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# Architectural transitions in *Vibrio cholerae* biofilms at single-cell resolution

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Many bacterial species colonize surfaces and form dense 3D structures, known as biofilms, which are highly tolerant to antibiotics and constitute one of the major forms of bacterial biomass on Earth. Bacterial biofilms display remarkable changes during their development from initial attachment to maturity, yet the cellular architecture that gives rise to collective biofilm morphology during growth is largely unknown. Here, we use high-resolution optical microscopy to image all individual cells in *Vibrio cholerae* biofilms at different stages of development, including colonies that range in size from 2 to 4,500 cells. From these data, we extracted the precise 3D cellular arrangements, cell shapes, sizes, and global morphological features during biofilm growth on submerged glass substrates under flow. We discovered several critical transitions of the internal and external biofilm architectures that separate the major phases of *V. cholerae* biofilm growth. Optical imaging of biofilms with single-cell resolution provides a new window into biofilm formation that will prove invaluable to understanding the mechanics underlying biofilm development.

biofilm | community | self-organization | emergent order | nematic order

Bacteria assemble into communities, termed biofilms, which are embedded in a secreted polymer matrix and often coat liquid–air or liquid–solid interfaces (1–4). Some biofilms are beneficial to human health, for example as part of the healthy gut and skin microbiota (5, 6) or in wastewater treatment systems (7). Other biofilms, however, cause serious problems in oral hygiene, chronic infections, and prosthetic contamination (8–10) and act as fouling agents in industrial flow systems (11). In all contexts, biofilms can be difficult to control due to their resilience against chemical and physical stresses (12, 13), including antibiotic treatment (14). Because of their ubiquity and their relevance to medicine and industry, the formation of biofilms has been studied intensively, with an emphasis on the genes, regulatory mechanisms, and transport properties that underlie transitions from planktonic growth to surface attachment (15–17), to proliferation and matrix secretion (2, 18), and finally to dispersal (19, 20). A basic understanding of several regulatory circuits and secreted matrix components governing biofilm formation has been developed (21–25). Nonetheless, the physical, biological, and chemical factors that interact to determine the biofilm architecture remain largely unknown.

Internal and global biofilm architectures are presumably consequences of emergent interactions between individual cell growth, physiological differentiation, secreted proteins, polymers and small molecules, and microenvironmental heterogeneity (21, 26–34). Attempts to dissect the individual and combined contributions of these factors to biofilm growth have increasingly relied on examination of bacterial communities in microfluidic devices that mimic central features of natural environments (11, 35, 36). Although sophisticated methods for fabricating biofilm microenvironments are available, a significant barrier to progress has been the lack of techniques capable of resolving all individual cells residing inside biofilms. Thus, the vast majority of studies to date have been limited to visualizing 3D biofilms as connected “clouds” of biomass, although some studies have used fixed samples to obtain cellular resolution (37–40). We therefore know little about the organiza-

tional principles that convert individual cell behavior into macroscopic growth and collective properties of biofilms.

Here, we develop and use experimental techniques to investigate at single-cell resolution the 3D architecture of biofilms containing thousands of cells. By using a customized spinning-disk confocal microscope that enables 3D imaging at high axial resolution with low-light doses and by combining this instrument with bespoke image analysis software, we were able to visualize and segment all individual cells in thousands of *Vibrio cholerae* biofilms grown on submerged glass surfaces under flow containing nutrients. From these data, we could construct ensemble averages of biofilm structure during every phase of growth. We discovered that the internal community architecture and global biofilm morphology undergo several distinct transitions, which manifest as changes in the relative arrangements of individual cells over the course of biofilm development. From these data, we identified four fundamental phases of biofilm growth, each characterized by its own unique architecture: 1D growth of 1–6 cells, 2D growth of 20–100 cells, 3D growth of 200–1,000 cells with low local order, and highly ordered growth of communities with more than 2,000 cells. These phases can be explained by transitions in the physical dimensionality of the particular biofilm combined with changes in local cell density. Of the three known *V. cholerae* matrix proteins RbmA, Bap1, and RbmC, only deletion of RbmA substantially perturbs cellular orientations and the overarching biofilm architecture. We thus provide, to our knowledge, the first steps toward resolving how the 3D biofilm architecture results from the interactions of the constituent cells.

## Significance

Bacterial biofilms are ubiquitous in the environment and serve beneficial roles in microbiota communities in the context of eukaryotic hosts and in industrial applications, yet biofilms of pathogenic bacteria can also cause devastating infections. Biofilm architectures are usually studied at a coarse morphological level, and consequently, little is known about the internal biofilm architecture and how it emerges. Here, we use an optical imaging technique to visualize every cell inside thousands of *Vibrio cholerae* biofilms to discover architectural transitions and the major phases of *V. cholerae* biofilm growth.

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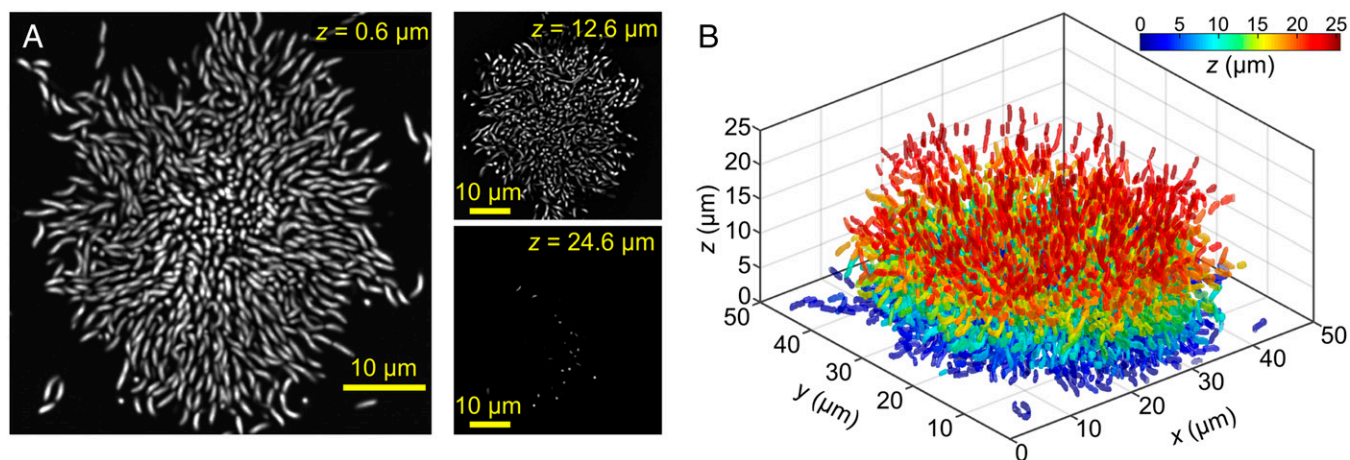
The authors declare no conflict of interest.

See Commentary on page 3711.

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**Fig. 1.** *V. cholerae* wild-type biofilm at single-cell resolution. (A) Planar cross-sections through the biofilm at heights  $z = 0.6 \mu\text{m}$ ,  $12.6 \mu\text{m}$ , and  $24.6 \mu\text{m}$ . (B) All individual cells from A were automatically segmented into separate 3D objects, which are color-coded in panel B according to the heights of their centers of mass using the color scale indicated at the top right. Panels A and B show the identical biofilm, which was grown until time  $t = 24.5 \text{ h}$  and contains  $N = 4,543$  cells. Global morphological parameters for this particular biofilm are indicated by the red data points in Fig. 2.

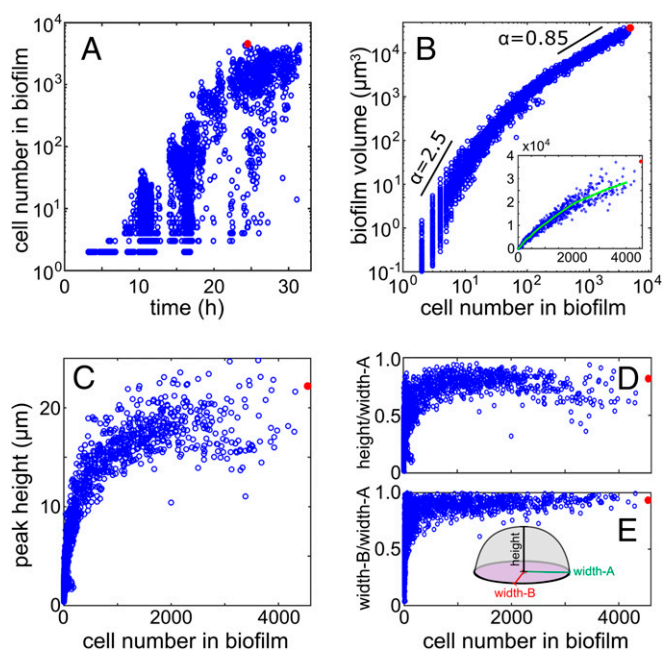
## Results and Discussion

Fluorescent proteins expressed from the *V. cholerae* chromosome do not provide a sufficient signal for live-cell imaging at single-cell resolution, even when the fluorescent proteins are expressed at levels just below those that inhibit growth. We therefore grew biofilms for different times in microfluidic flow channels and stained the biofilms in situ with a nucleic acid dye that we added to the medium immediately before imaging. This protocol makes it possible to investigate biofilm architecture at different growth stages. Using spinning disk confocal microscopy, we were able to resolve all individual cells inside biofilms. By applying our Matlab-based 3D image analysis software, we could localize and distinguish all cells in biofilms up to  $\sim 30 \mu\text{m}$  in height, as shown in Fig. 1A (see *Materials and Methods*). From these gray-scale images, we obtained binary 3D images of each cell via image-processing techniques that are based on spatial band-pass filtering. A complete reconstruction of the positions and orientations of all cells in a biofilm with  $N = 4,543$  cells is shown in Fig. 1B, where each cell is colored according to its distance from the substratum.

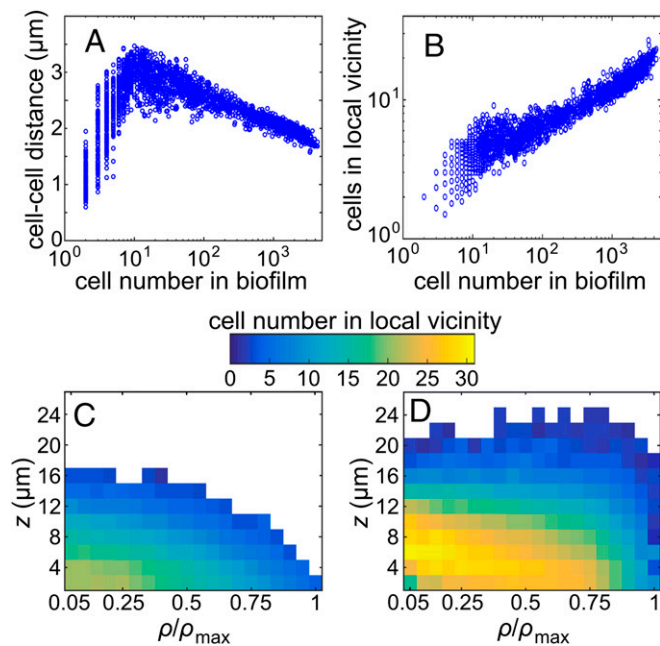
Recent work has shown that *V. cholerae* biofilms grow clonally from single founder cells or founder cell clusters (41) into microcolonies. Here, we use the term “biofilm” to refer to these individual cell clusters on the glass substrate rather than the collection of all cell clusters on the surface. These biofilms strongly resist attachment and invasion by motile planktonic cells (42), although they permit increased surface attachment upon partial digestion of their extracellular matrix (43). Predominantly, clonal growth of biofilms also occurs in the infant rabbit model of *V. cholerae* infection (44), suggesting that clonal growth with strong segregation between different cell lineages is the dominant form of biofilm development for *V. cholerae*. Motivated by these findings, we used low seeding densities in our flow channels to ensure that we exclusively imaged biofilms that had arisen from single founder cells. To verify that biofilms originated from single founder cells in our experiments, we seeded our flow chambers with a mixture of three derivatives of wild-type *V. cholerae*, differing only in the fluorescent proteins expressed from the chromosome (mTFP, mKO, or mKate2). All biofilms were composed entirely of cells expressing the same fluorescent protein, as shown in Fig. S1, indicating that the biofilms were monoclonal and that attachment of planktonic cells to existing microcolonies was either rare or absent in our experiments.

**Biofilm Morphology and Internal Architecture During Growth.** Using the high-resolution microscopy technique noted above, we im-

aged a total of 2,429 *V. cholerae* biofilms of different sizes. This large number of 3D biofilm images allowed us to reconstruct ensemble averages to characterize morphological features, cell



**Fig. 2.** The growth laws and global morphology of *V. cholerae* wild-type biofilms. Each of the 2,429 data points in the subpanels represents a different biofilm. The red data point is the biofilm shown in Fig. 1. (A) Number of cells in biofilms as a function of incubation time. (B) The biofilm volume, calculated as the convex hull containing all of the cells inside each biofilm, shows a transition in slope around  $N = 10^2$ . The *Inset* shows the same data on a linear-linear scale. The green line depicts the running average of the data. (C) The peak height of the biofilm is the  $z$ -component of the center of the highest cell and reaches a maximum at  $\sim 20 \mu\text{m}$  under the growth conditions tested. (D and E) Fitting an ellipse to the base of the biofilm in the XY plane yields a “width-A” and “width-B” for the biofilm, corresponding to the major and minor axes of the ellipse, respectively. For small  $N$ , ellipse fitting only provides qualitatively accurate results, whereas for large  $N$ , the ellipse fits are highly accurate. Panel D shows that the height of the biofilm remains lower than the width-A (a ratio height/width-A = 1 would correspond to a hemisphere), whereas the cross-section in the XY plane becomes nearly circular as the cell number in the biofilms increases.



**Fig. 3.** Cell density depends on position inside biofilms and changes during *V. cholerae* biofilm growth. (A) The average distance to the center of mass of the nearest five neighboring cells from any individual cell center of mass in the biofilm, averaged over all cells in the entire biofilm, strongly depends on the total number of cells in the biofilm. (B) The cell number in the local vicinity, averaged over all cells in each biofilm and plotted as a function of the number of cells in the biofilm, shows a steady increase in cell density with biofilm size. The local vicinity in this figure is defined as a sphere of radius  $r = 3 \mu\text{m}$  around the center of each cell. (C and D) Spatially resolved average cell number in the local vicinity. To offset the effect that the cells in the bottom-most layers have fewer neighbors due to geometric constraints, the biofilm was mirrored at the  $z = 0$  plane before calculating the cell number in the local vicinity in panels C and D. For panel C, 45 biofilms with  $N = 600\text{--}800$  cells were averaged, using cylindrical coordinates. For panel D, 15 biofilms with  $N = 3,500\text{--}4,000$  cells were averaged.

shapes, as well as relative and absolute cell arrangements throughout biofilm growth.

The resulting “growth laws” (45–47) for *V. cholerae* wild-type biofilms are summarized in Fig. 2. We found that the biofilm incubation period is a poor assessment parameter in ensemble averaged experiments, as shown in Fig. 2A, because dispersal and reattachment of cells to the glass substrate results in biofilms with a range of different ages and sizes at a given chamber incubation time. However, the global morphological features, such as biofilm volume (Fig. 2B), height (Fig. 2C), width (Fig. 2D and E), and free surface area (Fig. S2), collapse tightly when measured as a function of the number of cells  $N$  in the biofilm. This finding implies that the cell number  $N$  is the key parameter that determines the biofilm architecture, and we therefore characterize our measurements in terms of  $N$  for the remainder of this work.

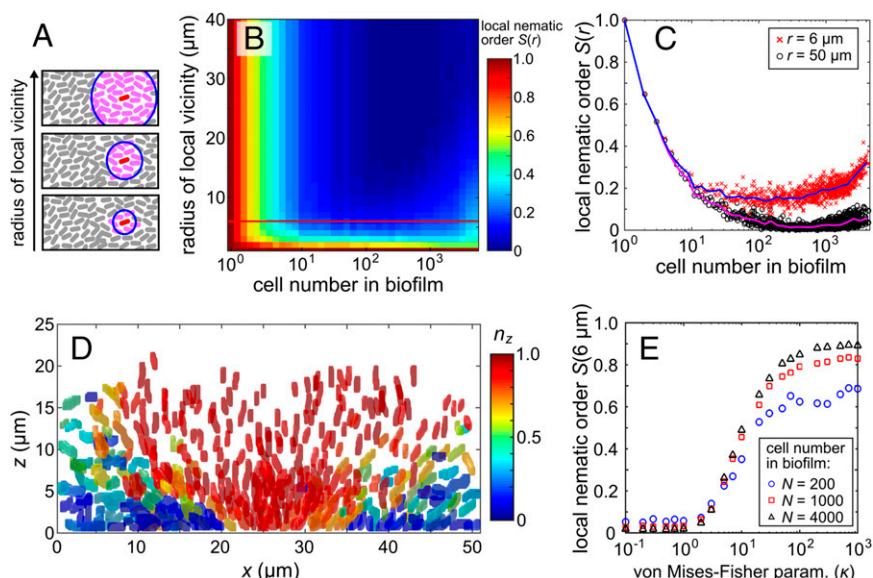
We find that the biofilm volume  $V$  increases more rapidly in biofilms with a low cell number than in biofilms with a large  $N$  (Fig. 2), indicating that an architectural or physiological transition occurs in the biofilm community at approximately  $N = 100$  cells (Fig. 2B). The relationship between  $V$  and  $N$  can be captured by a simple power law  $V = c N^\alpha$ , where  $c$  is a constant. For bacterial growth in well-mixed suspension cultures, a value of  $\alpha = 1$  would be expected due to a 1:1 correspondence between cell number and population biovolume. However, in the case of biofilms, we measure  $\alpha = 2.5$  for  $N < 100$ , whereas  $\alpha = 0.85$  for  $N > 100$ . The rapid accumulation of biovolume for  $N < 100$  results from a significantly larger mean cell center–cell center distance in small biofilms, which

reaches a maximum of  $\sim 3 \mu\text{m}$  for biofilms with  $N = 10$  (Fig. 3A). For biofilms with a large number of cells, the exponent  $\alpha$  is less than unity, indicating that the biofilms compact with increasing cell number, which is reflected by reduced cell–cell distances (Fig. 3A), higher cell density (Fig. 3B), and smaller cell sizes (Fig. S3A and B). All cell–cell distances and cell sizes were measured in 3D. The underlying mechanisms that drive the dramatic changes in biofilm expansion rate as a function of  $N$  are unknown. We speculate that the significant changes in cell–cell spacing between small and large biofilms are due to strong temporal variation in the extracellular matrix composition and/or production levels per cell.

Global biofilm shape also undergoes a clear transition between small and large cell number. Following initiation, biofilm height rapidly increases (Fig. 2C), saturating at  $\sim 25 \mu\text{m}$  in our experimental conditions. Thus, the ratio of biofilm height to width and the ratio of their two cross-sectional widths (found by fitting an ellipse to the biofilm base) show that, initially, biofilms are highly asymmetric. However, for biofilms with  $N > 500$  cells, the biofilm cross-section becomes nearly circular (Fig. 2E), and the height-to-width ratio approaches a steady state of 0.8 (Fig. 2D). In contrast to *Bacillus subtilis* biofilms (27), cell death does not seem to play a major role in determining the global architecture for *V. cholerae* biofilms (Fig. S4).

The most striking features of the architectural transitions we observed in growing *V. cholerae* biofilms concerned the internal arrangement of the cells. To quantify such structural transitions, we defined the cellular local nematic order parameter  $S(r) = \langle 3/2(\mathbf{n}_i \cdot \mathbf{n}_j)^2 - 1/2 \rangle$ , where  $\mathbf{n}_i$  is the orientation vector of a focal cell (see Fig. 4A),  $\mathbf{n}_j$  represents the orientation vectors of all other cells in the local vicinity, and  $\langle \dots \rangle$  indicates an average, as described below. The local vicinity is defined as a 3D sphere of radius  $r$  around the center of the focal cell, as shown in Fig. 4A. The average local nematic order for each cell in its vicinity is subsequently averaged over all cells inside the biofilm to obtain a single order parameter  $S(r)$ , which quantifies the degree of cellular alignment in the entire biofilm. The value  $S = 1$  corresponds to perfect alignment of cells with their neighbors, whereas  $S = 0$  corresponds to a random cellular arrangement. Fig. 4B shows that the average local order  $S(r)$  depends on the size of the local vicinity, with  $r = \infty$  corresponding to the average global cellular order inside a biofilm (in practice, we set  $r = 50 \mu\text{m}$  to capture the global order). For a value  $r = 6 \mu\text{m}$ , which corresponds to a few cell lengths, the associated local order parameter  $S(6 \mu\text{m})$  directly reflects the characteristic internal architectural transitions that biofilms undergo during growth (Fig. 4C): In very small biofilms, cells are nearly perfectly aligned. This high local order is rapidly lost during growth, and for  $N > 10$  cells, the local order reaches  $S(6 \mu\text{m}) \sim 0.2$  and remains at this low, but not random, level for two orders of magnitude of population growth. The global cellular order drops to  $S(50 \mu\text{m}) \sim 0$  for  $N > 10^2$ , indicating globally heterogeneous cellular orientations. However, for very large biofilms ( $N > 2,000$ ), the local order  $S(6 \mu\text{m})$  increases sharply (see Fig. 4C), indicating that an architectural transition to high local cellular order occurs as biofilms grow to large population sizes. The characteristic internal cellular ordering for such large biofilms is shown in Fig. 4D: Globally the cells become oriented such that they appear to radiate from roughly the center of the biofilm’s basal plane. In the center of the biofilm, cells are aligned vertically relative to the substratum, transitioning gradually to fully parallel orientations relative to the base.

**Physical Aspects of Biofilm Internal Architecture.** *V. cholerae* cells can be coarsely approximated as growing and replicating rod-like objects with an aspect ratio of  $\sim 2.5$  (cell length/width; see Fig. S3B). Whereas in planktonic cultures *V. cholerae* has a characteristic curved cell shape, inside our biofilms, we observed predominantly straight, rod-shaped cell bodies (Fig. 1A and Fig. S5). In soft matter physics, it is well known that aligned nematic states of objects with aspect ratios  $> 1$  form in dense suspensions due to steric and entropic constraints (48, 49). Nematic ordering is generally counteracted



**Fig. 4.** Transitions in local and global ordering of cells during wild-type *V. cholerae* biofilm growth. (A) Schematic drawing illustrating the definition of the local vicinity. The local vicinity is defined as a sphere of radius  $r$  around the center of each cell (denoted in blue; cells in the local vicinity are shown in pink). (B) The local nematic order parameter  $S(r)$  is color-coded and displayed for different radii  $r$  of the local vicinity, as a function of cell number in the biofilm. The red horizontal line in panel A indicates one value of  $r$  for which a more detailed graph is shown in panel C. (C) The biofilm architecture undergoes a transition in cell ordering as biofilms grow from  $N = 1$ – $10$  cells (a loss of order) and a further transition for biofilms for  $N > 2,000$  cells (a gain in order). Solid lines are running averages of the red or black data points. (D) An XZ-slice through the center of a biofilm with  $N = 3,975$  cells shows the internal architecture. Cells are colored according to their values of  $n_z$ , the  $z$ -component of their orientation vector. (E) Biofilm local order parameter  $S$  from simulations for  $r = 6 \mu\text{m}$ . A “hedgehog”-type cellular arrangement corresponds to a von Mises–Fisher parameter  $\kappa \gg 1$ , whereas complete disorder occurs for  $\kappa < 1$ . Our experimental data from panel C show that, for the simulated values of  $N$ , the local order parameter  $S$  ranges from 0.14–0.35, indicating that, for *V. cholerae* biofilms,  $\kappa = 3$ – $10$ , which signifies an approximately hedgehog-type cellular arrangement.

by orientational fluctuations. In molecular liquid crystals, these fluctuations are due to thermal rotational diffusion, which becomes large enough to disrupt the ordered phase around  $\sim 50$ – $250^\circ\text{C}$ , depending on pressure, concentration, and chemical composition of the liquid crystal molecules and solvent fluid (50–52). In suspensions of motile bacteria, cellular alignment and collective motion (53–56) are suppressed by orientational fluctuations due to variations in flagellar motor activity and cell–cell collisions that reorient cells with respect to one another (57). The factors that promote or inhibit nematic ordering in growing, nonmotile bacterial colonies remain largely unknown, yet recent experiments (58, 59) have suggested that for *Escherichia coli* growing in artificially confined 2D colonies, nematic alignment occurs at high area fractions due to a self-generated, growth-induced expansion flow. Our data for *V. cholerae* biofilms now allow us to quantify the emergence of orientational order in three dimensions, presumably as it occurs in natural settings.

We find that during growth and replication in biofilms, the average cell–cell distance between nearest neighbors first increases for cell numbers  $N \lesssim 10$ , before this distance steadily decreases for all biofilms with  $N \gtrsim 10$ , implying that cell densities inside of biofilms steadily increase (Fig. 3A). The increase of cell density is confirmed by directly counting the number of cells within a local neighborhood of a reference cell (Fig. 3B), resulting in volume fractions of  $\phi = 0.46 \pm 0.05$  in the densest regions of biofilms with  $N \sim 4,000$  cells (Fig. S5). At such high-volume fractions, systems of passive spherocylinders have been predicted to approach a phase in which isotropic regions and highly ordered crystalline domains coexist (60). In biofilms, both cell–cell adhesion and the extracellular matrix that fills the space between cells strongly inhibit rotational and translational diffusion of the bacterial cells, thereby effectively lowering the Brownian temperature. This reasoning suggests that the significantly increased cell density in large biofilms should promote local alignment. Our data indeed confirm that local orientational ordering increases at large cell numbers (Fig. 4C), with cells arranging in hedgehog-like configurations (Fig. 4D).

To quantify the orientational ordering, we compared the experimentally observed cell orientations with simulations of a basic stochastic biofilm model. In these simulations, we constructed model biofilms of different sizes  $N$  by sampling cell positions  $\mathbf{x}$  uniformly in a 3D parabolic cap centered around the  $z$  axis. For a given number of cells  $N$ , the height and diameter of the paraboloid were matched to the corresponding experimentally determined values (Fig. 2). The individual cell orientations  $\mathbf{n}$  were sampled from a spherical normal distribution  $f(\mathbf{n}) = C(\kappa) \exp(\kappa \mathbf{R} \cdot \mathbf{n})$ , where  $C(\kappa)$  is the normalization constant,  $\mathbf{R} = \mathbf{x}/|\mathbf{x}|$  is the radial unit vector pointing from the coordinate origin to the cell position, and  $\kappa$  is a distribution parameter that determines the average deviation of the orientation vector  $\mathbf{n}$  from the radial direction  $\mathbf{R}$ . The exponential orientation distribution  $f(\mathbf{n})$ , also known as the von Mises–Fisher distribution, defines the least biased cell orientation model as  $f(\mathbf{n})$  maximizes the Shannon entropy on a sphere for a given mean orientation  $\mathbf{R}$ . The resulting stochastic biofilm model converges to a perfect radially symmetric hedgehog in the limit of a large von Mises–Fisher parameter  $\kappa \gg 1$ , whereas  $\kappa = 0$  yields a uniform distribution on a sphere, corresponding to completely disordered cell orientations. The numerically generated model biofilms were analyzed with the same methods and metrics as the experimental biofilms. This analysis shows that for biofilms with  $N = 200$ – $4,000$  cells, the experimentally obtained values of the local order parameter  $S$  are recovered for von Mises–Fisher parameters in the range  $\kappa = 3$ – $10$  (Fig. 4E). The fact that  $\kappa \sim 10$  is required to match the experimental data for  $N \sim 4,000$  confirms quantitatively that large biofilms exhibit approximately hedgehog-like orientational order.

The measured cellular aspect ratio of  $\sim 2.5$  (Fig. S3B) does not suffice to explain nematic ordering on purely thermodynamic grounds (48). We therefore hypothesize that cellular ordering inside biofilms is not a thermal process in which an isotropic phase of diffusing nematic objects is compressed until it transitions to orientational order. Instead, the biofilm matrix strongly suppresses rotational and translational diffusion so that the cellular ordering takes place at near-zero “effective temperature.” In addition to

the absence of Brownian diffusion, there are two additional nonequilibrium effects that likely play important roles: First, the increase in cell number occurs due to cell growth by elongation and division, which is a directed process biased to favor local alignment, and second, the growth of cells inside an adhesive matrix leads to an increased effective pressure on the cells that favors nematic order. The observed ordering in our *V. cholerae* biofilms is consistent with recent experiments examining 2D confined colonies of *E. coli*, which have similar aspect ratios to *V. cholerae* (58, 59). Together, these findings suggest that a combination of local densification and radial expansion flows that occur as a consequence of cell division can trigger the 3D ordering transitions we observe in naturally growing biofilms.

**Biofilm Architectures with Altered Matrix Production.** The secreted matrix is a defining feature of biofilms. To investigate its role in shaping global biofilm architecture, we analyzed mutants lacking individual biofilm matrix proteins (RbmA, Bap1, and RbmC), a mutant that is a matrix hyperproducer [carrying the *vpvC*<sup>W240R</sup> allele (61)], and mutants locked in either the low- or high-cell density quorum-sensing states (*luxO*<sup>D47E</sup> and  $\Delta$ *luxO*, respectively). The proteins RbmA, Bap1, and RbmC are known to localize heterogeneously within biofilms, and their individual roles in biofilm structure have recently been examined (42, 43, 62–67). RbmA is implicated in binding mother and daughter cells to each other and to polysaccharide components of the matrix. Bap1 is involved in attaching cells to the surface, and RbmC forms envelopes surrounding clusters of cells. RbmA also plays an ecological role in restricting access to existing biofilms by planktonic cells (42). Mutants in quorum-sensing components were investigated because quorum sensing regulates matrix production in *V. cholerae* (23, 68). Specifically, a strain that mimics the quorum-sensing low-cell-density state (*luxO*<sup>D47E</sup>) is a matrix overproducer, whereas a strain that mimics the high-cell-density state ( $\Delta$ *luxO*) displays decreased matrix production. In every strain we tested, the global morphological biofilm features were remarkably similar to those of the wild type, with respect to volume (Fig. S6), height (Fig. S7), and height/width ratio (Fig. S8), and depended on cell number *N*.

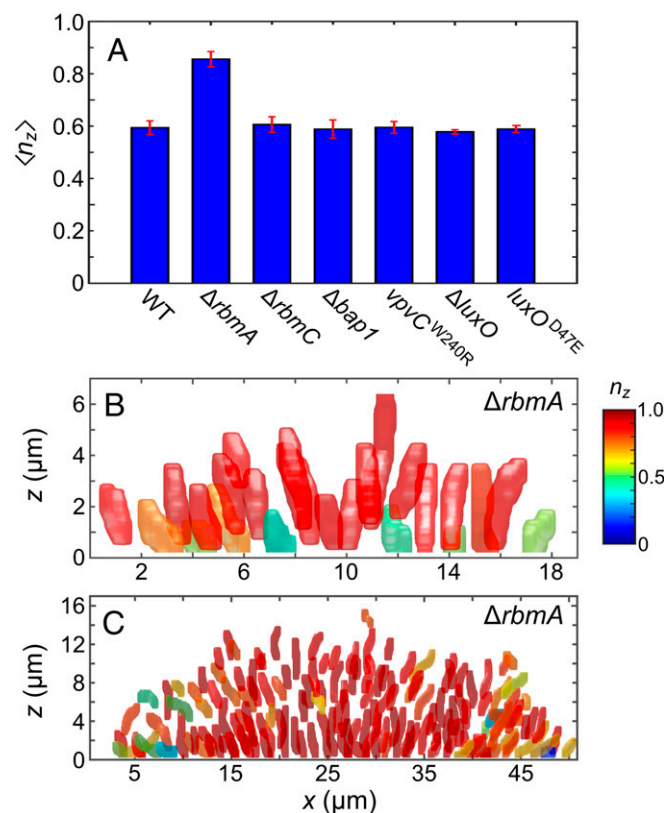
We also compared the internal structures of biofilms formed by the different mutants to that of the wild type. Fig. 5*A* shows the average of the *z*-component of the cellular orientations  $n_z$  in biofilms with cell numbers in the range of  $N = 1,500$ – $2,500$ , a size range for which the orientational order in wild-type biofilms is not particularly high (see Fig. 4*C*). The internal architectures of strains lacking the matrix protein RbmC or Bap1, the matrix hyper-producing strain, and the strains locked in low- or high-quorum-sensing states are similar to the wild-type architecture (Fig. 5*A*). The  $\Delta$ *rbmA* strain, however, exhibits a significantly different internal structure, shown in detail in Fig. 5*B* and *C*. In contrast to the wild type and the other strains analyzed here, the majority of cells in  $\Delta$ *rbmA* biofilms are vertically oriented and consequently display a high orientational order at much smaller biofilm sizes. Even the surface-attached cells, which are predominantly horizontal in the wild type (Fig. 4*D*), are primarily vertical relative to the substratum in the *rbmA* mutant, revealing a new function for RbmA in surface attachment. Because the initial substrate-attached cells are vertically oriented, the consecutive layers of cells in  $\Delta$ *rbmA* biofilm growth are also primarily vertical, as shown in Fig. 5*C*.

**Phases of *V. cholerae* Biofilm Growth.** Based on our results, we propose that biofilm growth of *V. cholerae* on submerged glass surfaces in the presence of flow can be separated into four distinct phases, illustrated in Fig. 6. In phase I ( $N = 1$ – $6$  cells), the cells grow in a 1D line and are highly ordered. All cells in this growth phase are attached to the glass substratum (Fig. 6*A* and *B*). This line of cells buckles into an in-plane 2D arrangement for  $N \geq 6$ , presumably when cell–surface adhesion becomes stronger than bacterial pole–pole adhesion. In phase II ( $N = 20$ – $100$  cells), cells are mostly

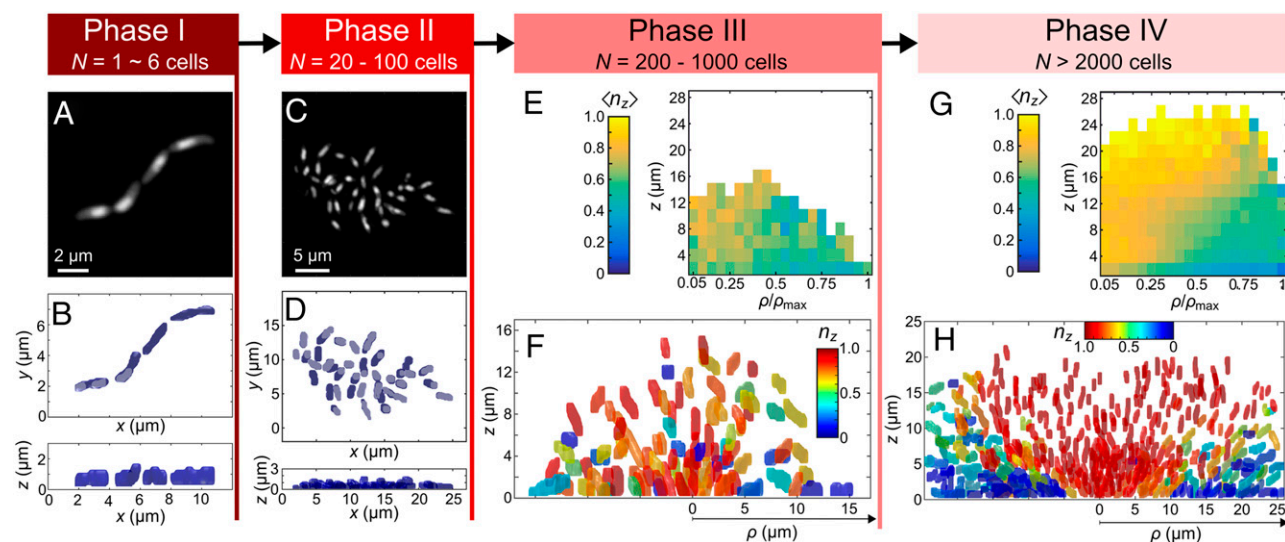
disordered locally and are growing in a 2D colony, with all cells still attached to the glass substratum (Fig. 6*C* and *D*). This 2D colony buckles into a 3D colony, presumably when the cell–surface adhesion force becomes smaller than the force that cells exert when they push against each other due to cell growth (69). In phase III ( $N = 200$ – $1,000$  cells), the 3D colony is still locally disordered (Fig. 6*E* and *F*) and at relatively low cell density (Fig. 3*B*). As the cell density inside the biofilm increases (Fig. 3*A* and *B*), the biofilm undergoes an ordering transition to phase IV ( $N \geq 2,000$  cells), which is characterized by high local and long-range cellular alignment, with the architecture shown in Fig. 6*G* and *H*. In phase IV, cells at large radial coordinate  $\rho$  and low *z* coordinates are located primarily horizontally, whereas cells in the center are primarily vertical (Fig. 6*H*). High cellular order and a radial cellular orientation inside biofilms likely provide a growth advantage compared with random cellular orientations, as radially oriented cells produce daughter cells at the outer edges of the 3D biofilm where the nutrient concentration is highest. Random cellular orientations, in contrast, will lead to nematic frustration of growth and fewer offspring directed to the high-nutrient regions.

## Conclusion

By imaging and analyzing the position, size, and orientation of all individual cells inside *V. cholerae* biofilms, we were able to measure



**Fig. 5.** The internal biofilm architecture of the *V. cholerae*  $\Delta$ *rbmA* mutant strongly deviates from that of wild-type *V. cholerae*. (A) The average *z*-component of each cell orientation,  $n_z$ , is shown for *V. cholerae* wild type; the matrix mutants  $\Delta$ *rbmA*,  $\Delta$ *rbmC*, and  $\Delta$ *bap1*; the matrix hyperproducing mutant *vpvC*<sup>W240R</sup>; and the quorum-sensing mutants  $\Delta$ *luxO* and *luxO*<sup>D47E</sup>. Error bars indicate the SD from at least three different biofilms with  $N = 1,500$ – $2,500$  cells for each strain. Detailed global and morphological comparisons between the biofilms of these mutants are provided in Figs. S6–S8. (B and C) The segmented cells in an XZ-slice through a biofilm with  $N = 114$  cells (B) and  $N = 1,658$  cells (C) are shown, in which individual cells are colored according to their orientation along the *z*-direction,  $n_z$ , using the same color scale as in Fig. 4*D* and Fig. 6.



**Fig. 6.** The different phases of *V. cholerae* wild-type biofilm growth. (A and B) Biofilms in phase I consist of highly ordered  $N = 1\sim 6$  cells growing along a line, and all cells are bound to the glass substratum. Panel A shows a representative image, and panel B shows the top and side views of the segmented cells in the community. (C and D) Biofilms in phase II consist of  $N = 20\sim 100$  cells, which form a 2D community with low local order, in which all cells remain in contact with the glass substratum. (E and F) Biofilms in phase III consist of  $N = 200\sim 1,000$  cells that form a disordered 3D community. Panel E shows the average magnitude of the  $z$ -component of the cellular orientation vector  $\langle n_z \rangle$ . For the heat map, 26 biofilms with  $N = 500\sim 600$  cells were averaged and the results plotted using cylindrical coordinates with radial coordinate  $\rho$ , normalized by the maximum cylindrical radius of the biofilm  $\rho_{\max}$ . Panel F shows all detected cells in an XZ-slice through a biofilm with  $N = 510$  cells; individual cells are colored according to their value of  $n_z$ , as indicated in the color bar. (G and H) Biofilms in phase IV are highly ordered 3D communities with  $N > 2,000$  cells. Panel G shows  $\langle n_z \rangle$  averaged for 11 biofilms with  $N = 3,800\sim 4,600$  cells, using cylindrical coordinates. Panel H shows the cells in an XZ-slice through a biofilm with  $N = 3,975$  cells; individual cells are colored according to their orientation along the  $z$ -direction,  $n_z$ . Biofilms with cell numbers in between these four growth phases are undergoing transitions from 1D to 2D growth, from 2D to 3D disordered growth, or from 3D disordered to ordered growth, respectively.

biofilm growth laws and we discovered architectural changes that occur during growth. The parameters that describe the internal and external biofilm architectures collapsed to a single curve, when measured as a function of cell number in the biofilm. Little variation was found between the biofilm structural parameters, when measured as a function of cell number for the wild type and mutants with varying degrees of matrix production levels, mutants defective in quorum-sensing regulation, and mutants deficient in production of specific matrix proteins. The experimentally observed global and internal biofilm architectures during and between the growth phases can, in part, be accounted for using mechanical arguments. However, mechanical effects alone are not sufficient to determine the entire biofilm architecture, as a mutant that does not produce the biofilm matrix protein RbmA exhibits a dramatically different biofilm architecture than the wild type and other mutant strains. Additional analyses to quantify the forces involved in *V. cholerae* surface adhesion (16, 70), cell-cell adhesion, and cell-matrix interaction strength are needed to develop accurate simulations of biofilm growth (71-74). Such simulations will be required to distinguish the effects of forces resulting from cell growth and division from those due to matrix production and adhesion in developing biofilms.

## Materials and Methods

**Strains and Media.** All *V. cholerae* strains used in this study are derivatives of the wild-type *V. cholerae* O1 biovar El Tor strain N16961. *V. cholerae* deletion mutations were engineered using pKAS32 harbored in *E. coli* S17-1 $\lambda$ pir. All strains were grown in LB medium supplemented with appropriate antibiotics at 37 °C for strain construction. Biofilm experiments with *V. cholerae* were performed in M9 minimal medium, supplemented with 2 mM MgSO<sub>4</sub> and 100  $\mu$ M CaCl<sub>2</sub>, MEM vitamins, 0.5% glucose, and 15 mM triethanolamine (pH 7.1). A detailed strain list is provided in Table S1.

**Flow Chamber Biofilm Experiments.** *V. cholerae* biofilms were grown in microfluidic flow chambers (dimensions were 500  $\mu$ m wide, 100  $\mu$ m high, and 7 mm long) made from polydimethylsiloxane bonded to glass coverslips by an oxygen plasma, with four channels on a single coverslip. Microfluidic channels were designed and constructed according to standard soft lithography tech-

niques. Channels were inoculated with *V. cholerae* strains that had been grown overnight at 37 °C in liquid LB medium under shaking conditions. Following inoculation of the channels, the cells were given 1 h to attach to the surface of the channel before a flow of 2  $\mu$ L/min M9 medium (formulation as described above) was initiated to wash away nonadherent cells and to remove LB growth medium from the channels. The flow rate was then set to 0.1  $\mu$ L/min, corresponding to a mean flow speed of 33  $\mu$ m/s in the channel until the end of the experiment. Flow rates were controlled using a syringe pump. Immediately before imaging, the inflowing medium was exchanged from simple M9 to M9 containing 10  $\mu$ M SYTO 9 nucleic acid stain (Life Technologies).

**Microscopy and Image Processing.** Images were acquired with a Yokogawa CSU-X1 confocal spinning disk unit mounted on a Nikon Ti-E inverted microscope, using a 100 $\times$  oil objective with N.A. 1.4, a 488-nm laser (Coherent Sapphire), and an Andor iXon EMCCD camera, cooled to -90 °C. To obtain sufficient magnification for automated cell segmentation, a 1.5 $\times$  lens was placed between the CSU-X1 and the Nikon Ti-E side port. The distances between the spinning disk, the tube lens, and the camera sensor were fine-tuned to provide sufficient  $z$ -resolution for the single-cell segmentation. Point-scanning confocal microscopy with an adjustable pinhole size could also be used to obtain sufficient optical resolution. The magnification was 0.1  $\mu$ m per pixel in the XY plane and 0.2  $\mu$ m step size in the  $z$ -direction. To avoid residual movement of immersion oil due to changing the objective position during the  $z$ -scan, we introduced a 1-s lag time between changing the focal height and exposure. The exposure time was 100 ms per image. To segment the biofilms into individual cells from the 3D images, custom code was written in Matlab. Briefly, images were treated separately for each  $z$ -plane, band-pass filtered, thresholded, and after that, binary objects were connected between the different  $z$ -planes to obtain 3D volumes for each cell inside the biofilm. By manually verifying the segmentation of 10 biofilms of  $\sim 200$  and  $\sim 500$  cells, we determined that the algorithm correctly segments 95% of the cells, whereas the remaining 5% are clusters of  $>2$  cells, of which 63% are clusters of two cells.

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