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ORIGINAL ARTICLE

Prey bacteria shape the community structure of their predators

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Although predator–prey interactions among higher organisms have been studied extensively, only few examples are known for microbes other than protists and viruses. Among the bacteria, the most studied obligate predators are the *Bdellovibrio* and like organisms (BALOs) that prey on many other bacteria. In the macroscopical world, both predator and prey influence the population size of the other's community, and may have a role in selection. However, selective pressures among prey and predatory bacteria have been rarely investigated. In this study, *Bacteriovorax*, a predator within the group of BALOs, in environmental waters were fed two prey bacteria, *Vibrio vulnificus* and *Vibrio parahaemolyticus*. The two prey species yielded distinct *Bacteriovorax* populations, evidence that selective pressures shaped the predator community and diversity. The results of laboratory experiments confirmed the differential predation of Bacteriovorax phylotypes on the two bacteria species. Not only did Bacteriovorax Cluster IX exhibit the versatility to be the exclusive efficient predator on *Vibrio vulnificus*, thereby, behaving as a specialist, but was also able to prey with similar efficiency on *Vibrio parahaemolyticus*, indicative of a generalist. Therefore, we proposed a designation of versatilist for this predator. This initiative should provide a basis for further efforts to characterize the predatory patterns of bacterial predators. The results of this study have revealed impacts of the prey on Bacteriovorax predation and in structuring the predator community, and advanced understanding of predation behavior in the microbial world.
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Introduction

Predation has been studied extensively among animals (Sinclair *et al.*, 2003; Finke and Denno, 2004); however, in the microbial world it has not been well investigated (Jurkevitch, 2007). Of the microorganisms, viral and protistan predation have received greatest attention (Wildschutte *et al.*, 2004; Jurkevitch, 2007; Danovaro *et al.*, 2008). Among the obligate predatory bacteria, the most studied are several genera known collectively as the *Bdellovibrio* and like organisms (BALOs), which prey on many Gram negative bacteria (Guerrero *et al.*, 1986; Schoeffield and Williams, 1990; Jurkevitch *et al.*, 2000). Contrary to the higher forms where predation and multiplication are not directly linked, the bacterial prey for the BALOs not only serves as a food source but also a growth and multiplication chamber. The predatory cycle of BALOs is unique in being biphasic with an extracellular 'hunt' phase, in which the organisms search for and attack its prey, and an intracellular phase. The latter phase begins when, following attack, the predator penetrates through the prey cell's outer membrane into its periplasmic space where it grows, multiplies and lyses the prey (Rendulic *et al.*, 2004) (Figure 1).

The *Bacteriovorax* are in the BALO group and are ubiquitous in salt–water environments (Pineiro *et al.*, 2008). As obligate predators, the growth of wild-type *Bacteriovorax* and other BALOs requires co-cultivation with a prey bacterium, which negates their characterization by the many cultural methods that demand pure cultures. Therefore, few methods are available for distinguishing between BALO isolates. One approach, comparative analysis of 16S rRNA sequences, has enabled identification and detection of specific *Bacteriovorax* phylogenetic clusters or operational taxonomic units (OTUs) within populations (Pineiro *et al.*, 2004). This has made possible geographical distribution studies that have found that some OTUs occur widely and in diverse environments; whereas, others are restricted to certain ecosystems (Pineiro *et al.*, 2007). Such

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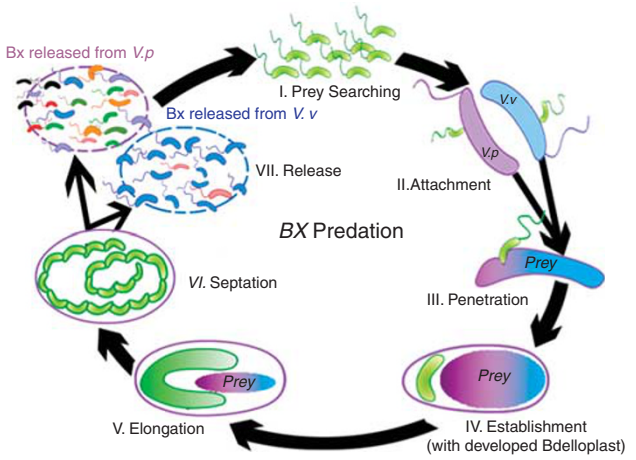

Figure 1 Schematic diagram showing typical *Bacteriovorax* (Bx) life cycle (I to VII), and the major finding of this study that Bx predation on two preys, Vp and Vv, yielded two distinct predator communities (VII) consisting of different Bx OTU clusters represented by the different colors, and defined by 16S rRNA gene sequences. Step I shows unknown environmental Bx community searching for prey.

findings have advanced knowledge of *Bacteriovorax,* and raised new questions about environmental parameters that account for the varied distribution patterns of different OTUs. Salinity and temperature are among such parameters (Williams and Falkler, 1984; Pineiro *et al.*, 2007) and there is speculation on a role for the prey (Sutton and Besant, 1994; Pineiro *et al.*, 2004). In this scenario, different prey species select for distinct populations of *Bacteriovorax* OTUs, and thereby influence the presence, distribution and services of the predators in nature.

Unfortunately, there have been few, if any, investigations on the impact of prey on the shaping of the predator community. Studies have examined the influence of BALO isolates on the bacteria prey community. In one study, in which a single BALO isolate was inoculated into a suspension of several bacterial species, the predator was observed to preferentially prey on certain bacteria (Rogosky *et al.*, 2006). However, to show the influence of any selective pressure of the prey on the selection of predators, requires a corollary study to investigate the response of multiple *Bacteriovorax* OTUs to a single prey species. No such studies have been reported.

In this study, we describe an investigation on the responses of different native *Bacteriovorax* OTU populations in environmental water samples to two prey bacteria, *Vibrio vulnificus* and *Vibrio parahaemolyticus*. As the two tested prey are of the same genus, we hypothesized that different *Bacteriovorax* clusters would respond similarly to them, which surprisingly was not found to be the case. The significance of this may have broad implications for the distribution and role of BALOs in bacterial mortality and community structure.

Materials and methods

Sampling strategy

To test the hypothesis, water samples were collected from sites in three different bodies of water located in Florida (USA): Dry Bar in Apalachicola Bay on three occasions, the eastern coast of the Gulf of Mexico and Atlantic Ocean coastal waters of northern Florida (Supplementary Table 1).

Sampling protocols

Ten litres of bottom water were retrieved from both sides of a NERR research vessel (25-foot, C-Hawk) using a sterile sampler at a depth of approximately 1.74 m. Physiochemical parameters (temperature, salinity, dissolved oxygen and pH) were measured and recorded on site (Supplementary Table 1). Following collections, the samples were stored on ice and transported to the laboratory at Florida A&M University and cultured for *Bacteriovorax.* Within 6 h the samples were used to establish the microcosms described below.

Bacterial strains and growth conditions

The two bacterial species used as prey were as follows: *V. vulnificus* FLA042 (*Vv*), a capsulated spontaneous rifampicin-resistant mutant of a virulent environmental strain MLT403, provided by Dr Paul Gulig, University of Florida and *V. parahaemolytics* strain P-5 (*Vp*) obtained from our laboratory stock collection, and is the most widely used prey bacterium for cultivation of *Bacteriovorax* from salt water systems (Schoeffield and Williams, 1990; Rice *et al.*, 1998; Pineiro *et al.*, 2004). Prey suspensions to establish the enrichment microcosms or for plating for *Bacteriovorax* recovery, were prepared by adding 5 ml of 70% artificial sea water (Instant Ocean, Aquarium Systems, Inc., Mentor, OH, USA) (pH 8, salinity 22 p.p.t.) to an overnight culture of either *Vv* grown on Luria-Bertani (LB) culture plates (Difco, Sparks, MD, USA) or *Vp* on sea water yeast extract agar (Kaneko and Colwell, 1973). The colonies were suspended in the liquid and the suspension was transferred into a sterile tube for subsequent use.

Establishment of laboratory microcosms

Immediately after being transported to the laboratory, water samples were mixed and filtered through a 0.8 μm filter (Nalgene, 0974025AA, Rochester, NY, USA) to remove debris and larger organisms, such as some protists. Five hundred ml of the filtrate was dispensed into each of four 2 l Erlenmeyer flasks for the microcosm-enrichment experiments. For subsequent analysis by denaturing gradient gel electrophoresis (DGGE), another 500 ml of the filtrate was filtered through 0.1 μm filter (Whatman, 111105, Florham Park, NJ, USA) to capture the microbial populations including *Bacteriovorax* on the filters, which were stored at −20 °C.

To complete the microcosms for the enrichment of *Bacteriovorax*, suspensions of *Vv* and *Vp* were spiked as prey into the respective flasks, described above to yield an optical density (OD) measurement of 0.7 at 600 nm. This corresponds to approximately 5×10^8 cells ml^{-1} as enumerated by plate count on LB-rif agar plates (LB agar with 50 μg ml^{-1} rifampicin (Sigma Chemical Co., St Louis, MO, USA)). The two control microcosms established to monitor the OD of the prey without interference from *Bacteriovorax* or other microorganisms, consisted of equal volumes of prey in sterilized environmental water. The microcosm flasks were shaken at room temperature and monitored at 24 h intervals through 120 h, by OD measurements (at 600 nm) in 48-well microtiter plates by an Absorbance Microplate Reader (Biotek, Winooski, VT, USA).

Quantification of prey bacteria
The viable prey bacterial counts in the test and control flasks were conducted at selected time points, by spread plating in duplicate 0.1 ml of serial 10-fold diluted samples onto LB-rif or sea water yeast extract agar agar plates for culture of *Vv* and *Vp,* respectively. The plates were incubated at 37 °C for two days, and colony-forming units were counted and recorded.

Isolation of predominant Bacteriovorax *strains in microcosms*
Samples from the test microcosms were cultured for *Bacteriovorax* using the double agar overlay technique (Williams and Falkler, 1984). Before (pre-spike) and immediately (0 h) after addition of the prey bacteria, 5 ml of sample were inoculated into Pp20 top agar tubes, with 1 ml of prey bacteria (*Vv* or *Vp*) and plated onto large culture plates (150×15 mm). At subsequent time points, samples from the test microcosms were diluted by a series of 10-fold dilutions. One ml of the dilutions was inoculated into 3.5 ml of molten Pp20 top agar tubes with 500 μl of prey. The contents of the tubes were mixed and overlaid onto Pp20 bottom agar plates. The plates were incubated at room temperature for up to 8 days. Randomly selected plaques were examined by fluorescence microscopy for typical *Bacteriovorax*-like cells. Plaque-forming units were counted and recorded. Plates with approximately 30 or less plaques were selected for further processing, as these represented the predominant culturable *Bacteriovorax* OTU within the microcosm at the time of plating. Materials from 80 to 100% of the plaques on these plates were collected with sterile micropipette tips, and each was inoculated respectively into a 1.5 ml eppendorf tube containing 50 μl of autoclaved MiliQ water, and stored at -20 °C for subsequent PCR analysis.

PCR amplification and phylogenetic assay
The tubes containing the selected *Bacteriovorax* plaques were boiled at 100 °C for 20 min. Ten μl of the suspension was PCR amplified using *Bacteriovorax*-specific primers, Bac-676F (5′-ATTTCGCATG TAGGGGTA-3′) and Bac-1442R (5′-GCCACGGCTTC AGGTAAG-3′) (Davidov *et al.*, 2006) by puReTaq Ready-To-Go PCR Beads (GE Healthcare Bio-Sciences, Little Chalfont, UK). All amplifications were performed under the following thermal conditions: initial denaturation at 95 °C for 3 min, followed by 34 cycles of 96 °C for 3 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min in an iCycler thermocycler (Bio-Rad, Hercules, CA, USA). PCR products were analyzed by electrophoresis for amplicons of approximately 760bp, purified with the QIAquick PCR-Purification Kit (Qiagen, Chatsworth, CA, USA) and sequenced with Bac-676F primer at the DNA Sequencing Laboratory at Florida State University. DNA sequences and homology searches were analyzed with the Basic Local Alignment Search Tool server, from the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov). Sequences were also analyzed using the Chimera_ Check, version 2.7 from the RDP-II Web site (Cole *et al.*, 2003). These sequences have been submitted to the GenBank databases under accession numbers GU350212–GU350224.

To compare the diversity of *Bacteriovorax* clusters with the two preys, Shannon–Weaver index was calculated using Estimates program (http://viceroy.eeb.uconn.edu/estimates) with 50 randomizations.

Analysis by denaturing gradient gel electrophoresis
For DGGE analysis (Muyzer *et al.*, 1993), 20 ml samples were removed from the test microcosm at 24 h intervals and centrifuged at 11 952 *g* for 25 min at 4 °C. The supernatant fluid was discarded and the resulting cell pellet was preserved at −20 °C. Subsequently, total DNA from the cell pellets and the bacteria previously concentrated on filters from the pre-spiked microcosm water samples were extracted using the QIAGEN Kit (QIAamp DNA Mini Kit), according to the manufacturer's protocol. PCR amplifications were performed with puReTaq Ready-To-Go PCR Beads using universal bacterial primers, GM5F (5′-CCTACGGGAGGCAGCAG-3′) with a GC clamp attached to its 5′ end and 907R (5′-CCGTCAATTCCTTTGAGTTT-3′) (Muyzer *et al.*, 1995). The amplified DNA was quantified by a NanoDrop Spectrophotometer (ND 1000, Thermo Fisher Scientific, Wilmington, DE, USA), and standardized to an equal concentration. Fifteen μl of PCR products were then run on a 6.5% polyacrylamide gel with a denaturing gradient of 30–60%. The gel was run in 1x TAE buffer at 60 °C and 100 V for 16 h on a Dcode Universal

Mutation Detection System (Bio-Rad), then stained with SYBR green (Molecular probes, Invitrogen, Eugene, OR, USA) for 60 min and photographed using Geldoc (Bio-Rad). DGGE bands were excised and the DNA was eluted in 50 µl of ultrapure Milli-Q water overnight at 4 °C. The eluted DNA was again re-amplified using the universal bacterial primer GM5F and 907R without the GC clamp, and sequenced as previously described.

Electron microscopy of microcosm bacteria
For electron microscopic studies, 10 ml samples were removed at the 48 h time point from both *Vv* and *Vp* microcosms and fixed (Koval and Bayer, 1997). Briefly, the cells were centrifuged for 20 min at 11 952 *g*, resuspended in 1 ml of 0.1 M sodium phosphate buffer (pH 7) and centrifuged for 15 min at 10 600 *g*. The pellet was resuspended in 2 ml of 0.1 M cacodylate buffer containing 2% glutaraldehyde and 1% formaldehyde, both diluted from 25% (v/v) and 16% (v/v) stock solutions, respectively. After 60 min at 4 °C and centrifugation at 10 600 *g*, the pellet was overlaid with cacodylate buffer, and an aliquot of samples was stained with uranyl acetate and examined with a Hitachi H-7600 transmission electron microscope (Hitachi High Technologies, America, Inc., Pleasanton, CA, USA).

Challenging Vv *and* Vp *with selected* Bacteriovorax *clusters*
To confirm the results of the previous microcosm experiments, which suggested that the *Vv* and *Vp* selected for specific *Bacteriovorax* phylotypes, a laboratory experiment was designed to test the predation efficiency and growth rate of *Bacteriovorax* Clusters IX and IV against *Vv* and *Vp*. Six microcosms were established consisting of four test flasks and two control flasks. Each of the test flasks contained 100 ml sterilized artificial sea water and equal concentrations of either *Bacteriovorax* Cluster IX or IV (average concentration 1.10×10^6 copies ml^{-1}) and prey, *Vv* or *Vp*, respectively (average concentration 7.47×10^7 colony-forming units ml^{-1}). The same prey concentrations were inoculated into sterilized artificial sea water, as a control to monitor their population excluding the effect of *Bacteriovorax* predation. Cultures were incubated at 27 °C on a shaker for 40 h to monitor the population dynamics between the predator and prey, and their respective abundances at selected time points. The prey in the test and control microcosms were monitored by measurements of OD values every 4 h and by bacterial plate counts at 0, 12, 20 and 40 h.

The *Bacteriovorax* present in the cultures were enumerated by quantitative real-time PCR (Zheng *et al*., 2008). Briefly, 1 ml samples were removed at 4 h intervals and genomic DNA was extracted using the QIAamp DNA Mini kit (Qiagen), with a final product of 100 µl eluted. *Bacteriovorax*-specific primer set, 519F (5′-CAGCAGCCGCGGTAATAC-3′) and 677R (5′-CGGATTTTACCCCTACATGC-3′) was used for quantification of the *Bacteriovorax*. Quantitative real-time PCR analysis was performed by using the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad). The quantitative real-time PCR reaction mixtures (25 µl) were composed of 12.5 µl of iQ SYBR Green Supermix (Bio-Rad), 1 µl of each primer (5 pmol $µl^{-1}$), 1 µl of sample DNA and 9.5 µl of MiliQ water. Thermal cycling conditions were: 2 min at 94 °C, followed by 45 repeats of 30 s at 94 °C, 10 s at 62 °C and 10 s at 72 °C. Each sample was measured in triplicate and negative controls (no template) were included. A 10-fold dilution series of plasmid containing a fragment of the *Bacteriovorax* 16S rRNA gene, was used in the quantitative real-time PCR assay to construct the standard curve (correlation coefficient >0.99).

Statistical analysis
The abundance of predator and prey (log transformed) was analyzed by analysis of variance to detect significant differences among the numbers of bacteria in the various microcosm treatments. When analysis of variance tests were passed, the Holm–Sidak test was performed. The *T*-test was used to compare two groups of treatments when normality and equal variance tests were passed. All statistical analyzes were performed using the Sigmastat, version 3.5, software package.

Results

Predator–prey interactions in microcosm studies
In the five independent experiments with laboratory microcosms, the predator and prey responses exhibited similar patterns (Figure 2). In all cases the inoculated prey, *Vv* or *Vp,* decreased with a corresponding increase in *Bacteriovorax* numbers,

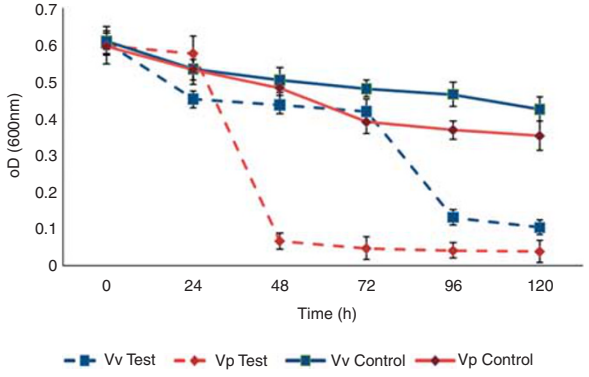

Figure 2 Kinetics of the lysis of *Vv* and *Vp* by *Bacteriovorax* (Bx) over time in test (with Bx), and control (without Bx) microcosms as measured by optical density. Values are means for the five microcosm experiments. Error bar represents the standard error of the mean ($N=5$).

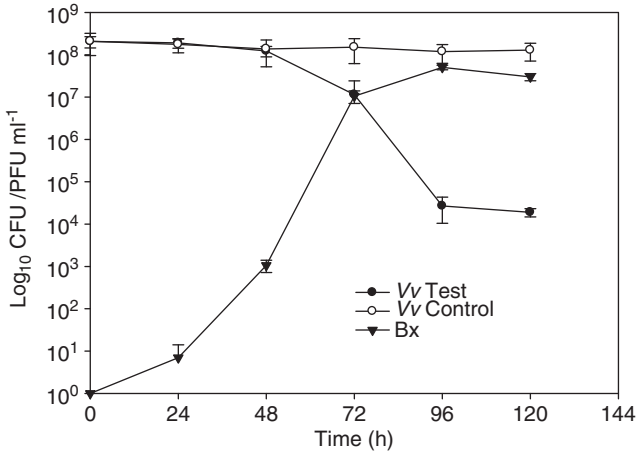

Figure 3 Numbers of *Vv* and *Bacteriovorax* (Bx) in samples taken at different time intervals from the microcosm established with Atlantic Ocean water, and *Vv* showing the typical predator-prey response, which was observed in all five experiments. The control contained prey cells without Bx. Bars indicate standard error.

indicating the predation of the prey by the predator in the microcosms. The difference between the two prey was that the greatest decrease was observed in the *Vp* microcosms after 24 h, whereas, in the *Vv* microcosms, a significant decrease (*t*-test, $P<0.01$) was not observed until after 72 h (Figures 2 and 3). The OD measurements of microcosm samples corresponded to the colony-forming units counts of *Vv* on LB-rif agar plates, and *Vp* on the sea water yeast extract agar plates. At the initiation of the microcosm experiments, the *Bacteriovorax* population that could grow on *Vv* or *Vp* ranged from below detectable levels up to 3 plaque-forming units $5\,ml^{-1}$. However, the predators responded quickly to the inoculated prey resulting in a thousand-fold increase within 24 to 48 h, and typically their numbers peaked at approximately 10^8 plaque-forming units ml^{-1} at 96 h. This increase in *Bacteriovorax* resulted in a 2–4 log prey reduction (Figure 3).

Identification of Bacteriovorax *16S rRNA phylogenetic clusters isolated on the two prey*

The results revealed that irrespective of the source from which the microcosm water was collected, the predominant *Bacteriovorax* isolates, recovered on *Vv,* typically belonged to Cluster IX and occasionally Cluster X (Figures 4a–e). Only in a single case, isolates of Cluster III were detected (Figure 4d). In the first microcosm experiment with water from Dry Bar, all plaques analyzed growing on *Vv* at the 24 h sample were of Cluster X. Plaques from the later time points could not be confirmed to be *Bacteriovorax* (Figure 4a). In the latter four *Vv* microcosm experiments established with waters from Dry Bar, Gulf of Mexico and the Atlantic Ocean, the *Bacteriovorax* isolates detected throughout the term of the experiment consistently belonged to Clusters IX and X. This was in contrast to the *Vp* microcosms, in which shifts among different *Bacteriovorax* clusters from one sampling interval to the next (Figures 4c–e) were frequently observed. Up to six different cluster types were observed over the duration of one experiment (Figure 4d). For the first Dry Bar experiment, the predominant *Bacteriovorax* detected on *Vp* at all time points were Cluster V (Figure 4a). Cluster X, which was predominant at 24 h in the *Vv*-enriched microcosm, was not found in the *Vp* microcosm (Figure 4a). For the second Dry Bar experiment, all the predominant isolates from both *Vv* and *Vp* microcosms were of Cluster IX (Figure 4b). In the third experiment using Dry Bar waters, all *Bacteriovorax* isolates from the *Vv* microcosm were Cluster IX, whereas, five *Bacteriovorax* clusters were recovered from the *Vp* microcosm (Figure 4c). Dramatic shifts in *Bacteriovorax* clusters detected were also observed in the Gulf of Mexico and ocean water *Vp* microcosms (Figures 4d and e). However, Cluster IX, which predominated in the *Vv*-enriched microcosm, was not detected in the *Vp* ocean microcosm and only once in the Gulf of Mexico microcosm (Figures 4d and e). Over the five experiments, a total of eight *Bacteriovorax* OTUs were detected on the two preys. In *Vp* microcosms the Shannon–Weaver index of *Bacteriovorax* biodiversity was higher for all the sampling sites than the index for *Vv* microcosms. The lower index indicates that only a few OTUs accounted for most of the *Bacteriovorax* biomass (Table 1). In Dry Bar #1 and #3 *Vv* microcosms, the index value was zero indicating that the *Bacteriovorax* OTUs detected were all identical.

These results show that the interactions of native environmental *Bacteriovorax* with *Vv* and *Vp* yielded different *Bacteriovorax* OTU populations, and suggest that *Vv* is preferential for Cluster IX. This distinct and rather drastic difference in the *Bacteriovorax* OTU communities from the two prey bacteria was not anticipated.

DGGE analysis of microcosm bacterial communities

DGGE targeting the 16S rRNA genes was used to analyze the bacterial community structure in the microcosms at selected time intervals. The prominent bands in the DGGE gel were excised and sequenced to confirm bacterial species composition. The greater the numbers of a particular bacterium in the sample, the more intense its band appears in the gel. Neither *Vv* nor *Vp* was detected by DGGE in the original water sample before establishing the microcosms. After the microcosms were amended with the prey, an intense band was observed. For the *Vv* microcosm, the prey population gradually diminished with time (Supplementary Figure 1 and Figure 2). For the *Vp* microcosm, the prey band was not detected after 24 h (Supplementary Figure 3) confirming that predation on *Vp* was more rapid than observed on *Vv*. Meanwhile, in both the *Vv* and *Vp* microcosms, the intensity of the *Bacteriovorax* band increased with time. The bands representing other

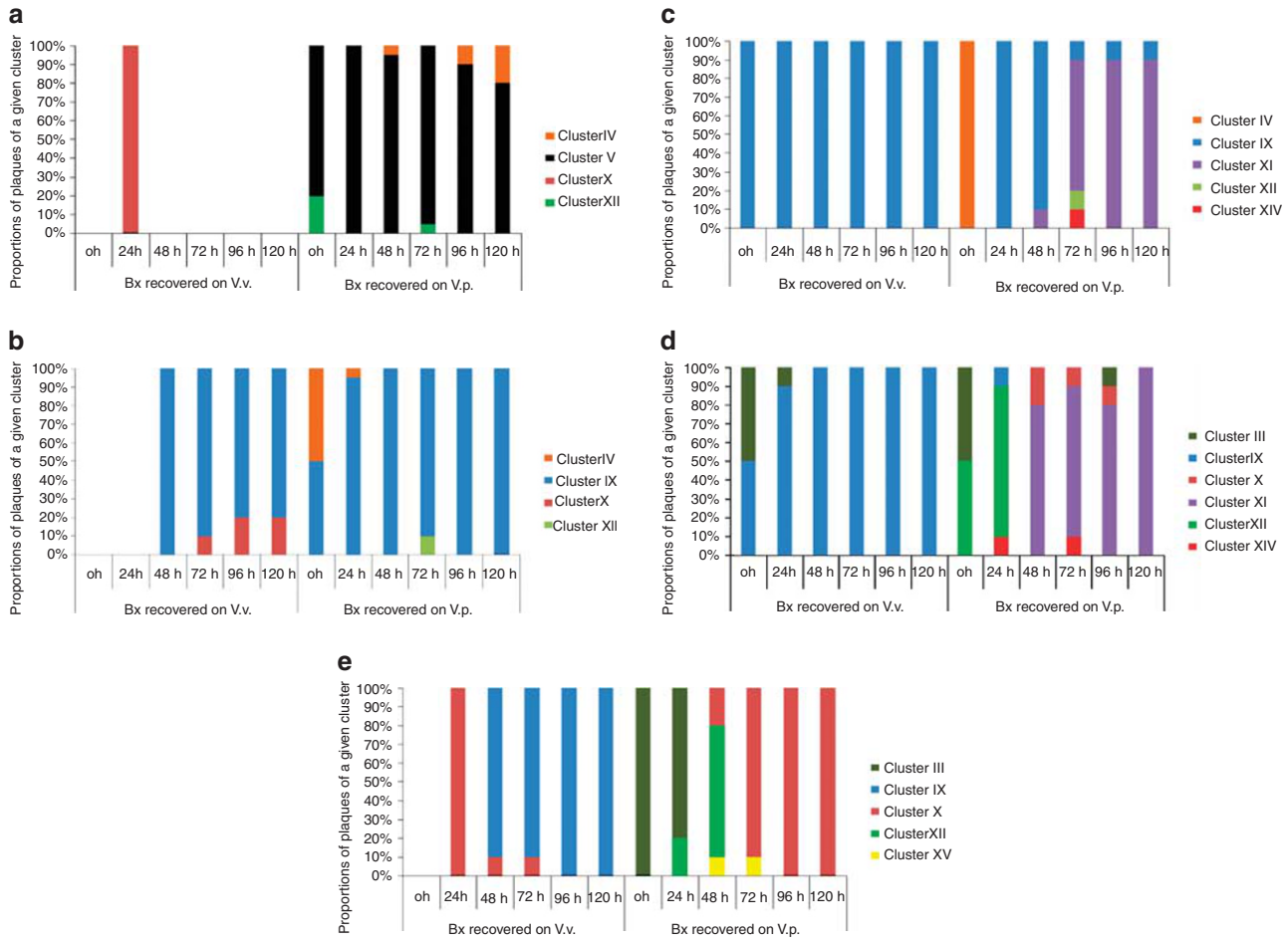

Figure 4 Predominant *Bacteriovorax* operational taxonomic units recovered at various time intervals from the *Vv* (left) and *Vp* microcosms (right) established with water samples from Dry Bar (**a**, **b** and **c**), Gulf of Mexico (**d**) and Atlantic Ocean (**e**). Clusters based on 16S rRNA gene sequence similarity are numbered consistently with previous reports (Davidov and Jurkevitch, 2004; Pineiro *et al*., 2007, 2008).

Table 1 Diversity index of *Bacteriovorax* in microcosms from different sites using *Vv* and *Vp* as prey

Sites	*Shannon–Weaver index*	
	Vv	*Vp*
Dry Bar #1	0	0.41
Dry Bar #2	0.08	0.37
Dry Bar #3	0	1.16
Gulf of Mexico	0.32	1.18
Atlantic Ocean	0.55	1.1

major bacteria present in the *Vv* microcosm at the initial time point, either disappeared or in some cases increased in intensity (Supplementary Figure 1). The predominant *Bacteriovorax* OTUs shown by DGGE analyses were consistent with the results from the culture methods (Figure 4, Supplementary Figures 1, 2 and 3).

Electron microscopic examination of microcosm samples

Examination of *Vv* and *Vp* microcosm samples, respectively, by transmission electron microscopy revealed the attachment of *Bacteriovorax* cells to both the preys (Supplementary Figure 4), and the predator inside prey cells forming the bdelloplast. These observations are further confirmation of predation. Observing that the *Vv* cells are longer but smaller in width than the *Vp* cells, raises the question of the impact of prey size on observed differences in *Bacteriovorax* OTU predation on the two preys.

Challenge of Vv *and* Vp *with selected* Bacteriovorax *clusters*

When *Bacteriovorax* Clusters IV and IX were, respectively, inoculated into independent cultures of *Vv* and *Vp*, each was able to reduce the abundance of the two preys as measured by plate count and OD readings, confirming the potential of *Bacteriovorax* to control both the preys. The reductions in plate counts were significant (analysis of variance, $P<0.001$, Holm–Sidak, $P<0.001$) at 40 h for all treatments. The abundance of *Vv* and *Vp* in the control microcosms remained stable (not significantly different, analysis of variance, $P>0.5$).

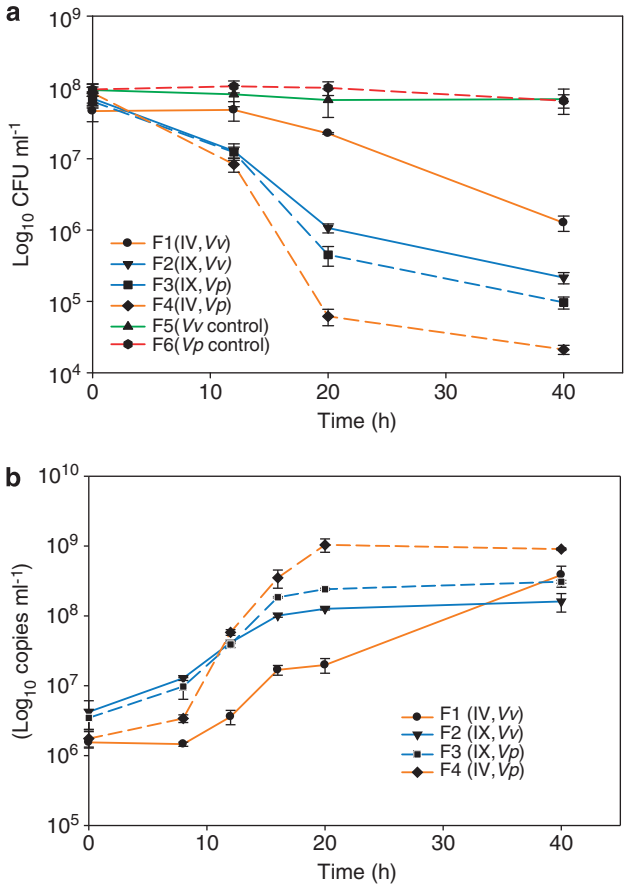

Figure 5 Kinetics of the lysis of *Vv* and *Vp* by *Bacteriovorax* (Bx) predation and the growth of bx on these prey. Bx microcosm flasks designated F1 and F2 contained *Vv* and Bx Clusters IV and IX, respectively. F3 and F4 consisted of *Vp* as prey and the respective Bx Clusters IX and IV. F5 and F6 represent the control microcosms consisting solely of *Vv* or *Vp* respectively without the interference of any predator. (**a**) Prey counts showing the effects of Bx Clusters IX and IV on population dynamics of *Vv* or *Vp* in experimental microcosms. (**b**) Growth dynamics of Bx Clusters IV and IX on *Vv* and *Vp* over a 40 h period as measured by QPCR assay. Values are means for triplicate samples. Error bars represent the standard deviation of the mean ($N=3$).

Both the killing of prey and growth rates of Cluster IV against *Vp* were significantly higher than that against *Vv,* in the first 20 h of incubation (*t*-test, $P<0.01$). At the 20-h time point, Cluster IV reduced the *Vp* by 3.13 logs, but the *Vv* by only 0.31 logs. After 20-h incubation, Cluster IV abundance in the *Vp* microcosm levelled off reaching about 9.1×10^8 copies $ml^{-1,}$ and the killing rate decreased from 0.12 per hour at maximum to 0.03 per hour because of the low number of remaining prey. In contrast, the predation rate of *Bacteriovorax* Cluster IX was not significantly impacted by the type of prey in the first 12 h (*t*-test, $P=0.837$). Cluster IX reduced the *Vv* cells 2.5 logs after 40 h, whereas Cluster IV reduced them by only 1.5 logs (Figure 5a). Corresponding to the decrease in prey, *Bacteriovorax* Cluster IX grew at a much faster rate on *Vv* than Cluster IV in the first 8 h of the experiment (*t*-test, $P<0.01$), whereas no such difference was observed on *Vp* (Figure 5b).

Discussion

The results of this study reveal new insights into the responses of native environmental *Bacteriovorax* communities to different bacterial species. For the first time it has been shown that specific *Bacteriovorax* OTUs vary in their predation on different bacteria, likely due to selective pressures exerted by the prey that yield predator progeny populations that vary in strain richness and composition. This was demonstrated in the responses of native *Bacteriovorax* populations in water samples collected from different ecosystems to two *Vibrio* species, and showed that the interaction of *Bacteriovorax* to specific prey is not random. With a single exception, only two phylotype Clusters, IX and X, were consistently observed to be the predominant *Bacteriovorax* OTU produced in *Vv*. To the contrary, multiple clusters were typically detected from *Vp*. This observation was consistent over multiple experiments conducted in three different water bodies, and was confirmed in a laboratory experiment using selected *Bacteriovorax* isolates in dual culture with the two preys. These findings were surprising as we had expected no difference in the specific *Bacteriovorax* clusters grown on the two *Vibrio* species, as they are closely related, being in the same genus. This establishes Clusters IX and X as keystone predators of *Vv*.

Although the specific reasons for the observed differential predation were not definitively determined, electron microscopic examination of the predator–prey interactions revealed the *Vv* to be considerably smaller in width than the *Vp* cells. Prey size has been reported to be a factor in predation for some microbes (Pernthaler, 2005). The small size of the *Vv* may exclude some *Bacteriovorax*. However, unique properties of *Bacteriovorax* Clusters IX and X, which allow them to overcome the prey exclusion, may explain the differential predation efficiency on the two preys but further investigation is needed. Preferential predation of BALOs on certain bacteria species has been reported (Rogosky *et al.*, 2006); however, no known selective mechanisms such as receptor sites or chemotaxis toward a specific prey have been identified.

Another distinction observed between the responses of the *Bacteriovorax* in the *Vv* and *Vp* microcosms during the 5-day period of the experiment was the stability of the predator population. For the *Vv* microcosms, typically the predominant *Bacteriovorax* cluster type observed initially at 24 h remained relatively stable throughout the duration of the experiments. This was in contrast to the *Vp* microcosms, in which shifts in the predominant *Bacteriovorax* cluster populations were observed with up to six phylotype clusters in one experiment (Figure 4d). The reasons for the shifts in the *Vp* microcosms are not known, but may be because of differences in growth rate over time among the various cluster types, which we confirmed in the laboratory experiment.

When the predation behaviors of BALO clusters from our study are considered in the context of the conventional classification scheme of parasitoid or predators (Symondson *et al.*, 2003), we found that Cluster IX possesses some of the properties of both generalist, which preys upon a broad range of prey, and specialist which typically have unique properties to prey upon a narrow spectrum of prey. However, until now there has been no evidence to categorize the BALOs in this scheme. The observation that Cluster IX not only has the versatility to be the exclusive efficient predator on *Vv*, thereby, behaving as a specialist, but is also able to prey with similar efficiency on *Vp* (Figure 5), is indicative of a generalist. Thus we propose, based on its behavior pattern, that an appropriate designation for Cluster IX is that of a versatilist. The advantage of being a versatilist is apparent, it can prey efficiently on bacteria that other predators typically cannot, thereby, minimizing competition, yet its predation is not restricted to just a single prey.

Although Cluster IX can prey on *Vp* as does Cluster IV, it is less efficient. This reduced efficiency in predation may be a cost to Cluster IX for the benefits it gained as a versatilist. This explains our observation in the ecological studies. When fed the selective prey (*Vv* narrower), versatilist had the advantage to predominate the predator community, whereas with non-selective prey such as *Vp*, they competed with other *Bacteriovorax* clusters resulting in shifts among the predators. Which cluster predominates may be determined by the number and type of the prey present at that time.

If we assume that the selective pressure exerted by the *Vv* prey is its small width making it difficult for BALOs to enter into the cell, then it could be expected that when Cluster IX predator encounters *Vp* not having the width restriction it would prey with equal efficiency as cluster IV. However, this was not observed to be the case. Although speculative, this may suggest that in its adaptation to prey on *Vv*, cluster IX was altered in some way that made it less efficient at preying on other prey. The adaptation made by *Vv* to resist predation by most BALO predators and the counter adaptation of cluster IX to prey on it at relatively high efficiency are very specific events. For two such highly specific and coordinated events to have occurred in two different organisms, raises the possibility of co-evolution.

The superiority of *V. parahaemolyticus* strains in quantitatively recovering *Bacteriovorax,* has made it the most widely used prey bacterium for cultivation of the predators from salt waters systems (Schoeffield and Williams, 1990; Rice *et al.*, 1998; Pineiro *et al.*, 2004). However, until now the question as to if *V. parahaemolyticus* or any other bacterium is capable of capturing the total population of the *Bacteriovorax* community in the environment has remained unanswered. The results from our study show that not all of the *Bacteriovorax* clusters were detected on either *Vp* or *Vv*. This reveals the limitations of prey bacteria in recovering with equal efficiency all BALO OTU's.

Although the significance of *Bacteriovorax* community structure and diversity is only beginning to be recognized (Chauhan *et al.*, 2009), in general, biodiversity has been reported to be an important factor in ecosystem function and responses to environmental changes (Bell *et al.*, 2005; Fuhrman, 2009). Regarding services of BALO populations in the environment, diversity is likely an important factor, especially relative to their potential function in controlling and shaping bacterial communities through selective mortality, which may be dependent upon the specific predator OTUs present. This hypothesis is consistent with the observation in this and other studies (Davidov *et al.*, 2006; Rogosky *et al.*, 2006) that not all *Bacteriovorax* OTUs are equal in their ability to prey on various bacteria. For example, based on the results reported here, the presence of Clusters IX and X in an environmental niche would be expected to be more important factors, than other clusters in contributing to the mortality of *Vv* populations in that situation.

The results of this study have revealed important features, not previously described, of the significance of the prey on *Bacteriovorax* predation and in structuring the predator community. Based on these results, the classification of BALO predators using the conventional classification scheme has been initiated and should provide a basis for further efforts to advance this area. It is of interest to determine in future studies which of these classifications, generalist, specialist or versatilist will prevail with other prey bacteria. Understanding BALO–prey interactions will lead to a greater comprehension and appreciation for the diversity of predation among all organisms.

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