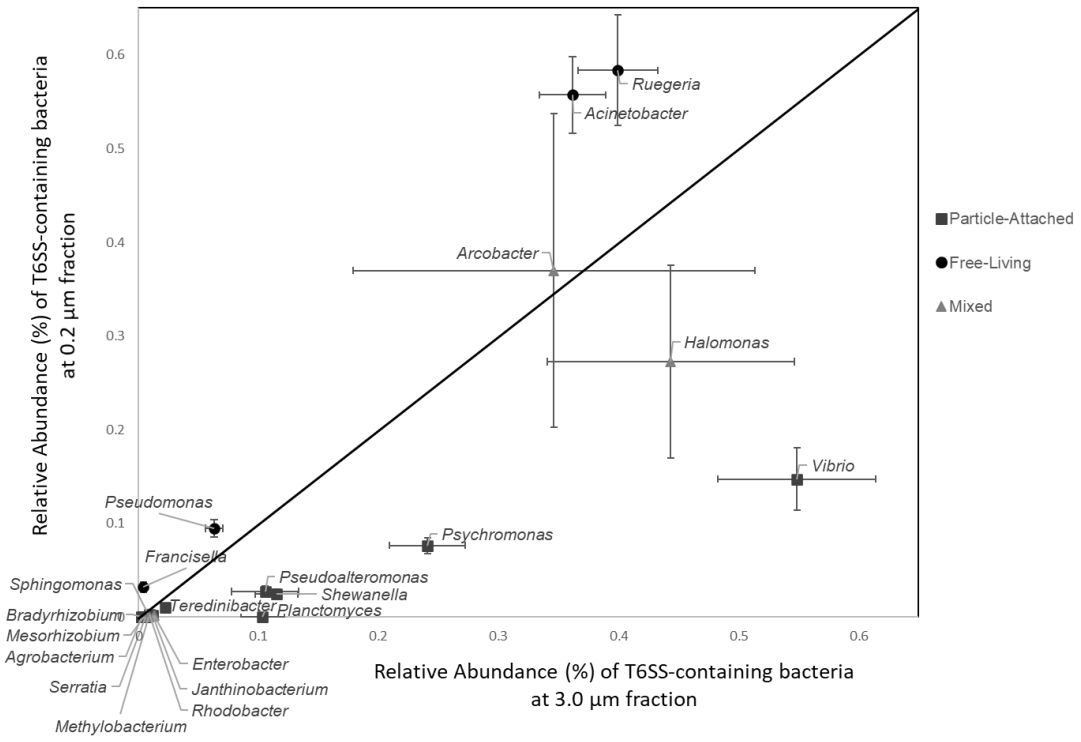

Fig. 3. Prevalence of predatory bacteria in the particulate vs. free-living fraction. Bacterial genera are plotted by averaging their relative abundance across all samples from 2014 and 2015 at both 3.0 µm (x-axis) and 0.2 µm (y-axis) size fraction. Error bars represent standard error of the mean. Particulate-attached or free-living lifestyle was determined by comparison of the means using Two-way Student's T test (table 1, $p<0.05$) between abundance at each filter size across all time points.

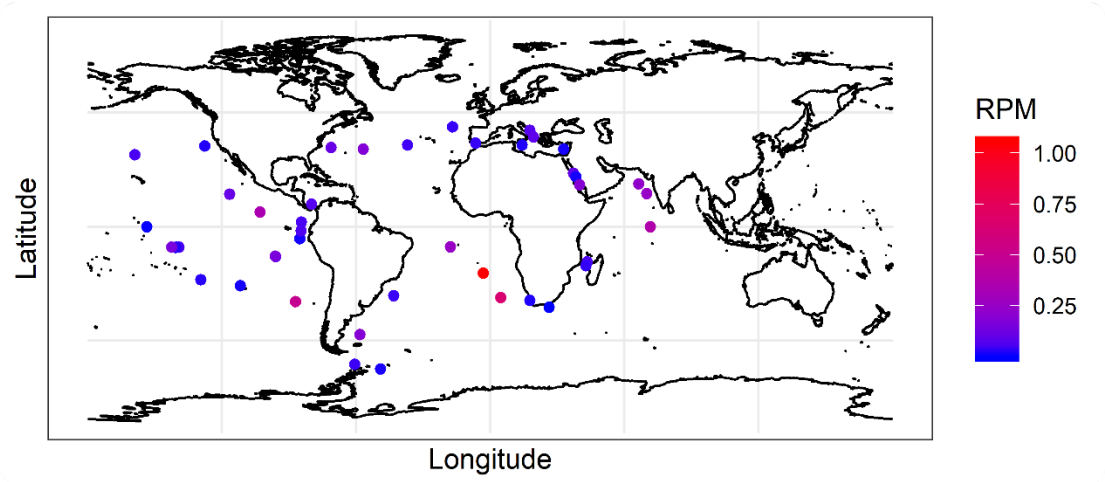

Fig. 4. Heatmap of global distribution of *hcp* and *vgrg* gene sequences. Charted in reads per million, calculated from TARA Oceans metagenome dataset. Hotspots of T6SS abundance appear in the South Atlantic and South Pacific gyres, known areas with extremely low biomass and low nutrient concentration. Reads matching known *hcp* and *vgrg* gene sequences were acquired using BLAST+ 2.9.0. Map was built using data from R Natural Earth. Code is available as supplemental materials.

Table 1. Total relative abundances of bacteria of interest, and results of two-way student's t-tests to determine most common lifestyles for those bacteria. Total relative abundance here is the percent of all Illumina NGS reads across all samples of a filter size belonging to each predatory genus out of total returned reads. Two-way t-test was performed between raw abundances of a single genera at each filter size. "Attached" genera showed a tendency for greater abundance in the 3.0 µm filter size, while "Free-living" genera showed a tendency for greater abundance in the 0.2 µm filter size ($p<0.05$). "Mixed" genera did not show a significant statistical tendency for either lifestyle based on available data ($p>0.05$).

Class	Genus	Mean Relative Abundance (%) and Standard Error				Lifestyle by T-Test	P-value
		3.0 µm fraction	3.0 µm SE	0.2 µm fraction	0.2 µm SE		
Planktomycetia	Planctomyces	0.1034	0.0178	0.0000	NA	Attached	0.0001
Alphaproteobacteria	*Bradyrhizobium*	0.0028	0.0012	0.0000	NA	Attached	0.0111
	Mesorhizobium	0.0045	0.0009	0.0000	NA	Attached	0.0001
	Methylobacterium	0.0082	0.0068	0.0000	NA	Mixed	0.1146
	Agrobacterium	0.0028	0.0011	0.0000	NA	Attached	0.0063
	Ruegeria	0.3993	0.0331	0.5836	0.0591	Free	0.0040
	Rhodobacter	0.0091	0.0028	0.0026	0.0016	Attached	0.0210
	Sphingomonas	0.0075	0.0031	0.0037	0.0025	Mixed	0.1712
Betaproteobacteria	*Janthinobacterium*	0.0114	0.0051	0.0022	0.0018	Attached	0.0402
Epsilonproteobacteria	*Arcobacter*	0.3461	0.1461	0.3700	0.1676	Mixed	0.4562
Gammaproteobacteria	*Acinetobacter*	0.3616	0.0276	0.5574	0.0410	Free	0.0001
	Enterobacter	0.0133	0.0073	0.0017	0.0006	Mixed	0.0605
	Francisella	0.0041	0.0016	0.0320	0.0058	Free	0.0001
	Halomonas	0.4433	0.1419	0.2728	0.1029	Mixed	0.1599
	Pseudoalteromonas	0.1056	0.0279	0.0274	0.0062	Attached	0.0033
	Pseudomonas	0.0631	0.0071	0.0948	0.0092	Free	0.0036
	Psychromonas	0.2409	0.0316	0.0764	0.0086	Attached	0.0001
	Serratia	0.0059	0.0014	0.0000	NA	Attached	0.0001
	Shewanella	0.1149	0.0180	0.0245	0.0052	Attached	0.0001
	Teredinibacter	0.0221	0.0033	0.0105	0.0013	Attached	0.0006
	Vibrio	0.5486	0.0659	0.1474	0.0330	Attached	0.0001

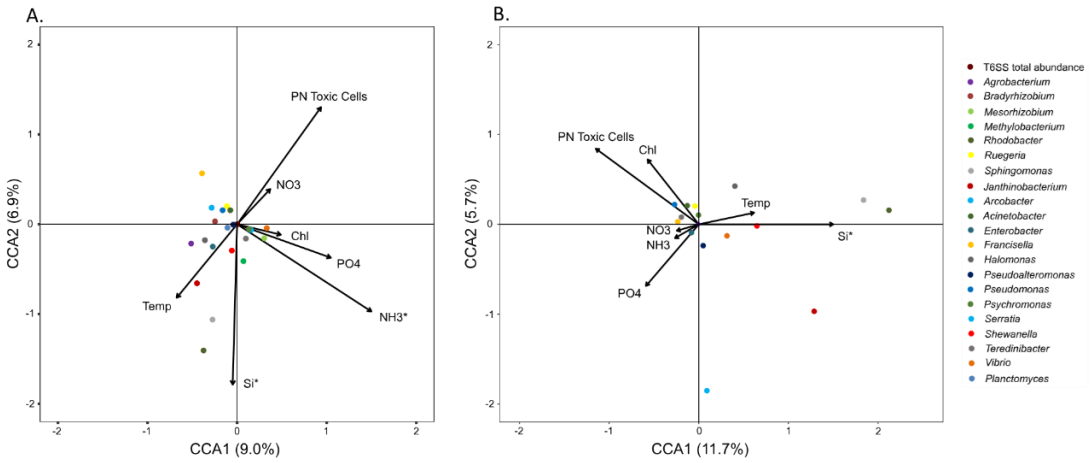

Fig. 5. Canonical correspondence analysis (CCA) of bacterial relative abundance and environmental conditions from Santa Cruz Wharf time series samples. Graphs show bacterial response by genera with T6SS to overlaid environmental variables. A. attached (3.0 µm filter fraction) community CCA. B. free-living (0.22 µm filter fraction) community CCA. Circles: response of bacterial relative abundance to environmental factors, colored by genera. *: significant environmental variables ($p < 0.05$). Considered environmental factors were toxic Pseudo-nitzschia (PN Toxic Cells) cells/mL, nitrate (NO3), ammonia (NH3), phosphate (PO4), and silicate (Si) concentrations, chlorophyll concentration (chl), and water temperature (temp). R code available in supplemental materials.

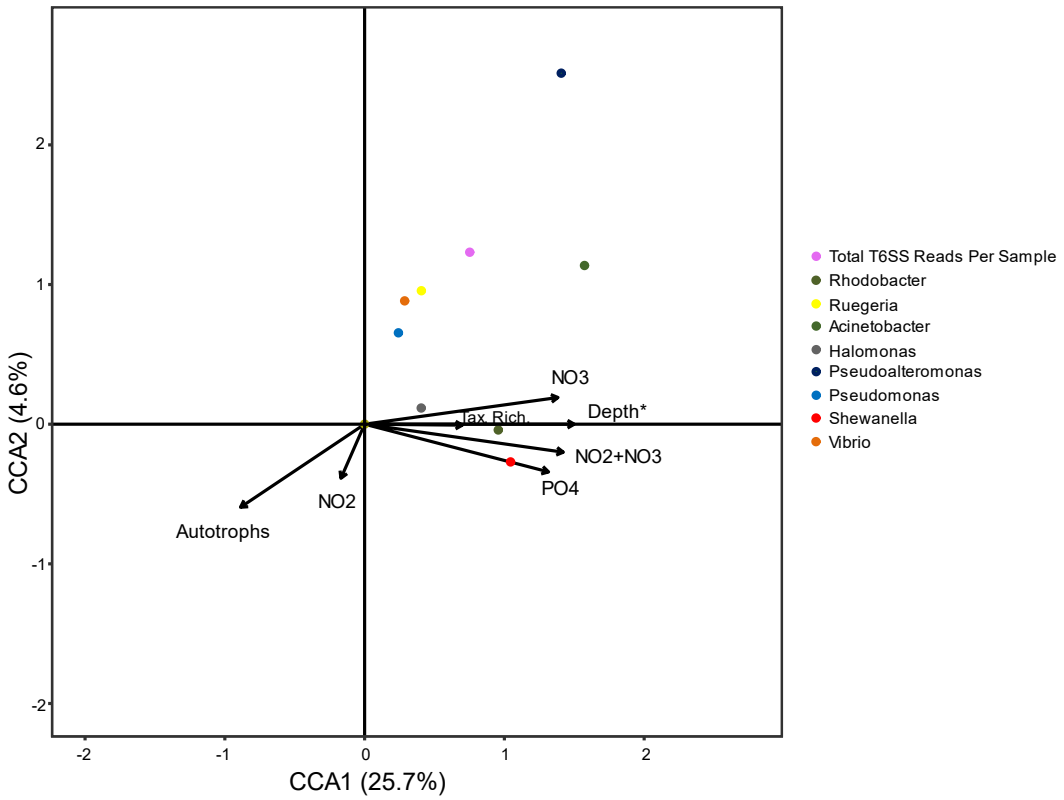

Fig. 6. Canonical correspondence analysis (CCA) of T6SS RPM and environmental variables from TARA Oceans metagenome samples. Graph denotes overall response of *hcp* and *vgrg* reads and response of site totals to overlaid environmental conditions. Colored circles: T6SS vgrG and hcp RPM response in relation to environmental factors, colored by genera. *: significant environmental variables ($p < 0.05$). Environmental variables considered include concentrations of bacteria and autotroph cells, Nitrate (NO3), nitrogen dioxide and nitrate in combination (NO2+NO3), and phosphate (PO4), taxonomic richness (Tax.Rich.) and sample depth (Depth), R code available in supplemental materials.

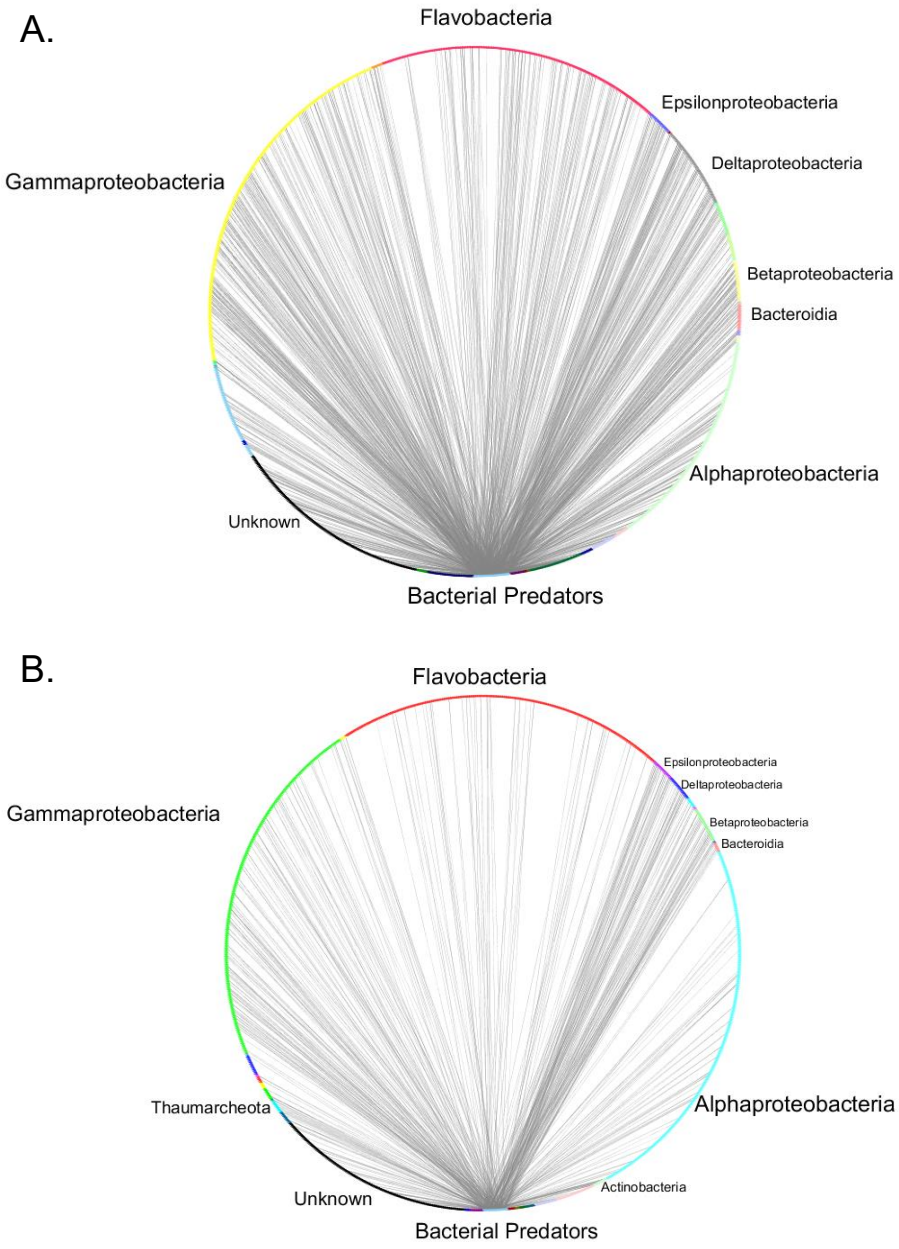

Fig. 7. Network diagrams of potential predation activity. A. Particulate-attached fraction (3. 0 µm). B. Free-living fraction (0.2 µm). Interactions were determined using time-lagged cross correlational analysis, with a time lag of 1 week. T6SS bacterial predators are at the base of each network diagram ("Bacterial Predators") while remaining OTUs are grouped and labeled by class. Each network connection (grey lines) indicates the relationship of one predator to one other bacterial OTU which showed significant negative correlation with predator abundance ($p < 0.05$). These lines indicate possible predatory relationships, indicating bacterial OTUs that decreased in relative abundance following high predator relative abundance or showed increase in relative abundance following low predator relative abundance.

Chapter 3: Conclusions and Future Directions

This research shows that T6SS-containing predatory bacteria are common and potentially ecologically important members of marine microbial communities. These predators were present in every sample, local and global, and increased as a proportion of the bacterial community under specific environmental conditions. It appears that predators can be grouped by the environmental variables they respond to, with at least two distinct groups appearing in our analysis. The first group is tied most closely with nutrient concentrations and chlorophyll, while the second group responds with silicate concentration and water temperature. There also remain a number of predatory genera that do not respond to the factors considered in this study. The group of predators responding with silicate and temperature may include members coming from deep water with upwelling plumes, as silicate is a marker for upwelled waters in Monterey Bay (31), and some of these same bacteria, *Rhodobacter* and *Shewanella*, are seen more commonly in deep waters in worldwide samples. However, when considering the component of water temperature and that these bacteria make up an extremely low portion of the community, it seems probable that these predators are being introduced by rainwater runoff and increasing in relative abundance in the marine community following rains along the California coast. The group of predators responding to nutrients and autotrophs is more ecologically interesting and contains the genera that are most abundant through the SC Wharf time-series, including *Ruegeria, Acinetobacter*, *Arcobacter*, *Vibrio*, *Psychromonas*, and *Pseudomonas*. Our analyses of both local time-series data and worldwide sampling data indicate that these bacterial predators increase in relative abundance compared to the rest of the bacterial community under limited nutrient and carbon resources. Resource limitation for bacteria can take many forms but appears to generally be associated with decreased abundance or biomass of primary producers.

T6SS in bacteria is likely an adaptation to temporal and spatial resource limitation, providing a means of resource acquisition and allowing predatory bacteria to thrive under

these shifting conditions compared to the rest of the bacterial population. From the data presented here, we expect that the energetic cost of production of T6SS outweighs its potential benefits when available nutrients are abundant. In times of depleted nutrients and carbon sources, however, it appears that access to T6SS provides a competitive advantage. T6SS bacterial predators increase in proportion of the bacterial population during these times, indicating that T6SS helps these predators outcompete their neighbors through acquisition of resources and reduction of local competition.

In times and places with depleted nutrients and few autotrophs, little material is available for bacterial consumption from the environment, and competition for resources must necessarily be high. With patterns of increased bacterial competition for limited resources, the evolution and/or acquisition of T6SS grants a competitive advantage to non-obligate bacterial predators. These predators are able to survive times and places of high nutrient availability through uptake from the environment and kill their neighbors to acquire nutrients when they aren't otherwise readily available. T6SS, then, likely provides an evolutionary strategy that is beneficial to these predators even though they do not use the system at all times. Maintaining this acquired system of predation requires genetic upkeep of up to 13 core genes required for T6SS function (37), but this analysis suggests that cost is offset by the benefits to survival in nutrient-deplete conditions.

Additional work needs to be done to fully understand the ecological impacts of T6SS predatory bacteria in marine ecosystems. Studying the expression of T6SS-associated genes from available metatranscriptomes or metaproteomes, for instance, is essential to determine when T6SS is being produced and employed by bacterial predators. Our study has provided evidence that bacteria with T6SS thrive in times and places with low nutrient and carbon availability. Hence, it might be inferred that predators with T6SS predate during those times to gather nutrients and reduce local competition. Transcriptomics or proteomics, on the other hand, will allow the direct measurement of times and places where T6SS is being employed

by bacterial predators. Moreover, additional time-series samples can also increase coverage of relative abundance data and will reduce the effects of any anomalous conditions on our analyses, such as the “warm blob” that persisted in the northern Pacific Ocean in 2014 (65), and increase the statistical power of the analyses performed. Also, the potential predatory interactions explored through time-lagged cross correlation should be studied either in situ or in the laboratory to confirm predator-prey interactions are occurring between these organisms. Taken together, these follow-up studies will be a rigorous test of the inferences made from the data collected and reported in this study and can confirm that T6SS predators specifically, and bacterial predators in general, play a significant role in microbial community dynamics.

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