

ORIGINAL ARTICLE

Environmental fluctuation governs selection for plasticity in biofilm production

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Bacteria can grow in a free-swimming state, as planktonic cells, or in surface-attached communities, termed biofilms. The planktonic and biofilm growth modes differ dramatically with respect to spatial constraints, nutrient access, population density and cell–cell interactions. Fitness trade-offs underlie how successfully bacteria compete in each of these environments. Accordingly, some bacteria have evolved to be specialists in biofilm formation, while others specialize in planktonic growth. There are species, however, that possess flexible strategies: they can transition between the molecular programs required for biofilm formation and for planktonic growth. Such flexible strategies often sacrifice competitive ability against specialists in a given habitat. There is little exploration of the ecological conditions favoring the evolution of the flexible biofilm production strategy for bacteria in competition with specialist biofilm producers or specialist non-producers. Here, we study the human pathogen *Vibrio cholerae*, a flexible biofilm-former, as well as constitutive biofilm-producing and non-producing mutants. We assess the fitness of these strains under biofilm conditions, planktonic conditions and conditions that demand the ability to transition between the two growth modes. We show that, relative to the specialists, the wild type is superior at dispersal from biofilms to the planktonic phase; however, this capability comes at the expense of reduced competitive fitness against constitutive biofilm producers on surfaces. Wild-type *V. cholerae* can outcompete the constitutive biofilm producers and non-producers if habitat turnover is sufficiently frequent. Thus, selection for phenotypic flexibility in biofilm production depends on the frequency of environmental fluctuations encountered by bacteria.

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Introduction

Bacteria frequently live in surface-attached communities, called biofilms, which are embedded in a secreted polymer matrix (Hall-Stoodley *et al.*, 2004; Nadell *et al.*, 2009; Flemming and Wingender, 2010). The biofilm mode of bacterial growth is integral to the natural ecology of bacteria, and is additionally important in industry and medicine (Hall-Stoodley *et al.*, 2004). Within the biofilm environment, cells are often immobilized, obtain nutrients passively by diffusion, and interact intimately with near-neighbors (Stewart and Franklin, 2008; Stewart, 2012; Nadell *et al.*, 2016). The biofilm and planktonic niches differ fundamentally. In the latter, cells are often motile, actively seek resources via chemotaxis, and occur at distances far from neighboring cells (Stocker *et al.*, 2008; Stocker and Seymour,

2012). Shifts in the expression of up to 10% of the genome are common when bacteria transition between the planktonic and biofilm modes of growth (Whiteley *et al.*, 2001; Beenken *et al.*, 2004; Mikkelsen *et al.*, 2007). In the case of biofilms, a substantial energetic investment is often required to produce the extracellular matrix (Nadell and Bassler, 2011). In the case of planktonic cells, assembling and operating flagella and mounting the chemosensory apparatus are metabolically expensive (Martínez-García *et al.*, 2014). Thus, fitness trade-offs are incurred for each lifestyle, such that phenotypes conferring competitive success in biofilm environments carry selective disadvantages in the planktonic phase, and *vice versa* (Nadell and Bassler, 2011; Madsen *et al.*, 2015; Oliveira *et al.*, 2015).

Given that determinants of fitness differ in biofilm versus planktonic environments, one might expect bacteria to specialize in order to thrive in one environment or the other. Indeed, some bacterial species produce biofilms but are never actively motile, while other species appear incapable of making biofilms (Yawata *et al.*, 2014). However, many bacteria—including common model organisms—are capable of switching between the biofilm and

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planktonic modes of growth. As opposed to their niche-specialist counterparts, such strains have plastic strategies (Snell-Rood, 2013): they invest in the regulatory machinery required to alternate between the different lifestyles depending on the circumstances. This remarkable flexibility in biofilm formation is well known, and, in the literature, is often assumed to be required for bacteria to successfully endure environmental variation. However, this assumption has not been explicitly tested experimentally, and, moreover, the minimal conditions that selectively favor flexible biofilm-production strategies when bacteria compete with other bacteria that possess specialist strategies have not been explored. Here we provide the first direct examination of the conditions favoring flexible versus fixed biofilm production.

Isolates of *Vibrio cholerae*, the etiologic agent of the human disease cholera, show broad variation in their propensities to make biofilms (Yawata *et al.*, 2014; Chowdhury *et al.*, 2016): some strains are constitutive biofilm-producers, some strains make little to no biofilms, while other strains including well-studied model strains are flexible strategists. With respect to *V. cholerae* strains that are flexible in their biofilm-forming capabilities, planktonic growth and biofilm formation are both crucial for successful proliferation within and outside of human hosts (Nelson *et al.*, 2009; Fong *et al.*, 2010; Utada *et al.*, 2014; Teschler *et al.*, 2015). There is little underlying understanding for the wide variation in biofilm production strategies observed in *V. cholerae* strains in particular, or across different bacterial species more broadly. Here, we examine a commonly studied wild isolate of *V. cholerae* that is capable of flexible biofilm production, as well as two derived strains, one that produces biofilm matrix constitutively and one that is incapable of biofilm matrix production. Using a variety of competition experiments, we define how these different strategies confer evolutionary fitness, and we identify the environmental conditions under which flexible biofilm production is favored by selection.

Materials and methods

Strains and media

All *V. cholerae* strains used in this study are derivatives of the wild-type *Vibrio cholerae* O1 biovar El Tor strain C6706. Additional mutations were engineered into this *V. cholerae* strain using *Escherichia coli* S17- λ pir carrying pKAS32 or its derivatives (Skorupski and Taylor, 1996). All strains were grown in lysogeny broth (LB) medium at 37 °C with shaking overnight. Unless otherwise indicated, biofilm experiments were performed in M9 minimal medium, supplemented with 0.5% casamino acids, 2 mM MgSO₄, and 100 μ M CaCl₂. All media contain 6 μ g ml⁻¹ polymyxin B. Although *V. cholerae* C6706 is resistant to polymyxin B, we found that low doses,

similar to other antibiotics (Hoffman *et al.*, 2005), encourage biofilm growth. A detailed strain list is provided in Supplementary Table 1.

Statistics

Four biological replicates were performed for each experiment unless indicated otherwise; error bars correspond to standard errors throughout the text. For biomass quantification, at least 6 different locations (technical replicates) with *xy* dimensions of 250 μ m \times 250 μ m were sampled and averaged for each biological replicate. For cell counting, after placing liquid samples on microscope slides, 10 different locations with *xy* dimensions of 250 μ m \times 250 μ m were sampled for each biological replicate. All statistical analyses are unpaired *t*-tests with Welch's correction.

Biofilm growth procedure for isolated strains

V. cholerae strains were grown overnight at 37 °C in liquid LB medium with shaking, back-diluted 30-fold and grown for an additional 2 h with shaking in M9 medium until early exponential phase (OD₆₀₀ = 0.2–0.4). The OD₆₀₀ of each culture was measured and the culture was back-diluted into fresh M9 medium to a final OD₆₀₀ of 0.033. The inoculum was then transferred into planar microfluidic devices measuring 1 cm in length, 400 μ m in width and 60 μ m in height. After 2 h of incubation, fresh medium was provided continuously with a syringe pump at a flow rate of 0.6 μ l min⁻¹ (corresponding to an average flow velocity of 417 μ m s⁻¹ inside the chamber). The biofilms were grown at ambient room temperature (25–30 °C). To measure biofilm population sizes, the biofilms were imaged by confocal microscopy at *z*-intervals of 3 μ m, to ensure minimal overlap between consecutive optical sections. The images obtained in this manner were thresholded and converted to cell counts per unit area using protocols developed previously (Drescher *et al.*, 2014; Nadell *et al.*, 2015). In brief, bacterial biomass was distinguished from matrix material and background fluorescence. Each cross-sectional image was thresholded and converted to cell counts based on the average size of a bacterial cell, which was measured separately in a monolayer sample. Biofilm growth involving dispersal mutants was performed in medium containing 10 μ M CaCl₂ instead of 100 μ M to promote increased VPS-dependent biofilm growth (Bilecen and Yildiz, 2009).

Procedure for dispersal assay

After 12 h of growth, biofilms were imaged for population size quantification. Immediately after imaging, the outlet tubing of the microfluidic channel was cut to a minimal length, and the flow was interrupted for 2 h. A fast flow pulse (5 μ l min⁻¹, corresponding to a flow velocity of \sim 3.5 mm s⁻¹) was

applied briefly (~2 min; which was sufficient for the effluent to become optically clear) to flush out all cells that had dispersed during the nutrient limitation period. The biofilms remaining in the microfluidic channel were imaged again at the original locations. The liquid drop exiting the effluent tubing was collected, diluted in series into M9 medium (without nutrients), plated onto LB agar and allowed to grow overnight at 30 °C. Colony-forming units were counted manually and used to calculate the total cell number in the original effluents. The biomass quantification procedure described above was used to assess the biomass prior to and after nutrient limitation.

Microscopy

Microscopy was performed with a Borealis confocal system with a Yokogawa CSU-X1 confocal spinning disk unit (Tokyo, Japan) mounted on a Nikon Ti-E inverted microscope (Tokyo, Japan). Images were acquired with a 60 × water objective with a numerical aperture of 1.2 and captured with an Andor iXon 897 EMCCD camera. Three diode lasers (445 nm, 543 nm and 592 nm) were used sequentially along with customized dichroic filters (Chroma, Marlborough, MA, USA) for detection, providing good spectral separation for the three fluorescent proteins used in the study. To image fine structures within individual biofilm clusters, an additional ×1.5 magnification lens was placed between the microscope and the spinning disk, and the z-step size was adjusted to 0.2 μm. All experimental images presented in this work are raw images from this step, rendered by Nikon Elements software.

Biofilm and dispersal competition assay

We competed the WT *V. cholerae* strain carrying constitutively expressed *mTFP1*, the extracellular polymeric substances (EPS⁺) strain with constitutively expressed *mKate2* and the EPS⁻ strain with constitutively expressed *mKO*, all of which were inserted into the chromosomal *lacZ* locus. Previous work has established that the production of these fluorescent proteins does not affect the growth rate of *V. cholerae* (Nadell and Bassler, 2011; Drescher et al., 2014; Nadell et al., 2015). Overnight cultures were first grown in the presence of glass beads (4 mm, MP Biomedicals, Santa Ana, CA, USA) and vigorously shaken, back-diluted 30-fold and grown with shaking in M9 medium with glass beads to OD₆₀₀ = 0.2–0.4. The regrown cultures were transferred to Eppendorf tubes with smaller glass beads (acid-washed, 425–500 μm, Sigma, St Louis, MO, USA), and vigorously bead-bashed on a vortex mixer. These steps were necessary to disassemble cell clusters that had formed in the liquid cultures, if any, for accurate OD₆₀₀ measurements and OD₆₀₀ equalization. An inoculum with a total OD₆₀₀ of 0.1, regardless of the composition, was prepared after the

measurement. The inoculum was then transferred into planar microfluidic chambers and incubated for 2 h, after which fresh medium was provided continuously with a syringe pump. For biofilm competitions, images were taken of biofilms in the microfluidic chambers after 16 h of growth. The image-processing-based biomass quantification procedure described above was used separately for each strain in the co-cultures, and subsequently, to define each strain's population frequency. In the case of dispersal competitions, after 12 h of biofilm growth, flow was interrupted for 2 h, followed by a fast flow pulse to flush out all dispersed cells (see above). Effluents were collected, mixed with 80% glycerol and temporarily stored at –80 °C. The frequency of each strain in the effluent was measured later by two methods: (1) cell counting via microscopy. In this case, 5 μl of effluents were placed between coverslips and agar pads made from M9 medium without nutrient. (2) serial dilution and plating. In this case, a gel imager (Image Quant LAS 4000) with different filter sets was used to differentiate the strains on LB plates, and to count the number of colonies of each strain.

Liquid growth curve and competition

Overnight cultures of each strain grown in LB were back-diluted 30-fold into M9 medium, and regrown for 2 h. The regrown cultures were back-diluted again into 15 ml M9 medium with glass beads in a 50 ml centrifuge tube to yield an initial OD₆₀₀ of 0.0004. The cultures were then grown on a shaker at 30 °C, and were additionally manually shaken and mixed by vortex every hour. Every 2 h, OD₆₀₀ was measured. For competitions in liquid, cultures were prepared as described above for biofilm competition experiments, to a total OD₆₀₀ of 0.3 per inoculating mixture. 10 μl of the inoculum was added to 500 μl of fresh M9 medium with small glass beads in a 1.5 ml Eppendorf tube, which was subsequently rotated end over end at 30 °C. Every 4 h, the culture was mixed by vortex. After 8 h of liquid growth, 10 μl of this culture was back-diluted into 500 μl of fresh M9 medium with small glass beads. After 16 h of growth in total, the liquid culture was temporarily frozen after addition of 80% of glycerol. The strain frequencies in the culture were later analyzed by imaging samples of the culture by confocal microscopy.

Experiments simulating a fluctuating environment

The biofilm growth procedure was performed as described above, with an initial inoculum containing a 1:1:1 mixture of WT, EPS⁺ and EPS⁻ strains at a total OD₆₀₀ of 0.1. For the zero-disturbance experimental treatment, biofilm samples were imaged at 34–36 h. For the one-disturbance experimental treatment, biofilms were imaged after 12 h of biofilm growth. The imaging duration was 2 h, after which

flow was interrupted. The outlet tubing was removed, and new tubing was used to connect the outlet of the initial chamber to a new empty microfluidic chamber. After 2 h of nutrient limitation, flow was resumed to pump the dispersed cells from the initial chamber to the new chamber. The new chamber was incubated for 2 h, and then fresh medium was provided. After another 12 h of biofilm growth, biomasses were again imaged. For the three-disturbance experimental treatment, the identical procedure was performed as described above for the one-disturbance treatment, except all the time scales were halved and the disturbance and chamber recolonization procedure was repeated 3 times instead of once. The total durations of the experiments for all three-disturbance conditions are roughly identical (35–36 h).

Results

Biofilm production, planktonic growth and dispersal

To explore the fitness consequences of constitutive versus flexible biofilm production strategies, we measured the biofilm and planktonic growth properties of three *V. cholerae* strains: the wild type (designated WT), which is able to transition between biofilm and planktonic modes of growth (McDougald *et al.*, 2012; Teschler *et al.*, 2015); a strain that constitutively produces extracellular matrix due to a point mutation (*vpvC*^{W240R}) that results in elevated levels of the second messenger molecule c-di-GMP

and, in turn, hyper-secretion of the biofilm matrix (designated EPS⁺; Beyhan and Yildiz, 2007); and a strain that is unable to produce extracellular matrix due to deletion of the *vpsL* gene, required for synthesis of a key matrix polysaccharide (designated EPS⁻; Fong *et al.*, 2010). When grown in isolation, the EPS⁺ strain is the strongest biofilm-producer, accumulating threefold more biomass in microfluidic channels than the WT and 10-fold more biomass than the EPS⁻ strain, which only forms a loosely attached monolayer (Figure 1a).

In planktonic cultures, which were periodically shaken with beads to ensure that cells did not grow in clusters (Nadell and Bassler, 2011), the WT and the EPS⁻ strain grew at indistinguishable rates that were ~20% higher than the growth rate of the EPS⁺ strain (Figure 1b). In liquid, the WT strain represses biofilm-associated genes (Beyhan *et al.*, 2007; Papenfort *et al.*, 2015), and hence, grows at the same rate as the EPS⁻ strain. Importantly, the growth rate differences between our three strains could be attributed to their differences in matrix secretion, rather than differences in c-di-GMP levels. Specifically, deletion of the *vpsL* gene in the strain with elevated c-di-GMP level restored the growth rate to that of the WT in liquid medium (Supplementary Figure 1). These results are consistent with our previous report documenting that extracellular matrix production enhances fitness on surfaces but at a cost to maximum growth rate in liquid (Nadell and Bassler, 2011). In sum, WT cells are inferior to

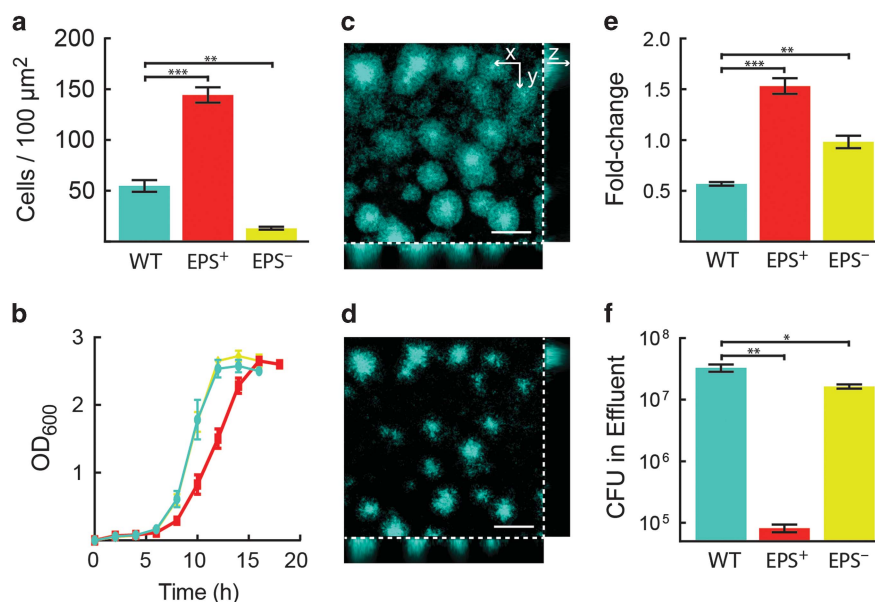


Figure 1 Growth characteristics of WT, EPS⁺ and EPS⁻ *V. cholerae* strains. In all panels, WT is teal, EPS⁺ is red and EPS⁻ is yellow. $n=4$ biological replicates for all experiments. (a) Number of biofilm cells per unit area of all strains after 16 h growth under biofilm-forming conditions. (b) Growth curves in shaken liquid culture. (c and d) Representative confocal images of biofilms formed by WT *V. cholerae* cells constitutively expressing *mTFP1*, before (c) and after (d) nutrient limitation for 2 h. The cross-sectional *xy*-view at 3 μm above the surface is shown in the upper left large panel and two orthogonal *z*-projections are shown on the bottom and right. Scale bar: 50 μm. (e) Fold-change in biofilm cell counts after 2 h nutrient limitation. (f) Colony-forming units (CFU) in the effluents collected immediately after the nutrient limitation period. Data are represented as mean ± s.e.m. All statistical comparisons are unpaired *t*-tests. *** denotes $P<0.0005$, ** denotes $P<0.005$, and * denotes $P<0.05$.

EPS⁺ cells under biofilm-forming conditions, and they do not have an advantage over EPS⁻ cells in planktonic growth.

Previous work has suggested that WT *V. cholerae* biofilm-dwelling cells are primed for dispersal when they become nutrient-limited (for example, when fluid flow over biofilms is halted; Nielsen *et al.*, 2006; Müller *et al.*, 2007). We measured how differences in EPS production among our three strains influence dispersal using two assays (Figures 1c–f). Biofilms of each strain were grown in isolation, after which (1) their biomasses were measured before and after nutrient limitation in the microfluidic channels, and (2) the total numbers of dispersed cells were measured in the effluent downstream of each biofilm immediately following nutrient limitation. The WT biofilm biomass declined by ~50% following nutrient limitation (Figure 1e); the WT biofilm clusters shrank in size and they appeared to lose cells from both the outer periphery and from the interior (Figures 1c and d). EPS⁺ biofilms, by contrast, continued to grow before nutrient was completely depleted, yielding a ~50% increase in biomass by the conclusion of the experiment (Figure 1e). EPS⁻ cells produced monolayer biofilms that did not significantly change in size after nutrient limitation (Figure 1e). The effluents from the nutrient-limited biofilms contained 2-fold and 400-fold more WT cells than EPS⁻ and EPS⁺ cells, respectively (Figure 1f). These data indicate that the EPS⁻ strain produces monolayer biofilms from which newly divided daughter cells detach continuously, whereas EPS⁺ cells are severely impaired for dispersal due to over-production of the matrix (Nadell and Bassler, 2011). The WT strain, by contrast, produces sufficient matrix to accumulate 3D biofilms while also effectively dispersing following nutrient limitation. WT dispersal depended on secretion of the extracellular enzyme RbmB, which has previously been implicated in EPS digestion (Fong and Yildiz, 2007), as well as extracellular proteases including HapA and IvaP that target the protein components of the *V. cholerae* biofilm matrix (Supplementary Figures 2 and 3; Smith *et al.*, 2015).

Pairwise and three-way competition between WT, EPS⁺ and EPS⁻ strains

The above results establish that WT, EPS⁺ and EPS⁻ strains of *V. cholerae* differ in their relative abilities to grow in the planktonic phase and to form and disperse from biofilms when in mono-culture. We investigated the consequences of these differences using pairwise and three-way competitions in the different environments, that is, in continuous liquid culture, continuous biofilm culture and in the biofilm dispersal assay. We varied the initial strain ratios systematically and measured the final population composition after 16 h (Supplementary Data 1). The data were used to construct phase plane diagrams, from which one can infer the stable

population steady state in each environmental condition.

When grown in shaken liquid co-culture, WT and EPS⁻ cells remain at the same ratio at which they were inoculated (Supplementary Figure 4). The right edge in Figure 2a defining all two-strain ratios of the WT and the EPS⁻ strain is thus a line of neutrally stable equilibria. However, WT and EPS⁻ cells are both competitively superior to the constitutively matrix-producing EPS⁺ strain in shaken liquid (Figure 2a and Supplementary Figure 5). Specifically, in liquid cultures containing mixtures of all three strains, WT and EPS⁻ strains outcompete the EPS⁺ strain, such that the relative ratio of WT to EPS⁻ changes minimally while the EPS⁺ population fraction declines toward zero (Figure 2a). This result is consistent with our above growth rate measurements (Figure 1b). When grown in biofilm-forming conditions, by contrast, uniform positive selection occurs for the EPS⁺ strain (Figure 2b and Supplementary Figure 5), that is, EPS⁺ cells approach 100% of the biofilm population starting from any initial condition. Notably, although the WT produces matrix and forms biofilms, it is outcompeted by the EPS⁺ strain in biofilm co-culture (Supplementary Figure 6). The WT strain, in turn, outcompetes the EPS⁻ strain in co-inoculated biofilms (Figure 2b and Supplementary Figure 6). Thus, each strain's fitness in the biofilm-forming environment tracks with its level of extracellular matrix production relative to its competitors (Xavier and Foster, 2007; Nadell *et al.*, 2008). Indeed, matrix-secreting strains of a variety of bacterial species spatially self-segregate from, laterally displace, and outcompete strains that produce less or no matrix (Xavier and Foster, 2007; Nadell and Bassler, 2011; Kim *et al.*, 2014; Madsen *et al.*, 2015; Nadell *et al.*, 2015; Schluter *et al.*, 2015; Drescher *et al.*, 2016; Irie *et al.*, 2016; Kragh *et al.*, 2016; Yan *et al.*, 2016).

To test the ability of the different strains to transition between biofilm and planktonic states in a competitive context, we assessed the composition of the effluent immediately following nutrient limitation of biofilms co-inoculated with different ratios of the three strains. The WT clearly dominates in direct competition with EPS⁺ and EPS⁻ cells (Figure 2c, Supplementary Figures 5 and 7). Presumably, the WT outcompetes the other strains because it can produce sufficient matrix to form a 3D biofilm structure during the growth period, and subsequently it can digest matrix components and disperse a large fraction of its biofilm biomass into the liquid phase during the nutrient limitation period. Consistent with this interpretation, WT cell clusters shrink during the nutrient limitation, whereas the EPS⁺ cell clusters do not (Figures 2d and e). Overall, our experiments suggest that the WT is inferior in the biofilm phase when in competition with stronger matrix-producers, but the WT is superior in its ability to transition between the biofilm and planktonic growth modes.

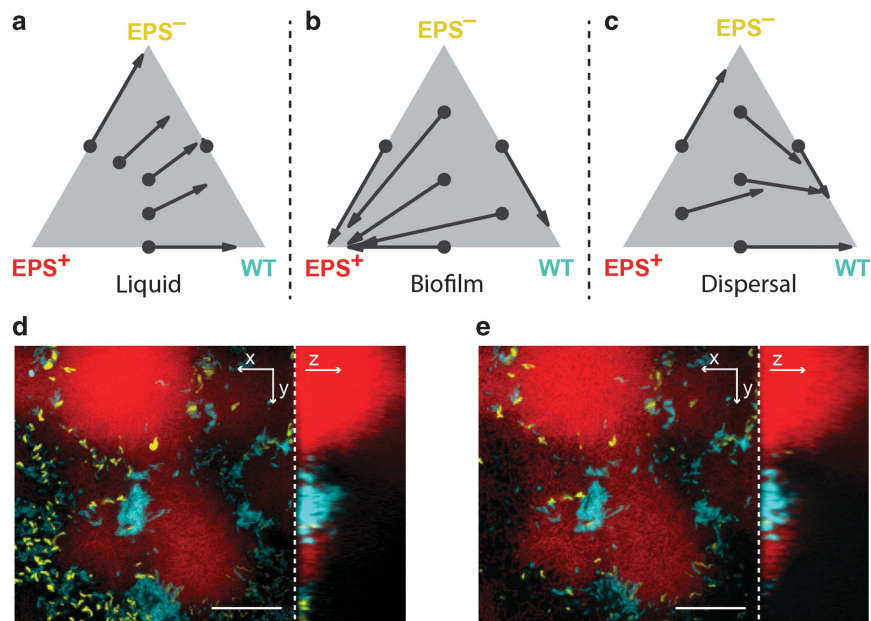


Figure 2 Competition of *V. cholerae* strains with different biofilm production strategies. In all panels, teal, red and yellow correspond to WT, EPS⁺ and EPS⁻ cells, respectively. (a–c) Three-way competition between WT, EPS⁺ and EPS⁻ strains in shaken liquid culture (a), under biofilm-forming conditions (b) and under biofilm dispersal conditions (c). The composition of each initial inoculum is represented by the black dots on the ternary plots. The average final compositions were measured and are denoted by the end points of each arrow. The full data are presented in Supplementary Figure 5. (d and e) Representative 12 h biofilm structures before (d) and after (e) 2 h of nutrient limitation, starting from a 1:1:1 mixture of WT, EPS⁺ and EPS⁻ inoculum of cells. The cross-sectional *xy*-view of the bottom cell layer is shown on the left and the *yz* side view is shown on the right. Scale bar: 20 μm. Note the decrease in size of the WT biofilms from d to e.

A flexible biofilm formation-dispersal strategy is favored in fluctuating environments

The above experiments examine competition under selection in a single habitat. The natural environment in which *V. cholerae* resides is subject to temporal variation in habitat type and quality, as well as sudden influxes or depletions of resources (Teschler *et al.*, 2015; Conner *et al.*, 2016). We explored the consequences of differential matrix production and dispersal abilities in alternating environments. To do this, we grew 1:1:1 mixtures of the WT, EPS⁺ and EPS⁻ strains under biofilm-forming conditions for a fixed total amount of time, and we varied the number of disturbance and biofilm re-colonization events between treatments (either 0, 1 or 3). For each disturbance event, fluid flow was interrupted to deplete nutrients from the biofilm cells prior to pulsing the biofilm effluent into another microfluidic chamber. The goal was to simulate the natural transition of *V. cholerae* from an existing biofilm with diminishing resources to a new region rich with resources with an intervening planktonic phase. Biofilms were imaged immediately prior to each disturbance event, and again at the end of the experiment (Figure 3).

When there were no disturbance events over 36 h, the EPS⁺ strain approached 97% of the population (Figure 3a, left). With the introduction of one-disturbance event, the population fraction of the EPS⁺ strain declined to 93%, the WT strain rose to a detectable minority at 7% and the EPS⁻ strain remained nearly undetectable (Figure 3a, middle).

When three-disturbance events occurred over 36 h, the WT comprised 75% of the population and was increasing at the conclusion of the experiment (Figure 3a, right). We expect WT cells to approach fixation in experiments with longer duration or higher disturbance rate, but these could not be performed at present due to technical constraints.

Figure 3b illustrates the time-resolved frequencies of WT, EPS⁺ and EPS⁻ at 4 time points during the experimental condition of three-disturbance/re-colonization events over 36 h. WT increased in frequency at every sampling point. The frequency of EPS⁺ cells trended downward overall, but oscillated from one-disturbance event to another, which appeared to be due to fluctuations in the initial population density after re-colonization of new biofilm chambers. The EPS⁻ strain increased in frequency initially, but over the course of the experiment, declined to a minority fraction of 14% of the population. We presume this decrease is due to the fact that the EPS⁻ strain is outcompeted by WT and EPS⁺ cells during biofilm growth, ultimately reducing its ability to seed new chambers following disturbance.

These results show that the WT capability to form and disperse from biofilms is selectively favored under high variation and turnover in habitat type over time, which is consistent with environmental conditions known to be important for *V. cholerae* in its natural state (Conner *et al.*, 2016). In the environment, *V. cholerae* resides on and consumes chitin particles with finite lifetimes (Meibom *et al.*, 2004; Stocker and Seymour, 2012; Drescher *et al.*,

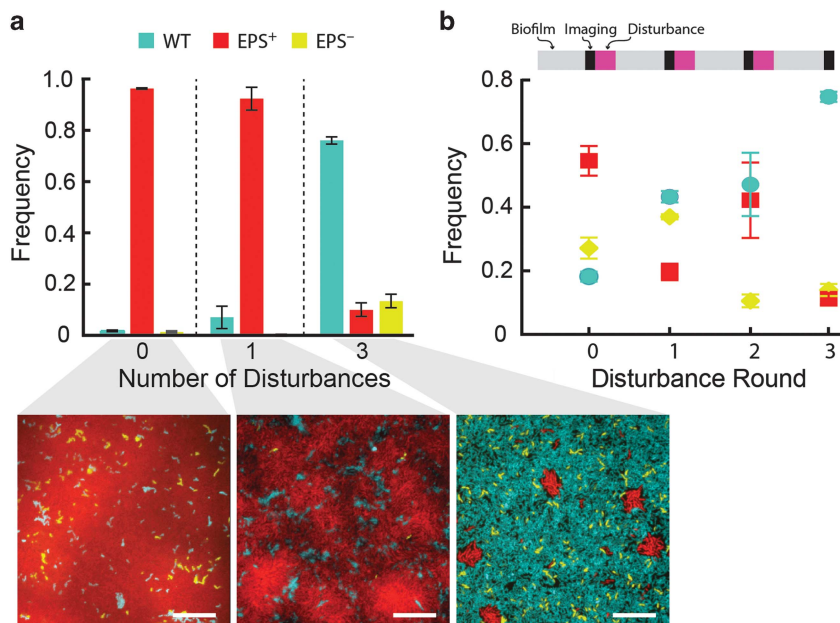


Figure 3 A fluctuating environment selects for WT *V. cholerae* with a plastic biofilm production strategy. Cultures were initiated with a 1:1:1 mixture of WT:EPS⁺:EPS⁻ cells in microfluidic devices. Chambers were subjected to a period of nutrient limitation (1/6 of the biofilm growth time) after which, cells were allowed to disperse to a new chamber. In all panels, WT is teal, EPS⁺ is red and EPS⁻ is yellow. (a) Final strain composition of cultures in competition experiments with different disturbance frequencies. Below: representative biofilm images taken of the bottom cell layer after competition with continuous nutrient provision (left), with disturbance events every 12 h (middle) and with disturbance events every 6 h (right). Images are single optical sections through the basal layers of the biofilms. Scale bar: 20 μ m. (b) Frequency of the WT, EPS⁺ and EPS⁻ strains during competition with disturbance events every 6 h. The timeline of the experiment is displayed in the bar above the data: light gray corresponds to biofilm-forming conditions in a microfluidic device; black refers to a sampling period when biofilms were imaged, and magenta corresponds to a disturbance event in which cells were depleted for nutrients for one hour. Following that, biofilm effluents were used to colonize new chambers for 1 h, followed by another phase of biofilm growth. WT: teal circles; EPS⁺: red squares; and EPS⁻ cells: yellow diamonds. Data are represented as mean \pm s.e.m ($n = 3-4$).

2014). During infection, *V. cholerae* establishes biofilms in the host intestine and then disperses after causing the lumen to become flooded with fluid (Fong *et al.*, 2010; Millet *et al.*, 2014; Teschler *et al.*, 2015; Sengupta *et al.*, 2016). Both of these natural environments involve repeated transitions between the planktonic and biofilm growth modes. Our experiments show that the plastic biofilm production strategy of WT *V. cholerae* is particularly well suited to such fluctuating environments.

Discussion

Understanding the conditions that select for phenotypic flexibility and diverse habitat occupation, as opposed to fixed specialization in one particular habitat, is of long-standing interest in ecology and evolution (Wilson and Yoshimura, 1994; Kassen, 2002; Egas *et al.*, 2004). A major factor that limits the evolution of generalist behaviors is the compromised ability to compete against specialists in any particular environment (Devictor *et al.*, 2008; Ali and Agrawal, 2012; Lowe and McPeck, 2014). Here, we demonstrate that WT *V. cholerae* transitions effectively from the biofilm to the planktonic state using matrix-degrading enzymes, but this capability comes at the expense of its competitive ability against constitutive biofilm producers on surfaces. This

compromise is offset when disturbance events are frequent, which allows WT *V. cholerae* cells to become the majority and, we expect, competitively eliminate constitutive biofilm producers and non-producers over the long run. Our results suggest that the temporal stability of environments in which particular *V. cholerae* isolates reside is key to determining whether or not biofilm regulation will be favored by natural selection. It is important to note that strains of *V. cholerae* and other *Vibrio* spp. that regulate matrix production as well as strains that make minimal biofilms or that constitutively secrete matrix have been isolated in nature (Yawata *et al.*, 2014; Chowdhury *et al.*, 2016). We predict that an important determinant underlying this variation in matrix production strategy is the lifetime of resource patches (Nadell *et al.*, 2008), with longevity favoring constitutive phenotypes and rapid turnover favoring flexible matrix production.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

CDN and JY initiated this project. JY and CDN constructed the strains. JY performed the experiments. JY, CDN and BLB analyzed the data. CDN, JY and BLB wrote the manuscript.

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