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Report

Solutions to the Public Goods Dilemma in Bacterial Biofilms

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Summary

Bacteria frequently live in densely populated surface-bound communities, termed biofilms [1–4]. Biofilm-dwelling cells rely on secretion of extracellular substances to construct their communities and to capture nutrients from the environment [5]. Some secreted factors behave as cooperative public goods: they can be exploited by nonproducing cells [6–11]. The means by which public-good-producing bacteria avert exploitation in biofilm environments are largely unknown. Using experiments with *Vibrio cholerae*, which secretes extracellular enzymes to digest its primary food source, the solid polymer chitin, we show that the public goods dilemma may be solved by two very different mechanisms: cells can produce thick biofilms that confine the goods to producers, or fluid flow can remove soluble products of chitin digestion, denying access to nonproducers. Both processes are unified by limiting the distance over which enzyme-secreting cells provide benefits to neighbors, resulting in preferential benefit to nearby clonemates and allowing kin selection to favor public good production. Our results demonstrate new mechanisms by which the physical conditions of natural habitats can interact with bacterial physiology to promote the evolution of cooperation.

Results and Discussion

Bacteria often depend on insoluble substrates for growth, including during infection and environmental biomass degradation [1, 12, 13]. To scavenge nutrients in such contexts, bacteria use secreted digestive enzymes, which degrade polymers into soluble units that can be imported and catabolized [12]. These soluble, nutrient-rich products can be captured by neighboring cells that do not themselves invest in the production of digestive enzymes [6–8]. Social evolution theory predicts that, for a given cost-to-benefit ratio of public good production, the benefits of a cooperative behavior must be sufficiently directed to other cooperating individuals for cooperation to remain evolutionarily stable against exploitation [14–16]. The way in which this selective mechanism is manifested in microorganisms can vary widely depending on the system [17], and examples range from active discrimination that selectively benefits cooperators [18] to metapopulation dynamics that maximize between-group genetic variance and minimize within-group genetic variance [15, 19]. Most experimental research examining social evolution in bacterial populations has been performed with liquid cultures, which eliminate population spatial structure that is ubiquitous in realistic environments [1, 2], and is predicted to be critical for the evolution of cooperation [20–24]. Social evolution has been studied in radially expanding colonies on agar, but no general mechanisms have been found that show how enzyme-producing cells avoid exploitation by nonproducers in biofilms [8–11, 25, 26]. Given that biofilms are the dominant form of bacterial life in nature and that many bacterial species secrete digestive enzymes, clarifying the means by which cooperative cells avert exploitation in biofilms is a major outstanding problem.

We explored the public goods dilemma using the biofilm-forming bacterium *Vibrio cholerae*. Notorious for causing pandemic disease in humans, *V. cholerae* is also adept at colonizing and consuming chitin in its native marine habitat. Chitin, a polymer of *N*-acetylglucosamine (GlcNAc), is the predominant structural material of arthropods and fungi and the second most abundant biomaterial on Earth behind cellulose [27]. To digest chitin, *V. cholerae* secretes diffusible chitinases that degrade the solid polymer into soluble GlcNAc and GlcNAc oligomers, which can be imported and catabolized [27]. Here, we show that chitinase secretion is exploitable by mutants that do not secrete chitinases, and by combining experiments and theory we identify general mechanisms that solve this public goods problem in natural habitats.

Chitinase Production Is an Exploitable Cooperative Behavior

We first established that chitinase secretion carries a fitness cost by competing wild-type *V. cholerae* with an isogenic mutant harboring deletions of the two primary chitinase genes (*chiA-1* and *chiA-2*) in well-mixed liquid media conditions. The chitinases encoded by *chiA-1* and *chiA-2* have both been shown to be diffusible extracellular enzymes [28]. When the two strains were grown in artificial sea water supplemented with $(GlcNAc)_2$, which induces chitinase production in the wild-type [27] (Figure 1A) but can be imported and catabolized by both strains, wild-type producer cells were outcompeted with a relative growth rate difference of 0.46% ± 0.08% (Figure 1B). The growth rate difference can be increased by induction of chitinase expression with a synthetic expression system (Figures S1A and S1B available online). In both of the above experiments, we distinguished between the wild-type and the mutant using different fluorescent protein expression constructs integrated onto the chromosome. These fluorescent protein markers did not influence the experimental outcome, as competition was neutral when strains that only differed in the fluorescent protein marker were competed against each other (Figure S1C). In an additional control experiment, the chitinase producer and nonproducer were grown in artificial seawater supplemented with GlcNAc, which does not activate chitinase production (Figure 1A), and competition was again neutral (Figure 1B). The cost of chitinase production suggests that nonproducers could exploit and outgrow producers on chitin, when the chitinases are required for growth.

We tested this prediction by competing wild-type and the *chiA-1, chiA-2* null mutant in artificial seawater with chitin

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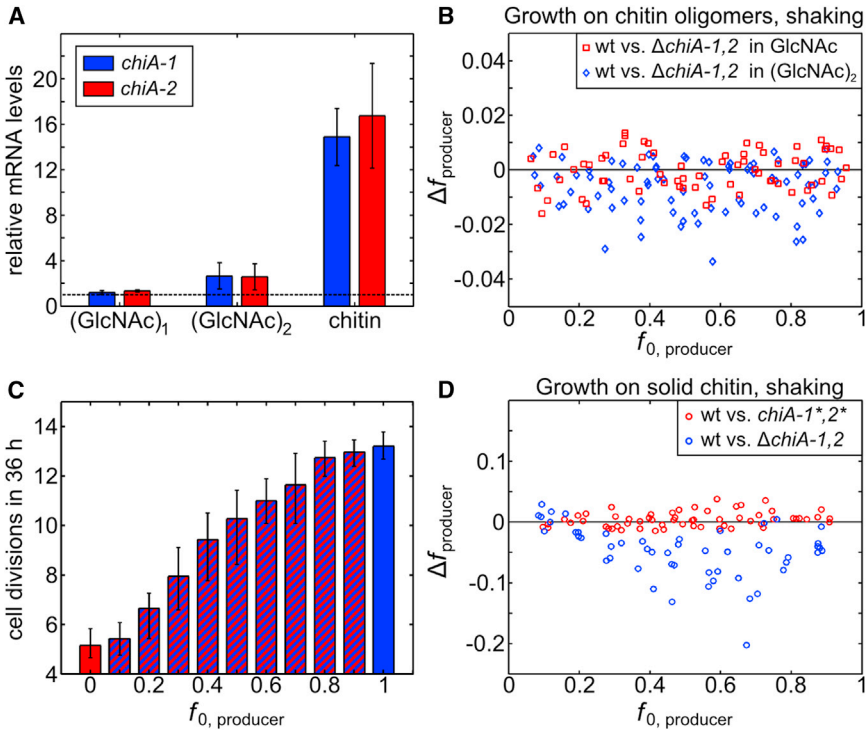

Figure 1. *V. cholerae* Faces a Public Goods Dilemma when Digesting Chitin

(A) Transcript levels of *chiA-1* and *chiA-2* in response to different substrates [27]. The black dashed line represents no change in expression relative to growth in glucose. Error bars indicate the SD.

(B) Wild-type in competition with chitinase nonproducers (Δ*chiA-1,2*) in liquid cultures supplemented with GlcNAc (red) and $(GlcNAc)_2$ (blue). $\Delta f_{producer}$ is the change in frequency of the chitinase producer in the population after ten cell divisions, and $f_{0,producer}$ is the initial seeding frequency of the producers.

(C) Cell divisions in shaking cultures grown on solid chitin. Red and blue bars are data from pure cultures of the Δ*chiA-1,2* mutant and the wild-type (WT), respectively, and striped bars represent cocultures at different frequencies of the WT. Error bars are the range of means from $n = 4$ independent replicas.

(D) Wild-type in competition with chitinase nonproducers (blue) and mutants that produce inactive chitinase proteins (red) in mixed liquid with chitin flakes. $\Delta f_{producer}$ was measured after 36 hr.

See also Figure S1.

flakes as the sole source of carbon and nitrogen. The cultures were shaken to distribute products liberated from chitin digestion. In these conditions, the population growth rate increased when the initial proportion of producers increased [19] (Figure 1C). When chitinase producers were seeded with initial frequencies of $f_{0,producer}>0.2$, they again decreased in frequency over the course of population growth, but now with a relative growth rate deficit of 4.1% ± 0.4% (Figure 1D, blue circles). This growth rate difference is an order of magnitude larger than that found above, which can be explained by the increased expression of chitinases by the wild-type when exposed to chitin relative to GlcNAc oligomers (Figure 1A). In populations with $f_{0,producer}<0.2$, we observed a modest increase in producer frequency (Figure 1D), which could lead to a mixed equilibrium of chitinase producers and nonproducers. These populations underwent relatively few cell divisions in 36 hr (Figure 1C). Liberated nutrient concentrations must have therefore been quite low, so that incomplete mixing of the public goods may have enabled producers to avoid exploitation. Competition was neutral when wild-type was competed against a strain producing chitinase proteins whose active sites were mutated (Figure 1D, red circles; see also Figure S1D), confirming that the cost of producing chitinase proteins is indeed responsible for the wild-type losing in competition with the chitinase nonproducer. Our results establish that chitin digestion via chitinase secretion is a public goods problem: nutrients liberated by chitinase producers (carrying either one or two chitinases; Figure S1E) can be used by nonproducers, which, in turn, grow more rapidly than producers because they do not pay the metabolic cost of chitinase production.

Population Spatial Structure and the Public Goods Dilemma

Most realistic bacterial environments are not uniformly mixed, which leads to population spatial structure and heterogeneity in biofilm composition and physiological activity [1, 2]. It has been hypothesized that such spatial structure alone suffices to avert the public goods dilemma [20, 21, 24, 29]. To test whether *V. cholerae* biofilms grown on chitin flakes in nonmixed liquid exhibit different competitive dynamics than those in shaken culture, we inoculated chitinase producers and isogenic nonproducers onto chitin flakes in unshaken artificial seawater, varying the initial frequencies of the two strains. We found that under these conditions, the public goods problem is mitigated, but not solved completely. When the wild-type is initiated at low frequency, it outcompetes the nonproducer, but when initiated at high frequency, the wild-type is outcompeted by nonproducers (Figure 2A). This regime of negative frequency-dependent selection again indicates that chitinase producers and nonproducers could coexist under some circumstances [30, 31]. However, all sequenced environmental isolates of *V. cholerae* and other *Vibrio* species possess multiple chitinases (Figure S2A), which would not be expected if chitinase null mutants have an advantage over chitinase producers in natural environments.

Spatial separation of producers from nonproducers could potentially minimize exploitation, but even when using inoculation densities 10^3-fold lower than in marine environments [13], such that the average distance between cells was ~350 μm, we found that nonproducers retained the ability to exploit chitinase producers (Figures 2A and S2D). Extending the distance between producers and nonproducers therefore did not solve the public goods problem. The high bacterial concentrations known to exist throughout the marine environment [13] make extreme population bottlenecks, which could lead to pure chitinase-producer populations, unlikely. When we competed immotile chitinase producers against immotile nonproducers (both were *flaA* mutants; Figure 2A), we found that movement to sites of chitin digestion is not required for nonproducers to exploit producers. This counterintuitive result can be explained by noting that diffusion homogenizes

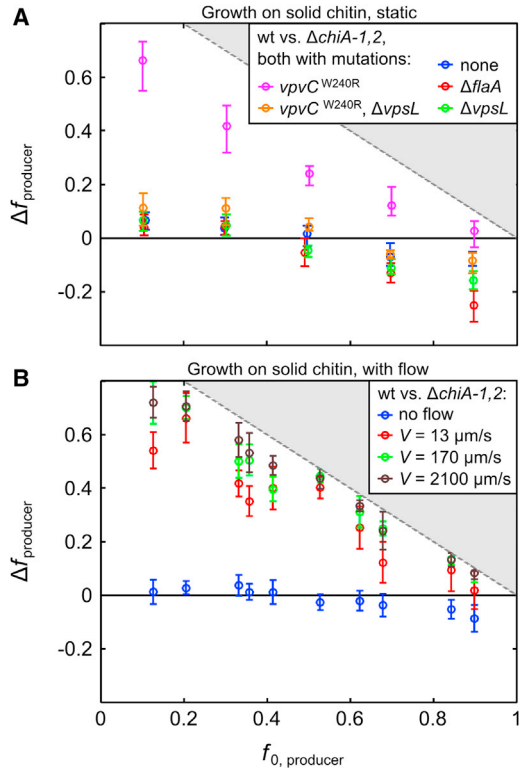

Figure 2. Solutions to the Public Goods Dilemma

(A) Wild-type in competition with chitinase nonproducers in nonshaken liquid with chitin flakes. Either the two strains had no additional mutations (blue) or both carried identical additional mutations as indicated in the legend. Δ*flaA* mutants have no flagellum, Δ*vpsL* mutants cannot make the biofilm matrix, and Δ*vpvC*^{W240R} mutants are matrix hyperproducers. The dashed line indicates the maximum $\Delta f_{producer}$ for a given $f_{0,producer}$, i.e., fixation of the producers. The strains were initially seeded with 10^3 colony-forming units (cfu)/ml. Error bars represent the range of means from $n = 4$ independent replicas.

(B) Wild-type in competition with chitinase nonproducers in microfluidic chambers subjected to a range of flow speeds. Error bars indicate the SD. See also Figure S2.

GlcNAc concentration gradients in the experimental chambers on time scales of <5 hr (see the Supplemental Experimental Procedures, "Mathematical Analysis 1"), which is significantly shorter than the 180 hr for which we competed the chitinase producers and nonproducers. Thus, nutrients liberated by chitinase producers were distributed throughout the sample chambers and accessible to all cells in the system.

Forming Thick Biofilms Solves the Public Goods Dilemma

Considering the ubiquity of chitinase genes in natural vibrio isolates, we hypothesized that conditions restricting diffusion of liberated nutrients might exist that shift selection in favor of chitinase producers, irrespective of their frequency in the population [4, 22]. Some wild *V. cholerae* isolates harbor mutations that promote extracellular matrix secretion [32, 33], and we found that spontaneous matrix hyperproducers occasionally arose during our experiments. To investigate whether matrix hypersecretion interacts with the competitive dynamics of chitinase production, we competed chitinase producers and nonproducers both carrying a *vpvC*^{W240R} allele that causes increased production of extracellular matrix [32], leading to thick biofilms (Figure 3A). This phenotype is due to the secretion of extracellular matrix, because a *vpvC*^{W240R} strain in which an essential gene for matrix secretion (*vpsL*) is deleted does not form thick biofilms (Figure 3A). For strains producing thick biofilms, the public goods problem is solved completely: chitinase producers outcompete nonproducers at every initial frequency (Figure 2A).

To understand how biofilm thickness can affect the availability of public goods, we developed a simplified mathematical model (see the Supplemental Experimental Procedures, "Mathematical Analysis 2") to calculate how much GlcNAc escapes from biofilms of different thicknesses growing on chitin. This model, whose parameters were measured experimentally (Figures S3C–S3F), reveals that increasing the biofilm thickness from a single layer to five cell layers leads to a 10^5-fold decrease in the escaping flux of soluble nutrients from the biofilm (Figure 3B). In a multilayered and densely packed biofilm, GlcNAc molecules have a very low probability of diffusing out of the biofilm without being consumed by the cells within it. The strong effect of biofilm thickness arises because the concentration of liberated GlcNAc oligomers is significantly lower than the concentration at which uptake saturates, so that the GlcNAc concentration inside the biofilm decreases exponentially with distance from the chitin surface (see the Supplemental Experimental Procedures, "Mathematical Analysis 2"). Cells that are not near the base of the biofilm of chitinase producers or that reside outside of the biofilm therefore experience very low concentrations of GlcNAc oligomers. The concentrations of GlcNAc oligomers outside the biofilms were too low (<< μM) to be measured accurately with mass spectrometry. In addition to causing thick biofilm formation, matrix overexpression generates greater spatial segregation between producers and nonproducers (Figure 3C), allowing chitinase-producing cells to restrict benefits to each other [4, 21, 24]. This system reflects a novel means by which bacterial physiology can enable kin selection to favor a cooperative phenotype, related to invertase production and sucrose consumption by clumps of yeast [34]. Importantly, we observed that spontaneous thick-biofilm-forming mutants are strongly positively selected (Figure 3D).

Our mathematical model predicts that the effect of biofilm thickness on the ability of public goods to escape is robust to changes in the model parameters. Thus, thick biofilms presumably also solve the public goods dilemma in other species. For example, matrix hyperproducing ("mucoid") mutants of *Pseudomonas aeruginosa* dominate the infectious populations in the lungs of nearly all cystic fibrosis patients [35], where *P. aeruginosa* uses exploitable extracellular compounds for growth [6, 36]. We speculate that these mucoid strains have a growth advantage because their thick biofilms prevent nonproducers from exploiting secreted extracellular enzymes and siderophores.

Flow Solves the Public Goods Dilemma

Only some natural *V. cholerae* isolates carry mutations that cause thick biofilm formation [33], perhaps due to ecological costs including reduced dispersal ability [23]. We therefore suspected that other conditions might exist under which the chitinase public goods dilemma could be solved.

Natural biofilms, such as those on marine snow or in the human intestine, are often subjected to flow [1, 13, 37], which could remove soluble nutrients released by chitin digestion and thereby limit access to liberated nutrients to clusters of chitinase producers. Using microfluidic chambers

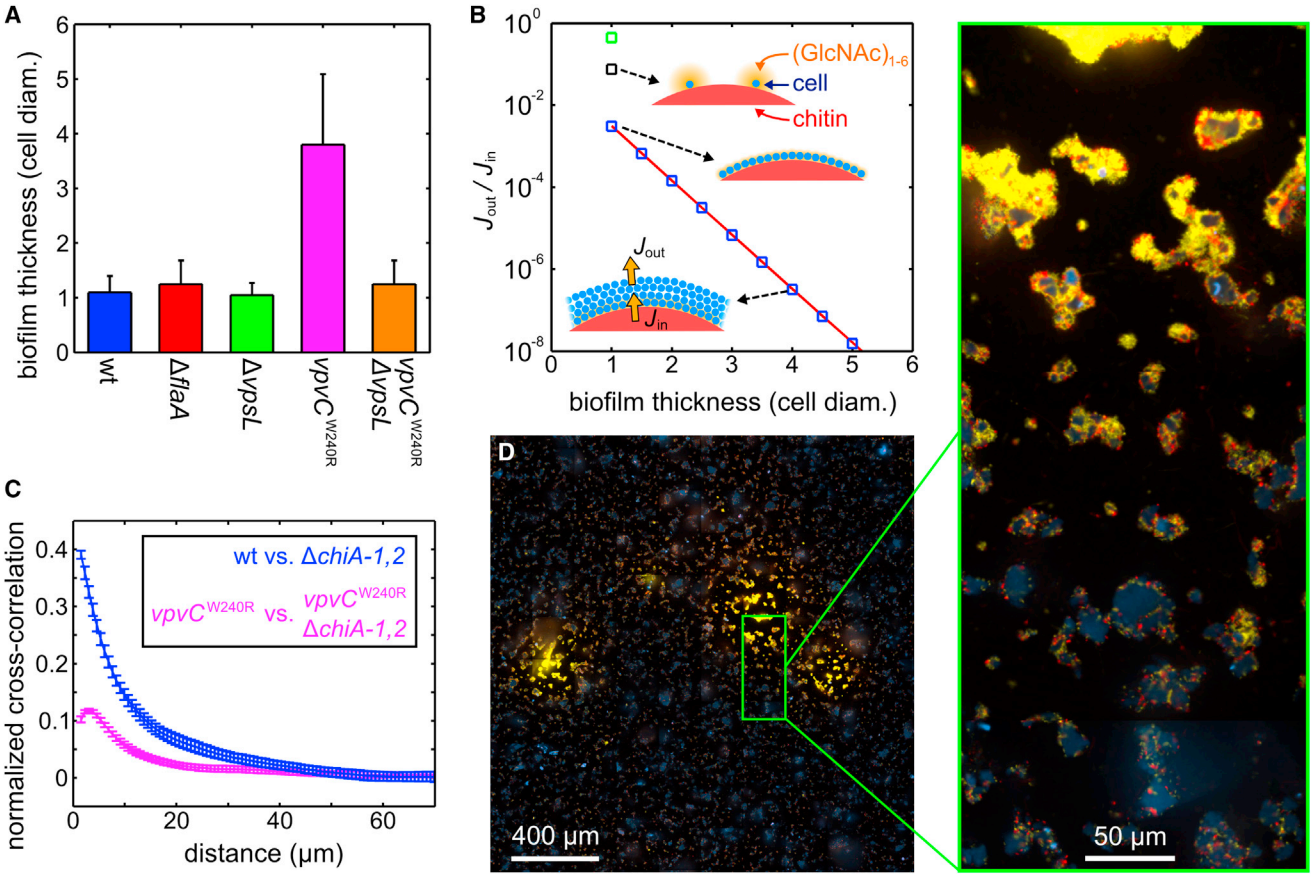

Figure 3. Thick Biofilms Localize Public Goods Close to the Producers

(A) The maximum biofilm thickness that developed over 180 hr for different strains. Error bars indicate the SD.

(B) Model calculation of the flux of GlcNAc molecules that escape from the biofilm (J_{out}) as a function of biofilm thickness. J_{out} is normalized by J_{in}, the flux of GlcNAc molecules that enter the biofilm at the chitin surface due to the action of the chitinases. Evaluation of the model with biofilm densities that are 10% and 1% of the confluent density yielded the black and green data points, respectively.

(C) Lower values of the normalized cross-correlation indicate higher spatial segregation of chitinase producers and nonproducers. The cross-correlation was evaluated for competitions of the wild-type and the Δ*chiA-1,2* mutant (blue) and for competitions in the *vpvC*^{W240R} background (magenta) 108 hr after inoculation. Error bars indicate the SD.

(D) Confocal image of a population of chitinase producers (yellow) competing against the Δ*chiA-1,2* mutant (red) on chitin (blue) in nonshaken liquid, initially seeded at $f_{0,producer} = 0.5$. Figure S3G shows this image at full resolution. There are three regions with significant growth, which are dominated by producers. The inset illustrates that in these regions, the chitinase producers exhibited the matrix hyperproducer phenotype (thick biofilms), due to spontaneous mutations, and they outcompeted the Δ*chiA-1,2* mutant.

See also Figure S3.

(Figure S2B), we tested this hypothesis by competing wild-type *V. cholerae* with isogenic chitinase nonproducers on chitin flakes subjected to flow. For a broad range of flow speeds that span the sinking speeds of marine snow, chitinase producers outcompeted nonproducers, regardless of initial frequency (Figure 2B). Micrographs reveal that, even in thin biofilms, producers can outcompete nonproducers in the presence of flow (Figures 4A and 4B). The lowest flow rate we tested corresponds to the sinking speed of a spherical chitin particle with a radius of ~4 μm. Most of the particulate organic matter in marine environments comprises larger particles that experience higher flow speeds, which favors producers [8, 13].

To understand how flow affects competition between producers and nonproducers, we take note of the Péclet number, which is a dimensionless number that quantifies the importance of molecular transport by flow, compared to molecular transport by diffusion [4, 38, 39]. The Péclet number is defined as $Pe = Ua/D$, where U is the flow speed, a is the typical size of a chitin flake, and D is the molecular diffusion constant of GlcNAc oligomers. At high Pe, the fluid establishes a thin boundary layer across which there is a large GlcNAc concentration gradient. By Fick's law, this strong gradient facilitates rapid molecular transport away from the surface of the biofilm. For the flow speeds we tested (13 μm/s, 170 μm/s, and 2,100 μm/s), our mathematical modeling reveals that the GlcNAc concentration above the biofilm is significantly reduced compared to the concentration in the absence of flow (Figures 4C and 4D and see the Supplemental Experimental Procedures, "Mathematical Analysis 3"). At the biofilm surface, the GlcNAc concentration is also decreased in the presence of flow (Figure 4E). All cells therefore experience a reduced concentration of the public goods, which is selectively disadvantageous to the chitinase mutants because these cells do not benefit from chitinases digesting chitin in their immediate vicinity. Flow is thus another novel

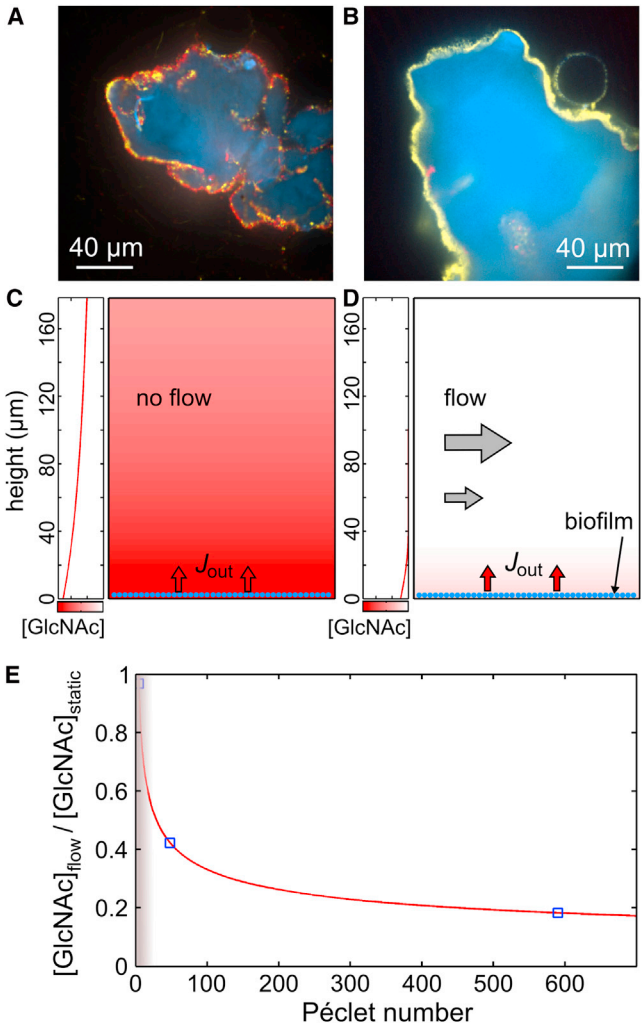

Figure 4. Flow Removes Public Goods and Minimizes Access for Nonproducers

(A) Microscope image acquired for a competition of the WT (yellow) versus the Δ*chiA-1,2* mutant (red) without flow (Pe = 0) on solid chitin (blue). The competition was seeded with $f_{0,producer}$ = 0.5.

(B) Microscope image acquired for competitions of the same strains and $f_{0,producer}$ as in (A), but with flow at 2,100 μm/s (corresponding to Pe = 590).

(C) In the absence of flow, mathematical models show that the flux of GlcNAc molecules escaping from the biofilm (J_{out}) sets up a long-range concentration gradient.

(D) Fast flow over the biofilm generates a boundary layer, which causes rapid transport of GlcNAc to the bulk fluid, leading to a lower GlcNAc concentration at the biofilm surface for the same flux J_{out}. The displayed concentration profile was calculated for flow speeds corresponding to Pe = 590.

(E) Model calculation for the ratio of the GlcNAc concentration at the top of the biofilm with flow and without flow. Blue squares indicate points on the curve for the Péclet numbers at which competitions were performed in Figure 2B. The shading close to Pe = 0 indicates that the model is valid for Pe >> 1.

See also Figure S4.

mechanism that enables kin selection to operate in favor of cooperation.

Both Solutions to the Public Goods Dilemma Are Unified by Inclusive Fitness Theory

Our experiments and models show that thick biofilm growth and fluid flow both lead to drastic reductions in the distance over which an enzyme-secreting cell provides benefit to its neighbors. Furthermore, copious production of biofilm matrix increases spatial segregation between enzyme producers and nonproducers. Hamilton's relatedness coefficient with respect to enzyme secretion, i.e., the correlation between the genotype of enzyme producers and the genotypes of those cells that receive a benefit from enzyme production [14, 15, 17], is therefore increased by forming thick biofilms and by fluid flow. Interestingly, growth of thick biofilms and flow lead to a similar outcome via opposite means: the former allows cooperators to sequester all liberated sugars for themselves, and the latter flushes away the majority of liberated sugars such that exploitative cells cannot access them.

Conclusions

Chitin digestion is a crucial process in global carbon and nitrogen cycling [12]. It is estimated that 10^{11} metric tons of chitin are shed annually as dead organic matter in the form of marine detritus, and yet the amount of chitin found on the sea floor is negligible [13, 27]. Our work reveals general mechanisms enabling chitinase production to remain evolutionarily stable in nature. As the selective mechanisms we discovered do not rely on any feature that is specific to *V. cholerae* or to chitin, we predict that these solutions to the public goods dilemma are relevant to other microbial communities in environmental, medical, and agricultural contexts.

Experimental Procedures

Competition Experiments

V. cholerae strain N16961 (O1 El Tor) and all mutants derived from this strain were grown in defined artificial sea water supplemented with 2 mM GlcNAc, 2 mM $(GlcNAc)_2$, or 0.15 mg/ml solid chitin from crab shells at ~30°C. Competing strains expressed either mKO or mKate2 fluorescent proteins constitutively, and an equal number of replicas were performed with fluorescent markers reversed. For competitions in well-shaken liquid, the frequencies of producers and nonproducers were measured by direct cell counts using a fluorescence microscope. Strain frequencies in biofilms on solid chitin flakes were measured using confocal laser-scanning microscopy.

Mathematical Analyses

Analytical models that describe the molecular transport of the GlcNAc oligomers are based on the advection-diffusion equations, which were solved in different geometries to model GlcNAc transport through a biofilm of variable thickness, and the GlcNAc concentration above a biofilm subjected to different flow speeds.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.10.030.

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