RESEARCH ARTICLE

In situ grazing resistance of Vibrio cholerae in the marine environment

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Abstract

Previous laboratory experiments revealed that Vibrio cholerae A1552 biofilms secrete an antiprototaxial factor that prevents Rhynchononas nasuta from growing and thus prevents grazing losses. The antiprototaxial factor is regulated by the quorum-sensing response regulator, HapR. Here, we investigate whether the antiprototaxial activity is ecologically relevant. Experiments were conducted in the field as well as under field-like conditions in the laboratory to assess the grazing resistance of V. cholerae A1552 and N16961 (natural frameshift mutation in hapR) biofilms to R. nasuta and Cafeteria roenbergensis. In laboratory experiments exposing the predators to V. cholerae grown in seawater containing high and low glucose concentrations, we determined that V. cholerae biofilms showed increased resistance towards grazing by both predators as glucose levels decreased. The relative resistance of the V. cholerae strains to the grazers under semi-field conditions was similar to that observed in situ. Therefore, the antipredator defense is environmentally relevant and not lost when biofilms are grown in an open system in the marine environment. The hapR mutant still exhibited some resistance to both predators and this suggests that V. cholerae may coordinate antipredator defenses by a combination of density-dependent regulation and environmental sensing to protect itself from predators in its natural habitat.

Introduction

In the last 20 years, cholera has occurred in areas that have been free from outbreaks for almost a century (for a review, see Tauxe et al., 1994). The recent increases in occurrences may be due to the fact the Vibrio cholerae El Tor biotype (cause of the seventh and current pandemics) may be more environmentally fit than the Classical biotype (etiological agent for the first six pandemics), and thus has replaced the Classical biotype in the environment. This highlights the need to better understand the factors that affect the occurrence and survival of V. cholerae in the environment. Researchers have begun to use remote sensing to determine whether they can identify correlations between cholera outbreaks and ocean parameters (e.g. phytoplankton and zooplankton blooms, seawater temperature, nutrient concentration) in an attempt to predict outbreaks (Lobitz et al., 2000). Many of the studies monitoring V. cholerae in the marine environment have focused on the effect of nutrient availability (Singleton et al., 1982) and on interactions of V. cholerae with copepods (Huq et al., 1983; Pruzzo et al., 2008). Several studies have shown that V. cholerae attaches preferentially to biotic surfaces such as copepods in the marine environment (Heidelberg et al., 2002; Mueller et al., 2007), while others have demonstrated a preference for planktonic growth of V. cholerae in the water column (Worden, 2006), in which case the bacterial cells experienced heavy grazing pressure by protozoa.

The interactions of bacteria and protozoa are considered to be one of the oldest predator–prey interactions in nature (Cavalier-Smith, 2002). Grazing by phagotrophic protists is one of the main mortality factors of bacteria in marine and freshwater systems (Azam et al., 1983; Hahn & Höfe, 2001; Matz & Jürgens, 2001) and a major selective force for the evolution of bacterial defense strategies (Matz & Kjelleberg, 2005). Predation can alter bacterial morphology and community structure through direct (predation; Hahn & Höfe, 1999; Jürgens et al., 1999) and indirect (nutrient recycling;
Sherr et al., 1982; Pernthaler et al., 1997) interactions. Bacteria have evolved different defense strategies including general avoidance (e.g. motility) and direct consumer effects (e.g. digestive resistance, toxin production) (Matz & Kjelleberg, 2005).

The majority of microorganisms in natural habitats occur as surface-attached communities called biofilms (Davey & O’Toole, 2000), which function to protect cells in the community from a variety of stresses. The biofilm architecture and bacterial-produced extracellular polymeric substances offer important protection against various stresses such as antimicrobial agents (Gilbert et al., 1997) and grazing (Parry, 2004; Weitere et al., 2005). Biofilm formation as well as toxin production are controlled by density-dependent bacterial gene regulation or quorum sensing (QS) in many bacterial species (Hammer & Bassler, 2003; Turovskiy et al., 2007). For example, in the pathogens Pseudomonas aeruginosa and V. cholerae, QS regulates the production of toxins that have been shown to kill predators, resulting in grazing resistance (Matz et al., 2004, 2008). While several studies have assessed the interactions of protozoa and V. cholerae in the suspended state and planktonically in mesocosms (Macek et al., 1997; Worden, 2006), surprisingly little is known about the impact of protozoa on the occurrence of attached V. cholerae.

In laboratory studies, we have shown that microcolony formation in biofilms of V. cholerae (Matz et al., 2005) and P. aeruginosa (Matz et al., 2004) protected against grazing losses. Further, it has been demonstrated that biofilms have antiprotzoal activity (Matz et al., 2004; Weitere et al., 2005). Matz et al. (2005) demonstrated that biofilms of V. cholerae A1552 wild-type strain could prevent the benthic grazer Rhynchomonas nasuta from growing, while biofilms of a QS mutant (lacking the response regulator, HapR) were grazed. In this study, we investigate the efficacy of the grazing resistance of biofilms observed in laboratory experiments in an ecologically relevant context. The survival of V. cholerae biofilms under grazing pressure was tested in situ in environmental diffusion chambers (McFeters & Stuart, 1972), where massive dilution effects occur from the surrounding seawater. The grazing assays were performed in the marine environment over a period of 10 days and survival and persistence in the presence of two marine flagellates, R. nasuta and Cafeteria roenbergensis, was assessed.

Materials and methods

Strains and culture conditions

Vibrio cholerae A1552 wild type, V. cholerae hapR [isogenic genetically modified organism (GMO) lacking the hapR gene encoding the QS response regulator], V. cholerae N16961 (natural hapR frameshift mutant) and Escherichia coli B were routinely cultured on Luria–Bertani agar containing 2% NaCl (LB20) or grown in LB20 broth overnight at 37 °C with shaking (200 r.p.m.). The benthic grazer, R. nasuta, was isolated from the field site at the Sydney Institute for Marine Science (SIMS), treated with an antibiotic cocktail (streptomycin, spectomycin, gentamycin, tobramycin, ampicillin and kanamycin at 150 μg mL⁻¹) and serially diluted for many generations to remove the natural contaminating bacterial community. Rhynchomonas nasuta and the predominantly planktonic flagellate, C. roenbergensis (Bicosocicida, Baltic sea, isolated by A.P. Mylnikov), were maintained axenically in 0.5 × nine salts solution (NSS, Vääätänen, 1976) supplemented with heat-killed P. aeruginosa PAO1 as prey at room temperature, and transferred to a fresh medium every 2 weeks.

Environmental chamber setup

Four replicate experiments were performed during the period of January 2008 to May 2009. Environmental diffusion chambers (McFeters & Stuart, 1972) were suspended in the marine environment at SIMS (see Table 1 for details). The chambers (volume 28.3 mL) were sealed with membranes (Supor® – 100 membrane filters, 0.1-μm pore size, 90 mm, PALL Life Science) that were permeable to seawater, but retained bacteria and protozoans inside the chambers.

Table 1. Environmental chamber field experiments

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*V. c. represents Vibrio cholerae strains.

1E. c. represents Escherichia coli.

1Number of chambers containing individual bacterial strains and grazers. Each chamber held one bacterial strain and one grazer.
These were further suspended in a mesh-lined crate to prevent puncture from larger marine animals. The chambers were modified to hold a glass slide as a substratum for biofilm formation. Cover slips were attached to the slide with silicone glue and one cover slip was removed for protozoan counting and one for staining and imaging by confocal laser scanning microscopy (CLSM; Leica DMRB, Leica, Switzerland). For each treatment, at least three, in one case four autoclaved chambers, were inoculated with 28.5 mL of bacteria (overnight cultures diluted to 10⁷ cells mL⁻¹) and protozoa suspension (10⁴ cells mL⁻¹) in 0.5 x NSS and incubated in the marine environment for 10 days (see Table 1 for details). Following incubation in the marine environment, chambers were collected and immediately transferred to the laboratory in seawater. Although a thin biofilm was sometimes detectable on the outside of the chambers, diffusion of seawater was not impeded as evidenced by the rapid exchange of seawater observed when the chambers were removed. Protozoan numbers inside chambers were determined by microscopy (Leica DM LB, Leica) and suspended bacterial numbers were determined by dilution drop plates (Hoben & Somasegaran, 1982). The abundance of V. cholerae and absence of contamination was verified by plating on selective CPC agar (Massad & Oliver, 1987) as well as LB20 agar. Microscopy was used to verify the absence of protozoan contamination at the end of the experiments. Glass slides with the cover slips were stored in 2% glutaraldehyde at 4°C until staining and imaging. For staining, the cover slips were detached from the glass slides, washed three times in sterile phosphate-buffered saline (PBS) and stained with propidium iodide (100 μg mL⁻¹). Stained biofilms were incubated for 10 min in the dark, followed by washing with PBS (three times). The cover slips were inverted on a drop of PBS and ‘clay feet’ on a glass slide and the sides were sealed with liquid candle wax. CLSM images were analyzed using IMAGE J (http://rsbweb.nih.gov/ij/).

**Grazing assays performed under field-like conditions in the laboratory**

To quantify protozoan dynamics on the V. cholerae biofilms in a nondestructive environment under conditions similar to those in the field, we performed experiments in the laboratory where the diffusion chambers were placed in a circuit with traditional biofilm flow cells (Christensen et al., 1999) connected with silicon tubing. A peristaltic pump circulated water from the chambers into the flow cells in which protozoan succession on the V. cholerae biofilms could be quantified and back into the chambers. The chambers were held in large plastic containers (25 L) in fresh seawater, which was changed twice daily. Four chambers for each experiment were inoculated with 28.5 mL of a suspension of 10⁷ cells mL⁻¹ of an overnight culture of V. cholerae strains in 0.5 x NSS and 10⁵ cells mL⁻¹ of R. nasuta or C. roenbergensis. Protozoan abundance was determined by microscopic observation of flow cells over 5 days.

**Effect of glucose concentration on persistence of V. cholerae under grazing pressure**

Because of seasonal differences in the results of our experiments, we investigated the influence of different carbon concentrations on the persistence of V. cholerae biofilms under grazing pressure, as nutrient levels would be expected to differ between these seasons. Overnight cultures of V. cholerae strains were inoculated (10⁵ cells mL⁻¹) in triplicate into 24-well microtiter plates (Sarstedt, Newton) in sterile filtered seawater containing 0.1% or 0.001% sterile filtered glucose as a carbon source. Rhynchosomonas nasuta (10⁴ cells mL⁻¹) was inoculated 2 h later to give the bacteria time to attach. As a control, flagellates were inoculated into the same medium, but with heat-killed P. aeruginosa as a food source. Experiments were run for 3 days at room temperature with shaking (60 r.p.m.). Protozoan numbers were determined by microscopy and the biofilm biomass was measured as described previously (O’Toole et al., 1999). Briefly, biofilms were washed three times with PBS, stained with 0.3% crystal violet for 10 min and washed three times with PBS. The biofilm was destained with 96% ethanol and A₄₉₀nm was read (Wallac 1420 Multilabel Counter, Perkin Elmer Life Sciences).

**Statistical analyses**

Statistical analyses were performed using SPSS 17.0 software. Pair-wise comparisons were performed using t-tests. Multiple comparisons were performed using one- or two-factorial ANOVAs. The Tukey–HSD test was used as a post hoc test after significant group effects were detected by ANOVA. In the case of nonhomogenous variances, data were log(x+1) transformed before the analyses. If the variances were still nonhomogenous after transformation, a nonparametric Kruskal–Wallis H-test for comparing multiple groups or the Mann–Whitney U-test for pair-wise comparisons was applied.

**Results**

**Protozoan abundance on V. cholerae and E. coli biofilms in the marine environment**

After 10 days in the field, protozoa could be detected on all biofilms grown in the environmental chambers, with the abundance being dependent on the time of year (generally, the abundance of protozoa was higher in autumn than in summer). Surprisingly, in midsummer (Fig. 1a), the number of the surface-feeder, R. nasuta, was significantly higher on the biofilms of the A1552 wild-type strain than on the
biofilms of the N16961 natural hapR mutant strain (t-test \( P < 0.01 \)). Because this strain carries a frameshift mutation in the QS response regulatory gene, hapR, it was expected that this strain would support a higher abundance of flagellates than the wild-type A1552 strain, as has been shown with the isogenic A1552 hapR mutant in laboratory studies (Matz et al., 2005), even though these two strains are not isogenic. The counts of suspended V. cholerae A1552 and N16961 were similar to each other (Fig. 1b), but were significantly higher (\( P < 0.001 \)) in the grazed chambers when compared with the grazer-free controls.

Figure 2a shows the abundance of the suspension feeder, C. roenbergensis, on V. cholerae A1552 and N16961 biofilms after 10 days of exposure in the field (experiments were performed during the middle to the end of summer). The abundance of C. roenbergensis was 10 times higher on the V. cholerae N16961 biofilms, in contrast to R. nasuta abundances, which were 20-fold higher on the A1552 wild-type biofilms (Fig. 1a). The abundance of suspended V. cholerae A1552 in the chambers was higher in the grazed chamber than in the nongrazed control (Fig. 2b), while the opposite was observed for N16961, where the number of suspended cells was higher in the nongrazed chamber (Fig. 2b). The differences in planktonic cell numbers were significant (\( P < 0.05 \)). These results are similar to those observed in laboratory experiments, where the isogenic hapR mutant strain supported strong growth of the flagellate, resulting in reduced bacterial cell numbers, while the opposite was true for the wild-type strain. Biofilm biomass did not differ significantly between the grazed and the ungrazed treatments or between strains (data not shown).

Experiments with R. nasuta or C. roenbergensis inoculated in diffusion chambers in the marine environment with either V. cholerae A1552 or N16961 biofilms were also performed at the end of autumn (Fig. 3). In the chambers containing C. roenbergensis, the trend is the same as for the experiments performed at the end of summer (Fig. 2), with a higher abundance of grazers on the N16961 biofilms. For
chambers containing *R. nasuta*, there was a lower abundance of grazers on the N16961 strain compared with the A1552 strain biofilms (Fig. 3a), but the difference was not as pronounced as in the experiments performed in midsummer (Fig. 1a).

Counts of suspended *V. cholerae* A1552 exposed to *R. nasuta* in the chambers at the end of autumn (Fig. 3b) differed from the previous series of experiments performed in midsummer (Fig. 1b). The abundance of suspended *V. cholerae* A1552 in the chambers containing *R. nasuta* was higher than the abundance of N16961 (9.2 × 10⁵ and 5.5 × 10⁴, respectively; Fig. 3b), while in the earlier experiments, they were similar (3.6 × 10⁷ and 1.8 × 10⁷, respectively; Fig. 1b). In the chambers with *C. roenbergensis*, the number of suspended N16961 was higher than that of A1552 (Fig. 3b), similar to the previous experiment (Fig. 2b). Again, biofilm biomass did not differ significantly between treatments or strains, indicating that the biofilms were not significantly grazed (data not shown).

To compare flagellate growth on *Vibrio* biofilms to a non-*Vibrio* biofilm that was previously shown to support growth of the protozoa, we exposed *E. coli* B and *V. cholerae* A1552 to both grazers in the field in autumn (Fig. 4). Both *R. nasuta* and *C. roenbergensis* numbers were higher on the *E. coli* B biofilms compared with the *V. cholerae* biofilms after 10 days in the field (Fig. 4a). While 0.32 ± 0.29 *R. nasuta* mm⁻² could be detected on the *V. cholerae* A1552 biofilms, *E. coli* biofilms harbored 50 times more. The abundance of *C. roenbergensis* was 2.5 times higher on the *E. coli* biofilms than the *V. cholerae* A1552 biofilms (Fig. 4a). Two-factorial ANOVA revealed highly significant differences between the grazers (**P** < 0.001). (b) Abundance (CFU mL⁻¹) of suspended *V. cholerae* A1552 (white bar) and *E. coli* B (black bar) in environmental chambers after 10 days in the marine environment. The mean values are shown (±SD, n = 3).

Fig. 3. Abundance of (a) the flagellates *Rhynchomonas nasuta* and *Cafeteria roenbergensis* on *Vibrio cholerae* A1552 (white bar) and N16961 (black bar) biofilms and (b) the abundance (CFU mL⁻¹) of suspended *V. cholerae* A1552 (white bar) and N16961 (black bar) from environmental chambers after 10 days of co-culture with the protozoan in the marine environment. The mean values are shown (±SD, n = 3). Note the logarithmic y-scale. Two-factorial ANOVA revealed no significant differences.

Fig. 4. Abundance of (a) the flagellates *Rhynchomonas nasuta* and *Cafeteria roenbergensis* on *Vibrio cholerae* A1552 (white bars) and *Escherichia coli* B (black bars) biofilms in environmental chambers after 10 days in the marine environment. *Rhynchomonas nasuta* appeared on the *V. cholerae* A1552 biofilms in very low abundances (0.32 ± 0.29 ind mm⁻²). Two-factorial ANOVA revealed highly significant differences between the grazers (**P** < 0.001). (b) Abundance (CFU mL⁻¹) of suspended *V. cholerae* A1552 (white bar) and *E. coli* B (black bar) in environmental chambers after 10 days in the marine environment. The mean values are shown (±SD, n = 3).
with R. nasuta, which is opposite to what was previously observed at the end of autumn (Fig. 3b).

**Abundance of R. nasuta and C. roenbergensis on V. cholerae biofilms under semi-field conditions**

The isogenic V. cholerae A1552 hapR mutant strain is a GMO and cannot be exposed to the natural marine environment; thus, in order to compare all three Vibrio strains, we designed grazing assays in the laboratory under conditions that simulated the field experiments as closely as possible. The abundance of R. nasuta and C. roenbergensis on the three V. cholerae biofilms was monitored for 5 days in the flow cells (Fig. 5). While R. nasuta appears on the biofilm in the flow cells in low numbers from day 2 onwards and slowly increases thereafter (Fig. 5a), C. roenbergensis is abundant in high numbers from day 1 onwards (Fig. 5b). The difference in the abundance of R. nasuta on the three different V. cholerae strains was significant (H-test \( P = 0.02 \)). Rhynchomonas nasuta was, expectedly, most abundant on the V. cholerae A1552 hapR mutant biofilms as seen previously in laboratory experiments (Matz et al., 2005) and was not detected in high numbers on either the V. cholerae N16961 or the A1552 biofilms after day 2 (Fig. 5a). For C. roenbergensis, there were differences in the abundance of the grazer on the Vibrio strains for the first 3 days, but the abundances thereafter were similar on all the strains (Fig. 5b).

**Effect of glucose concentration on the persistence of V. cholerae biofilms under grazing by R. nasuta**

The growth of R. nasuta on V. cholerae biofilms supplemented with high and low carbon concentrations revealed that biofilms grown under low carbon concentrations (0.001%) supported a lower abundance of grazers than those grown at a higher glucose concentration (0.1%) regardless of the strain (Fig. 6). The growth of R. nasuta on the A1552 biofilms was 2.8 times higher if the biofilms were grown on the higher glucose concentration, while for the hapR mutant strain, the increase was 1.5 times more and for N16961, 1.6 times higher. A two-factorial ANOVA revealed significant influences of the strain \( (P = 0.003) \) and the glucose concentration \( (P = 0.002) \) on the growth of R. nasuta. A post hoc test revealed significant differences between the growth of R.
nasuta on A1552 compared with growth on the isogenic hapR mutant biofilm ($P = 0.004$) and growth on N16961 compared with A1552 hapR biofilms ($P = 0.014$). No significant differences in growth on A1552 and N16961 were found. The growth of the flagellates on medium with either high or low glucose supplied with heat-killed bacteria was not significantly different (data not shown). The biofilm biomass was not significantly different on the two glucose concentrations (data not shown).

Discussion

Most studies investigating predator–prey interactions in biofilms have been performed in laboratory settings under strictly controlled conditions. Our previous results indicated that V. cholerae A1552 wild-type biofilms grown in 24-well microtiter plates were toxic to the benthic grazer, R. nasuta, and resulted in flagellate death, while feeding on the hapR QS mutant biofilm resulted in positive growth (Matz et al., 2005), indicating that a factor regulated by HapR is responsible for protozoan killing. The current study was designed to investigate whether V. cholerae biofilms grown in situ where there is a large dilution effect due to surrounding seawater could inhibit protozoan growth, i.e. whether the toxicity seen in the laboratory was an artifact due to the concentration effects of the microtiter experiments. In these field experiments, we used the biofilm feeder R. nasuta as well as C. roenbergensis, which is primarily a suspension feeder, but also attaches to the biofilm. The results presented here reveal that V. cholerae A1552 wild-type biofilms prevent predation-associated loss of biofilm biomass in the marine environment where there is a large dilution effect due to surrounding seawater and thus support the concept that the antipredator activity observed in the microtiter experiments is ecologically relevant.

Protozoan abundance varied on biofilms of different V. cholerae strains in the field as well as under semi-field conditions

The abundances of R. nasuta and C. roenbergensis varied on different V. cholerae and non-Vibrio strains and at different times of the year. While the abundance of R. nasuta was significantly lower on V. cholerae N16961 than on the wild-type strain A1552 in summer (Fig. 1a, $P < 0.01$), the difference in abundance on the two strains in autumn was not significant (Fig. 3a). The predominantly planktonic grazer, C. roenbergensis, appeared on the N16961 biofilms in higher numbers in midsummer and late autumn (Figs 2a and 3a). In general, the numbers of C. roenbergensis on biofilms of all strains were higher than the numbers of R. nasuta, indicating that the surface-grazing flagellate R. nasuta might be more negatively affected by V. cholerae than the suspension feeding C. roenbergensis. This may be due in part to the fact that C. roenbergensis can escape the biofilm and feed on planktonic bacteria, but may also indicate that the biofilm affects the grazers differently.

The fact that V. cholerae N16961 supported lower abundances of the obligatory benthic-feeding R. nasuta than A1552 was surprising as N16961 is a QS mutant and thus, it was expected that the QS-regulated antiprotazoal activity (seen in A1552 in previous laboratory studies) would not be expressed. This suggests that there may be a QS-independent pathway for expression of traits that lead to biofilm persistence; however, other differences between the strains or the nutritional quality may also account for this difference in grazer abundance, as these strains are not isogenic. It has been shown previously that P. aeruginosa expresses QS-regulated lethal factors, which play a key role in grazing protection of late biofilms, while QS-independent upregulation of the type III secretion system is important as an immediate response to predation (Matz et al., 2008).

Because of the unexpected result of higher numbers of R. nasuta on the wild-type V. cholerae strain A1552 than on the QS mutant strain, we compared the growth of the grazer on a non-Vibrio strain, E. coli B, in the field. Both R. nasuta and C. roenbergensis occurred on the E. coli biofilms in higher abundances than on the V. cholerae A1552 strain (Fig. 4a), but the differences in abundance were not significant.

Under semi-natural conditions, the same trend for low grazer abundances on the V. cholerae A1552 and N16961 strains when compared with the A1552 hapR mutant strain was observed (Fig. 5). While abundances on the A1552 hapR biofilm were higher for both R. nasuta and C. roenbergensis, the number of grazers on biofilms of the N16961 strain was similar to the A1552 strain. Thus, the results obtained in the field and under semi-field conditions were similar. The N16961 strain has a frameshift mutation in the hapR gene and thus was considered to be more susceptible to losses to predation than the wild-type A1552. Matz et al. (2005) showed that the hapR gene controls antiprotazoal factor(s) that, when secreted, prevented flagellate grazing. The field experiments reported here indicate that that this activity might be ecologically relevant and that there may be hapR-independent defensive mechanisms expressed in situ. Previous results have shown that P. aeruginosa uses both QS-dependent and QS-independent mechanisms for predation resistance and that these mechanisms operate under different physiological and environmental conditions (Matz et al., 2008); therefore, the same types of responses may be expressed by V. cholerae during grazing.

Grazing resistance increases as carbon levels decrease

The differences in protozoan growth on the biofilms in midsummer and autumn may be due to differences in DOC levels in the seawater. Thus, we investigated the influence of prey grown on different nutrient concentrations on grazer
numbers. When the glucose concentration was higher, the flagellate growth rates were at least 1.5 times higher for all three *V. cholerae* strains, while in controls (flagellates with heat-killed bacteria), there was no effect of glucose concentration on the flagellate growth rate. The greatest difference in the growth rates between the high and low glucose biofilms was for the A1552 strain. Interestingly, there was a difference in numbers of grazers on the *hapR* mutant biofilms grown under different nutrient concentrations, indicating that under low nutrient conditions, *QS*-independent grazing resistance occurs.

It is known from planktonic studies that in nutrient-rich environments with a high abundance of metazooplankton, the phagotrophic protists experience a high grazing pressure (e.g., Jürgens, 1994; Corno et al., 2008), allowing the bacterial community to increase in numbers. This is in part due to sloppy feeding and from the excretion of the recycled zooplankton nutrients, which then become available for the bacterial community (e.g., Lampert, 1978; Pernthaler et al., 1997; Corno & Jürgens, 2006). Thus, when nutrient levels are high in the environment, grazing pressure on the bacterioplankton decreases while available nutrient increases, resulting in rapid growth of the bacterioplankton (Cole et al., 1988; Simek et al., 2003).

In contrast, in nutrient-poor environments, the impact of grazers on the bacterial community is greater, resulting in significant changes in the composition of the bacterial community (Jürgens et al., 1999; Hahn & Höfe, 2001). The greater effectiveness of the prevention of grazing losses of *V. cholerae* grown under low nutrient concentrations suggests an adaptation to higher grazing pressure in natural food webs with less available nutrients. In fact, top-down control (of the grazers) is generally more pronounced in resource-limited areas (Simek et al., 2003). This pattern of defense metabolite production fits with the resource availability hypothesis, which states that in environments with low resource availability, plants with low growth rates and high levels of defense will be favored, while in environments with high resource availability, plants with fast growth and lower defense levels will be favored (Coley et al., 1985). Thus, when nutrients are available, *V. cholerae* is able to grow more quickly than it is eliminated by predation, but when nutrients are limited, resources may be shifted from growth to defense metabolites.

The results presented here show that *V. cholerae* biofilms are protected from grazing losses in situ where there are large dilution effects due to the surrounding seawater environment. While the biofilm biomass remained stable under grazing pressure, the planktonic biomass increased for both strains when the benthic feeder, *R. nasuta*, was present on the biofilm. This may be due to cells leaving the biofilm for the planktonic phase to ‘escape’ predation and to increased nutrient availability due to nutrient recycling by predator feeding. The lower abundances of flagellates on N16961 biofilms were unexpected as this strain has a frameshift mutation in *hapR*, which has been shown to be required for the antiprotzoal activity of the A1552 strain expressed in the laboratory (Matz et al., 2005). These data indicated that there is potentially *QS*-independent antipredation activity exhibited by this strain, but does not rule out the possibility that there may also be other strain differences that account for the grazing protection. This work is the first to show that the protection against grazing losses expressed by *V. cholerae* is ecologically relevant and further highlights the advantages of surface-associated growth in environmentally relevant contexts. The data also clearly show that *V. cholerae* expresses traits that prevent grazing-induced loss of biomass that are regulated by the cell–cell signaling pathway, *hapR*, as well as in response to environmental conditions (e.g. nutrients) and thus, such traits could play important roles in the persistence of *V. cholerae* in the environment within predator-resistant biofilms.

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