Microbial Herd Protection Mediated by Antagonistic Interaction in Polymicrobial Communities

Megan J. Q. Wong, Xiaoye Liang, Matt Smart, Le Tang, Richard Moore, Brian Ingalls, Tao G. Dong

Department of Ecosystem and Public Health, University of Calgary, Calgary, AB, Canada; Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, AB, Canada; Department of Applied Mathematics, University of Waterloo, Waterloo, ON, Canada; Department of Microbiology, Immunology, and Infectious Diseases, University of Calgary, Calgary, AB, Canada; Snyder Institute for Chronic Diseases, University of Calgary, Calgary, AB, Canada

ABSTRACT

In host and natural environments, microbes often exist in complex multispecies communities. The molecular mechanisms through which such communities develop and persist, despite significant antagonistic interactions between species, are not well understood. The type VI secretion system (T6SS) is a lethal weapon commonly employed by Gram-negative bacteria to inhibit neighboring species through the delivery of toxic effectors. It is well established that intraspecies protection is conferred by immunity proteins that neutralize effector toxicities. In contrast, the mechanisms for interspecies protection are not clear. Here we use two T6SS-active antagonistic bacterial species, Aeromonas hydrophila and Vibrio cholerae, to demonstrate that interspecies protection is dependent on effectors. A. hydrophila and V. cholerae do not share conserved immunity genes but could coexist equally in a mixture. However, mutants lacking the T6SS or effectors were effectively eliminated by the competing wild-type strain. Time-lapse microscopic analyses showed that mutually lethal interactions drive the segregation of mixed species into distinct single-species clusters by eliminating interspersed single cells. Cluster formation provides herd protection by abolishing lethal interactions inside each cluster and restricting the interactions to the boundary. Using an agent-based modeling approach, we simulated the antagonistic interactions of two hypothetical species. The resulting simulations recapitulated our experimental observations. These results provide mechanistic insights regarding the general role of microbial weapons in determining the structures of complex multispecies communities.

IMPORTANCE

Investigating the warfare of microbes allows us to better understand the ecological relationships in complex microbial communities such as the human microbiota. Here we use the T6SS, a deadly bacterial weapon, as a model to demonstrate the importance of lethal interactions in determining community structures and the exchange of genetic materials. This simplified model elucidates a mechanism of microbial herd protection by which competing antagonistic species can coexist in the same niche, despite their diverse mutually destructive activities. Our results also suggest that antagonistic interactions impose strong selection that could promote multicellular organism-like social behaviors and contribute to the transition to multicellularity during evolution.

The healthy human microbiome consists of hundreds of microbial species occupying a variety of body sites. These microbes are not passive passengers but play significant roles in maintaining health (1–3). Microbiotic dysbiosis has been implicated in various infectious and chronic diseases, including vaginosis, diabetes mellitus, and immunological disorders (2, 3). Modulation of the microbiota has shown great promise in treating diseases, as exemplified by the effective treatment of Clostridium difficile-infected patients with fecal transplants (4, 5). Despite the tremendous progress, the mechanisms that control the composition of the microbiota and dynamic changes in response to disruptions (e.g., infections and dietary changes) are largely unknown. Although sequencing data can provide a general view of the microbiota at the species level, a full understanding of the complexity and functions of the microbiota requires appreciation of how these species interact with each other at the level of the microniche they coocupy and how they form structured communities. Considering the diversity of weapons microbes have developed for competition, antagonistic interactions no doubt represent a significant proportion of microbial experiences in polymicrobial niches. However, the processes by which antagonistic microbes establish community structures and coexist are not well understood.

The type VI protein secretion system (T6SS) is a multicomponent molecular weapon commonly employed by Gram-negative bacteria (6–10). About 25% of Gram-negative pathogens possess one or more T6SS clusters in their genomes (11), which indicates that the T6SS plays an important role in bacterial fitness in multispecies communities. Unlike systems that produce diffusible inhibitory molecules (e.g., antibiotics) or require surface-bound receptors (e.g., phage), the T6SS injects multiple toxic effectors into neighboring cells in a contact-dependent manner (7, 10). Each antibacterial effector can be neutralized by a genetically linked
immunity protein that confers protection against effector delivery between sister cells (7, 12, 13). In addition, the T6SS has been shown to play an important role in vivo in multiple species, including human-symbiotic *Bacteroidetes* species (14–16). As a lethal weapon, the T6SS has been shown to mediate kin recognition in the multicellular bacterium *Proteus mirabilis*, which can develop elongated swarmer cells and form distinct boundaries (known as Dienes lines) between different *P. mirabilis* strains (17–20). Nonidentical *P. mirabilis* strains are unable to infiltrate each other’s clusters, a phenomenon attributed to T6SS-mediated killing and lack of shared immunity proteins [18]. In comparison with intraspecies immunity protein-dependent protection, it is not clear how antagonistic species (normally lacking specific immunity genes) can coexist within a shared niche.

We previously characterized the T6SS antibacterial activities of two waterborne pathogens, *Vibrio cholerae* strain V52 (13) and *Aeromonas hydrophila* strain SSU (21). *V. cholerae* possesses four antimicrobial effectors, namely, VgrG3, which functions as a lysozyme (13, 22); the lipase TseL (13, 23); VasX, which has a colicin-like domain (13, 24); and the hydrolase TseH (25), which seems to play only a minor role in interbacterial killing (25). *A. hydrophila* has one known effector, TscC, which, like VasX, carries a colicin-like domain (21). Interestingly, we noticed that a 1:1 mixture of *V. cholerae* and *A. hydrophila* exhibited little CFU decrease of either species, despite the constitutive T6SS activity of *V. cholerae* and *A. hydrophila* and the lack of shared immunity proteins. Moreover, *V. cholerae* and *A. hydrophila*, which share a mutual host in aquatic environments (26), exhibit almost identical growth rates and similar genomic arrangements of their T6SS genes (27). Consequently, the T6SS-mediated interactions between *V. cholerae* and *A. hydrophila* serve as a useful model to elucidate the strategies that account for the coexistence of antagonistic species. We demonstrate that, unlike immunity protein-mediated intraspecies protection, toxic T6SS effectors confer protection by intoxicating the attacking species. Mutants that are either defective in the T6SS or missing effectors are sensitive to the T6SS-mediated toxicity of the other species. Time-lapse microscopic analyses show that T6SS is required to establish a defensive boundary that separates mixed antagonistic species. Using agent-based simulation models (28), we confirmed that antagonistic interactions generically result in the establishment of microbial community structures, which confer herd protection by shielding cells inside each cluster from lethal activities (while interspersed single cells are rapidly eliminated from the community).

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** *V. cholerae, A. hydrophila,* and their derivative T6SS mutants are listed in Table 1. Deletion mutants were constructed using crossover PCR and homologous recombination, as described previously (29). *V. cholerae vasK*Kan was constructed by transforming the *V. cholerae vasK* mutant with a pBAD18-Kan plasmid to gain kanamycin resistance. Strain C6706K carries a kanamycin resistance transposon inserted in the *lacZ* gene (30). Cultures were grown in LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl) aerobically at 37°C. The following antibiotics and chemicals were used wherever appropriate: streptomycin (100 μg/mL), kanamycin (50 μg/mL), ampicillin (100 μg/mL), chloramphenicol (25 μg/mL for *Escherichia coli* and 2.5 μg/mL for strains SSU and V52), and arabinose (0.1% [wt/vol]). Gene expression vectors were constructed as described previously (31). All constructs were verified by sequencing.

**Table 1 Strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V. cholerae V52</strong></td>
<td>Serotype 37 clinical isolate from Sudan</td>
<td>8</td>
</tr>
<tr>
<td>vasK*</td>
<td>T6SS null mutant lacking vasK gene</td>
<td>8</td>
</tr>
<tr>
<td>cIPv</td>
<td>T6S2 lacking VCA0116</td>
<td>32</td>
</tr>
<tr>
<td>tseL</td>
<td>Deletion mutant of VCA1418</td>
<td>32</td>
</tr>
<tr>
<td>vasX</td>
<td>Deletion mutant of VCA0020</td>
<td>32</td>
</tr>
<tr>
<td>vgrG3</td>
<td>Deletion mutant of VCA0123</td>
<td>32</td>
</tr>
<tr>
<td>tseL vasX</td>
<td>Double deletion mutant</td>
<td>13</td>
</tr>
<tr>
<td>tseL vgrG3</td>
<td>Double deletion mutant</td>
<td>13</td>
</tr>
<tr>
<td>vasX vgrG3</td>
<td>Double deletion mutant</td>
<td>13</td>
</tr>
<tr>
<td><strong>V. cholerae C6706K</strong></td>
<td>Transposon insertion in lacZ, conferring kanamycin resistance</td>
<td>30</td>
</tr>
<tr>
<td><strong>A. hydrophila SSU</strong></td>
<td>Diarrheal isolate</td>
<td>27</td>
</tr>
<tr>
<td>vasK*</td>
<td>T6SS null mutant, with in-frame deletion of vasK</td>
<td>21</td>
</tr>
<tr>
<td><strong>tseC</strong></td>
<td>ORF2402 deletion mutant</td>
<td>21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD18-Cm</td>
<td>Conferring chloramphenicol resistance</td>
<td>48</td>
</tr>
<tr>
<td>pBAD18-Kan</td>
<td>Conferring kanamycin resistance</td>
<td>48</td>
</tr>
<tr>
<td>pBAD24</td>
<td>Conferring ampicillin resistance</td>
<td>48</td>
</tr>
<tr>
<td>pBAD18-siGFP</td>
<td>For expression of siGFP</td>
<td>33</td>
</tr>
<tr>
<td>pBAD18-Kan-VCA0021</td>
<td>For expression of immunity protein</td>
<td>13</td>
</tr>
<tr>
<td>pBAD24-mCherry2</td>
<td>For expression of mCherry2 protein</td>
<td>33</td>
</tr>
</tbody>
</table>

*Bacterial cell killing assay.* Killing assays were performed as described previously (32). Briefly, 1 ml of exponential-phase cultures (optical density at 600 nm [OD_{600}] of 0.8) was centrifuged at 10,000 × g for 2 min, and the resultant pellet was resuspended in 100 μL of fresh LB medium. Killer and prey cells were mixed at different ratios in a final volume of 20 μL, and each mixture was incubated for 3 h at 37°C on a LB agar plate. Cells were recovered from the mixture and resuspended in 1 μl of LB medium. The survival of prey cells was quantified by serial dilution in LB medium and plating on LB medium containing appropriate antibiotics.

**Fluorescence microscopy.** Overnight cultures were diluted 1:100 (vol/vol) in fresh LB medium and grown to an OD_{600} of 0.2. Arabinose (0.1% [wt/vol]) was added to induce the expression of plasmid-borne superfolder green fluorescent protein (sfGFP) and mCherry2 (Table 1). Cells were collected by centrifugation after induction for 1.5 h and were concentrated to an OD_{600} of 10. Samples were spotted on a 1% (wt/vol) agarose pad containing 0.01% (wt/vol) arabinose and 10% (vol/vol) LB medium (33). Cells were imaged at room temperature. Fluorescence signals of sfGFP and mCherry2 were detected using a fluorescein isothiocyanate (FITC) filter set and a tetramethyl rhodamine isothiocyanate (TRITC) filter set, respectively. A Nikon Ti-E inverted microscope equipped with a perfect focus system and a CPI Plan Apo Lambda ×100 oil objective lens was used for imaging. Fiji software was used for all image analysis and manipulation (34). Images from a time series were normalized to the same mean intensity, as described previously (33).

**Agent-based modeling and simulation.** The agent-based model simulates the temporal behavior of agents on a square lattice. Periodic boundary conditions are assumed. At each time point, each lattice point (i) is empty, (ii) is debris filled, or (iii) contains an agent (cell) of type A or type B. The simulation proceeds in uniform time steps of 2 min. At each time step, a randomized list of all nonempty lattice points is constructed. Activities at those lattice points are resolved in sequence according to their order in the list. The resolution of a cell’s lattice site begins with a death check (details below). Cells that die are converted to debris; the debris site...
is assigned a latency period drawn from the normal distribution with a mean of 40 and a standard deviation of 5 \( \text{i.e., } N(40,5) \). Cells that do not die perform two actions, i.e., division and shooting (details below). The order in which these two events occur is chosen randomly for each cell. At its birth, each cell is assigned a time-to-division value (in minutes) drawn from \( N(20,2.5) \). At a division event, a cell that has reached its division time checks its eight neighboring lattice points for empty sites. If there are any empty sites, then it replicates (a daughter of the same type appears) in a randomly chosen empty neighboring site. Both cells are then assigned a new time-to-division value. When a shooting event occurs, a shooting capacity \( C \) is chosen from \{0,1,2,3,4,5\}, an empirical value corresponding to the number of needles in a T6SS-active cell at a given time (35). Targets are then chosen at random from the eight neighboring lattice points. If a targeted site contains a cell of the opposite type, then that cell is marked as fatally wounded and is converted to debris after a duration drawn from \( N(5,1) \). Shooting has no effect on empty or debris-filled sites, and it does not affect cells of the same type as the shooter. Debris sites are resolved as follows: a debris element is removed (the lattice site becomes empty) once the latency period has ended. All times are measured in minutes. For initialization of the simulation, the lattice is populated with cells and debris as desired. Each cell is assigned a time-to-division value sampled from the uniform distribution of 0 to 20. The algorithm was implemented in Python 2.7. The source code is available upon request.

**RESULTS**

**V. cholerae and A. hydrophila**

**V. cholerae and A. hydrophila**

**reliance on T6SS for survival in coinfections.** Because \( V. cholerae \) and \( A. hydrophila \) share very similar growth characteristics and T6SS activity levels but possess distinct effector-immunity modules, we were interested in testing whether \( V. cholerae \) and \( A. hydrophila \) could coexist. When exponential-phase wild-type \( V. cholerae \) and \( A. hydrophila \) were mixed at a 1:1 ratio, they survived equally well despite lacking the protection of immunity proteins (Fig. 1A and B). The T6SS null vasK mutants coding for a T6SS membrane complex protein of \( V. cholerae \) or \( A. hydrophila \), however, exhibited 1,000-fold decreases in survival when exposed to wild-type \( A. hydrophila \) or \( V. cholerae \), respectively. At a 10:1 killer/prey ratio, the survival of the vasK mutants of \( V. cholerae \) and \( A. hydrophila \) was reduced 10\(^6\)-fold (Fig. 1A and B). Wild-type \( V. cholerae \) was reduced only 10\(^4\)-fold when it was outnumbered by \( A. hydrophila \), whereas wild-type \( A. hydrophila \) was reduced 100-fold when it was outnumbered by \( V. cholerae \) (Fig. 1A and B). It should be noted that the vasK mutant, which is defective in the T6SS but still expresses functional immunity proteins, is known to be resistant to wild-type T6SS activities (13, 36). These data collectively indicate that the T6SS is essential for the survival of \( V. cholerae \) and \( A. hydrophila \) in mixed populations.

**Dependence of competitive fitness on T6SS effectors.** Because \( A. hydrophila \) and \( V. cholerae \) do not share conserved immunity proteins that would confer protection to the delivered T6SS effectors, we reasoned that the protection of \( A. hydrophila \) and \( V. cholerae \) by T6SS might be due to effector activities. To test this, we coinoculated wild-type strains and a series of single and double effector mutants of \( A. hydrophila \) and \( V. cholerae \) at 1:1 ratios. Consistent with our prediction, we found that, although the survival of \( V. cholerae \) single effector mutants lacking TseL, VasX, or VgrG3 did not differ significantly from that of wild-type \( V. cholerae \), gene for \( V. cholerae \), and its T6SS null vasK mutant (TS6\(^{-}\)) attacked by \( A. hydrophila \). (B) Survival of wild-type \( V. cholerae \) (TS6\(^{+}\)) and its T6SS null vasK mutant (TS6\(^{-}\)) attacked by \( V. cholerae \). (C) Survival of \( V. cholerae \) effector mutants against killing by \( A. hydrophila \). (D) Survival of the \( A. hydrophila \) tseC effector mutant against killing by \( V. cholerae \). Survival of prey cells was determined by serial dilutions on selective medium. (E) Survival of the \( A. hydrophila \) vasK mutant attacked by the \( V. cholerae \) vgrG3 tseL double mutant. The \( A. hydrophila \) vasK mutant was complemented with either an empty vector or the plasmid-borne tsL\(^{V} \) gene, encoding the immunity protein for \( V. cholerae \) effector VasX (the remaining functional T6SS effector of the \( V. cholerae \) vgrG3 tseL double mutant). Killer and prey cells were mixed at a ratio of 1:1. In panels A and B, the killer/prey ratio is indicated; representative results from at least three separate experiments are shown. In panels C and D, assays were performed in triplicate by mixing killer and prey cells at a ratio of 1:1. Statistical significance was calculated using Student’s t test. *, \( P \) value of <0.05, in comparison with the wild-type strain.
erae, double mutants lacking any two effectors showed significantly reduced survival (Fig. 1C). It should be noted that all of the tested effector mutants, except for the vasX vgrG3 double deletion mutant, still possessed functional T6SS antimicrobial activities (13). Similarly, the survival of the tseC mutant of A. hydrophila was reduced significantly (1,000-fold) in comparison with wild-type A. hydrophila (Fig. 1D). To confirm that the observed killing between V. cholerae and A. hydrophila was mediated by T6SS effector activities, we tested whether a V. cholerae immunity gene could confer protection to the A. hydrophila vasK mutant. Because there are multiple effectors in both V. cholerae and A. hydrophila, we decided to focus on one V. cholerae effector-immunity pair, VasX-TsiV2, by transforming the A. hydrophila vasK mutant with a plasmid-borne arabinose-inducible tsiV2 gene. Expression of the tsiV2 gene effectively protected the A. hydrophila vasK mutant from killing by the V. cholerae tseL vgrG3 mutant, which can specifically deliver VasX but not the other two effectors (Fig. 1E). Therefore, unlike immunity proteins that confer intraspecies protection, T6SS effector proteins are required for competitive fitness between different species.

**T6SS-mediated cluster formation of V. cholerae and A. hydrophila.** We then sought to investigate how the distinct T6SS systems of V. cholerae and A. hydrophila, which are capable of mutual destruction, could promote coexistence. Using microscopic analysis, we noticed that, in a random mixture, wild-type A. hydrophila was killed by V. cholerae if outnumbered and vice versa (Fig. 2A and B; also see Movie S1 in the supplemental material), consistent with the results of killing assays on plates (Fig. 1). Further, A. hydrophila and V. cholerae eventually formed segregated clusters. In contrast, the T6SS mutants of A. hydrophila and V. cholerae coexisted as a mixed community (Fig. 2C; also see Movie S2 in the supplemental material). When wild-type V. cholerae was mixed with the T6SS mutant of A. hydrophila, the mutant could survive only when there was no contact (Fig. 2D; also see Movie S3 in the supplemental material). When two V. cholerae and A. hydrophila clonal clusters established direct contact, extensive cell death was observed at the interface (Fig. 2E; also see Movie S4 in the supplemental material).

Based on these observations, we propose the following conceptual model. The competitive fitness ($f$) of a T6SS organism is dependent on the potency of each effector ($e$), the number of effectors ($n$), and the T6SS shooting frequency ($a$) (Fig. 3A). Because T6SS cells often possess multiple effectors, we used the propensity of shooting of a given effector ($p$) to account for how likely each T6SS ejection is to contain that effector as “ammunition”; this might depend on the effector’s affinity for the T6SS or its production rate. In the case of V. cholerae and A. hydrophila interactions, loss of effectors or T6SS activity results in reduced $f$ and increased susceptibility to T6SS killing (Fig. 1C and D). The T6SS shooting frequency ($a$) is determined by the number of contraction events per cell per unit time. Because the mutant of clpV (the gene that encodes the ATPase that disassembles the contracted sheath for recycling) exhibits less active T6SS dynamics in V. cholerae (37), we reasoned that the clpV mutant, with reduced contraction activities, would be less competitive. Indeed, we found that the clpV

---

**FIG 2** Microscopic analysis of the interactions between A. hydrophila (sfGFP-labeled) and V. cholerae (mCherry2-labeled) strains. (A) Outnumbered A. hydrophila cells killed by V. cholerae (arrows). (B) Outnumbered V. cholerae cells killed by A. hydrophila (arrows). (C) Coexistence of the vasK mutants of A. hydrophila and V. cholerae. (D) A. hydrophila vasK mutant killed by V. cholerae. (E) Killing at the interface of A. hydrophila and V. cholerae populations. Cells that move toward and establish contact with the opposing population are likely killed. Arrows, a killed V. cholerae cell that formed a spheroplast and burst. The space that cell had been occupying, likely filled by debris, remained empty for about 1 h until another V. cholerae cell moved to occupy it. (F) Impaired survival of the V. cholerae (VC) clpV mutant, which is subject to T6SS killing by A. hydrophila (AH), in comparison with wild-type V. cholerae. The killer/prey ratio was 2:1.
FIG 3 Model depicting T6SS-mediated fitness and interactions. (A) T6SS-mediated fitness, determined by effector activities, the number of effectors, and contraction dynamics. In the equation, \( a \) is the shooting frequency and \( n \) is the number of effectors; for each \( i = 1, 2, \ldots n \), \( e_i \) is the potency of effector \( i \) and \( p_i \) is the propensity for shooting of effector \( i \). (B) Establishment of segregated clusters from a random mixture of two T6SS killer strains. X, cell death. Due to stochastic firing, a green cell kills a neighboring red cell and takes its place. Red cells in the vicinity are then outnumbered and killed. This triggers a series of events in which the local red population could be eliminated. Similar events may also occur to isolated green populations, eventually resulting in separation into kin-only clusters. (C) Interactions at the interface of two killer populations. When a green cell moves toward the opposing red cell population, due to cell division or motility, the green cell is outnumbered and killed. Similar events can occur at multiple locations along the interface and with any advancing red cells. This results in the demarcation of two T6SS-active communities. In some instances, cell debris left by dead cells forms a physical barrier that can, at least temporarily, separate killer cells.

FIG 4 T6SS-mediated cheater cell protection. (A) Wild-type \( V. cholerae \) (VC) conferring protection against \( A. hydrophila \) (AH) T6SS killing to its vasK mutant. The V52 vasK-Kan mutant carrying a pBAD18-Kan plasmid was incubated with wild-type \( A. hydrophila \) or the \( A. hydrophila \) vasK mutant, in the presence or absence of wild-type \( V. cholerae \) or the \( V. cholerae \) vasK mutant. Survival of the \( V. cholerae \) vasK-Kan strain was enumerated by serial plating on kanamycin medium. (B) \( V. cholerae \) protecting C6706, a T6SS-inactive cholera pandemic isolate, from \( A. hydrophila \) T6SS. C6706K, \( V. cholerae \), \( A. hydrophila \), and their vasK mutants were co-incubated similarly, as indicated. C6706K carries a kanamycin resistance transposon inserted in the lacZ gene. Survival of C6706K was also examined by serial plating on kanamycin plates. All strains were grown in LB medium aerobically to an \( \text{OD}_{600} \) of 0.8, mixed at a killer/protector/prey ratio of 2:1:1, and coincubated on LB agar plates for 5 h. Student’s \( t \) test was used to evaluate statistical significance. Means and standard errors are indicated. The assays were repeated three times.

Enhancement of survival of T6SS-inactive cells by mutual antagonism. Maintaining an active T6SS is likely costly to cells, and mutants expressing the immunity genes but not assembling the T6SS structures may still survive inside the protective clusters, a form of “cheating” behavior (38). These cheater cells may arise due to spontaneous mutations inside each cluster or may be present in the initial mixture with the wild-type strain (prior to being exposed to lethal interactions). We tested whether a \( V. cholerae \) T6SS null vasK mutant could be protected by wild-type \( V. cholerae \) against \( A. hydrophila \)-mediated attack. To differentiate prey cells from killer cells, the \( V. cholerae \) vasK mutant was transformed with a plasmid conferring kanamycin resistance, named \( V. cholerae \ vasK-Kan. The \( V. cholerae \) vasK-Kan mutant was then coincubated with \( V. cholerae \) wild-type or \( V. cholerae \) vasK strains and \( A. hydrophila \) wild-type or \( A. hydrophila \) vasK strains. We found that the survival of the \( V. cholerae \) vasK-Kan strain was significantly increased by the T6SS-dependent protection of \( V. cholerae \) (Fig. 4A).

Some \( V. cholerae \) isolates, including the seventh pandemic \( V. cholerae \) strain C6706, are phenotypically T6SS inactive due to transcriptional control (39). We postulated that these \( V. cholerae \) strains may also be protected by T6SS-active \( V. cholerae \). Previous analyses showed that strain C6706 encodes the same effector-immunity modules and is resistant to the T6SS of \( V. cholerae \) (13). We tested whether strain C6706 could be protected by \( V. cholerae \) from \( A. hydrophila \) attack. Indeed, the recovery of C6706K mixed with wild-type \( V. cholerae \) was significantly greater than that of C6706K mixed with the \( V. cholerae \) vasK strain, indicating T6SS-dependent protection (Fig. 4B).
clusters is a general feature of contact-dependent antagonistic interactions. The specific initial condition was obtained by randomly filling the lattice with a type A (green)/type B (red)/empty (white) ratio of 0.2:0.2:0.6. Yellow lattice points, debris. The simulation duration was 24 h. Over time, the mixed population is segregated into two clusters (see Movie S6 in the supplemental material). This proof-of-principle model is thus consistent with our experimental results; antagonistic interactions separate the competing species on a square lattice (see Materials and Methods for details). At each time point, each lattice point (i) is empty, (ii) is debris filled, or (iii) contains an agent (cell) of type A or type B. To simplify the process, we supposed that the two cell types have equal killing abilities. The simulation proceeds in uniform 2-min time steps. At each time step, cells have probabilities of dividing and of shooting their neighbors. Cell death and debris formation follows from successful shooting events. Model parameters were assigned values to arrive at reasonable estimates of system behavior. The temporal simulations provide qualitative confirmation of our experimental results; antagonistic interactions separate the two mixed species into clonal clusters over time and stabilize them for long periods (Fig. 5; also see Movie S6 in the supplemental material). This proof-of-principle model is thus consistent with previous models describing unilateral contact inhibition (41–43) and provides a framework for more detailed analysis, which could involve calibrating the model with respect to specific features of the interacting populations.

DISCUSSION

In a complex multispecies community, individual microbes establish various types of interactions with neighboring species, which ultimately determine the dynamic changes in structure and the relative abundance of individuals that constitute the community. The mechanisms of interactions, or the rules of engagement, when two distinct antagonistic species encounter each other in complex communities are not fully understood. Here we present a conceptual model for the competitive fitness and extensive interactions of mutually destructive killer species (Fig. 3). We show that T6SS effectors are the key determinants of competitive fitness and they confer protection in interspecies competition. The contributions of effectors to fitness are additive; the absence of two or more effectors in V. cholerae reduces the V. cholerae fitness to the T6SS null mutant level (Fig. 1C). Our data suggest that antagonistic interactions are the driving force for the acquisition of additional effector modules, which presumably promotes fitness, expands the range of susceptible species, and reduces the chances of target cells developing resistance. Indeed, environmental isolates of the same species have been shown to carry distinct effector-immunity modules (36, 44). The presence of multiple T6SS systems and associated effectors in T6SS organisms is likely reflective of the complex environments these species have encountered during evolution.

Our data demonstrate that bacteria employ the T6SS to establish protective clusters by maintaining mutually destructible battlefront interfaces (Fig. 2D; also see Movie S4 in the supplemental material). In contrast, the T6SS mutants of A. hydrophila and V. cholerae lost the ability to form separate clonal clusters and instead maintained mixed populations. In order to focus on lethal interaction-dependent community changes, we intentionally set up the competition at a very high cell density (OD600 of 10) to allow rapid and sufficient contact between cells; this is distinct from growth-mediated cluster formation, during which cells mixed at lower initial densities grow into clusters before establishing contact with the other species. Despite the difference, the T6SS is critical for maintaining clonal clusters during antagonistic interactions. It was demonstrated previously that sparsely seeded Burkholderia thailandensis cells developed into clusters and outcompeted a Pseudomonas putida strain in a flow chamber biofilm but the T6SS-1 mutant failed to survive (45).

In contrast to previous studies investigating the intraspecies interactions of preformed communities (17, 18), our experimental and agent-based modeling results demonstrate the process of cluster segregation from random initial mixtures during antagonistic interspecies interactions. Unlike previous reports that attributed the intraspecies incompatibility to lack of immunity proteins, our results show that different species can achieve equilibrium due to immunity protection but to dynamic killing interactions at the interfaces of clusters, which are formed and maintained by antagonistic interactions.

Debris released by killed cells can function as a physical barrier that can delay or prevent further contact between species and thus promote survival of the less aggressive species. This effect may allow clusters to replace dead cells at the interface with freshly divided cells. The survival of such a cluster would then be dependent on multiple factors, including the speed of killing, the rate of growth, and the time of establishing direct contact. Indeed, a recent study showed that nonkiller E. coli may survive T6SS-mediated V. cholerae attacks if the E. coli colonies have established a sufficiently large domain prior to contact (43). The killing in the previous study was unilateral, while our model focuses on two mutually destructible species. In a random mixture, E. coli, similar to the T6SS null mutants, can be efficiently eliminated from the mixed population by V. cholerae or A. hydrophila upon direct contact (13, 21).

Interspecies microbial relationships (e.g., in the gut microbiota and in other natural environments) are tremendously complex. The T6SS investigated in this study represents only one of many different forms of antagonistic interactions; others include the production of bacteriocins (46) and the contact-dependent inhibition system (40, 47). Importantly, the selection for clonal clusters imposed by antagonistic interactions may allow microbes to develop multicellular organism-like social behaviors, including cooperation and cheating (38). Here we present an example of cheating behavior in which both the T6SS null mutant and the T6SS-inactive pandemic strain C6706 were protected by V. cholerae from A. hydrophila attack, which suggests that cells that have reduced their energetic burden by inactivating T6SS delivery can directly benefit from the herd protection conferred by the contributing kin cells. We must be cognizant of the limitations of our
simple experimental design and model simulations. Further investigation will involve more complex multispecies environments and more sophisticated models that take into account differences in growth rates, killing strength, contact efficiency, and other parameters. Such studies will continue to provide insights toward understanding antagonistic interactions. Nonetheless, our simplified model highlights the importance of antagonistic interactions in determining community structures and diversifying clonal populations, which may be a factor driving the transition to multicellularity.

ACKNOWLEDGMENTS

This work was supported by a Canadian Institutes of Health Research operating grant, an Alberta Livestock and Meat Agency grant, and a Canadian Natural Sciences and Engineering Research Council discovery grant to T.G.D. T.G.D. was also supported by a Government of Canada Research Chair award, a Canada Foundation for Innovation grant, and an Alberta Innovation and Advanced Education grant. M.W. was supported by an Ecosystem and Public Health scholarship. L.T. was supported by an Alberta Innovates Health Solutions postdoctoral fellowship. M.S. and B.I. were supported by a Canadian Natural Sciences and Engineering Research Council discovery grant. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

We thank the Advancing Canadian Wastewater Assets program for providing infrastructure and equipment support. We thank Alex Le, Christy Catalano, Linh Lam, Jessica Li, and other members of the Dong laboratory for providing reagents and general support.

We declare no conflicts of interest.

FUNDING INFORMATION

This work, including the efforts of Le Tang, was funded by Alberta Innovates - Health Solutions (AIHS). This work, including the efforts of Taq G. Dong, was funded by Alberta Livestock and Meat Agency (ALMA). This work, including the efforts of Taq G. Dong, was funded by Canada Research Chairs (Chairs de recherche du Canada). This work, including the efforts of Brian Ingalls and Taq G. Dong, was funded by Gouvernement du Canada | Natural Sciences and Engineering Research Council of Canada (NSERC). This work, including the efforts of Taq G. Dong, was funded by Gouvernement du Canada | Canadian Institutes of Health Research (CIHR). This work, including the efforts of Taq G. Dong, was funded by Canada Foundation for Innovation (CFI).

REFERENCES


