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Antagonistic Interactions among Marine Bacteria Impede the Proliferation of *Vibrio cholerae*

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Changes in global climate have raised concerns about the emergence and resurgence of infectious diseases. *Vibrio cholerae* is a reemerging pathogen that proliferates and is transported on marine particles. Patterns of cholera outbreaks correlate with sea surface temperature increases, but the underlying mechanisms for rapid proliferation of *V. cholerae* during ocean warming events have yet to be fully elucidated. In this study, we tested the hypothesis that autochthonous marine bacteria impede the spread of *V. cholerae* in the marine environment. It was found that some marine bacteria are capable of inhibiting the growth of *V. cholerae* on surfaces and that bacterial isolates derived from pelagic particles show a greater frequency of *V. cholerae* inhibition than free-living bacteria. *Vibrio cholerae* was less susceptible to antagonism at higher temperatures, such as those measured during El Niño-Southern Oscilliation and monsoonal events. Using a model system employing green fluorescent protein-labeled bacteria, we found that marine bacteria can directly inhibit *V. cholerae* colonization of particles. The mechanism of inhibition in our model system was linked to the biosynthesis of andrimid, an antibacterial agent. Antibiotic production by the model antagonistic strain decreased at higher temperatures, thereby explaining the increased competitiveness of *V. cholerae* under warmer conditions. These findings suggest that bacterium-bacterium antagonism is a contributing mechanism in regulating the proliferation of *V. cholerae* on marine particles.

Vibrio cholerae is a ubiquitous member of the bacterial community in temperate and tropical marine coastal waters. Pathogenic biotypes of *V. cholerae* are annually responsible for more than 300,000 cases of cholera, 6,500 of which are lethal, in over 50 countries worldwide (40, 41). Numerous studies have established a seasonal pattern for cholera outbreaks (6, 21, 31). *V. cholerae* abundance (22, 23) and cholera outbreaks increase with warming sea surface temperatures such as are observed during El Niño-Southern Oscillation events (30, 38). Thus, there is concern that global climate warming will increase the frequency and geographical distribution of cholera epidemics (7, 10, 15).

The ecology of *V. cholerae* is intimately coupled with its attachment to particles, and these interactions are considered important in its transmission from aqueous environments to humans (7, 8). The association of *V. cholerae* with both phytoplankton (17, 19, 20) and zooplankton (18, 24) is well documented. *V. cholerae* can reach such high abundance (10^4 to 10^5) on larger particles that a single particle is sufficient to provide an infectious dose to humans (7). Chironomid egg masses can harbor high concentrations of *V. cholerae* and serve as reservoirs for the bacterium (4).

Marine particles are hot spots for microbial activity, and molecular phylogenetic analysis has established that the dominant species of particle-attached bacteria are different from those free living in the surrounding seawater (3, 9, 34). The intense enzymatic activity of these "particle specialists" on organic matter creates high-nutrient plumes that help support heterotrophic bacterial growth (2, 25, 37). Particle-associated bacteria also aggressively employ antagonistic interactions against other bacteria, perhaps to limit competition in these nutrient-rich microenvironments (14, 26). Considering the pervasive nature of antibiosis, we tested the hypotheses that antagonistic interactions between marine bacteria and *V. cholerae* impede the latter from colonizing and proliferating on organic-rich surfaces and that elevated water temperatures affect such interactions.

MATERIALS AND METHODS

Source of organisms. *Vibrio cholerae* isolates Vc N16961 and Vc 0395 were gifts from John J. Mekalanos (Harvard Medical School). Isolates Vc O1, Vc O139, Vc NonO1, and Vc 1st case in Mexico are all part of an unpublished strain collection of Marcial Leonardo Lizzaraga-Partida (CICSESE, Ensenada, Mexico). Isolates Vc SIO and Vc TP are environmental strains isolated from coastal waters in La Jolla, CA (33). The serogroup, serotype, and biotype for each isolate are presented in Table 1. All other marine bacterial isolates, representing a phylogenetically diverse group of cultivable marine bacteria including *Bacteroidetes*, *Alphaproteobacteria*, and *Gammaproteobacteria*, were from the bacterial culture collection of Farooq Azam (Scripps Institution of Oceanography) and are described elsewhere (26).

GFP mutagenesis. Wild-type SWAT3 (SWAT3-wt) (GenBank accession no. AF366022), a particle-derived *Vibrio* strain, was mutagenized using the protocol of Stretton and coworkers (39). Briefly, a triparental conjugation with SWAT3-wt Rif$^+$, *Escherichia coli* SM10(pLOFKm*gfp*), and *E. coli* 2073 was performed, and screening for green fluorescent protein (GFP) fluorescence was conducted on ZoBell 2216 (42) plates supplemented with rifampin (50 μg ml^{-1}). Approximately 2,000 mutants were examined by epifluorescence microscopy (excitation, 480 nm; emission, 520 nm), leading to the identification of 150 isolates with strong GFP signals on surfaces. Picked GFP mutants were tested in antagonism assays (see below) to identify mutants that retained or lost inhibitory effects against the growth of *V. cholerae*.

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TABLE 1. *Vibrio cholerae* isolates used in this study

Strain	Serogroup	Serotype	Biotype
Vc N16961	O1	Inaba	El Tor
Vc O1	O1	Unknown	Unknown
Vc O139	O139	Unknown	Unknown
Vc NonO1	Non-O1/O139	NA[a]	NA
Vc O395	O1	Ogawa	Classical
Vc 1st case	O1	Unknown	Unknown
Vc TP	Non-O1/O139	NA	NA
Vc SIO	Non-O1/O139	NA	NA

[a] NA, not applicable.

Antagonism assays. Cultures of *V. cholerae* and marine bacterial isolates were grown overnight at 20°C in ZoBell 2216 medium (1.5% Bacto Peptone and 0.5% yeast extract in 100-kDa-filtered seawater). Cultures were diluted in fresh ZoBell 2216 to an optical density (OD) at 600 nm of 1. Soft agar lawns of *V. cholerae* (3 ml 0.6% ZoBell agar inoculated with 30 μl of diluted *V. cholerae* cultures) were overlaid onto firm ZoBell plates (1.5% agar). Ten microliters of diluted marine bacterial cultures was spotted in a three-by-three grid on top of the lawns, and the plates were incubated at either 20°C or 30°C overnight. Antagonistic interactions were scored when zones of inhibition were observed. Assays were performed in triplicate, and only when inhibition was observed in all three assays were isolates scored as positive for inhibition.

Particle colonization assay. Six model particles were prepared by spotting 6 μl of 1% agarose amended with ZoBell 2216 onto Teflon-coated glass microscope slides (Cell-Line, Portsmouth, NH). The agarose had been first inoculated with either an antagonistic GFP mutant (SWAT3-4) or a nonantagonistic GFP mutant (SWAT3-111). Slides were submersed in 100-kDa-filtered seawater (tangential flow filtration) (0.1-μm Supor Membrane LV Centramate; Pall Corp, EastHills, NY) inoculated with or without *V. cholerae* N16961. Replicate slides were removed after 0, 5, and 16 h; fixed with 2% borate-buffered formalin; and DAPI (4′,6′-diamidino-2-phenylindole) stained. *V. cholerae* abundance was determined by subtracting SWAT3 (GFP) counts from total bacterial (DAPI) counts for all six particles by epifluorescence microscopy. A second set of experiments was conducted with agarose particles amended with freeze-thaw-killed cells of the dinoflagellate *Lingulodinium polyedrum* in place of ZoBell 2216. Replicate slides were removed after 0, 5, 16, 24, and 32 h.

Determination of SWAT3 allelochemicals. SWAT3-wt was cultured in ZoBell 2216 medium (39 1-liter cultures) at 23°C on a rotary shaker for 4 days. Ethyl acetate extraction of the whole culture broths yielded 1.95 g of extract that potently inhibited *V. cholerae* growth (a paper disk impregnated with 500 μg extract provided a 27-mm zone of inhibition). Purification of the active substance was achieved by bioassay-guided fractionation using column chromatography (Amberchrom CG-161m; gradient, methanol [MeOH]-H_2O) and reverse-phase high-pressure liquid chromatography (HPLC) (45 to 80% MeOH in H_2O over 20 min at 10 ml min^{-1}; Waters Xterra RP18 5-μm, 19- by 100-mm column). The purified antibiotic (34 mg) was identified as andrimid by comparison of ^1H and ^{13}C nuclear magnetic resonance and mass spectra to previously published data (11).

Andrimid production by SWAT3 mutants. SWAT3-4, SWAT3-111, and SWAT3-wt were cultured in 1 liter of ZoBell 2216 broth at 23°C on a rotary shaker for 4 days. Each culture broth was extracted with two 500-ml portions of ethyl acetate. The organic layers were dried over Na_2SO_4, filtered, and concentrated in vacuo to yield 84.3 mg (SWAT3-wt), 85.8 mg (SWAT3-4), and 74.7 mg (SWAT3-111). Extracts were dissolved at 3.3 mg ml^{-1} in MeOH, and the andrimid concentration was measured by comparing analytical HPLC peak areas to a standard curve (10-μl injections, 45 to 80% MeOH in H_2O over 20 min at 1 ml min^{-1}, UV detection at $\lambda = 292$; Waters Xterra RP18 5 μm, 3.0- by 100-mm column). To create the standard curve, serial dilutions of pure andrimid in methanol (three replicates per concentration) were analyzed by analytical HPLC (same conditions as described above). Averaged andrimid peak areas were then plotted versus concentration.

Broth dilution assay. Overnight cultures of *V. cholerae* N16961 were diluted (OD at 450 nm of 0.043) and treated with fourfold serial dilutions of andrimid. Tetracycline and methanol served as an antibiotic standard and a solvent control, respectively. The treated cultures ($n = 10$ each) were incubated at 24°C, 28°C, or 30°C, and optical density was measured at 630 nm after 24 h. Percent inhibition was calculated based on the following equation: percent growth = 100% × $(OD_{sample} - OD_{blank})/(OD_{solvent\ control} - OD_{blank})$.

TABLE 2. Effect of temperature on growth inhibition of *Vibrio cholerae* by marine bacteria

V. cholerae strain	% Inhibition[a] at:			
	20°C		30°C	
	Attached	Free living	Attached	Free living
Vc N16961	22.9	3.2	20.0	0
Vc O1	22.9	3.2	8.6	0
Vc O139	17.1	6.5	17.1	0
Vc NonO1	22.9	6.5	17.1	0
Vc 395	31.4	16.1	20.0	3.2
Vc 1st case	25.7	6.5	14.3	0
Vc TP	22.9	3.2	11.4	3.2
Vc SIO	20.0	3.2	31.4	22.6

[a] Percentages of particle-attached ($n = 35$) and free-living ($n = 31$) bacterial isolates that inhibited the growth of *V. cholerae* in a modified Burkholder inhibition assay. Each marine bacterial isolate was tested against the eight *V. cholerae* strains shown at both 20°C and 30°C. The marine bacterial isolates were spotted onto lawns of *V. cholerae* growing within ZoBell 2216 agar and then incubated overnight. Antagonistic strains were operationally assigned as those that inhibited the growth of a *V. cholerae* strain by at least 2 mm from the edge of the colony.

Temperature dependence of SWAT3-wt antibiotic biosynthesis. SWAT3-wt was cultured in 100 ml ZoBell 2216 broth on a rotary shaker for 4 days at either 24°C ($n = 5$) or 30°C ($n = 5$). SWAT3-wt entered stationary phase after approximately 20 h at both temperatures. The whole culture broths were then extracted with ethyl acetate, dried over Na_2SO_4, filtered, and concentrated in vacuo. Each extract was dissolved at 3.3 mg ml^{-1} in methanol and analyzed by C_{18} HPLC as described above. Andrimid peak areas were averaged and then compared to a standard curve to calculate compound production at each temperature.

RESULTS

Inhibitory interactions. Direct competition assays performed on agar plates with 66 marine bacterial isolates (representing a phylogenetically diverse group of cultivable marine bacteria including *Bacteroidetes*, *Alphaproteobacteria*, and *Gammaproteobacteria*) against eight strains of *V. cholerae* showed that a range of bacteria could impede the growth *V. cholerae* (Table 2). At 20°C, a mean of 23% of the marine bacteria derived from particles inhibited the growth of *V. cholerae*, while only 6% of free-living bacteria showed such antagonistic behavior. Clinical and environmental strains of *V. cholerae* were equally inhibited, including both the classical and El Tor serotypes. Far fewer antagonistic interactions were ob-

TABLE 3. Effect of temperature on growth inhibition of *Vibrio cholerae* by SWAT3

Vibrio cholerae strain	Distance (mm)[a] at:	
	25°C	30°C
Vc N16961	5	Hazy zone
Vc O1	6	None
Vc O139	6	None
Vc NonO1	5	None
Vc 1st case	4	Hazy zone
Vc TP	7	Hazy zone
Vc SIO	4	Hazy zone

[a] Marine isolate SWAT3-wt, a particle-derived *Vibrio* sp., was assayed against clinical and environmental strains of *V. cholerae* at both 25°C and 30°C using a modified Burkholder inhibition assay. Results were determined after 14 hours. The reported distance is the clear zone that extends from the edge of the growing SWAT3-wt colony to where the *V. cholerae* lawn becomes opaque. Hazy zone indicates minimal inhibition, with a lawn clearing of less than 2 mm.

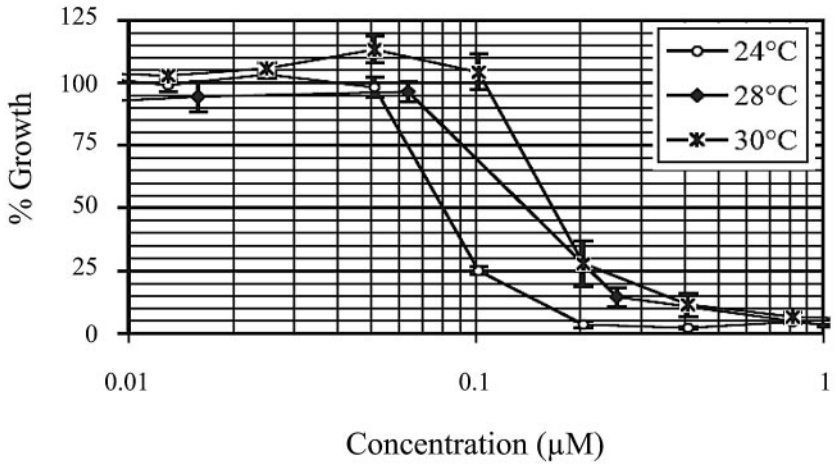

FIG. 1. Sensitivity of *Vibrio cholerae* N16961 to andrimid as a function of temperature. Broth dilution assays were conducted at 24°C, 28°C, and 30°C with a 24-h incubation ($n = 10$; error bars are one standard deviation).

served at 30°C (Table 2). An exception to this trend was observed with an environmental isolate, Vc SIO, which was more susceptible to growth inhibition at the higher temperature.

SWAT3, a particle-derived marine *Vibrio* strain, was selected as a model organism for a detailed study of its temperature-dependent mechanism of bacterium-bacterium antagonism against *V. cholerae*. SWAT3 was chosen due to its potent growth inhibition against the panel of *V. cholerae* strains at 25°C but markedly diminished effects at 30°C (Table 3).

Mechanism of antagonism. Bioassay-guided fractionation of SWAT3 culture extracts led to the identification of andrimid (11, 12) as the responsible antagonistic agent against *V. cholerae*. Andrimid potently inhibits *V. cholerae* growth (50% inhibitory concentration of 80 nM at 24°C). To investigate the temperature dependence of the observed antagonism, SWAT3-wt was cultured for 4 days in 100 ml ZoBell 2216 medium at 24°C or 30°C. SWAT3-wt produced an average of 5.8 mg liter^{-1} andrimid at 24°C, while its biosynthesis decreased to 0.7 mg liter^{-1} at 30°C. Growth rates of SWAT3 were not significantly different at these two temperatures (by analysis of variance, df = 1, $F = 0.032$, and $P = 0.874$). The sensitivity of *V. cholerae* to andrimid over the same temperature range was not significantly different (Fig. 1). Therefore, the variable SWAT3 antagonism toward *V. cholerae* is due to decreased antibiotic production at the warmer temperature.

Mutant generation and characterization. Since particles are focal points for *V. cholerae* proliferation in the environment, the effects of bacterium-bacterium antagonism on the ability of *V. cholerae* strain N16961 to colonize model particles was examined. SWAT3 was again selected as the model strain in

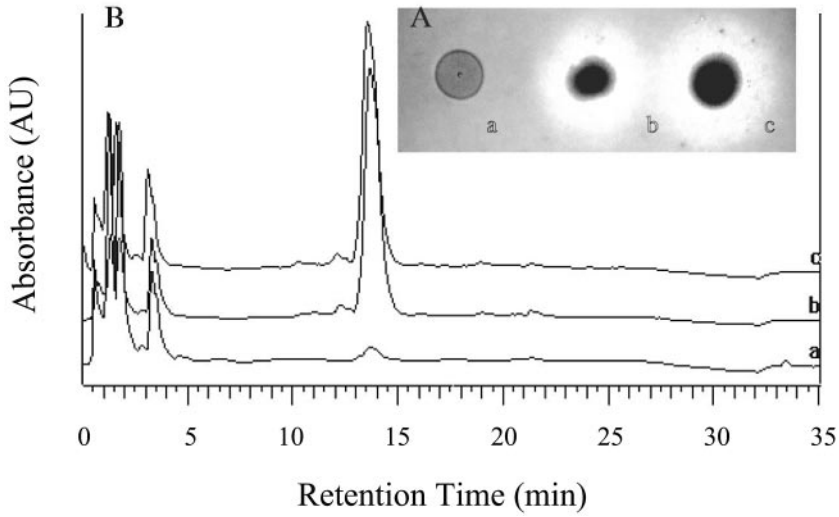

FIG. 2. (A) Inhibition of *V. cholerae* by SWAT3 isolates**.** SWAT3-111 (a), SWAT3-4 (b), and SWAT3-wt (c) were spotted onto lawns of *V. cholerae* N16961and incubated overnight at 20°C. Clear zones around SWAT3-4 and the wild type indicate inhibition of *V. cholerae* growth. (B) Production of the antibiotic andrimid by SWAT3 and its mutants. HPLC analysis was conducted on culture extracts of the three SWAT3 strains. The large peak at 14 min shows the presence of andrimid. The antagonistic activity of SWAT3-4 and SWAT3-wt against *V. cholerae* correlates with the production of this antibiotic. AU, arbitrary units.

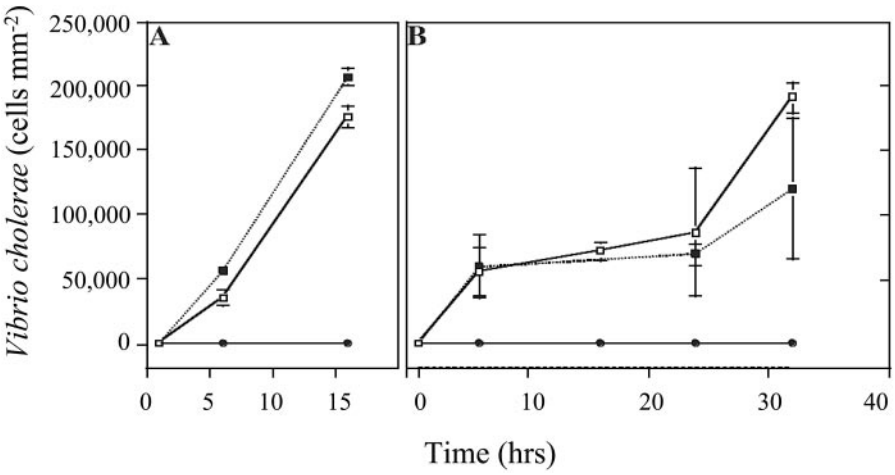

FIG. 3. Inhibition of *Vibrio cholerae* colonization of model particles by an antagonistic marine bacterium. Model agarose particles were inoculated with either SWAT3-4 (circles) or SWAT3-111 (filled squares). Sterile control particles are indicated as open squares. After overnight incubation, *V. cholerae* N16961 was inoculated into the seawater in which the particles were immersed. Replicate model particles ($n = 3$) were removed at the time points indicated, fixed, and stained with DAPI. Particle-attached *V. cholerae* was identified as those bacteria lacking fluorescence and counted. The error bars represent standard deviations. (A) Particles amended with yeast extract and peptone; (B) particles embedded with freeze-thaw-killed cells of *Lingulodinium polyedrum*.

these studies. SWAT3 was first mutagenized with a transposon Tn*10* derivative containing a promoterless green fluorescent protein in order to both microscopically distinguish it from *V. cholerae* and knock out one of the genes necessary for antagonistic interactions (39). Of the 2,000 mutants screened, 150 had a strong GFP signal when grown on surfaces. GFP mutants SWAT3-4 and SWAT3-111 have growth characteristics comparable to those of the wild type, but only the former retained the antagonistic behavior of the wild type (Fig. 2A). Analysis of SWAT3-4 and SWAT3-111 culture extracts by analytical HPLC showed that SWAT3-111 no longer produced andrimid (Fig. 2B). Therefore, SWAT3-111 served as a control for bacterium-bacterium antagonism in our model system.

Colonization assay. Nutrient-enriched, 6-μl agarose particles were inoculated with one of the SWAT3 mutants and incubated overnight in petri dishes containing sterilized seawater. *V. cholerae* was then inoculated into the seawater surrounding the particles. The density of SWAT3 on the particles at the time of *V. cholerae* inoculation was 10^8 ml^{-1}, which falls within the range of bacterial density on marine particles (10^7 to 10^{10} ml^{-1} [1]). *V. cholerae* was also inoculated into a control petri dish with unseeded agarose particles. The antagonistic mutant (SWAT3-4) completely inhibited the colonization of particles by *V. cholerae*, while particles inoculated with the nonantagonistic mutant (SWAT3-111) had no inhibitory effect on *V. cholerae* particle colonization (Fig. 3A).

The colonization experiment was repeated with agarose particles embedded with freeze-thaw-lysed cells of the dinoflagellate *Lingulodinium polyedrum*, and patterns of colonization similar to those in the previous experiment were observed (Fig. 3B), although absolute growth rates were lower.

DISCUSSION

Vibrio cholerae is among the most studied marine heterotrophic bacteria. Factors such as temperature and salinity that regulate its distribution and abundance have been elucidated at the meso-scale and ocean basin levels (6, 7, 30). At smaller scales, its attachment to pelagic particles is well documented. To successfully colonize organic matter in the marine environment, *V. cholerae* must compete against the other 10 to 1,000 million phylogenetically diverse bacteria that reside within a cubic centimeter of a particle. Our findings here show that interspecies antagonistic interactions involving allelochemicals can influence particle colonization by *V. cholerae* and that these interactions can be temperature sensitive.

Previous studies of antagonistic interactions between marine bacteria have focused on isolates from pelagic particles, including marine snow (13, 14, 26, 27). Organic particles are sites of intense microbial activity (1, 32, 35, 37), and bacterial abundance increases with proximity to nutrient-rich particles, reaching concentrations up to three orders of magnitude greater than those in ambient waters (5). It is hypothesized that bacteria use chemically mediated defenses to compete for space and nutrients in these microenvironments (26). Long and colleagues found that an antibiotic molecule produced by a marine particle-associated *Alteromonas* sp. could influence bacterial community structure, which in turn could alter the remineralization of the organic matter on the particle (27). Grossart and colleagues have further suggested that interspecies antagonistic interactions are a microscale factor that can influence particle colonization rates (13).

This study found that a range of phylogenetically diverse bacteria have the potential to interfere with *V. cholerae* growth on surfaces. There was little difference is the level of inhibition observed between clinical and environmental strains of *V. cholerae*. While the model SWAT3 isolate is capable of producing the highly inhibitory compound andrimid, the mechanism of *V. cholerae* inhibition by the other isolates in this study has not yet been elucidated. Andrimid has been previously reported to be produced by several different *Gammaproteobacteria* (11, 28, 29, 36). Oclarit and

colleagues isolated a *Vibrio* sp. from a marine sponge that produced andrimid in laboratory culture (29). Extracts directly from the sponge indicated that andrimid was also produced in situ. Recently, it was shown that andrimid at the nanomolar level inhibits the acetyl coenzyme A carboxylase of gram-negative bacteria than at 20°C (12).

The *Vibrio cholerae* strains in this study were sensitive to far fewer antagonistic interactions at 30°C. The relationship between antagonism and temperature is particularly intriguing in view of the increased occurrence of cholera outbreaks during El Niño-Southern Oscillation events (31, 38). It has been proposed that climate changes are expanding the range of pathogenic organisms both spatially and temporally (16). In the case of cholera, climate-related factors such as sea surface temperature, salinity, and sea surface height have been correlated to outbreaks (6, 21, 30, 31). These factors can influence interspecific interactions between bacteria at the microscale, including the production of and/or resistance to antibiotics.

In conclusion, our results suggest that marine bacterium-bacterium antagonism is a contributing factor in regulating the proliferation of *V. cholerae* on particles. Importantly, autochthonous bacteria appear to become less inhibitory against *V. cholerae* at elevated temperatures. Hence, as sea surface temperatures increase due to changes in global climate, reduced competitiveness from other autochthonous microbes may contribute to increasing abundance and geographic spread of this and other pathogens.

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