VxrB Influences Antagonism within Biofilms by Controlling Competition through Extracellular Matrix Production and Type 6 Secretion

Jennifer K. Teschler,a Eva Jiménez-Siebert,b,d Hannah Jeckel,b,d Praveen K. Singh,c Jin Hwan Park,a Stefan Pukatzki,e Carey D. Nadell,f Knut Drescher,b,c,d Fitnat H. Yildizb

aDepartment of Microbiology and Environmental Toxicology, University of California, Santa Cruz, Santa Cruz, USA
bBiozentrum, University of Basel, Basel, Switzerland
cMax Planck Institute for Terrestrial Microbiology, Marburg, Germany
dDepartment of Physics, Philipps University Marburg, Marburg, Germany
eDepartment of Biology, The City College of New York, New York, New York, USA
fDepartment of Biological Sciences, Dartmouth College, Hanover, New Hampshire, USA

ABSTRACT The human pathogen Vibrio cholerae grows as biofilms, communities of cells encased in an extracellular matrix. When growing in biofilms, cells compete for resources and space. One common competitive mechanism among Gram-negative bacteria is the type six secretion system (T6SS), which can deliver toxic effector proteins into a diverse group of target cells, including other bacteria, phagocytic amoebas, and human macrophages. The response regulator VxrB positively regulates both biofilm matrix and T6SS gene expression. Here, we directly observe T6SS activity within biofilms, which results in improved competition with strains lacking the T6SS. VxrB significantly contributes to both attack and defense via T6SS, while also influencing competition via regulation of biofilm matrix production. We further determined that both Vibrio polysaccharide (VPS) and the biofilm matrix protein RbmA can protect cells from T6SS attack within mature biofilms. By varying the spatial mixing of predator and prey cells in biofilms, we show that a high degree of mixing favors T6SS predator strains and that the presence of extracellular DNA in V. cholerae biofilms is a signature of T6SS killing. VxrB therefore regulates both T6SS attack and matrix-based T6SS defense, to control antagonistic interactions and competition outcomes during mixed-strain biofilm formation.

IMPORTANCE This work demonstrates that the Vibrio cholerae type six secretion system (T6SS) can actively kill prey strains within the interior of biofilm populations with substantial impact on population dynamics. We additionally show that the response regulator VxrB contributes to both T6SS killing and protection from T6SS killing within biofilms. Components of the biofilm matrix and the degree of spatial mixing among strains also strongly influence T6SS competition dynamics. T6SS killing within biofilms results in increased localized release of extracellular DNA, which serves as an additional matrix component. These findings collectively demonstrate that T6SS killing can contribute to competition within biofilms and that this competition depends on key regulators, matrix components, and the extent of spatial population mixture during biofilm growth.

KEYWORDS Vibrio cholerae, biofilm, type six secretion system
biophysical properties of the matrix, which vary across strains and species, matrix secretion can also mediate spatial competition between different lineages that differentially produce it (1, 3). An extreme type of antagonistic interaction is caused by the type six secretion system (T6SS), which is found in the genomes of approximately 25% of sequenced Gram-negative bacteria. The T6SS requires cell-to-cell contact to deliver toxic effectors, resulting in the target cell’s death if it does not possess corresponding immunity factors (4), which is predicted to ultimately result in a competitive hierarchy that locally enriches for the attacking strain over susceptible strains (1, 5–7).

While cooperative interactions in biofilms have been investigated comprehensively, the impact of T6SS on biofilm formation is still being explored. For *V. cholerae* biofilms it has recently been shown that *Vibrio* polysaccharide (VPS)—a central component of the *V. cholerae* biofilm matrix—can protect cells against exogenous T6SS attack from other species without preventing *V. cholerae* from utilizing its own T6SS (8). Despite the VPS-based protection, T6SS killing can generate clonal patches of competing strains, even within initially well-mixed colonies (9). When *V. cholerae* mixed-strain biofilms are formed in confined spaces, killing via the T6SS eventually becomes limited due to the accumulation of dead cells along borders of strain groups (10). This consolidates spatial separation between the strains and allows T6SS-susceptible cells to coexist in biofilms along with a T6SS-active predator (10, 11). Additionally, the *V. cholerae* T6SS plays an important role in invading and displacing the microbiota to colonize the small intestine (12–15). Although regulation of the T6SS in *V. cholerae* has been studied in some detail (4), the role of T6SS regulators during biofilm formation is not well understood. Notably, the response regulator VxrB influences both the T6SS and biofilm matrix production, indicating that matrix production and T6SS activity may be intertwined in their effect on the ecology of antagonism during biofilm formation (12, 16).

The spatial distribution of T6SS activity within biofilms and the population dynamic consequences at cellular resolution are unknown. Similarly, though transcriptional profiling shows that T6SS genes are upregulated in biofilms (17), the interaction of T6SS activity and other mechanisms mediating competition in biofilms is not well characterized. Here, we characterize T6SS firing and killing within *V. cholerae* biofilms using high-resolution imaging, first focusing on the role of the response regulator VxrB and the role of particular biofilm components in altering competition dynamics. We then demonstrate a critical effect of the population structure and the degree of spatial mixing among strains for the efficacy of T6SS-mediated antagonism. Finally, we show that T6SS killing within biofilms creates an increased release of extracellular DNA (eDNA) in the vicinity of killed cells, which may have downstream consequences for biofilm architecture.

**RESULTS AND DISCUSSION**

**Direct visualization of T6SS activity in biofilms.** To better understand when and where the T6SS is assembled within biofilms, we grew *V. cholerae* biofilms in flow chambers, using strains harboring a functional VipA-sfGFP fusion (18), and imaged the three-dimensional biofilms using confocal microscopy. VipA, along with VipB, forms the tubular structure of the outer sheath of the T6SS apparatus (19), and VipA-sfGFP can be used to visualize T6SS structures formed in living cells (18). We compared T6SS assembly during biofilm formation by V52 wild-type, V52 ΔvasK, and V52 ΔvxrB strains (Fig. 1A). VasK is an ATPase required for T6SS firing (its absence abolishes T6SS secretion, but not assembly [20–23]); VxrB has been shown to influence T6SS and biofilm matrix production (12, 16).

BiofilmQ was used to count VipA foci, allowing for quantitative spatial analysis of VipA production in each strain background (24). Wild type biofilms showed extensive expression and assembly of VipA-sfGFP foci by 24 h of growth, with the highest signal registered roughly 10 μm below the outer boundary of the biofilm with the surrounding growth medium (Fig. 1B). We observed that the dual regulator for T6SS and biofilm matrix, VxrB, positively contributes to T6SS assembly during biofilm formation: the number of T6SS foci per biovolume was at least 2-fold lower in a ΔvxrB strain when
compared to a wild-type strain in biofilms at 24 h and 48 h after inoculation. We saw a similar decrease in the number of T6SS foci per biovolume for the \( \Delta \text{vasK} \) strain, in which the T6SS should assemble but not fire, compared to a wild-type strain (Fig. 1B). This is consistent with previous observations reporting that the loss of VasK leads to decreased transcription of T6SS genes (8) and suggests that there may be a positive regulatory feedback mechanism between T6SS firing and reassembly.

**T6SS killing can be observed within biofilms.** We next wanted to better understand how the T6SS impacted competition between strains during biofilm formation. To this end, we constructed a susceptible prey strain lacking the major T6SS effector/immunity protein pairs (referred to in figures as \( \Delta e_i \), which is equivalent to \( \Delta seltsiV1\Delta \text{vasxtsiV2}\Delta \text{vgrG3tsiV3} \)) (23, 25–27). The immunity genes are encoded directly downstream of their effector pair and code for proteins that can deactivate toxic effectors delivered by the T6SS, thus protecting the cell (25). The effector and immunity proteins are deleted together to prevent the endogenous effector production that would lead to self-lysis. The \( \Delta e_i \) strain is therefore susceptible to cell lysis by the T6SS attack from neighboring cells and has an inactive T6SS, as it also lacks the T6SS effector proteins. Pilot experiments indicated minimal T6SS-mediated killing of a \( \Delta e_i \) prey strain in the *V. cholerae* A1552 background by a wild-type (WT) *V. cholerae* A1552 predator strain. We therefore used *V. cholerae* V52 predator strains instead, as this strain background showed quantifiable killing of A1552 \( \Delta e_i \) prey cells. Control experiments in which WT A1552 and WT V52 were competed against each other in biofilms showed that the V52 strain background has a small intrinsic fitness disadvantage (Fig. S1 in the supplemental
material), which may be due to differences in their investment into extracellular matrix (3, 28, 29). However, when grown in liquid media, no growth difference between predator and prey strains was observed (Fig. S6). Since VasK is required for T6SS killing, we used a ΔvasK deletion mutant as a T6SS-inactive control predator (20–23). We then used liquid cultures composed of a predator strain and the Δei prey strain mixed at a 5:1 ratio (predator:prey) at OD₆₅₀ = 0.01 to inoculate flow chambers for subsequent mixed-strain biofilm growth. We imaged the resulting biofilms at different stages of biofilm formation: surface attachment (1 h), microcolony formation (6 h), and mature biofilms (24 h, 48 h) (Fig. 2A, Movies S1–S3). From these images, the ratio of predator:prey was measured by computational image analysis. The predator:prey ratio was used as a metric of their competitive interaction over time, and the number of rounded cells normalized by the total number of prey cells was taken as a metric for T6SS-dependent killing. Rounded cells are a commonly used metric for detecting T6SS-killing, as cells have been shown to round in response to T6SS attack prior to lysis (30). The biofilm filling fraction, which is a measure of the cell density inside biofilms, does not differ between the predator and prey strains (Fig. S2A). The Δei prey cells primarily localize in the deeper regions of the biofilm and are overgrown by the predator cells (Figure S2B1, C1, D1). Most of the biomass of the biofilm is located in the deeper region of the biofilm, and the upper regions are less dense (Figure S2B2, C2, D2).

**FIG 2** T6SS killing in mature biofilms. (A) Three-dimensional images of biofilms 48 h after inoculation. The *V. cholerae* predator strain is depicted in cyan, and the *V. cholerae* prey strain (Δei indicates that the T6SS effector immunity pairs have been deleted) is depicted in red. Image shows the bottom layer of the biofilm (lower portion) and a three-dimensional rendering of the mature biofilm (upper portion). Scale bar is 4 μm. (B) Predator to prey ratio in 1 h, 6 h, 24 h, and 48 h biofilms. (ns, no significance; **, P < 0.005; ***, P < 0.0001; n = 6). (C) Rounded prey cells normalized to total prey at the base of 48 h biofilms (***, P < 0.0001; n = 6).
Noting that the WT V52 strain is at a slight disadvantage in biofilms (Fig. S1), the roughly constant predator:prey ratio for the WT predator in competition with Δei prey over 48 h (Fig. 2A and B) implies that the WT predator obtained a fitness benefit from the T6SS activity. We could also directly detect killing of prey cells via cell rounding in the presence of WT predator cells (Fig. 2C). By comparison, the ΔvasK control predator strain showed no measurable killing of prey (Fig. 2C), and the ΔvasK strain showed a marginal decrease in relative abundance in competition with the Δei prey strain, as expected from the small differences observed in control experiments with competitions between the WT V52 and WT A1552 strains (Fig. 2B). The ΔvxrB strain decreased substantially in relative abundance in competition with the Δei prey strain (Fig. 2B), and produced less cell death in the prey strain compared to the WT T6SS-capable strain (Fig. 2C). This result suggests that the response regulator VxrB contributes to the ability of *V. cholerae* to compete within mixed-strain biofilms and positively contributes to T6SS killing within biofilms.

**VxrB contributes to protection against T6SS killing in biofilms.** VxrB has been implicated in both regulating T6SS attack as well as protection from T6SS killing (12, 31). To explore the effect of VxrB activity in the prey population, we used the same biofilm culture conditions described above, competing a T6SS+ wild-type predator and a T6SS− ΔvasK predator with a T6SS-susceptible Δei prey strain lacking vxrB (Fig. 3A). The biofilm filling fraction does not differ between the predator and prey strains (Fig. S3A). The ΔeiΔvxrB prey cells primarily localize in the deeper regions of the biofilm and are overgrown by the predator cells in a more prominent way than the Δei cells in Fig. 2, indicating a protective function of VxrB in T6SS-mediated killing in biofilms (Fig. S3B, C).

We found that in competition with a T6SS+ wild-type strain, a ΔeiΔvxrB prey strain fairs far worse than a Δei prey strain with VxrB intact (Fig. 2B, 3A, B). Specifically the T6SS+ predator increases from an initial ratio of 5:1 to a final ratio of ~20:1 (after 48 h) against a prey strain lacking VxrB. This result was dependent on the T6SS system being active in the predator, as a ΔvasK T6SS− strain competed neutrally, i.e., it did not appreciably change in ratio in competition with ΔeiΔvxrB prey. Curiously, however, the amount of prey cell rounding in experiments with a T6SS+ predator was not different between the Δei strain possessing or lacking VxrB (Fig. 3C), suggesting that there were similar levels of prey killing whether or not the prey strain was producing VxrB. This could potentially be due to limitations in our methodology, which does not quantify cells that have already lysed and lost fluorescent signal, or due to the somewhat protective nature of dead cell debris along the interfaces between predator and prey strains (10). We suggest that the competitive defect of the ΔeiΔvxrB prey strain can be primarily attributed to the loss of VxrB’s positive regulation of matrix production in the prey strain (16, 31).

**Biofilm matrix components play a protective role against T6SS killing in biofilms.** Our competition data above suggest that VxrB activity within prey bacteria may influence their susceptibility to T6SS via regulation of biofilm matrix production; to test this possibility further, we compared the competition and T6SS killing activity against prey cells with and without key components of the *V. cholerae* biofilm matrix. The exopolysaccharide VPS and the secreted protein RbmA are important structural components of *V. cholerae* biofilms and contribute to cell–cell packing and formation of three-dimensional biofilm structures (32–34). Previous work identified a protective role for *Vibrio* polysaccharide (VPS) against T6SS attack, while suggesting that biofilm proteins did not lend the same protection (8). However, the capacity of these matrix components to modulate T6SS defense during mature biofilm formation between strains coincubated within mixed biofilms is not yet clear. To examine this question, we deleted the two operons (vps-I and vps-II operons) encoding for vps genes in the Δei prey strain background. We also generated a rbmA deletion in the Δei prey strain background. We then competed these prey strains against a T6SS+ predator and a ΔvasK T6SS− control predator strain to isolate the effects of T6SS activity and differential matrix production on the susceptible prey (Fig. 4A). These comparisons were critical because differential VPS and RbmA production have been shown to contribute on their own to competitive success in mixed-strain biofilms of *V. cholerae* (3, 33, 35). The
Biofilm filling fraction does not differ between the predator and prey strains (Fig. S4A). The ΔeiΔvpsI-II prey cells primarily localize in the deeper regions of the biofilm and are overgrown by the predator cells in a more prominent way than Δei cells, suggesting a protective role of VPS production in T6SS mediated killing in biofilms (Figure S4B1, C1, D1, E1). An increased intercell spacing resulting from the deletion of the rbmA gene in the Δei prey also leads to a decrease in competitive capacity of the ΔeiΔrbmA prey, but it is not the predominant effect in matrix-mediated protection against the predator.

As noted in Fig. 2 and Fig. 3, a wild-type V52 T6SS+ predator does not appreciably increase in relative abundance over time against a Δei prey strain. By contrast, in competition with ΔeiΔvpsI-II prey, T6SS+ V. cholerae increased from an initial ratio of 5:1 to a final ratio of over 60:1 over 48 h; a T6SS- (ΔvasK) predator strain, on the other hand, increased only to a final ratio of 30:1 (Fig. 4A and B). This latter result reflects the competitive defect of prey that

**FIG 3** VxrB protects from T6SS killing in mature biofilms. (A) Three-dimensional images of biofilms 48 h after inoculation. The V. cholerae predator strain is depicted in cyan, and the V. cholerae prey strain (Δei indicates that the T6SS effector immunity pairs have been deleted) is depicted in red. Image shows the bottom layer of the biofilm (lower portion) and a three-dimensional rendering of the mature biofilm (upper portion). Scale bar is 4 µm. (B) Predator to prey ratio in 1 h, 6 h, 24 h, and 48 h biofilms. (*, P < 0.05; **, P < 0.005; ***, P < 0.0001; n = 6). (C) Number of rounded prey cells normalized to total area occupied by prey cells at the base of 48 h biofilms (ns, no significance; ***, P < 0.0001; n = 6).
Biofilm components provide protection from T6SS killing in mature biofilms. (A) Three-dimensional images of biofilms 48 h after inoculation. The *V. cholerae* predator strain is depicted in cyan, and the *V. cholerae* prey strain (∆ei indicates that the T6SS effector immunity pairs have been deleted) is (Continued on next page)
fail to produce VPS, the core matrix polysaccharide, when in coculture with matrix-replete but T6SS− competitors. The additional increase in relative abundance of T6SS+ predators against ΔeiΔvpsII prey, in turn, reflects the combined competitive defects of the prey’s failure to produce the core matrix polysaccharide VPS and the increased susceptibility to T6SS activity relative to a Δei prey background. This shows that in the absence of matrix production on the part of prey, its susceptibility to T6SS attack increased substantially. In other words, VPS contributes to competition both as a component of the biofilm matrix and as a defense from T6SS attack. This observation supports earlier work demonstrating that VPS is protective against T6SS killing (8) and, further, that VPS is protective within highly structured biofilms. T6SS killing of the ΔeiΔvpsII prey strain, as measured by rounded cells, was also higher than that for a VPS-producing prey strain, further supporting that VPS can protect against T6SS killing within biofilms.

By contrast with VPS null mutants, which are unable to produce three-dimensional biofilm architecture, V. cholerae cells lacking the matrix protein RbmA are still able to produce biofilms, but they are structurally less robust and have characteristically reduced cell–cell packing relative to biofilms of wild-type strains (3, 32, 36–40). In competition with ΔeiΔrbmA prey cells, T6SS+ V. cholerae increased from an initial ratio of 5:1 to a final ratio of over 14:1 over 48 h; a T6SS− (ΔvasK) control predator strain, on the other hand, increased only to a final ratio of 7:1 (Fig. 4A and B). Consistent with previous reports (3), our results suggest that ΔrbmA prey mutants have a mild competitive deficiency against wild type in the absence of T6SS activity, most likely due to their reduced structural strength, but that ΔrbmA mutants are substantially more susceptible to T6SS killing compared with a prey strain that is able to produce all matrix components. This interpretation was corroborated by the observation that T6SS-dependent killing as measured by prey cell rounding was significantly higher against a prey strain lacking RbmA than for a prey strain producing the full set of matrix protein components (Fig. 4C). Previous work demonstrated that loss of rbmA leads to increased invisibility of planktonic cells into biofilms and reduced protection from Bdellovibrio bacteriovorus, as looser cell–cell packing allows the predatory bacteria to infiltrate the biofilm (3, 37). We suggest that a similar mechanism may contribute to the enhanced T6SS killing of the ΔrbmA prey strain; as the biofilm matures, the more loosely packed ΔrbmA cells may allow for greater access to prey cells by predator cells, leading to a higher susceptibility to killing.

**Increased spatial strain mixing greatly increases the efficacy of T6SS attack.**

Our results above demonstrate that strong effects of T6SS-mediated killing can be seen under conditions in which prey bacteria are not producing biofilm matrix components, making them much more susceptible to killing than matrix-producing prey, which were less affected by the T6SS predator. We were surprised by the modest population dynamical effects of T6SS predation against the prey, and so we next explored how varying the population spatial structure might alter this result. Under the biofilm conditions used for the previously described experiments, the cell density of the initial seeding inoculum was relatively low (OD600 ~0.02). These conditions allow for individual bacteria to attach to a surface and form spatially separated microcolonies before coming into contact with neighboring colonies composed of clonal lineages, reducing the total amount of contact area between T6SS+ and susceptible prey. Theory suggests that the degree of spatial mixing between strains, which controls the amount of contact area between predator cells and susceptible prey, should be a critical influence on the relative success of contact-mediated antagonism (1, 9, 41). To test this concept in our system, we altered the degree of mixing of T6SS+ predator or ΔvasK T6SS− control predators and Δei prey cells by increasing the inoculum density used to initiate biofilm growth (inoculum OD600 = 1 in Fig. 5A, OD600 = 5 in Fig. 5B). These treatments increase the initial surface coverage of flow chambers with random distributions of predator and prey cells, with the degree of mixing and contact among predators and

**FIG 4 Legend (Continued)**

FIG 4 is depicted in red. Image shows the bottom layer of the biofilm (lower portion) and a three-dimensional rendering of the mature biofilm (upper portion). Scale bar is 4 μm. (B) Ratio of predator to prey in 1 h, 6 h, 24 h, and 48 h biofilms. (*) P < 0.05; ** P < 0.005; *** P < 0.0001; n = 6). (C) Number of rounded prey cells normalized to the total area occupied by prey cells at the base of 48 h biofilms (*** P < 0.0001; n = 6).
prey increasing with increasing inoculum density. The biofilm filling fraction does not differ between the predator and prey strains at 24h, when the biofilms are grown from different initial surface seeding densities (Fig. S5).

Measuring the biofilm competitive index (defined as the final ratio of predator to prey bacteria at time x divided by the initial ratio of predator to prey bacteria) of T6SS+ predators to Δei prey over 24 h of biofilm growth, we found that the competitive advantage of T6SS+ predators over prey increased substantially with increasing initial inoculum density and initial biofilm surface coverage (Fig. 5C, initial attachment patterns of cells shown in Fig. 5). As noted above, with an initial inoculum OD_{600} of 0.02, T6SS+ predators do not appreciably increase in relative abundance over Δei prey over the course of 24 h. By contrast, with initial inocula of OD_{600} = 1.0, the predator:prey ratio increases approximately 3-fold by 24 h, and with initial inocula of OD_{600} = 5.0, the predator:prey ratio increases approximately 4-fold by 24 h. These results were entirely dependent on T6SS activity, as control experiments with a T6SS– control predator (harboring a ΔvasK deletion) showed minimal change in the biofilm competition index, regardless of initial inoculum density.

**T6SS killing results in altered eDNA localization and release in biofilms.** Beyond influencing competition, T6SS killing within biofilms is expected to result in additional consequences from the cell lysis of the prey strain that may contribute to biofilm structure, available nutrients, or cell signaling. One of the expected by-products of T6SS killing is the release of eDNA via cell lysis. eDNA is an important structural component of the *V. cholerae* biofilm matrix (42) and additionally represents a potential source of nutrients and genetic material for horizontal gene transfer (43, 44). Therefore, we next analyzed the concentration of eDNA in our mixed strain biofilms.

Using the TOTO-3 to stain for eDNA under the high-density starting inoculum conditions (OD_{600} = 1), we imaged biofilms after 24 h of growth. In biofilms formed with the T6SS+ predator, we observed a similar pattern of enhanced eDNA at the border between predator and prey strains, indicating that cell death by T6SS killing leads to DNA release in the biofilm (Fig. 6A). This border protects against continued killing within confined biofilms, as the accumulation of cell debris acts as a physical barrier between predator and prey strains (10). We observed that the eDNA appeared to maintain a spherical shape, similar to rounded cells in the process of dying. While this can be partially attributed to loss of membrane integrity in dying cells, this is consistent with reports of intact eDNA released in *Pseudomonas aeruginosa* biofilms that, compared to fragmented eDNA, maintains a rounded shape and has different properties than the fragmented eDNA (45). While some eDNA was observed at the border between predator and prey strains in biofilms formed with a ΔvxrB predator, it was less than what was observed for the WT predator. This is consistent with the reduction in T6SS killing observed in biofilms formed with a VxrB predator (Fig. 2 and 5). In biofilms formed with a ΔvasK predator, eDNA was distributed throughout the biofilm (Fig. 6A). We quantified the spatial distribution of the eDNA signal and found that, consistent with our visual observations, the eDNA signal was highest at the interface between predator and prey strains in biofilms formed with a T6SS+ predator and drastically decreased in the ΔvxrB predator and ΔvasK predator backgrounds (Fig. 6B). These findings demonstrate that the T6SS can contribute to localized enhanced eDNA release within mixed-strain biofilms.

The ability to lyse target cells in addition to killing them makes the T6SS a more effective weapon, as it helps clear the “corpses barriers” that act as a protective wall for the cells inside these barriers (11). Fast-acting lytic toxins have been shown to contribute to more effective lysis and enhanced T6SS killing in bacterial communities. Taxonomic analysis of lytic toxins across bacterial species showed that *V. cholerae* encodes for two “slow-lysing” lytic toxins (VasX and TseH) (11). It is possible that delivery of a higher dosage of these slow-lysing toxins may hasten and enhance cell lysis, perhaps accounting for the decrease in killing and eDNA signal observed in a biofilm formed by a ΔvxrB predator.

Localized cell death has been shown to alter the structures of biofilms (42, 46–48), and eDNA is an important structural component of biofilms (49). Killing via the
Increased contact during the early stages of biofilm formation favors a T6SS+ predator strain. (A) Three-dimensional images of 24-h biofilms formed with a high cell density of the starting inoculum (starting OD$_{600}$ = 1.0), which results in increased early contact between predator and prey strains. The *V. cholerae* predator strain is depicted in cyan, and the *V. cholerae* prey strain (Δ*l* indicates that the T6SS effector immunity pairs have been deleted) is depicted in red. The image shows the bottom layer of the biofilm (lower portion) and a three-dimensional rendering of the mature biofilm (upper portion). The scale bar is 4 μm. (B) Three-dimensional images of 24-h biofilms formed with a very high density of the starting inoculum (starting OD$_{600}$ = 5.0) to enhance early contact between predator and prey strains. The *V. cholerae* predator strain is blue, and the *V. cholerae* prey strain is red. Image shows the bottom layer of the biofilm (lower portion) and a three-dimensional rendering of the mature biofilm (upper portion). (C) Biofilm competitive index (BCI) of WT predator strain competed against the prey strain, at 6 h, and at 24 h seeded with different inoculum densities (OD$_{600}$ = 0.02, 1.0, 5.0; n = 6). BCI is defined as the final ratio of predator to prey bacteria at a particular time (6 h and 24 h) divided by the initial ratio of predator to prey bacteria. (μ, P < 0.05; ***+, P < 0.0001). (D) Biofilm competitive index (BCI) of ΔvasK predator strain competed against the prey strain at 6 h, and 24 h seeded with different inoculum densities (OD$_{600}$ = 0.02, 1.0, 5.0; n = 6). BCI is defined as the final ratio of predator to prey bacteria at a particular time (6 h and 24 h) divided by the initial ratio of predator to prey bacteria.
T6SS has been shown to result in dead cell debris, which presumably includes eDNA, when a susceptible strain is present in *V. cholerae* biofilms (10). In other species, studies have found that lysed cells can be used as nutrients: *Bacillus subtilis* can "cannibalize" lysed cells, utilizing the debris for growth, and *P. aeruginosa* produces extracellular DNases to use eDNA from lysed cells as a nutrient source (50–52). It has been shown that *V. cholerae* can utilize eDNA as its sole phosphate source (42, 53), suggesting that it may use eDNA released via T6SS as a nutrient source, as observed for other species. T6SS-mediated eDNA release has previously been shown to facilitate horizontal gene transfer in *V. cholerae* grown on chitin, implying that strain interfaces where T6SS killing occurs have the potential to be evolutionary hotbeds (44, 54). eDNA has additionally been shown to interact with VPS in *V. cholerae* biofilms (55), suggesting that eDNA release via T6SS could also play a structural role within biofilms.

**Conclusion.** Here, we establish that the T6SS is actively fired and can kill susceptible competitors within biofilms. The response regulator VxrB enhances the ability of both predator and prey strains to compete in mixed-strain biofilms, likely via its coregulation of T6SS and biofilm matrix genes. The T6SS confers a competitive advantage during biofilm formation, allowing strains with an active T6SS to better maintain their foothold within the biofilm compared to strains lacking a T6SS, VxrB, or biofilm matrix components. This advantage is enhanced when competing strains have enhanced physical contact with one another during the early stages of biofilm formation. T6SS killing results in localized regions of cell death and eDNA release, although further work is needed to better understand the
contribution of eDNA to biofilm structure and emergent properties. This work provides new insights into how bacteria compete within complex communities and enhances our understanding of the contributions of the VxrB regulator, biofilm matrix components, and spatiogenetic structure to T6SS-mediated competition within *V. cholerae* biofilms.

**MATERIALS AND METHODS**

**Strains and growth conditions.** *V. cholerae* predator strains used are derivatives of the VS2 O37 serogroup strain (58). *V. cholerae* prey strains used are derivatives of the O1 biovar El Tor strain A1552 in which the three main effector immunity pairs have been deleted (referred to in figures as A1552*Δ*v) and their functions were restored using allelic exchange of the native open reading frame (ORF) with the truncated ORF, as previously described (58). Plasmids harboring fluorescent proteins were mated into *V. cholerae* using an *E. coli* strain harboring the conjugation plasmid pRK2013.

**Biofilm assays and staining.** Flow cells were inoculated by diluting overnight-grown cultures of *V. cholerae* strains harboring either sfGFP or mRuby3 by a 5:1 ratio of predator to prey. For biofilms with a starting inoculum of a high cell density, the OD<sub>600</sub> was normalized to an OD<sub>600</sub> of either 1.0 or 5.0, and cells were mixed at a 1:1 ratio of predator:prey to increase initial contact between the strains. Cells were then injected into an Ibidi m-Slide (0.2 g/L tryptone, 0.1 g/L yeast extract, 1% NaCl) at a rate of 7.5 mL/h and continued for up to 48 h. For biofilms in which eDNA was stained, a starting OD<sub>600</sub> of 1.0 was used, and strains were mixed at a 1:1 ratio before being injected into an Ibidi m-Slide. After biofilm formation (24 h), flow was halted, biofilms were gently washed with 1X phosphate-buffered saline (PBS) containing 0.01% Triton X-100, then stained with TOTO-3 (2.0 μg/mL; Invitrogen, Carlsbad, CA) for 30 min. Biofilms were then washed gently with PBS three times before imaging. Confocal laser scanning microscopy (CLSM) images of the biofilms were captured with a Zeiss 880 Confocal microscope equipped with the Airyscan Fast function. To obtain data for image analysis, at least three Z-stacks were taken at independent locations within at least two biofilm replicates (total n = 6–12).

**Biofilm imaging.** Live biofilms were imaged at 1 h, 6 h, 24 h, and 48 h with a 63× oil immersion, numerical aperture 1.4 objective using a Zeiss 880 Confocal microscope with Airyscan Fast. A 488-nm laser was used to excite sfGFP, and a 561-nm laser was used to excite mRuby3. For experiments where eDNA was imaged, a 647-nm laser was used to excite TOTO-3. Images were collected in either the sfGFP-channel or the mRuby3-channel, properties of VipA foci were quantified using BiofilmQ (total fluorescence intensity and size, for each VipA focus). The spatial distribution of foci in the biofilm was determined by counting the number of VipA foci per biovolume cube and normalizing with the corresponding

**Image analyses.** Quantitative image analysis was performed using BiofilmQ, a software tool that has been developed for measuring spatially-resolved biofilm properties (available at drescherlab.org/data/BiofilmQ) (24). Details about the BiofilmQ program can be found in the cited publication and website; below, a brief description of the individual analyses performed are included.

For the spatiotemporal quantification of sfGFP-tagged VipA in *V. cholerae*, a threshold was applied to the mRuby3 channel, which was manually selected for each image to adjust for different imaging conditions. To analyze and quantify the spatial distribution of the biovolume, the segmented biovolume in the mRuby3 channel was then sub-divided into cubes (side-length 0.5 μm) using BiofilmQ (24). To identify the individual foci, the edge detection algorithm based on seeded watershed segmentation, as described in (59) with modifications from (60), was applied to the sfGFP channel. Based on this segmentation of the sfGFP-channel, properties of VipA foci were quantified using BiofilmQ (total fluorescence intensity and size, for each VipA focus). The spatial distribution of foci in the biofilm was determined by counting the number of VipA foci per biovolume cube and normalizing with the corresponding
biovolume in the cube. A VipA focus was assigned to be localized within a particular cube if the focus’
centroid was within the cube volume.

Additionally, the spatial distributions of foci properties were measured by averaging the proper-
ties of the foci in each cube. Quantifications from cubes with a similar height above the substrate
were averaged to result in one pixel in the heatmaps shown in Fig. 1. Heatmaps of spatiotemporal
foci quantifications were obtained by averaging over the biological and technical replicates \((n = 6)\).
In these heatmaps, spatiotemporal pixels with less than 3 replicates were eliminated from the
graph.

For the measurements of the predator to prey ratios, the following steps were performed. The
488-nm and 561-nm channels were segmented, to determine the overall biovolume of each strain
within the biovolume. The biovolume of the predator was then divided by the biovolume of the
prey strain to determine the ratio of predator to prey for each biofilm that was acquired \((n = 6)\).
Statistical analysis of this data was performed using ANOVA and Dunnett’s multiple comparison
test.

For the quantification and localization of eDNA, the following steps were performed. Super-resolu-
tion images were cropped into quarters to reduce the data size for efficient computational proc-
essing. The 488-nm and 561-nm channels were segmented, and the nearest neighbor parameter
was run to determine the distance of prey from a predator. The fluorescent intensity parameter was
run for the 647-nm channel to assess the intensity of eDNA signal. The BiofilmQ visualization tool
was then used to create a “1.5D histogram,” plotting the distance of prey from predator along the x
axis and the fluorescent intensity of the eDNA signal along the y axis. A total of \(n = 12\) biofilm
images were quartered, analyzed, and plotted.

Imaris software (Bitplane) was used to manually count rounded cells at the base of biofilms. This
was done as follows. First, 3D biofilm images were cropped to include only a single Z-plane at the
base of the biofilm. The spots tool was used to manually label and count all circular cells, and the
surface tool was used to calculate the total area of the prey strain. To determine the ratio of round
cells to total prey present, the number of round cells was divided by the total area of prey strain
present \((n = 6)\). Statistical analysis of this data was performed using ANOVA and Dunnett’s multiple
comparison test.

For the quantification of the biofilm filling fraction, a foreground–background biovolume seg-
mentation was performed on each fluorescence channel using Otsu’s method for threshold determi-
nation with a manually adjusted sensitivity. To compute the filling fraction, the detected biovolume
was divided by the volume enclosed by the biofilm perimeter, which was calculated for each fluo-
rescence channel individually. To determine the biofilm perimeter, three morphological image proc-
essing operations were performed on every slice of the super-resolution images: dilation of the
detected biovolume using a disk structuring element with a 30-pixel radius \((1.3 \mu m)\), followed by a
hole-filling operation and three erosion steps using a disk structuring element with a radius of 10
pixels. The hull volume was corrected by subtracting the volume occupied by the other strain and
reincclusion of the volume of the analyzed strain.

The spatiotemporal characterization of the predator biovolume fraction was based on the segmented
data for the biofilm filling fraction quantification and was calculated by dividing, for each image slice of the
3D image stack, the area occupied by the predator by the total area detected for all bacterial cells. For the
total biovolume image fraction quantification, for each slice the area occupied by bacterial cells was
divided by the entire image area. For the analysis of biofilm-internal structures based on super-resolution images, 6
replicates are available for each condition, except for the \(\Delta vasK\) versus \(\Delta ei\) cocultures, where only 2 replicate
super-resolution images are available. For computing the heatmaps, averages for each heatmap pixel were
calculated for all biological replicates.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, PDF file, 0.2 MB.
**FIG S2**, PDF file, 0.3 MB.
**FIG S3**, PDF file, 0.3 MB.
**FIG S4**, PDF file, 0.3 MB.
**FIG S5**, PDF file, 0.2 MB.
**FIG S6**, PDF file, 0.3 MB.
**FIG S7**, PDF file, 0.3 MB.
**MOVIE S1**, AVI file, 5.1 MB.
**MOVIE S2**, AVI file, 4.7 MB.
**MOVIE S3**, AVI file, 3.8 MB.

**ACKNOWLEDGMENTS**

This work is supported by NIH R01 AI114261 grant to F.H.Y. This work was also supported
by grants from the European Union’s Horizon 2020 research and innovation program under
the Marie Skłodowska-Curie grant agreement No. 955910, the European Research Council.
REFERENCES

1. Nadell CD, Drescher K, Foster KR. 2016. Spatial structure, cooperation and competition in biofilms. Nat Rev Microbiol 14:589–600. https://doi.org/10.1038/nrmicro.2016.84.

2. Flemming H-C, Wingender J, Szweczyk U, Steinberg P, Rice SA, Kjelleberg S. 2016. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol 14:563–575. https://doi.org/10.1038/nrmicro.2016.94.

3. Nadell CD, Drescher K, Wingreen NS, Bassler BL. 2015. Extracellular matrix structure governs invasion resistance in bacterial biofilms. ISME J 9:1700–1709. https://doi.org/10.1038/ismej.2014.246.

4. Joshi A, Kostiauk B, Rogers A, Teschler J, Pukatzki S, Yildiz FH. 2016. Rules of engagement: the type VI secretion system in Vibrio cholerae. Trends Microbiol 22:2833–2842.

5. Buccio V, Nadell CD, Xavier JB. 2011. The evolution of bacteriocin production in bacterial biofilms. Am Nat 178:E162–E173. https://doi.org/10.1086/662668.

6. Kerr B, Riley MA, Bohannan BJM. 2002. Local dispersal promotes biodiversity in a rock–paper–scissors. Nature 418:171–174. https://doi.org/10.1038/nature00823.

7. Schwarz S, West TC, Boyer F, Chiang W-C, Carl MA, Hood RD, Rohmer L, Tolker-Nielsen T, Skerritt SJ, Mougous JD. 2010. Burkholderia Type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. PLoS Pathog 6:e1001068. https://doi.org/10.1371/journal.ppat.1001068.

8. Bosellini F, Grisoni A, Di Natale F, Benini D, Gatti F, Pignatelli S, Vorder Brueghel M, Forni P. 2017. Killing by Type VI secretion drives genetic phase separation and correlates with increased cooperation. Nat Commun 8:14371. https://doi.org/10.1038/ncomms14371.

9. Steinbach G, Grisan C, Ng SL, Hammer BK, Yunker PJ. 2020. Accumulation of dead cells from contact killing facilitates coexistence in biofilms. J R Soc Interface 17:20200486. https://doi.org/10.1098/rsif.2020.0486.

10. Smith WP, Vetter GE, Winter J, Ruyer T, Comstock LE, Basler M, Foster KR. 2020. The evolution of the type VI secretion system as a disintegration weapon. PLoS Biol 18:e3000720. https://doi.org/10.1038/s41591-020-00873-4.

11. McNaughton E, Bernhardt E, Thomas J, Kalzei A, Pottz J, Brown SP, Hammer BK, Yunker PJ, Ratcliff WC. 2017. Killing by Type VI secretion drives genetic phase separation and correlates with increased cooperation. Nat Commun 8:14371. https://doi.org/10.1038/ncomms14371.

12. Toska J, Ho BT, Mekalanos JJ. 2018. Expolysaccharide protects Vibrio cholerae from exogenous attacks by the type 6 secretion system. Proc Natl Acad Sci U S A 115:7997–8002. https://doi.org/10.1073/pnas.1804569115.

13. Bucci V, Nadell CD, Xavier JB. 2011. The evolution of bacteriocin production in bacterial biofilms. Am Nat 178:E162–E173. https://doi.org/10.1086/662668.

14. Kerr B, Riley MA, Bohannan BJM. 2002. Local dispersal promotes biodiversity in a rock–paper–scissors. Nature 418:171–174. https://doi.org/10.1038/nature00823.

15. Schwarcz S, West TC, Boyer F, Chiang W-C, Carl MA, Hood RD, Rohmer L, Tolker-Nielsen T, Skerritt SJ, Mougous JD. 2010. Burkholderia Type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. PLoS Pathog 6:e1001068. https://doi.org/10.1371/journal.ppat.1001068.

16. Teschler J, Cheng AT, Yildiz FH. 2017. The two-component signal transduction system VxrB Antagonism within Biofilms. mBio 8:215. https://doi.org/10.1128/mBio.00355-17.

17. Dimitriadis S, Ehrlich GD, Bassler BL, Foster KR. 2008. The Vibrio cholerae type VI secretion system VlpA promotes biofilm formation. Proc Natl Acad Sci U S A 105:17141–17146. https://doi.org/10.1073/pnas.0804569115.

18. Basler M, Pilhofer M, Henderson GP, Jensen GJ, Mekalanos JJ. 2012. Type VI secretion requires a dynamic contractile phage tail-like structure. Nature 483:182–186. https://doi.org/10.1038/nature10846.

19. Bönnemann G, Pietrosiuk A, Diemand A, Zentgraf H, Mogk A. 2009. Remodelling of VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion. EMBO J 28:315–325. https://doi.org/10.1038/emboj.2008.269.

20. Ma LS, Lin JS, Lai EM. 2009. An lcrF family protein, ImplM, is an integral inner membrane protein interacting with ImplK, and its Walker a motif is required for type VI secretion system-mediated Hcp secretion in Agrobacterium tumefaciens. J Bacteriol 191:4316–4329. https://doi.org/10.1128/JB.00029-09.

21. Basler M, Ho BT, Foster KR. 2020. The evolution of the type VI secretion system as a disintegration weapon. PLoS Biol 18:e3000720. https://doi.org/10.1038/s41591-020-00873-4.

22. Basler M, Ho BT, Mekalanos JJ. 2013. Identification of T6SS-dependent effector and immunity proteins by Trn-seq in Vibrio cholerae. Proc Natl Acad Sci U S A 110:2623–2628. https://doi.org/10.1073/pnas.1222783110.

23. Unterweger D, Kitaoka M, Miyata ST, Bachmann V, Brooks TM, Moloney L, Manera K, Kamal F, Burkinshaw B, Lam L, Provenzano D, Pukatzki S. 2014. The Vibrio cholerae type VI secretion system employs diverse effector modules for intraspecies competition. Nat Commun 5:3549. https://doi.org/10.1038/ncomms4549.

24. Dong TG, Ho BT, Yoder-Himes DR, Mekalanos JJ. 2013. Identification of T6SS-dependent effector and immunity proteins by Trn-seq in Vibrio cholerae. Proc Natl Acad Sci U S A 110:2623–2628. https://doi.org/10.1073/pnas.1222783110.

25. Provenzano D, Pukatzki S. 2014. The type VI secretion system VlpA promotes biofilm formation. mBio 8:215. https://doi.org/10.1128/mBio.00355-17.

26. Teschler J, Cheng AT, Yildiz FH. 2017. The two-component signal transduction system VxrB Antagonism within Biofilms. mBio 8:215. https://doi.org/10.1128/mBio.00355-17.

27. Unterweger D, Kitaoka M, Miyata ST, Bachmann V, Brooks TM, Mullins T, Kostkiu B, Provenzano D, Pukatzki S. 2014. The Vibrio cholerae type VI secretion system employs diverse effector modules for intraspecies competition. Nat Commun 5:3549. https://doi.org/10.1038/ncomms4549.

28. Basler M, Ho BT, Mekalanos JJ. 2013. Tit-for-tat: type VI secretion system counterattack during bacterial cell-cell interactions. Cell 152:884–894. https://doi.org/10.1016/j.cell.2013.01.042.

29. Herne S, Watanabe N, Stetzi MS, Manerka K, Kamal F, Burkiniash B, Lam L, Provenzano D, Pukatzki S. 2014. The Vibrio cholerae type VI secretion system VlpA promotes biofilm formation. Proc Natl Acad Sci U S A 111:1577–1582. https://doi.org/10.1073/pnas.1318904111.

30. Provenzano D, Pukatzki S. 2014. The type VI secretion system VlpA promotes biofilm formation. Proc Natl Acad Sci U S A 111:1577–1582. https://doi.org/10.1073/pnas.1318904111.
production, chlorine resistance, and biofilm formation. Proc Natl Acad Sci U S A 96:4028–4033. https://doi.org/10.1073/pnas.96.7.4028.
35. Fong JC, Karplus K, Schoolnik GK, Yildiz FH. 2006. Identification and characterization of RbmA, a novel protein required for the development of rugose colony morphology and biofilm structure in Vibrio cholerae. J Bacteriol 188: 1049–1059. https://doi.org/10.1128/JB.188.3.1049-1059.2006.
36. Yan J, Sharo AG, Stone HA, Wingreen NS, Basiller BL. 2016. Vibrio cholerae biofilm growth program and architecture revealed by single-cell live imaging. Proc Natl Acad Sci U S A 113:E5337–E5343. https://doi.org/10.1073/pnas.1611484113.
37. Wucher BR, Elsayed M, Adelman JS, Kadouri DE, Nadell CD. 2021. Bacterial predation transforms the landscape and community assembly of biofilms. Curr Biol 31:2643–2651.e3. https://doi.org/10.1016/j.cub.2021.03.036.
38. Drescher K, Dunkel J, Nadell CD, van Teefelen S, Granja I, Wingreen NS, Stone HA, Basiller BL. 2016. Architectural transitions in Vibrio cholerae biofilms at single-cell resolution. Proc Natl Acad Sci U S A 113:E2066–E2072.
39. Fong JC, Rogers A, Michael AK, Parsley NC, Cornell W-C, Lin Y-C, Singh PK, Hartmann R, Drescher K, Vinogradov E, Dietrich LE, Parth CL, Yildiz FH, 2017. Structural dynamics of RbmA governs plasticity of Vibrio cholerae biofilms. Elife 6:e2616. https://doi.org/10.7554/eLife.26163.
40. Giglio KM, Fong JC, Yildiz FH, Sondermann H. 2013. Structural basis for biofilm formation via the Vibrio cholerae matrix protein RbmA. J Bacteriol 195:3277–3286. https://doi.org/10.1128/JB.00374-13.
41. Granato ET, Meiller-Legrand TA, Foster KR. 2019. The evolution and ecology of bacterial warfare. Curr Biol 29:R521–R537. https://doi.org/10.1016/j.cub.2019.04.024.
42. Seper A, Fengler VHI, Roier S, Wolinski H, Kohlwein DS, Bishop AL, Camilli A, Reidl J, Schild S. 2011. Extracellular nucleases and extracellular DNA utilization of DNA as a nutrient source. Environ Microbiol 12:1621–1629. https://doi.org/10.1111/j.1462-2958.2009.06882.x.
43. Metzger LC, Stutzmann S, Scrignari T, Van der Henst C, Matthey N, Givskov M, Jelli E, Vaidya S, Blokesch M. 2016. Independent regulation of type VI secretion in Vibrio cholerae by TfxO and Tfir. Cell Rep 15:951–958. https://doi.org/10.1016/j.celrep.2016.03.092.
44. McDonough E, Lazinski DW, Camilli A. 2014. Identification of in vivo regulators of the Vibrio cholerae xds gene using a high-throughput genetic selection. Mol Microbiol 92:302–315. https://doi.org/10.1111/mmi.12557.
45. Metzger LC, Zinnaka Y, Carpenter CJ. 1972. An enterotoxin produced by non-cholera Vibrios. Johns Hopkins Med J 131:403–411.
46. Zinnaka Y, Carpenter CJ. 1972. An enterotoxin produced non-cholera Vibrios. Johns Hopkins Med J 131:403–411.
47. Liu X, Beyhan S, Lim B, Linington RG, Yildiz FH. 2010. Identification and characterization of a phosphodiesterase that inversely regulates motility and biofilm formation in Vibrio cholerae. Mol Microbiol 74:609–618. https://doi.org/10.1111/j.1365-2958.2009.06882.x.
48. Gloag ES, Turnbull L, Huang A, Vallotton P, Wang H, Nolan LM, Millili L, Hunt C, Lu J, Osath V, Monahan LG, Cavaliere R, Charles IG, Ward MP, Gee ML, Prabhakar R, Whitchurch CB. 2013. Self-organization of bacterial biofilms is facilitated by extracellular DNA. Proc Natl Acad Sci U S A 110: 11541–11546. https://doi.org/10.1073/pnas.1218896110.
49. Baize J-P, Aldecoa AL, Zafra O, Gonzalez-Pastor JE. 2017. Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities. Front Microbiol 8:1390. https://doi.org/10.3389/fmicb.2017.01390.
50. López D, Vlamakis H, Losick R, Kolter R. 2009. Cannibalism enhances biofilm development in Bacillus subtilis. Mol Microbiol 74:609–618. https://doi.org/10.1111/j.1365-2958.2009.06882.x.
51. Mulcahy H, Channon-Mazened L, Lewenza S. 2010. Pseudomonas aeruginosa produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source. Environ Microbiol 12:1621–1629. https://doi.org/10.1111/j.1462-2920.2010.02208.x.
52. Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, Koch B, Givskov M, Kjelleberg S. 2003. Cell death in Pseudomonas aeruginosa biofilm development. J Bacteriol 185:4585–4592. https://doi.org/10.1128/JB.185.15.4585-4592.2003.
53. Blokesch M, Darrieux G, Palmer D, Sperandio V. 2005. Architecture and motility of Pseudomonas aeruginosa biofilms. Environ Microbiol Rep 12:594–606. https://doi.org/10.1111/j.1758-2229.2012.12870.
54. Zinnaka Y, Carpenter CJ. 1972. An enterotoxin produced non-cholera Vibrios. Johns Hopkins Med J 131:403–411.