Maximizing Growth Yield and Dispersal via Quorum Sensing Promotes Cooperation in *Vibrio* Bacteria

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ABSTRACT Quorum sensing (QS) is a form of bacterial chemical communication that regulates cellular phenotypes, including certain cooperative behaviors, in response to environmental and demographic changes. Despite the existence of proposed mechanisms that stabilize QS against defector exploitation, it is unclear if or how QS cooperators can proliferate in some model systems in populations mostly consisting of defectors. We predicted that growth in fragmented subpopulations could allow QS cooperators to invade a QS defector population. This could occur despite cooperators having lower relative fitnesses than defectors due to favored weighting of genotypes that produce larger populations of bacteria. Mixed metapopulations of *Vibrio* QS-proficient or unconditional cooperators and QS defectors were diluted and fragmented into isolated subpopulations in an environment that requires QS-regulated public good production to achieve larger population yields. Under these conditions, we observed global invasions of both cooperator genotypes into populations composed of primarily defectors. This spatially dependent increase in cooperator frequency was replicated for QS cooperators when mixed populations were competed in soft agar motility plates under conditions that allowed cooperators to disperse and outcompete defectors at the population edge, despite being less motile in isolation than defectors. These competition results show that the coordinated growth and dispersal of QS cooperators to additional resources is heavily favored in comparison to unconditional cooperation, and that dispersal of cooperators by motility into new environments, examined here in laboratory populations, constitutes a key mechanism for maintaining QS-regulated cooperation in the face of defection.

IMPORTANCE Behaviors that are cooperative in nature are at risk of exploitation by cheating and are thus difficult to maintain by natural selection alone. While bacterial cell-cell communication, known as quorum sensing (QS), can stabilize microbial cooperative behaviors and is widespread in *Vibrio* species, it is unclear how QS can increase the frequency of cooperative strains in the presence of defectors without additional mechanisms. In this study, we demonstrate under multiple conditions that QS-mediated cooperation can increase in populations of *Vibrio* strains when cells experience narrow population bottlenecks or disperse from defectors. This occurred for both conditional cooperation mediated by QS and for unconditional cooperation, although conditional cooperators were better able to stabilize cooperation over a much wider range of conditions. Thus, we observed that population structuring allowed for assortment of competing genotypes and promoted cooperation via kin selection in microbes in a QS-dependent manner.

KEYWORDS *Vibrio harveyi*, quorum sensing, cooperation, evolution, communication, Simpson's paradox, *Vibrio*

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Explaining the ongoing persistence of cooperative behavior has been a long-standing challenge for evolutionary biology (1–3). Cooperative behaviors are not expected to be evolutionarily stable by natural selection alone and thus require additional mechanisms to explain their existence and persistence in nature (4, 5). Nonetheless, cooperation is widely found at all levels of biological organization, from subcellular molecular systems to multispecies communities. One well-studied class of cooperative behaviors is the production of public goods, costly products that increase individual fitness but whose resulting benefits can be shared between all members of a population, including nonproducers. Public good production provides an opportunity for under- or nonproducing defectors to cheat contributing cooperators in mixed populations, increasing their frequency while simultaneously destabilizing productivity of the whole population. This can in turn lead to a tragedy of the commons undermining the stability of the cooperative behavior (6–8).

An available mechanism contributing to the limitation of defectors is facultative production of public goods when the benefit to do so is increased or the cost is reduced (9, 10). Bacteria often regulate public good production in a condition-dependent manner by using a common form of chemical communication known as quorum sensing (QS). In members of the *Vibrionaceae* bacteria, including the marine bacterium *Vibrio harveyi* and the human pathogen *Vibrio cholerae*, the QS circuit consists of a phosphorelay pathway that is controlled by exogenous chemical signals known as autoinducers (11, 12). At low cell densities, when autoinducer concentrations are low, membrane-bound kinases funnel phosphate through this pathway, leading to phosphorylation of the response regulator LuxO, which represses expression of the high-cell-density master regulators LuxR for *V. harveyi* and HapR for *V. cholerae* via the induction of regulatory small RNAs (sRNAs) (11, 12). At high cell densities, autoinducer molecules rise to sufficient concentrations to be bound by their cognate receptors and thereby activate these receptors' phosphatase activity, leading to dephosphorylation of LuxO and expression of LuxR or HapR, which induces expression of many genes (12, 13). The *Vibrio* QS circuit is a powerful system to study the role of QS in coordinating cooperation because multiple mutant phenotypes can be readily generated. An unconditional cooperator (UC) genotype can be engineered through null mutations to *luxO*, which locks the cells into a high-density state, while a defector (D) genotype that is locked into a low-density, noncooperative state can be generated by null mutations to the *luxR* or *hapR* master regulator gene (Fig. 1). We have shown that in *V. harveyi*, the cooperative production of extracellular proteases, which are public goods susceptible to cheating by nonproducing defectors, can be stabilized even in well-mixed conditions by facultative expression via QS regulation (14). However, the unregulated protease production of the UC strain is exploited, resulting in a tragedy of the commons.

Although we previously showed that QS regulation stabilizes protease production, even the facultative QS cooperator genotype of *V. harveyi* was unable to invade a population of mostly defectors in an environment that required proteolytic activity for maximum productivity (14). Yet, QS-proficient *Vibrio* strains are common in natural environments, and multiple *V. cholerae* QS mutant strains that are QS defective or even strains locked in high-cell-density states have also been isolated (15). While different explanations, including stress response regulation, metabolic constraint, metabolic prudence, policing, and spatial structure, have been noted to promote cooperation in other bacterial species, primarily *Pseudomonas aeruginosa* (16–23), none of these strategies have been described for *Vibrio* species. Therefore, the question of how *Vibrio* QS is so prevalent in natural environments and whether conditions exist that enable *Vibrio* QS cooperators to successfully invade in populations composed mostly of defectors remains.

In this study, we demonstrate that QS-proficient *Vibrio* strains can invade a population predominantly composed of defectors in structured environments that promote population bottlenecks and/or dispersal, including experimental metapopulations and expanding motile colonies. One way this could occur is via Simpson's paradox (24, 25), a statistical phenomenon in which a given trend is evident in multiple independent

FIG 1 Phenotypes of strains used in the study. WT, wild type; UC, unconditional cooperator; D, defector; subscript letters refer to the species (Vh, *V. harveyi*; Vc, *V. cholerae*). Blue coloration of cells depicts the activation of QS-regulated *lux* gene expression that leads to bioluminescence.

subgroups (i.e., $A > B$), but the reverse trend is observed when the outcomes of all the groups are combined (i.e., $B > A$). For cooperative traits, Simpson's paradox (originally termed "trait group selection" in this context [26]) describes a situation wherein defectors outperform cooperators on local scales in subpopulations due to higher relative fitnesses (defined as a change in genotype frequency due to differences in realized growth rates), but cooperators ultimately outperform defectors on a global scale due to higher absolute fitnesses afforded by the cooperative trait (defined as a change in genotype abundance due to higher potential carrying capacities). We show that this weighting leads to increased growth of subpopulations that possess a higher proportion of cooperators driven by active dispersal events, such as bacterial motility that promote kin selection, by selecting for higher growth yields driven by cooperative traits. This can occur even when those genotypes possess relatively lower growth rates than defectors. Although Simpson's paradox has been demonstrated to increase cooperator frequency in simulated or engineered microbial systems (27, 28), we demonstrate here that this phenomenon occurs in a nonsynthetic phenotypic system in which cooperators and defectors differ primarily by growth yield rather than by growth rates. Our results offer a mechanism by which structured environments can promote maintenance of QS in *Vibrio* populations.

RESULTS

Cooperators and defectors of both *Vibrio* species examined exhibit similar patterns of growth in M9-casein medium. For the duration of the paper, we will use "WT" to refer to the wild-type facultative cooperating wild-type strains *V. harveyi* BB120 (WT_{Vh}) and *V. cholerae* C6706str2 (WT_{Vc}), "UC" to refer to the unconditional $\Delta luxO$ cooperator strains (UC_{Vh} or UC_{Vc}), and "D" to refer to the $\Delta luxR$ or $\Delta hapR$ defector strains (D_{Vh} or D_{Vc}, respectively) (Fig. 1). We have previously shown that when *V. harveyi* WT_{Vh} and D_{Vh} are competed in large populations of bacteria under well-mixed conditions where growth is dependent upon the induction of extracellular protease production by the QS system, the WT_{Vh} strain exhibits equivalent fitness to the D_{Vh} strain at all starting frequencies examined (14). Alternatively, a population of the UC_{Vh} strain suffers from a prisoner's dilemma and is invaded by both WT_{Vh} and D_{Vh} strains at all starting frequencies. This finding showed that QS could stabilize public goods in *V. harveyi*. Importantly, however, neither cooperating strain was able to invade populations of D_{Vh} cells, regardless of their introduced abundance. For *V. cholerae*, a previous

FIG 2 Growth of cooperator and defector strains in M9-casein medium. Growth was measured by observing the optical density at 600 nm (OD₆₀₀). Data for defectors is reported at 24, 48, and 72 h of growth to demonstrate differences between *V. harveyi* and *V. cholerae* defectors. Measures for cooperator strains did not vary between the days tested, so only the 24-hour measures are displayed here. Error bars are 95% confidence intervals on 5 biological replicates. WT, wild type; UC, unconditional cooperator; D; defector; and subscript letters refer to the species. Significance labels depicted represent (a) defector strains significantly less than both cooperator strains of both species, $P < 0.001$; (b) cooperator growth significantly differ between *Vibrio* species, $P < 0.001$; and (c) *V. cholerae* defector strain growth significantly differs between first and later days postinoculation, $P < 0.05$.

study had shown that the D_{Vc} mutant was able to invade the WT_{Vc} in minimal casein medium (29). Therefore, it was unclear how such functional QS systems in *Vibrio* species maintain their widespread abundance in the natural world.

To extend our previous study, we first increased the duration of growth in a minimal medium containing casein as the sole carbon source (M9-casein) and compared cooperator and defector growth in monoculture. The *V. harveyi* cooperator strains that can express the high-cell-density QS state (WT_{Vh} and UC_{Vh}) that results in upregulated extracellular protease production are able to grow to higher densities in this environment (Fig. 2). This is due to the QS-regulated production of extracellular proteases, which can digest the extracellular casein and liberate nutrients for growth. Alternatively, the D_{Vh} mutant is unable to grow to equivalent high densities in M9-casein, due to an inability to produce high levels of extracellular proteases, even at 48 and 72 h, compared to the levels produced by WT_{Vh} and UC_{Vh} (Fig. 2, left). However, low levels of D_{Vh} growth do occur due to small amounts of already liberated nutrients that do not require enzyme-mediated proteolysis (14). This analysis was next extended to analogous QS strains of the human pathogen *V. cholerae*. We observed that, similar to *V. harveyi* strains, WT_{Vc} and UC_{Vc} cooperator strains that could activate the high-density QS state exhibited higher absolute yields (Fig. 2, right). However, one difference we noted between the two *Vibrio* species was that D_{Vc} grew to higher cell densities by 48 and 72 h than D_{Vh} , although it still did not reach the densities of the cooperator strains.

We determined the growth rate of the three *V. harveyi* and three *V. cholerae* strains during low cell density (see Table S1 in the supplemental material). An analysis of growth during the first 6 h of the culture by quantifying CFU shows that the growth rates of the WT_{Vh} and D_{Vh} strains are statistically indistinguishable from one another but 74% and 66% greater, respectively, than that of the UC_{Vh} (Table S1). *V. cholerae* exhibited more rapid growth in this medium. The UC_{Vc} strain exhibited the lowest growth rate, although none of the three strains were statistically indistinguishable from each other, even though the D_{Vc} has been previously shown to rapidly invade WT_{Vc} in this medium (29). This suggests that an additional mechanism other than the growth rate may promote invasion of D_{Vc} during dual strain competition.

Cycles of population bottlenecks, fragmentation, and regrowth increase the frequency of *Vibrio* cooperator strains via Simpson's paradox, mediated by kin selection. Because QS cooperators can maximize population growth yield in M9-casein (Fig. 2), we hypothesized that environments that select for growth yield rather than growth rate could provide a mechanism by which the examined cooperative behaviors are enriched. To test this hypothesis, we initiated cycles of global cell mixing as a

FIG 3 Simpson's paradox results in increasing fractions of cooperators in metapopulations of *Vibrio* bacteria. Populations initiated with 10% cooperators (WT or UC) were serially passed through eight cycles of stringent bottlenecks and regrowth in partitioned subpopulations. All subpopulations were remixed to begin each subsequent experimental cycle. (A) For *V. harveyi*, the growth time between transfers is 3 days, and (B) for *V. cholerae* it is 2 days. The data points represent the mean from the 3 metapopulations after recombining all subpopulations at each cycle, and the lines show the cooperation frequency of each individual replicate metapopulation.

metapopulation (30), fragmentation into subpopulations with strong bottlenecks and regrowth, followed by recombining the subpopulations into a metapopulation and repeating the cycle. We hypothesized that defectors should be favored over cooperators on a local scale within mixed subpopulations due to higher growth rates, but subpopulations that by chance had more cooperators present would be favorably weighted once the metapopulation was regenerated, due to higher growth yields (26–28). In essence, we asked if growth rate or growth yield was favored under these conditions. This situation could constitute a Simpson's paradox, in which cooperators could increase in frequency in mixed populations without ever having relative fitnesses higher than those of their conspecific defectors if cells more frequently interact with their own genotype, a situation that would promote kin selection (31).

We initiated this experiment by mixing either the WT or UC strains into a predominantly (~90%) D population in M9-casein medium, followed by dilution and division into subpopulations in 96-well plates to promote assortment and inhibit gene flow between subpopulations. We fitted the number of wells with no growth to a Poisson distribution to estimate that each well received on average approximately 2 bacteria (see Fig. S1 in the supplemental material). In support of our hypothesis, we observed that both WT_{vh} and WT_{vc} strains invaded defectors in metapopulations when passed through these cycles of population bottlenecks, fragmentation, and regrowth (Fig. 3, black lines). More surprisingly, both the UC_{vh} and UC_{vc} strains were also able to invade defectors (Fig. 3, gray lines), despite having lower growth rates and consequently lower relative fitnesses that led them to be driven to extinction by defectors in larger well-mixed population conditions (Table S1) (14). The invasion of *V. cholerae* cooperator genotypes into populations of the D_{vc} defector contrasts with results of previous experiments performed in well-mixed liquid cultures with large populations (29) and demonstrates that this result is robust across different *Vibrio* species.

To more closely examine the dynamics of *V. harveyi* cooperator invasion, we determined final QS activity (measured by bioluminescence) and growth (measured by optical density at 600 nm [OD₆₀₀]) of all subpopulations (Fig. 4). After the first growth cycle, only a small fraction of subpopulations exhibited bioluminescence (14.1% in WT_{vh} metapopulations and 23.7% in UC_{vh} metapopulations produced normalized bioluminescence of >10⁶ relative light units [RLU]) because the populations were initiated with ~90% D_{vh} cells, which do not produce observable bioluminescence (Fig. 4A). However, following the final experimental cycle, the vast majority of subpopulations for both cooperator strain treatments exhibited significant bioluminescence (>10⁶ RLU), suggesting that they contained large numbers of cooperator cells (Fig. 4B).

FIG 4 Growth and luminescence of *V. harveyi* subpopulations. Phenotypic profiles of individual subpopulations of *V. harveyi* over the course of metapopulation experiments were determined by measuring QS activity (measured in proxy by bioluminescence normalized to cell growth [RLU/OD₆₀₀]) and growth (measured by OD₆₀₀) at the completion of the (A) first and (B) final growth cycle of the experiment. Each data point depicts a single well subpopulation.

The WT_{vh} treatment subpopulations produced homogeneous OD₆₀₀ values among subpopulations by the completion of the last cycle, with the vast majority of subpopulations reaching both higher densities (96.6% at an OD₆₀₀ of >0.5) and high bioluminescence (98.9% of subpopulations with normalized bioluminescence of >10⁶ RLU), with few subpopulations present at intermediate states of either variable (Fig. 4B). Alternatively, the UC_{vh} populations exhibited a wide range of OD₆₀₀ values. This outcome is likely a result of the WT_{vh} strain having a higher growth rate than the UC_{vh} (Table S1) and the UC_{vh} strain not reaching the maximum yield during the conditions of this experiment. As expected, the UC_{vh} strain produces more bioluminescence at lower cell densities, as can be seen when all transfers are plotted on the same graph (see Fig. S2 in the supplemental material), consistent with constitutive expression of high-cell-density behaviors that delay growth (14). Moreover, there was a positive correlation between total cell density in the metapopulation and cooperator frequency, demonstrating that for either genotype, increasing public good-producing cooperator numbers improved overall productivity in a manner expected of Simpson's paradox (see Fig. S3 in the supplemental material).

Swimming motility promotes cooperation for facultative cooperators in competitions against defectors. The observed increases of cooperator frequencies in our metapopulation experiments demonstrated that the cooperator strains can invade defector-majority populations via a Simpson's paradox. This is dependent upon sufficient separation from defectors to colonize new environments that allow maximization of growth yields. We hypothesized that swimming motility could also provide such a mechanism. Previous studies have demonstrated interconnections between swimming motility and quorum sensing, although these studies were not performed on M9-casein medium (32, 33). To test this hypothesis, we first compared the swimming capabilities of *Vibrio* strains in isolation by inoculating monocultures into M9-casein motility plates and measuring the total dispersal area. We observed in both species that defector strains dispersed more than either of the cooperator strains ($P < 0.05$; Fig. 5). However, the area that the defector strains, particularly strain D_{vh}, colonized was not as dense as the colonies produced by either cooperator strain (see Fig. S4B in the supplemental material), consistent with the idea that the defectors were not able to utilize the casein carbon source as effectively (Fig. 2).

To test if swimming motility could promote cooperator dispersal and an overall enrichment of cooperators via Simpson's paradox, we mixed populations of cooperator and defector strains in equal proportions and allowed them to compete in the M9-casein motility plates. Different regions of these colonies were sampled for cooperator frequency during growth. Because much larger seeding populations were used in these experiments (around 10⁶ cells) than in the metapopulation experiments, this experiment served as a much more stringent test of the potential for Simpson's

FIG 5 *Vibrio* strains exhibit a colonization-dispersal trade-off in M9-casein medium. Strains were seeded on motility (0.3%) agar plates with 1/1,000 defector strain carrying capacity initiating cells ($\sim 10^5$ to 10^6 cells). Motility was calculated by measuring the total area of the region over which cells had traversed from their initial inoculation spot, determined by the fringe of visible cell growth. Statistically significant differences are labeled as such: (a) D strain produced a significantly larger dispersal area than both conspecific cooperator strains, $P < 0.001$; (b) UC strain produced a significantly smaller dispersal area than both conspecific WT and D strains, $P < 0.01$; (c) mixed colony produced a significantly smaller dispersal area than the contained defector strain in monoculture, $P < 0.001$; (d) mixed colony produced a significantly larger dispersal area than the contained cooperator strain in monoculture, $P < 0.001$; (e) UC versus D dispersal areas significantly differ between species, $P < 0.05$.

paradox to drive an increase of cooperators. In mixed populations, the WT strains constituted the majorities of the cell populations on the colony edges at day 2, whereas they comprised relatively equivalent frequencies as the defector strains in the colony centers (Fig. 6 and Fig. S4). Alternatively, the UC strains were largely undetectable at the colony exterior and were outcompeted in the center (Fig. 6). This result is not due to the WT strains being generally more motile, as the D strains exhibited greater motility in monoculture (Fig. 5). The prevalence of the WT_{vh} strain at the colony exterior through the first 2 days is also visibly evident, as the edge of the motile colony is highly bioluminescent (Fig. S4). This was not evolutionarily stable under these conditions, as D_{vh} nonluminescent strains ultimately take over the population. However, as we argue in the Discussion, the conditions encountered by *Vibrio* strains in natural environments are likely more complex than the laboratory conditions tested here.

Unlike the metapopulation experiments, the UC_{vh} and UC_{vc} strains were outcompeted by the defector in all regions of the motile population at day 2 (Fig. 6). The low relative fitness of the $\Delta luxOU$ strain in these competitions is evident by visualizing bioluminescence of *V. harveyi* competitors, as the entire colony is dim or completely nonluminescent (Fig. S4). Thus, while the *Vibrio* WT and UC strains performed similarly

FIG 6 Motility plate competitions. Each cooperator strain of both *V. harveyi* and *V. cholerae* were competed against their conspecific defector strain in M9-casein motility plates. During growth, samples were taken from the center and edge of colony growth, diluted, and plated to determine the composition of these populations. At the completion of the experiment, the entire colony was harvested, homogenized, diluted, and plated to determine the composition of these populations. The frequency of the included cooperator strain is measured on the y axis. Error bars represent 95% confidence intervals for 4 biological replicates per strain mix.

well when passaged through population bottlenecks and were grown in isolated subpopulations, stark differences appeared when they were competed against defectors in motility plates.

Harvesting of entire dispersed colonies from motility plates on day 3 revealed that it is possible for the *V. cholerae* wild-type strain to significantly increase in frequency in the presence of D_{vc} ($P < 0.05$, Fig. 6). The WT_{vh} strain also showed no significant drop from its starting frequency of 50% throughout the dispersed colonies. However, the UC mutants of both *Vibrio* species exhibited low fitnesses, leading to strong drops in their frequencies following competitions against defectors (Fig. 6). The relative fitness of the competing strains versus defectors under all conditions tested is shown in Fig. S5 in the supplemental material.

As an additional control, we performed similar experiments with a nonmotile $\Delta flrA$ mutant of *V. cholerae* (34). Even though the nonmotile mutant can activate QS, in the absence of motility it was outcompeted by the D_{vc} strain on M9-casein motility agar plates, showing that active dispersal is required for increased frequency of the WT_{vc} strain on the colony edge and throughout the dispersed colony (see Fig. S6 in the supplemental material). Together, these experiments provide evidence that a functional QS system, combined with dispersal by flagellar-based motility, renders the QS cooperators of either *Vibrio* species more competitive against defectors, even allowing the *V. cholerae* WT strain to increase in frequency in large, premixed populations with defectors.

DISCUSSION

The results of this study provide evidence that cooperative genotypes can persist and even increase in frequency in the presence of defector genotypes when provided with appropriate environmental conditions. For *Vibrio* strains, this appears to be driven by both the growth advantages conveyed by QS regulation, as well as by the ability to disperse via flagellar motility. While previously published results demonstrated that cooperator strains of *V. harveyi* could persist in the presence of defectors in well-mixed conditions, cooperators did not overtake defectors under these conditions (14). Alternatively, *V. cholerae* defectors were able to outcompete the facultative cooperators in well-mixed M9-casein (29). We predicted that providing a means of assortment between competing strains could allow cooperators to invade defectors. Both the experimental metapopulation and motility competition results were implemented under conditions that fostered increased numbers of local interactions with neighboring cells, which are more likely to be kin than cells encountered in well-mixed populations, and these examples demonstrate kin selection in operation. Structure and limited dispersal serve as proxies for increased relatedness (1, 35, 36), but our results suggest that dispersal by cooperator strains was a necessary component of being competitive in the presence of defectors. This is reinforced by the observation that mutant strains incapable of producing flagella for swimming dispersal were not capable of invading defectors, while those that retained this functionality could (Fig. 6 and Fig. S6). While it seems counterintuitive to suggest that the ability to disperse should foster assortment of strains, it makes sense when considering that dispersal allows cells to navigate their environment to locate uncolonized patches of untapped resources and then increase their numbers via clonal reproduction, and differences in dispersal abilities between competing strains may exacerbate this effect.

In this work, we have demonstrated that Simpson's paradox can increase the frequency of cooperator *Vibrio* genotypes with functional QS systems in metapopulations of mixed cooperating and defecting genotypes when the ability to cooperate provides an advantage for population growth yields. Unlike previous studies demonstrating this paradox, our study examined a natural QS system regulating production of a public good important for fitness, rather than an engineered system consisting of only signal molecule production as a cooperative behavior (27) or simulated systems not concerning other cellular constraints (28). While these other studies have pursued the use of simplified systems that avoid possible associated pleiotropic effects, here we

explore a system that embraces all associated complexities and layers of pleiotropy that may have evolved between signaling, cooperation, and other coregulated behaviors. Another important distinction is that while these earlier examples primarily relied upon differences in growth rates between cooperators and defectors, here the key difference was that cooperators could achieve higher growth yields.

We predicted that growth under conditions of fragmented populations without gene flow between subpopulations would result in favored weighting of cooperator strains, mediated by Simpson's paradox, which can act to promote increases in cooperation due to differences in carrying capacities, and this is indeed what we observed (Fig. 3). These results are ecologically relevant because natural environments are frequently fragmented or structurally discontinuous, and differences in growth yields are likely to commonly occur among genotypes that elicit cooperative behaviors to differing degrees (37). Namely, it is relatively common for cooperative behaviors to positively influence population growth, and thus more cooperative genotypes should be able to achieve larger population sizes in environments where a cooperative behavior impacts an important trait like nutrient supply or accessibility.

To provide a more stringent test of Simpson's paradox promoting cooperation in *Vibrio* strains, we used larger starting populations of cooperators and defectors and allowed dispersal through the environment to be actively driven by the bacteria themselves using flagellar-based swimming, unlike previous studies examining competitions of cooperators and defectors during colonization and dispersal (38–42). Moreover, swimming motility is an individual behavior that cannot be cheated by nonproducers, unlike swarming motility, which has been extensively studied as a cooperative behavior (18). And unlike those results, cooperator strains are not completely dominated by defectors in the colony centers, or "homeland" (Fig. 6 and Fig. S4) (42), likely due to the pleiotropic effects that result from regulating other traits in addition to cooperative behavior with QS. Furthermore, colonies do not display the distinct stochastic sectoring patterns shown in these previous studies, but rather exhibit strongly uniform colony edges dominated by WT cooperators (Fig. S4).

Based on the results of previous studies in well-mixed conditions that did not include dispersal, we would expect D_{vh} strains to outcompete UC_{vh} strains and be equivalent with WT_{vh} strains (14), while D_{vc} strains should outcompete both cooperators (29). Interestingly, WT cooperator strains were capable of gaining large advantages on colony edges, despite being significantly less motile than defector strains when grown in monocultures, suggesting that the success of the WT strains resulted in part from the ability to more fully utilize resources rather than just from cooperator dispersal (Fig. 5 and 6 and Fig. S5). However, this advantage was not indefinitely maintained (Fig. S4), suggesting this could lead to altering cycles of invasion between cooperators and defectors. Although the laboratory conditions that we implement here are highly useful for inferring competitive dynamics, they likely do not fully mimic growth in the environment in several ways, including the abundance of resources, lack of spatial heterogeneity, and the time scale of competitions. Therefore, we expect that in the natural marine environment where *Vibrio* bacteria reside, resources are highly heterogeneous and populations sizes are much lower, and thus more reflective of the earlier time points of our assay.

Previous population genetic results for production of the public good siderophores in *Vibrio* strains demonstrated coexistence between producers and nonproducers (43), and our results similarly show mixed populations at the colony frontiers after several days of colony growth (Fig. 6 and Fig. S4). While trade-offs between colonization and dispersal in *Vibrio* strains have been reported, the flexibility in gene regulation and resulting physiological growth responses afforded to the WT strains by QS could help mediate this type of trade-off and provide strong competitiveness under a variety of selective pressures (40, 44). It is even possible that QS regulation could provide a more heterogeneous population-level response, in which subpopulations of cells invest more heavily in dispersal or growth (45–47).

Examining outcomes in motility plates confirmed our predictions that while UC strains

performed quite well in metapopulation experiments that had strong bottlenecks and diminished mixing of cooperators and defectors, they were less fit than defectors in more mixed populations (Fig. S5). Alternatively, for both *V. harveyi* and *V. cholerae*, the WT strains were more fit relative to defectors than UC strains were in the colony center, where the populations were larger and more well-mixed, and this improvement in competitive growth impacted its ability to reach and succeed at the colony edges (Fig. 6). Indeed, for both species, the WT strain was highly successful at the edges of the dispersed colonies, where it exhibited a majority throughout 2 days of competition (Fig. S6). This result is consistent with observations in other systems that cooperators can outpace defectors at the leading edge of range expansions (42, 48); however, those previous studies focused solely on growth effects in the absence of motility, while here, cooperator success depends upon both growth parameters and functional motility.

The composition of motility agar medium used in this study constitutes a uniform, resource-rich environment. A patchier and more varied environment, such as particles encountered in natural aquatic environments, could select for both the ability to disperse rapidly to reach new patches and also to grow maximally on patches of limited resource once colonized (49–52). In such environments, the defector strains could act as a “fugitive species,” while the cooperator strains would represent a strategy that possesses superior colonizing capabilities, resulting in their increased carrying capacities. Our results suggest that WT strains harness quorum sensing to best utilize both dispersal and growth, which would diminish the potential trade-off between them (40, 53, 54). Indeed, the quorum sensing system of *Vibrio* bacteria negatively regulates biofilm formation (55), and computational modeling predicts this regulatory network is designed to promote dispersal from a biofilm at the appropriate time (39). Fugitives and colonizers could also coexist in more complex environments capable of supporting multiple niches, the fugitive strains by dispersing more effectively to open patches or those occupied by populations of exploitable cooperators and the cooperator strains by being more fecund in this environment.

This complex scenario would be expected for aquatic *Vibrio* strain that are known to cycle between motile, planktonic lifestyles and formation of biofilms on chitin surfaces. Once attached to the chitin, the bacteria secrete extracellular chitinases, another form of public good that degrades the polymer, releasing *N*-acetylglucosamine monomers and oligomers for growth (56). Expression of chitinases is also regulated by quorum sensing, like the proteases that are studied here, although in the case of the former, the regulation is negative (57). Our results suggest that the patchy resource environment in which *Vibrio* bacteria often exist is ideal for maintaining cooperative traits, especially when coregulated with other traits, which could explain the prevalence and stability of the complex QS systems that have evolved in these bacteria. Our results also explain the observation that both locked low- and high-cell-density *Vibrio* mutants can be isolated from natural sources (15).

Together, we have demonstrated that cooperation can be favored in strains utilizing QS when assortment is sufficient to allow differences in growth and motility to be realized. One way in which this was observed was that competing strains underwent population bottlenecks and assortment before regrowth, allowing Simpson’s paradox to occur for both WT and UC cooperators. While bottlenecks have been appreciated as potentially important for maintaining cooperative behaviors in populations (58, 59), our findings show that even in large populations, cooperation can be promoted if there is sufficient assortment between cooperators and noncooperators through mechanisms such as bacterial motility.

MATERIALS AND METHODS

Strains and media. *V. harveyi* strain BB120 (wild type [WT], ATCC BAA-1116, recently reassigned as *Vibrio campbellii*, a sister taxon in the Harveyi clade, and to which we refer with the common-use designation *V. harveyi* in this study [60, 61]) and derivative strains JAF78 ($\Delta luxOU$ [62]) and KM669 ($\Delta luxR$ [63]) were grown at 30°C as indicated. *V. cholerae* strain C6706str2 (wild type [WT]) and derivative strains $\Delta luxO$ (64), $\Delta hapR$ (65), and $\Delta flrA$ (34) were grown at 35°C. Strains were grown in M9-casein, a liquid medium composed of M9 salts (Sigma-Aldrich or Beckton, Dickinson, and Company) supplemented with

sodium chloride (Macron) to 2% (wt/vol) and sodium caseinate (Sigma-Aldrich) as the sole carbon source at a concentration of 0.5% (wt/vol). We have previously found that noncooperator strains lacking protease production can grow to low levels in this medium, suggesting the casein contains nutrients that do not require public good proteolysis for utilization (14). Strains were streaked from frozen stocks, and individual colonies were inoculated into and passaged for 24 h in LB before growth in M9-casein medium. With the exception of motility plate competitions, all growth was conducted in liquid media. Viable cell counts were conducted on LB agar plates.

Metapopulation growth. Strains were passaged in monoculture in M9-casein medium for 24 h before mixing. During initial mixing, 6 biological replicates each of competing strains were washed, diluted to equivalent densities, and then combined to form 3 replicate cultures. Upon mixing, cells were diluted and divided into subpopulations in 96-well plates and incubated under shaking conditions for growth. Dilution estimates were made based upon OD₆₀₀ measurements, such that the target density was approximately 2 cells per subpopulation. Estimates of average starting subpopulation size were additionally corroborated by counting the number of empty wells (determined as wells with no significant increase in OD₆₀₀ over the medium control at the completion of the growth cycle) present and fitting that number to a Poisson distribution (as in reference 27; Fig. S1). Plates were grown for 3 days at 30°C for *V. harveyi* strains and for 2 days at 35°C for *V. cholerae* strains between transfers, to allow subpopulations to reach saturating densities. Plates were incubated on orbital shakers to facilitate even mixing within subpopulations. After the incubation period, growth (for both *V. harveyi* and *V. cholerae*) and bioluminescence (for *V. harveyi* only) of all subpopulations were measured with an Envision plate reader (PerkinElmer). Bioluminescence data are reported as normalized bioluminescence (RLU/OD₆₀₀). All subpopulations were combined into a metapopulation, and overall growth was measured using viable cell counts on LB agar plates. The frequency of cooperators versus defectors was determined by quantifying bioluminescent and nonluminescent colonies for *V. harveyi*, while the frequency of *V. cholerae* cooperators/defectors was determined by quantifying rugose colonies (indicative phenotype of the $\Delta hapR$ strain) and smooth colonies, indicative of the cooperator strains. This cycle was completed a total of 8 times.

Motility plates. Prior to plating, strains were grown for 16 h in LB medium and then transferred and subsequently grown in M9-casein medium for 24 h for acclimation. Competing strains were mixed in a 1:1 ratio, and approximately 10⁶ total cells were plated on the surface of M9-casein petri plates containing 0.3% agar. Cells were spotted in the center of the plate and incubated to allow populations to grow and disperse (34). *V. harveyi* plates were incubated at 30°C, and *V. cholerae* plates were incubated at 35°C. Plates were imaged daily with an Alphamager HP imaging system (ProteinSimple). Center samples were picked from the inoculation point on the motility plate, and edge samples were obtained from the visible boundary of the expanding colonies, sampled daily over the course of the experiment. When whole colonies were sampled on day 3, the entire dispersed colony was excised from the plate, suspended in 10 ml M9-casein liquid medium, vortexed until homogenized, and then diluted and plated to assess colony composition. Data presented in Fig. 5 were normalized to the maximum dispersal area from the inoculation point for a given species on a given day and then standardized to the dispersal fraction obtained by the WT strain of that species.

Statistical comparisons. All analyses were performed using R version 3.0.2. In all graphs containing them, error bars represent 95% confidence intervals. One-way analysis of variance (ANOVA) was used for comparisons between strains or competition treatments (Fig. 6 and Fig. S6). Statistical comparisons in Fig. 2 and 5 were completed by two-way ANOVA with Tukey's honestly significant difference (HSD) test for multiple comparisons. Comparisons between productivity and cooperator frequency were conducted via linear regression (Fig. S3).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00402-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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E.L.B. and C.M.W. designed the experiments, E.L.B. conducted the experiments, E.L.B. analyzed the data, and E.L.B. and C.M.W. wrote the paper.

We declare no conflict of interest.

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