

Emergence of three-dimensional order and structure in growing biofilms

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Supplementary Information

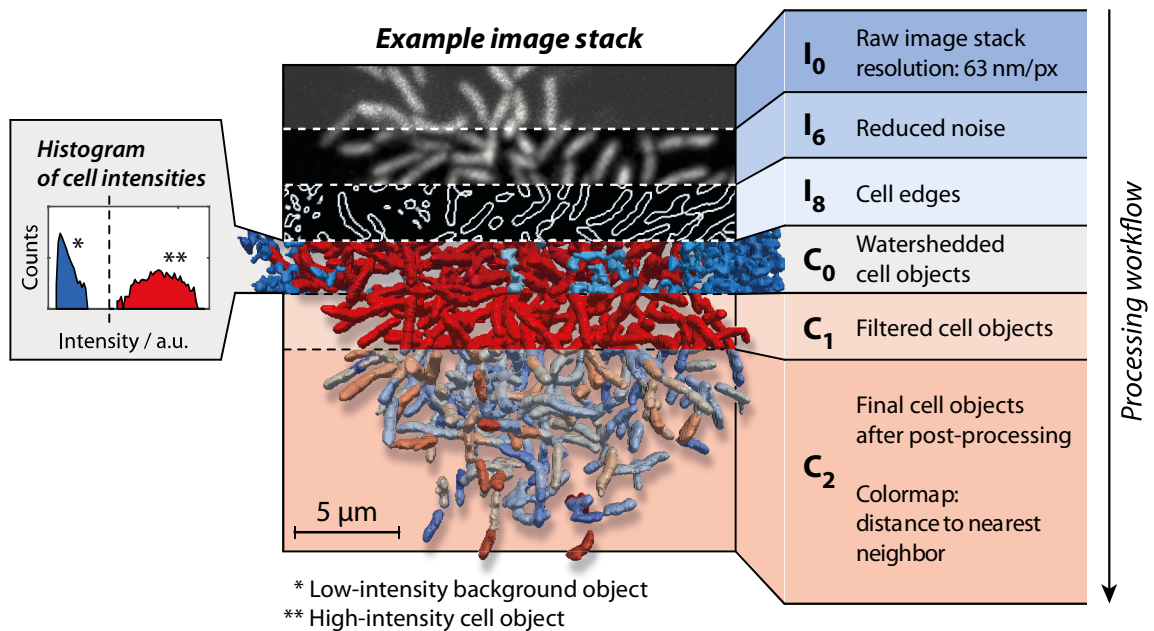
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1. Data Analysis

1.1. Image processing

Image processing was carried out using MatLab (MathWorks). In the following sections, raw data 3D image stacks are referred to as $I_0(t)$ and are matrices of dimension x, y , and z , which were acquired at a time point t (see Supplementary Fig. 1). Any intermediate processing result is labelled with consecutive indices $I_i(t)$. Prior to segmentation, subsequent image stacks were aligned along x, y , and z by image registration ($= I_1$). Floating cells which were not associated with a biofilm were removed by median filtering along z ($= I_2$). Image stacks that have been registered and for which floating cells have been removed, were then up-sampled along the z -dimension to obtain equal voxel side lengths ($= I_3$). Subsequently, noise was removed by filtering as follows. The eigenvalues of each xz - and each yz -plane were obtained by singular value decompositions and the lowest thirds of the calculated values were set to zero to reduce high frequency noise ($= I_4$). In addition, a 3D-convolution with an averaging kernel was performed ($= I_5$). Afterwards, the out-of-focus fluorescence was suppressed by slice-wise Top-hat filtering of the image stacks ($= I_6$, see Supplementary Fig. 1).



Supplementary Figure 1 – Image processing pipeline. Biofilms on spinning disc confocal fluorescence image stacks are processed as illustrated, to obtain 3D models of biofilms based on individual cells.

1.2. Cell detection

Image stacks, $I_6(t)$, were further processed to identify individual cells. First, the gamma value was adjusted to $\gamma = 3$ to enhance the range between signal and background ($= I_7$). Second, a 3D-edge detection was performed by convolving the data with a 3D Laplacian-of-Gaussian (LoG) kernel of 0.82 μm cube length (corresponding to 13 px) ($= I_8$). In the obtained matrices $I_8(t)$, zero values represent those locations where the cell's fluorescence intensity values drop approximately by 50% in comparison to its local intensity maxima. These zero crossings were determined to obtain closed surfaces representing the cellular outlines ($= I_9$). To distinguish between the “inside” ($=$ cell) and “outside” ($=$ background) of such a closed surface, a 3D-Gaussian filter kernel was applied to I_6 with the same spatial dimension as the LoG kernel used for the edge detection. The local maxima of the resulting image stack were identified ($= I_{10}$) and used as seeds to “flood-fill” the cell interior in I_9 , yielding the binary image I_{11} containing all cell clusters. Cell de-clumping was performed by 3D-watershedding. The intensity landscape used

for watershedding was obtained by enhancing the local maxima (I_{10}) inside I_6 by a factor of 10, followed by 3D-Gaussian smoothing and masking with I_{11} ($= I_{12}$). After watershedding, I_{12} was 3D-median filtered and binary cell objects ($= C_0$) were obtained and processed further, as described in the next sections.

1.3. Cell object processing

For the cell objects C_0 the average underlying intensities in stack I_7 were determined and assigned to each cell. As the edge-detection-based segmentation will also yield objects, which are background, a filtering step is required. Because the average intensities of such background objects (calculated based on I_7) are typically orders of magnitude lower than those of actual cells, the cell objects C_0 were filtered based on these intensity measurements in stack I_7 ($= C_1$). After neglecting background objects, segmentation results were corrected based on C_1 . Over-segmentation was corrected by merging very small cells below a certain volume threshold with neighbouring cells of largest contact area. Clumped cells in multicellular structures (characterized by large volume and low convexity) were dissected by fitting a Gaussian mixture distribution model (GMM) with N components (or number of underlying cells) to the corresponding voxel coordinate cloud. N was determined by counting the number of skeleton branches above a typical length threshold. The additional cells obtained after GMM clustering were checked for size. Very small objects were merged again with the neighbouring cell of largest contact area; the remaining de-clumped cells were used to replace the original multicellular structure. In addition, cells touching the image edges were removed. The cellular orientations and cell dimensions (length, height and width) were obtained by analysing the principal components (found by principal component analysis, PCA) of each cell volume (in other words: by fitting an ellipsoid into each cell). For additionally calculated cell features see the section on “Biofilm features”. Final cell objects are referred to as C_2 (see Supplementary Fig. 1).

1.4. Cell tracking

To determine the single-cell growth rate, the resulting cells were tracked over successive time points. For time point t_i , the parent of each cell was determined by comparison with time point t_{i-1} , and finding the corresponding cell for which volume overlap was maximized and deviations in cell orientation were minimized. All cells that were not related to the biofilm founder cells were excluded from further analysis (at low flow rates a layer of non-related cells is usually formed after longer imaging, *cf.* white cells in Fig. 1b,d and Supplementary Fig. 6), resulting in the analysis of purely clonal biofilms. The accuracy of the segmentation was investigated by segmenting synthetic microscopy image stacks as described in the section on “Validation of biofilm segmentation”.

1.5. Biofilm features

Name	Parameter	Unit	Description
Axial cell position	$height$	μm	Z-coordinate of each cell centroid.
Cell alignment with direction of flow	$angle(\mathbf{n}_{cell}, \mathbf{flow})$	rad	Angle between each cell's major axis (see <i>cell size and orientation</i>) and the direction of the flow.
Radial alignment	$angle(\mathbf{n}_{cell}, \mathbf{r})$	rad	Angle between each cell's major axis (see <i>cell orientation</i>) and the radial vector \mathbf{r} pointing from the centre of mass pinned down to the bottom of the biofilm ($z = 0 \mu\text{m}$) to the corresponding cell.
Vertical alignment	$angle(\mathbf{n}_{cell}, \mathbf{z})$	rad	Angle between each cell's major axis (see <i>cell orientation</i>) and \mathbf{z} .
Cell volume	v	μm^3	Cell volume as obtained by the segmentation.
Cell size	l, h, w	μm	Cell dimensions (length, height and width), as determined by principal component analysis (PCA) of the segmented cell (equivalent to fitting an ellipsoid into the cell and deriving the three eigenvalues which correspond to the lengths of the main axes).
Cell orientation	$\mathbf{n}_{cell} = (e_1, e_2, e_3)$		Vector of the cell's major axis determined by PCA (eigenvector with largest eigenvalue).
Distance to centre of mass	d_{centre}	μm	Assuming radially symmetric biofilm growth in spherical coordinates (which is a good estimate for the low flow regime) each cell can be described spatially by its distance to the centre of mass projected onto the $z = 0 \mu\text{m}$ plane.
Distance to nearest neighbour	$d_{nearest\ neighbour}$	μm	Euclidian centroid-to-centroid distance to the nearest cell.
Distance to surface	$d_{surface}$	μm	Shortest distance of a particular cell to the outer surface of the biofilm.
Local cell density	ρ_{local}	μm^{-3}	Number of cells in a vicinity defined as a sphere of radius $3 \mu\text{m}$ around a cell, normalized by the volume of the vicinity.
Local order (nematic order)	S		Nematic order parameter $S = \langle 3/2(\mathbf{n}_i \cdot \mathbf{n}_j)^2 - 1/2 \rangle$ in a vicinity defined as a sphere of radius $3 \mu\text{m}$ around a cell. \mathbf{n}_i and \mathbf{n}_j refer to the orientation vectors of cells i and j , respectively ¹ .
Single cell growth rate	$growth\ rate$	$\mu\text{m}^3\text{s}^{-1}$	Single cell growth rate dv_i/dt of cell i (fixed by cell tracking) with volume v_i as determined by comparing the cell volume in frame N and $N+1$ at times t_N and t_{N+1} : $\Delta v_i/\Delta t_N = (v_{i,N+1} - v_{i,N})/(t_{N+1} - t_N)$. Potential bias caused by over-/under-segmentation and/or cell dispersion/off-shearing was corrected by setting v_{N+1} to zero for cells with no children and setting v_N to zero for cells with no parents. Using this approach, the global biofilm volume $V(t)$, as determined by segmentation, matches $V(t) = \int \sum \Delta v_i dt$.

Supplementary Table 1 – List of calculated single-cell features.

Name	Parameter	Unit	Description
Aspect ratio	Z/XY		Biofilm height divided by average biofilm base radius.
Biofilm base circularity	B_{circ}		Deviation of biofilm base cross section from a circle $B_{circ} = 1-Z/XY $.
Global cell density	$\rho_{biofilm}$	μm^{-3}	Number of cells divided by the volume of the biofilm's convex hull.
Biofilm volume	V	μm^3	Sum of the volume of all individual cells

Supplementary Table 2 – List of calculated global biofilm features.

1.6. Optical flow

Pre-processed image stacks (I_5 , for details see section on “Image processing”) were down-sampled to 1/4 of the initial resolution. The optical flow (= biovolume velocity) vector field (u_x, u_y, u_z) of isolated growing biofilms was determined for each containing voxel using the Horn-Schunck method² implemented in MatLab by Mustafa *et al.*³. Afterwards, the optical flow was set to zero in the space that did not contain cells. To investigate the effect of the external flow rate on net biomass movement, in terms of moving biovolume, the total biovolume flow through defined planes was calculated (see Supplementary Fig. 2). For external flow of growth medium \mathbf{v}_{liquid} pointing in the positive y -direction, the total biovolume flow I_{BV}^{\parallel} through the xz -plane for different y -coordinates, and the total biovolume flow I_{BV}^{\perp} through the yz -plane for different x -coordinates, respectively, was calculated by summation over the biovolume fluxes through the corresponding planes (Eq. (1) and Eq. (2), Supplementary Fig. 2).

The biovolume flow I_{BV} through xz -planes (with plane normal vectors $\hat{\mathbf{n}}_y$) parallel to \mathbf{v}_{liquid} for different values of y_i is:

$$I_{BV}^{\parallel}(y_i, t) = \sum_{x,z,y=y_i} \hat{\mathbf{n}}_y \cdot (u_x(t), u_y(t), u_z(t)) \quad (1)$$

The biovolume flow I_{BV} through yz -planes (with plane normal vectors $\hat{\mathbf{n}}_x$) perpendicular to \mathbf{v}_{liquid} for different values of x_i is:

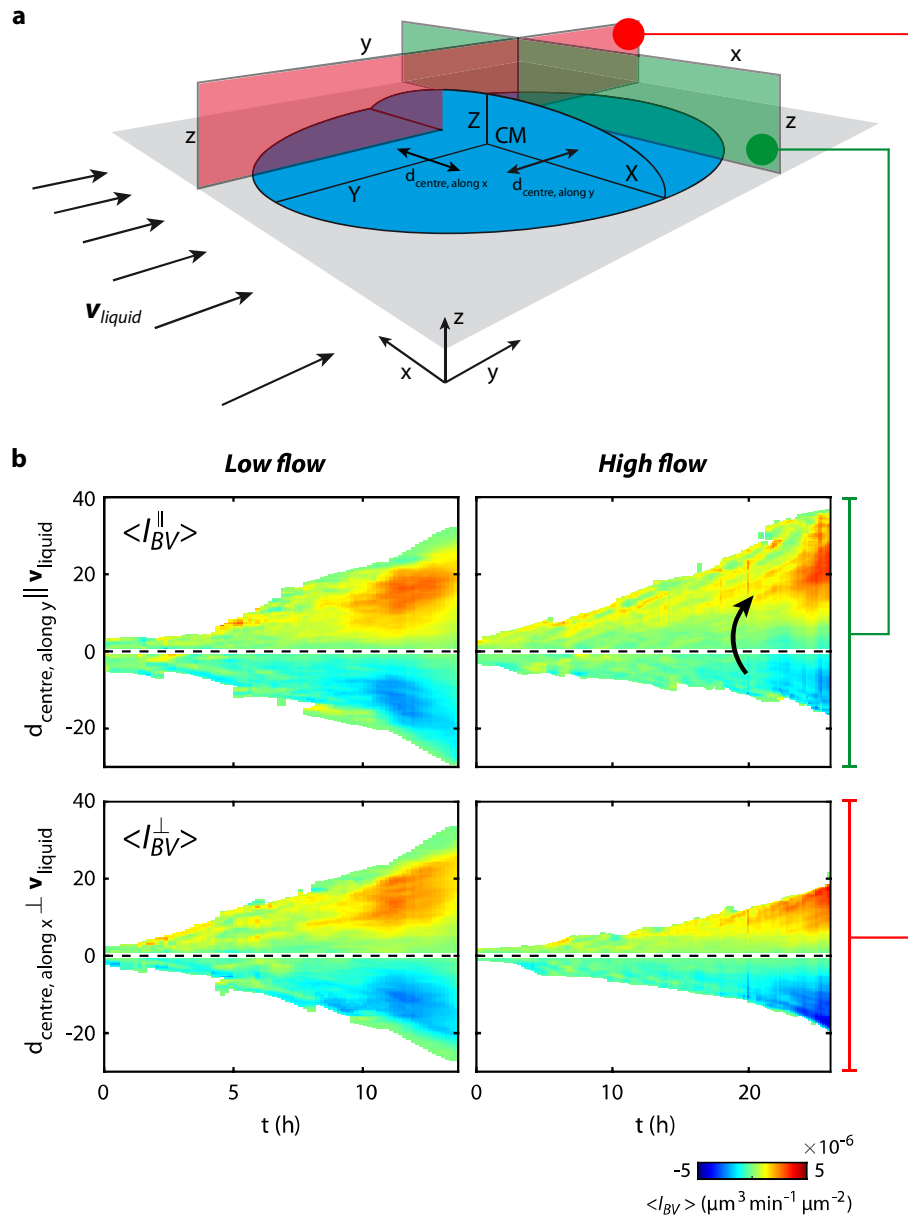
$$I_{BV}^{\perp}(x_i, t) = \sum_{x=x_i,z,y} \hat{\mathbf{n}}_x \cdot (u_x(t), u_y(t), u_z(t)) \quad (2)$$

To calculate the *biomass shift* (displayed in Fig. 3f of the main paper), the biovolume flow I_{BV} was summed up either along y or x -direction, was normalized by the sum of the absolute values, and the result was averaged over all time points:

$$biomass_shift^{\parallel} = \left\langle \frac{\sum_{y_i} I_{BV}^{\parallel}}{\sum_{y_i} |I_{BV}^{\parallel}|} \right\rangle \quad (3)$$

$$biomass_shift^{\perp} = \left\langle \frac{\sum_{x_i} I_{BV}^{\perp}}{\sum_{x_i} |I_{BV}^{\perp}|} \right\rangle \quad (4)$$

In Fig. 3f of the main paper, values are given as mean \pm standard deviation for $n = 3$ individual biofilms. For better visualization in Supplementary Fig. 2b, the *averaged* biovolume velocity through the xz - and yz -planes is shown, whereas for Fig. 3f the *summed* velocities were used.



Supplementary Figure 2 – Optical flow measurements of biomass movement inside a biofilm. **a**, The “optical flow” (= biovolume flow) of a growing biofilm (blue) through the green plane (perpendicular to the direction of flow \mathbf{v}_{liquid} , indicated with black arrows) and through the red plane (parallel to the direction of \mathbf{v}_{liquid}) was determined. **b**, In panel **a**, the positions of the red and green plane with respect to the centre of mass (CM) projected down to the bottom of the biofilm were varied (red plane: $d_{centre, \text{ along } x}$; green plane: $d_{centre, \text{ along } y}$). For each time point and value of d_{centre} the average biovolume flow through the corresponding plane is shown as coloured pixel in the heatmap. In the heatmap, red values indicate an average net flux in the positive x or y direction, whereas blue values indicate an average net flux in negative directions. The two heatmaps in the upper row show the biovolume flow with respect to the green plane perpendicular to the direction of the external flow \mathbf{v}_{liquid} with distance $d_{centre, \text{ along } y}$ to the centre. At high flow speed more biomass is moving in the direction of \mathbf{v}_{liquid} than at low flow speed, as indicated by the black arrow, and the asymmetry along the $d_{centre} = 0$ axis. The two heatmaps in the bottom row show the flux through the red plane parallel to \mathbf{v}_{liquid} (with distance $d_{centre, \text{ along } x}$ to the centre). The two heatmaps in the bottom row show that irrespective of the flow speed, biofilms grow symmetrically in the direction perpendicular to the flow, as indicated by the symmetric heatmaps around the $d_{centre} = 0$ axis.

1.10. 3D visualization

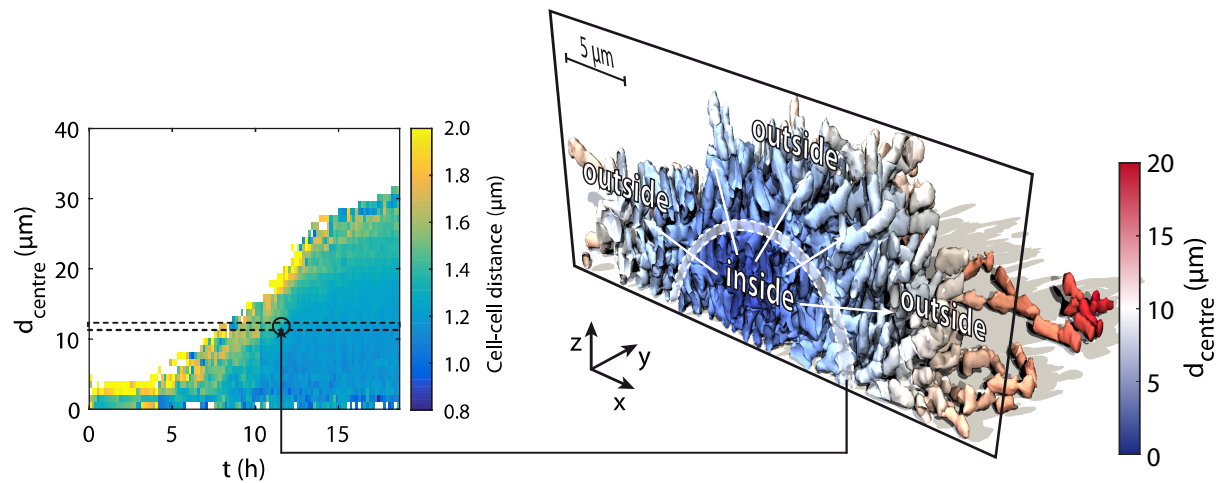
Segmented biofilms were exported using the mVTK library⁴ and rendered in ParaView 5.1.2 (Kitware) using OSPRay rendering with shadows.

Visualization of RbmA

FLAG-labelled RbmA was detected in an additional fluorescence channel. The fluorescence distribution was rendered as a semi-transparent cloud and overlaid with the reconstructed biofilm in ParaView (see Fig. 1c).

1.11. Space-time heatmaps of biofilm development

In Fig. 1, single cell parameters for the WT* and $\Delta rbmA$ mutant biofilms are visualized spatially and temporally resolved in heatmaps (standard deviations of the presented mean values are shown in Supplementary Fig. 5 and Supplementary Fig. 7, respectively). For these space-time heatmaps, the cells were binned with respect to a spatial descriptor (vertical height z , distance to surface $d_{surface}$, or distance to centre of mass d_{centre}) and the corresponding cell property values were averaged for this particular spatial descriptor across the biofilm at a particular time t , as shown schematically in Supplementary Fig. 3.



Supplementary Figure 3 – Illustration showing how a single tile in the heatmap (left panel) was generated. In this heatmap, cell-cell distance (color-coded) is measured as a function of the distance to the centre of mass (CM) of the biofilm pinned down to the bottom (d_{centre}), shown on the y-axis of the heatmap. For each time point, all cells inside the biofilm are grouped according to their distance to the CM, as indicated by the white dashed lines in the 3D rendering, approximately corresponding to the black dashed lines in the heatmap. For all cells having a similar value of d_{centre} the cell-cell distances are averaged (right panel) and visualized as a coloured tile in the left panel.

1.12. Liquid crystal (Q-tensor) representation of biofilms

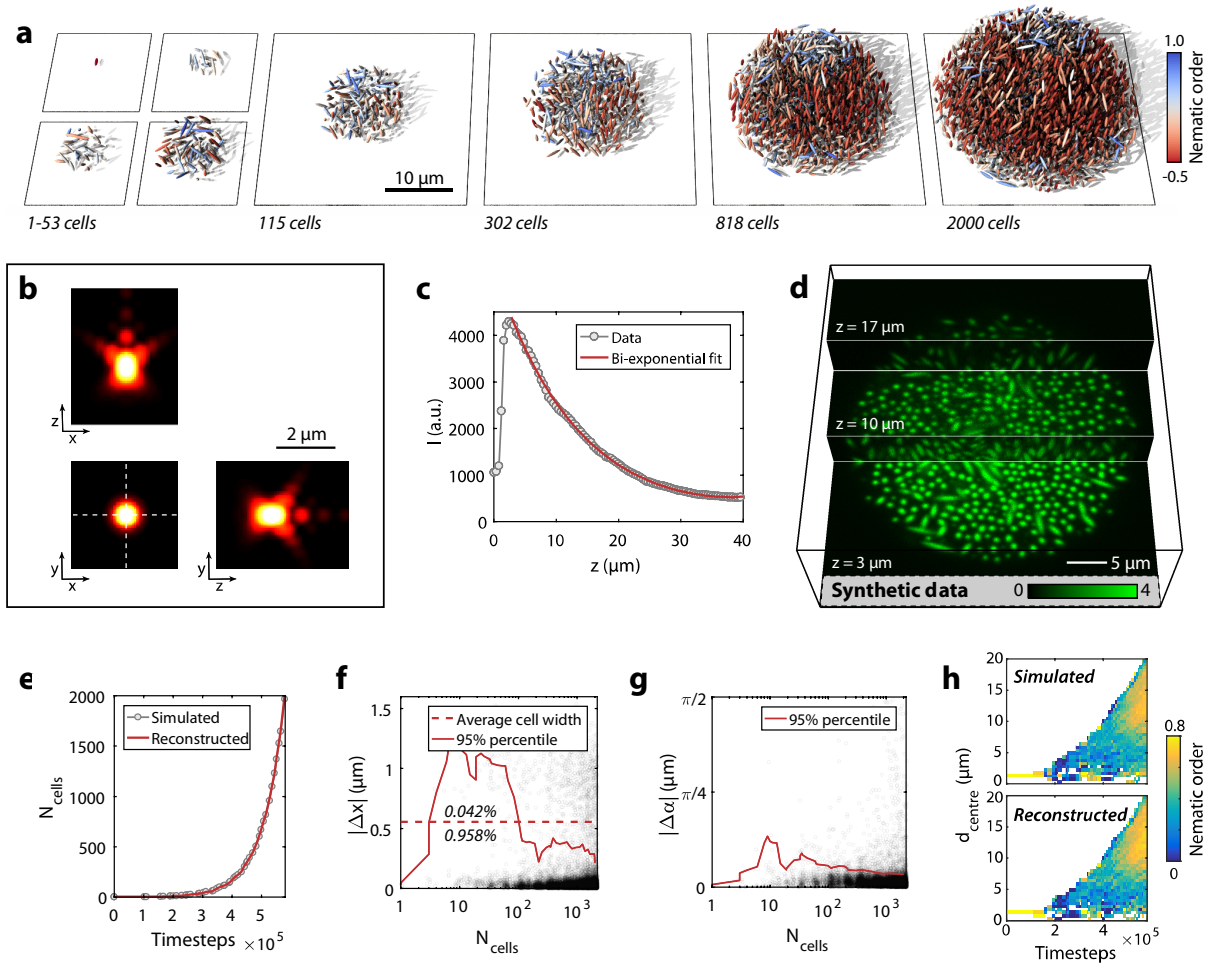
To visualize the average cellular local order and alignment, an evenly spaced 3D-grid with 5 μm spacing was overlaid on the biofilm. For each grid node the uniaxial tensorial order parameter $Q_i = 1/2(3\mathbf{n}_i \otimes \mathbf{n}_i - \mathbb{I})$ was calculated⁵ for each cell i in a 2.5 μm vicinity, where \mathbf{n}_i denotes the cell orientation vector. Based on the individual cell tensors the average Q-tensor and its eigenvalues and eigenvectors were determined for each node. In Fig. 3g the resulting values are represented as ellipsoids, where the largest eigenvalue and corresponding eigenvector are represented as the length and major axis orientation of each ellipsoid, respectively. The colour of each ellipsoid corresponds to the angle between the major axis vector \mathbf{n} of each ellipsoid and the direction of the flow.

1.13. Validation of biofilm segmentation

To benchmark the single-cell segmentation and tracking algorithms, the individual cell-based simulations (See section 3 of this document) were used to synthesize biofilm image stacks, which were convolved with the point spread function of the microscope we used and distorted by the addition of noise, to obtain data as similar as possible to the experimental data. In detail, a simulation up to $N = 2,000$ cells was performed using parameters which correspond to the $\Delta rbmA$ mutant (for biofilm renderings see Supplementary Fig. 4a, for a description of the simulation framework see the section on “Individual Cell-Based Simulations”).

The simulated biofilm data were converted into image stacks with a spatial resolution of 62.3 nm/pixel and a temporal resolution of approximately 10 min. The image stacks were blurred using a theoretical point spread function (PSF) corresponding to our microscopy setup (Supplementary Fig. 4b) (Huygens software, Scientific Volume Imaging) and down-sampled along z to match the axial resolution of the biofilm data (0.4 $\mu\text{m}/\text{pixel}$). Finally, the intensity levels were adjusted to the experimental data. To adjust the intensity levels, the average background intensity and the average cell intensity, depending on the axial imaging position z , were extracted from the experimental data. The average background intensity was independent of z ($I_{background} \approx 500$). To record the average cell intensity per plane, the corresponding intensity values were sorted and the highest 2000 values were averaged. The resulting curve $I_{cell}(z)$ was fitted with a bi-exponential function (Supplementary Fig. 4c), which was used to normalize the synthetic image stacks. Poisson-distributed noise was added to mimic noise due to photon detection inside the camera. The noise levels were estimated and generated using the algorithm proposed by Liu *et al.*⁶. The generated images (Supplementary Fig. 4d) were processed as described in the section on “Image processing”.

In Supplementary Fig. 4e-h the simulated dataset is compared with the reconstructed one in terms of cell number (Supplementary Fig. 4e), cell displacements (Supplementary Fig. 4f), differences in cellular orientation (Supplementary Fig. 4g), and internal order (Supplementary Fig. 4h). Based on this validation procedure, we determined that our image segmentation algorithms yielded accurate cell segmentation for $> 95\%$ of all cells.

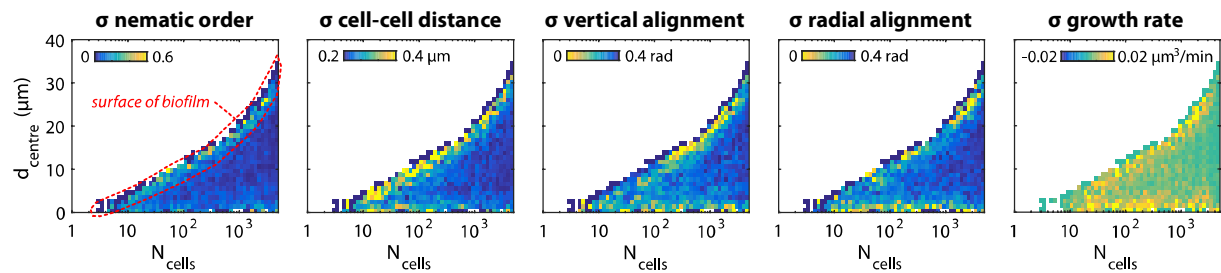


Supplementary Figure 4 – Validation of the single-cell segmentation algorithms using synthetic images. **a**, Time series of a rendered simulated biofilm, which was used to synthesize microscopic image stacks. **b**, Theoretical point spread function (PSF) of our experimental setup, used for convolution with the simulation data to obtain a more realistic dataset (see **d**). **c**, Experimentally determined typical average cell intensity curve $I_{\text{cell}}(z)$ versus the axial position z of the imaged plane. This intensity profile was used to introduce an intensity gradient along z in the synthetic image stacks (see **d**). **d**, Artificial image stacks obtained after convolution with the characteristic PSF (from **b**), intensity fading along z (from **c**), and distortion by typical Poisson-distributed detector noise. **e**, Simulated (grey) vs. reconstructed (red) cell numbers N_{cell} . **f**, Cell displacements Δx after reconstruction. The simulated and reconstructed data was overlaid. For each cell in the simulated dataset, the distance Δx to the closest cell in the corresponding reconstructed data was calculated. 95.8% of all reconstructed cells (for all time points) were closer than one cell width (dashed line) to the theoretical coordinates. Per time point, 95% of all cells deviated less than indicated by the solid red curve. **g**, Differences in cellular orientation (expressed as angle between the major axis of a cell in the simulated dataset and the major axis of the nearest cell in the reconstructed data $\Delta\alpha$). Per time point, 95% of all cells showed a difference in orientation smaller than indicated by the solid red curve. **h**, Spatially resolved distribution of the internal biofilm order of the simulated (top) and reconstructed (bottom) data.

2. Detailed Characterization of Biofilm Architecture

2.1. WT* biofilm structure

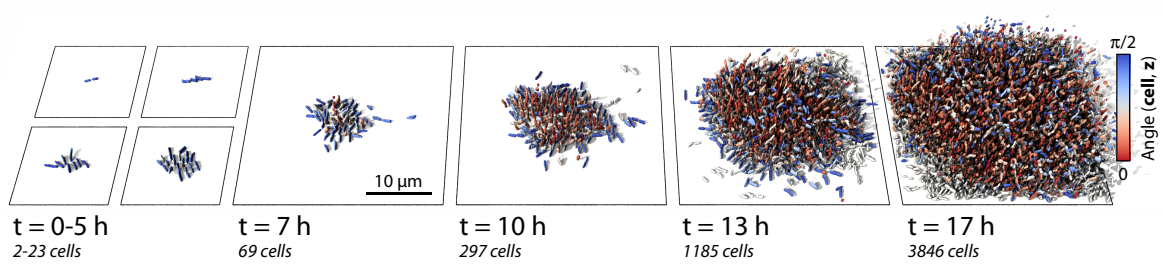
Following the growth dynamics of the WT* in an unperturbed low-flow environment, we found strong structural differences between the outer biofilm layer and the central part, and several distinct structural phases of the biofilm during growth (Fig. 1e). For each time point we characterized the biofilm spatially with respect to order, density, alignment, and growth to obtain a quantitative description of the WT* phenotype and standard deviations of the properties (Supplementary Fig. 5). Small biofilms of less than 50 cells were generally characterized by a relatively high order, and a low vertical alignment, indicating 2D growth. Then, a transition to three-dimensional growth occurred as indicated by an increasing vertical alignment. Generally, order and cell-cell spacing increased with distance from the biofilm centre for all biofilm sizes. The order at the centre of the biofilm decreased as the cell number increased up to biofilms with more than 1,000 cells, when the order in the centre increased again in agreement with previous structural analyses¹. This increase in order coincided with a local decrease in cell-cell spacing and a strong local increase in vertical cellular alignment. The radial alignment was higher at the outer parts of small- to medium-sized biofilms and decreased as the biofilms expanded. Surprisingly, the local growth rate, as measured by cell tracking, was constant in space and time, indicating that growth inside the observed biofilms was not limited by diffusion constraints of nutrients in our conditions.



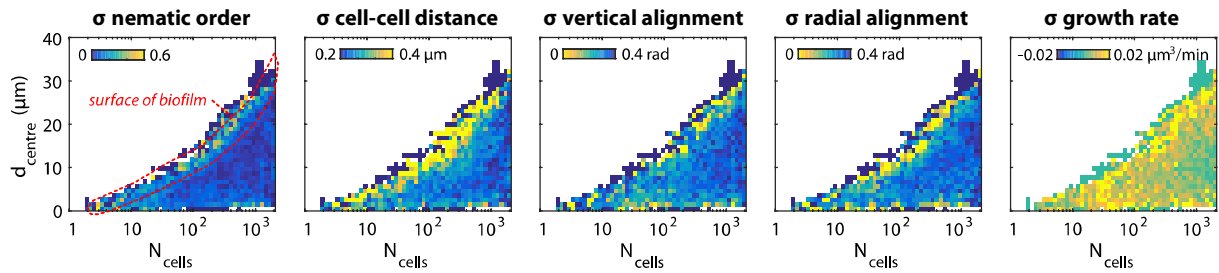
Supplementary Figure 5 – Standard deviations of WT*-data shown in Fig. 1e for $n = 4$ biofilms (characterization of WT* biofilm architecture in an unperturbed low-flow environment).

2.2. $\Delta rbmA$ biofilm structure

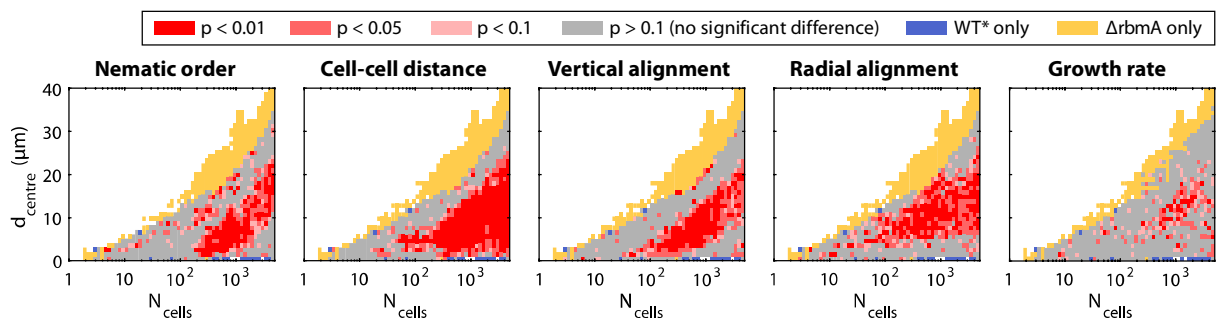
We internally perturbed our model system by varying the strength of the attractive interaction. This was achieved by modifying the extracellular RbmA levels with an arabinose-inducible promoter (strain KDV1082, with genotype $\Delta rbmA$, $P_{BAD-rbmA}$). Biofilms lacking RbmA were more fragile and displayed an increased cell-cell spacing and increased order, in agreement with previous studies (Supplementary Fig. 6, Fig. 1f, Supplementary Movie 2)^{1,7}. In comparison to the WT*, the order and vertical alignment inside the biofilm centre was higher, except in the bottom-most layer, where cells were on average oriented parallel to the surface (Fig. 1f). In summary, $\Delta rbmA$ mutant biofilms can be partitioned into three parts: the highly ordered core (i) is surrounded by a $\sim 5\mu\text{m}$ thick, disordered shell (ii), followed by the top outer layer (iii), which again has a slightly higher order. Cells inside the highly ordered core showed strong vertical alignment, whereas in the disordered shell and in the outer layer the vertical alignment was lost. The average cell-cell spacing was more than 30% higher than the WT*. Standard deviations of the properties in Fig. 1f are shown in Supplementary Fig. 7.



Supplementary Figure 6 – Time-resolved biofilm growth series of the $\Delta rbmA$ -mutant in a low-flow environment. Each cell is coloured according to the cellular alignment with the z -axis pointing away from the substrate plane.



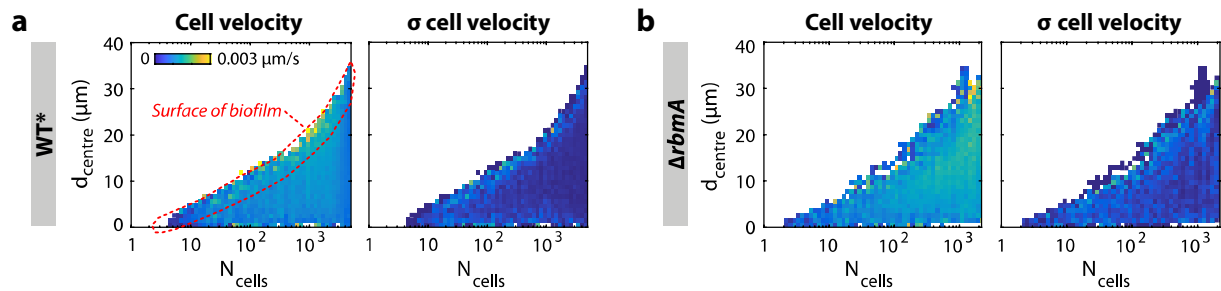
Supplementary Figure 7 – Standard deviations of $\Delta rbmA$ -data shown in Fig. 1f for $n = 4$ biofilms (characterization of $\Delta rbmA$ -mutant biofilm architecture in a low-flow environment).



Supplementary Figure 8 – Spatiotemporal differences between WT* and $\Delta rbmA$ biofilm architecture in a low-flow environment in relation to Fig. 1e,f. The data was compared using a two-tailed t-test, yielding p -values.

2.3. Cell motility inside biofilms

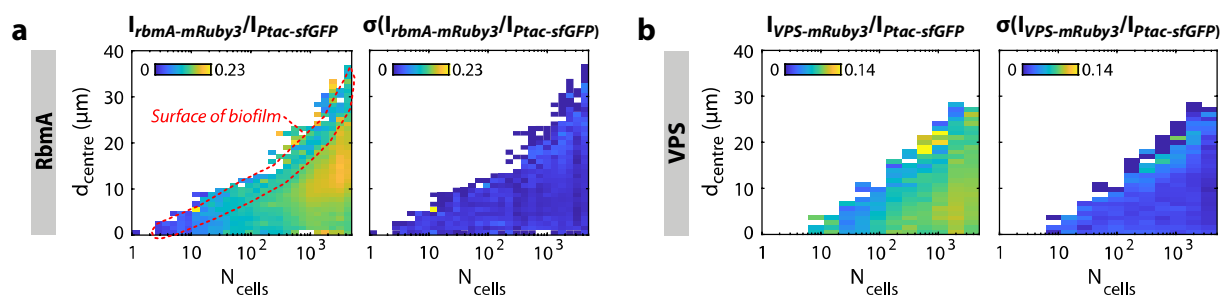
V. cholerae cells in rugose biofilms do not display flagellar or type IV pili mediated motility due to high levels of c-di-GMP⁸⁻¹⁰, which represses expression of flagella and type IV pili. Twitching motility based on type IV pili has also never been observed for *V. cholerae*¹¹. As shown in Supplementary Fig. 9, the individual cell speed inside WT* and $\Delta rbmA$ biofilms is on the order of nm/s, i.e. much lower than flagella driven motility ($\sim 50 \mu\text{m/s}$)¹¹ or pilus-based motility ($\sim 1 \mu\text{m/s}$)¹², and increases slightly with increasing biofilm sizes. These data indicate that cell movement in *V. cholerae* biofilms is dominated by passive cell displacement due to biofilm expansion rather than active cell motility.



Supplementary Figure 9 – Cell motility inside WT* and $\Delta rbmA$ biofilms in a low-flow environment. **a**, Average centroid velocity after linking cells in successive image stacks by cell tracking, and corresponding standard deviation for the WT* ($n = 4$ biofilms). **b**, Corresponding data for the of $\Delta rbmA$ mutant ($n = 4$ biofilms).

2.4. Spatiotemporal expression of *rbmA* and VPS in WT* biofilms

The *V. cholerae* rugose strain N16961, which was used during this study, is known to continuously produce extracellular matrix, including RbmA, even if the biofilms are small, due to this strain's intrinsically high c-di-GMP levels¹³. The expression of *rbmA* and VPS in an unperturbed low-flow environment were determined in the WT* (strain KDV1027 and KDV1218) using transcriptional fusions of *mRuby3* to the *rbmA* promoter or to the *vpsI* promoter, respectively. To measure *rbmA* and VPS expression in biofilms, the ratio of the background-subtracted *rbmA-mRuby3* fluorescence and *VPS-mRuby3* fluorescence, respectively, were divided by the background-subtracted signal of the constitutively expressed *sfGFP*, per cell (see Supplementary Fig. 10).

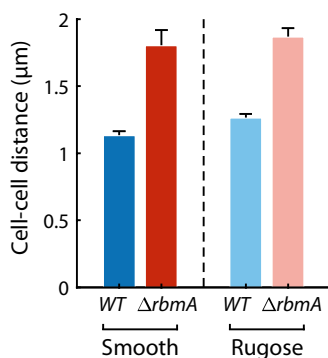


Supplementary Figure 10 – Spatio-temporal expression of *rbmA* and VPS in the WT* strain in a low-flow environment. **a**, Expression of *rbmA* (left: mean values, right: standard deviation, $n = 3$ biofilms). In each cell the fluorescence of *rbmA-mRuby3* was normalized by the signal of the constitutive P_{tac} -promoter-driven *sfGFP*-fluorescence signal. **b**, Expression of VPS (left: mean values, right: standard deviation, $n = 6$ biofilms). In each cell the fluorescence of *VPS-mRuby3* was normalized by the signal of the constitutive P_{tac} -promoter-driven *sfGFP* fluorescence signal.

2.5. Effect of *Vibrio* polysaccharide (VPS) on cell-cell interaction

To test whether VPS can contribute to cell-cell attraction, we compared the average cell-cell spacing in biofilms of cells with normal VPS production (smooth colony phenotype, $\Delta rbmA$, KDV383) with a VPS

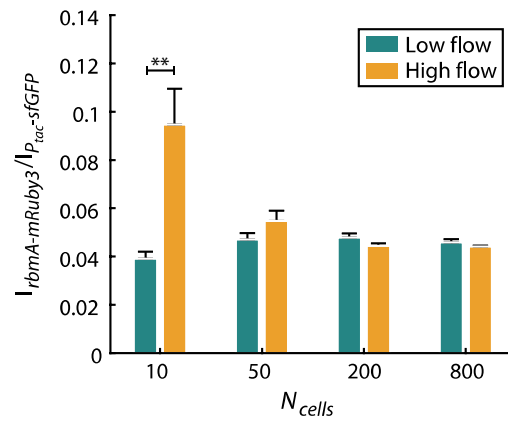
overproduction strain (rugose colony phenotype, $\Delta rbmA$, KDV692). Transcriptome comparisons of smooth and rugose strains indicate strong induction of VPS production in the rugose strain¹⁰. Comparing the smooth WT and the smooth $\Delta rbmA$ mutant (or the rugose WT and the rugose $\Delta rbmA$ mutant), we observe an increase in cell-cell spacing due to lack of RbmA. Comparing the cell-cell spacing for the smooth $\Delta rbmA$ and rugose $\Delta rbmA$ (VPS overproduction) strains, we find a similar cell-cell spacing (Supplementary Fig. 11, red bars), indicating that increased VPS production does not mediate an increased cell-cell attraction. In addition, data for the smooth and Rugose WT (KDV103, and, KDV615, respectively) show that enhanced VPS production even slightly increases the cell-cell spacing (Supplementary Fig. 11, blue bars). Increased VPS production therefore does not increase cell-cell attraction.



Supplementary Figure 11 – Effect of VPS overproduction on cell spacing in a low-flow environment. The “cell-cell distance” measurement refers to the average cell centroid-centroid distance. Rugose variants produce more VPS compared with smooth strains¹⁰. Enhanced VPS production does not lead to a decreased cell-cell spacing, indicating that enhanced VPS production is not causing an increased cell-cell attraction. Error bars correspond to the standard error ($n \geq 3$ biofilms).

2.6. Expression of *rbmA* at low vs. high shear rates

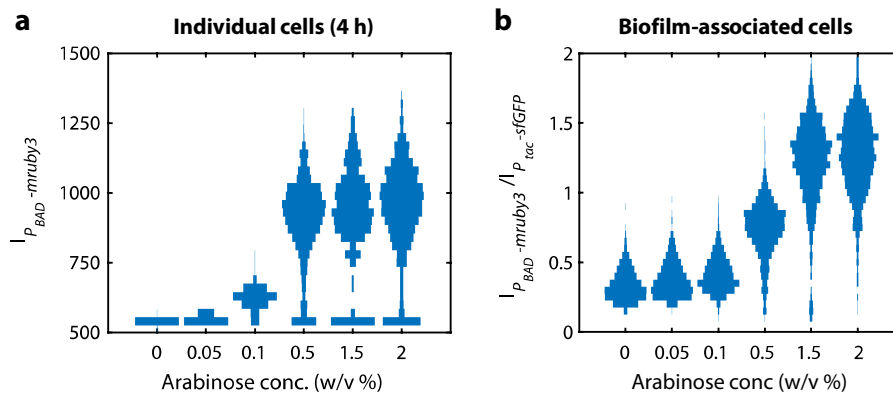
The expression of *rbmA* at low shear rate ($\dot{\gamma} = 2 \text{ s}^{-1}$) and high shear rate ($\dot{\gamma} = 2000 \text{ s}^{-1}$) was measured in the WT* strain (KDV1027) by calculating the ratio of the background-subtracted fluorescence of the *rbmA-mRuby3* transcriptional reporter, divided by the background-subtracted signal of the constitutively expressed sfGFP, per cell (see Supplementary Fig. 12). Individual cell measurements were averaged per time point and biofilm. Cells in biofilms growing at high shear rates were found to express *rbmA* at higher levels than those in low shear rates, for small biofilms of 10-50 cells, when all cells experience an increased level of shear and there is little deflection of the flow by cells and matrix on the biofilm surface.



Supplementary Figure 12 – Shear rate-dependent expression of *rbmA*, for different biofilm sizes. In each cell the fluorescence intensity signal of the *rbmA-mRuby3* transcriptional reporter was normalized by the signal of the constitutive P_{tac} -promoter-driven sfGFP fluorescence signal. Error bars correspond to the standard error ($n = 6$ biofilms). Statistical significance: ** is $p < 0.01$ (Mann–Whitney–Wilcoxon test).

2.7. Calibration of arabinose-induced expression of *rbmA*

The expression of *rbmA* (and therefore cell-cell attraction strength) was controlled by the arabinose-inducible promoter P_{BAD} which was reported to be bi-stable for cells in liquid culture^{14,15}. However, for using this expression system for our purposes inside biofilms, a homogenous expression pattern is required. Therefore, we analyzed the expression pattern of $P_{BAD-mRuby3}$ among individual cells (KDV1228) after 4 h of arabinose-induction (see Methods section in the main manuscript) in (a) shaken liquid cultures of individual cells, and (b) biofilm-associated cells (KDV1231) inside biofilms (300-500 cells) for different levels of arabinose (Supplementary Fig. 13). Bi-stable expression of P_{BAD} appears to be a characteristic behavior of individual cells in liquid culture, but not of biofilm-associated cells, as only few cells were found to be non-responsive to arabinose in biofilms (Supplementary Fig. 13).



Supplementary Figure 13 – Stability of the expression of the arabinose-inducible promoter P_{BAD} . **a**, Bi-stable expression of P_{BAD} inside individual cells in shaken liquid culture, 4 h after inoculation. **b**, In contrast, a homogeneous expression of P_{BAD} is observed for biofilm-associated cells. Data is shown as “violin plot” distributions.

3. Individual Cell-Based Simulations

3.1. Model description and implementation

The cells are modelled as interacting ellipsoids of half-length ℓ and half-width r , described by their position \mathbf{x} and orientation $\hat{\mathbf{n}}$. Because cells live at low Reynolds number ($Re \approx 10^{-4}$), we approximate the dynamics as over-damped, ignoring any inertial effects. Cells can interact with the wall boundary and other cells through interaction potential functions, U_{bdy} and V . Denoting the identity matrix by \mathbf{I} , the over-damped translational and orientation dynamics for a single cell are

$$\frac{d\mathbf{x}}{dt} = \mathbf{\Gamma}^{-1} \left[-\frac{\partial U_{bdy}}{\partial \mathbf{x}} - \frac{\partial V}{\partial \mathbf{x}} \right] \quad (5)$$

$$\frac{d\hat{\mathbf{n}}}{dt} = (\mathbf{I} - \hat{\mathbf{n}}\hat{\mathbf{n}}^T) \left[\mathbf{\Omega}^{-1} \left(-\frac{\partial U_{bdy}}{\partial \hat{\mathbf{n}}} - \frac{\partial V}{\partial \hat{\mathbf{n}}} \right) \right] \quad (6)$$

where $\mathbf{\Gamma}$ and $\mathbf{\Omega}$ are

$$\mathbf{\Gamma} = \gamma_m [\gamma_{\parallel} (\hat{\mathbf{n}}\hat{\mathbf{n}}^T) + \gamma_{\perp} (\mathbf{I} - \hat{\mathbf{n}}\hat{\mathbf{n}}^T)] \quad (7)$$

$$\mathbf{\Omega} = \omega_m \omega_R \mathbf{I} \quad (8)$$

Here, γ_m and ω_m are the typical translational and rotational drag coefficients for Stokes' drag in the extracellular matrix for a spheroid ($\gamma_m = 6\pi\mu_m r$, $\omega_m = 8\pi\mu_m l r^2$). Surface adhesion of the cells is captured by increasing the magnitude of the friction tensor $\mathbf{\Gamma}$ by a factor of 30 if the cells are within $1.5 \cdot r$ of the surface. γ_{\parallel} , γ_{\perp} and ω_R are dimensionless geometric parameters characterizing the longitudinal and transverse friction parameters that depend only on the aspect ratio $a = \ell/r$ of the cell, as given by Han *et al.*¹⁶:

$$\gamma_{\parallel} = \frac{8/3}{\frac{2a}{1-a^2} + \frac{2a^2-1}{(a^2-1)^{3/2}} \ln \left(\frac{a + \sqrt{a^2-1}}{a - \sqrt{a^2-1}} \right)} \quad (9)$$

$$\gamma_{\perp} = \frac{8/3}{\frac{a}{a^2-1} + \frac{2a^2-3}{(a^2-1)^{3/2}} \ln(a + \sqrt{a^2-1})} \quad (10)$$

$$\omega_R = \frac{2}{3} \frac{a^4 - 1}{a \left[\frac{2a^2-1}{\sqrt{a^2-1}} \ln(a + \sqrt{a^2-1}) - a \right]} \quad (11)$$

An important property of our proposed orientation dynamics is that $\hat{\mathbf{n}} \cdot d\hat{\mathbf{n}}/dt = 0$, such that the unit length of $\hat{\mathbf{n}}$ is conserved.

The interaction between a cell and the wall boundary is modelled with a repulsive interaction potential, U_{bdy} , that is proportional to the overlap between a cell and the wall boundary. The wall boundary is represented as a plane. To determine this overlap, an overlap coordinate, z_o , is introduced such that $z_o < 0$ implies no contact with the boundary and $z_o > 0$ implies contact with the boundary. The overlap coordinate is defined as

$$z_o = \ell |\hat{\mathbf{n}} \cdot \hat{\mathbf{N}}| + r - \hat{\mathbf{N}} \cdot (\mathbf{x} - \mathbf{S}) \quad (12)$$

where $\hat{\mathbf{N}}$ is the unit normal of the plane and \mathbf{S} is a point on the plane. In the simulations, we set $\hat{\mathbf{N}} = [0, 0, 1]$ and $\mathbf{S} = [0, 0, 0]$ such that the wall is an xy plane located at the origin. Thus, repulsion from the wall can be represented by

$$U_{bdy} = \begin{cases} 0 & \text{if } z_o \leq 0 \\ \epsilon_{bdy} \exp\left(\frac{z_o}{\sigma_{bdy}}\right) & \text{if } z_o > 0 \end{cases} \quad (13)$$

ϵ_{bdy} captures the magnitude of the cell-boundary interaction, and σ_{bdy} is a scale factor of order of the half-width of the cell.

V is the total potential of a single cell α for all the N pairwise cell-cell interactions between cell α and cell β ($V = \sum_{\beta=1, \beta \neq \alpha}^N U$). The interaction between cell α and cell β is governed by the cell-cell interaction potential introduced in the section ‘‘Cell-cell interaction potential’’.

The instantaneous cell length growth rate for a single cell is

$$\frac{d\ell}{dt} = \frac{\ell}{\tau_g} \ln(2) \quad (14)$$

where ℓ is the half-length of the cell at time t and τ_g is the growth time constant (obtained by experimental measurements). The cell width is constant throughout the simulation. Following the Adder model described by Taheri-Araghi *et al.*¹⁷, the length added between birth and division (ℓ_{add}) is constant for each cell. Thus, the cells divide when they grow an additional ℓ_{add} from their birth length. At division, a random number M is drawn from a normal distribution with mean 0.5 and standard deviation $\sigma_{birth\ size}$. Suppose ℓ_p is the length of the cell before division, then the birth lengths of the daughter cells are $M\ell_p$ and $(1 - M)\ell_p$ such that the length of the parent cell is conserved. The new orientations of the daughter cells are drawn from a von Mises-Fisher distribution, with mean direction corresponding to the direction of the parent cell and concentration parameter κ .

If we use r , $\tau_t = \frac{\gamma_m r^2}{\epsilon_0}$, and ϵ_0 as characteristic length, time, and energy scales, we can recast Eq. (5), (6) and (14) in dimensionless form

$$\frac{d\mathbf{x}^*}{dt^*} = \left[\frac{1}{\gamma_{\parallel}} (\hat{\mathbf{n}}\hat{\mathbf{n}}^T) + \frac{1}{\gamma_{\perp}} (\mathbf{I} - \hat{\mathbf{n}}\hat{\mathbf{n}}^T) \right] \left[-\frac{\partial U_{bdy}^*}{\partial \mathbf{x}^*} - \frac{\partial V^*}{\partial \mathbf{x}^*} \right] \quad (15)$$

$$\frac{d\hat{\mathbf{n}}}{dt^*} = (\mathbf{I} - \hat{\mathbf{n}}\hat{\mathbf{n}}^T) \left[\frac{3}{4a\omega_R} \left(-\frac{\partial U_{bdy}^*}{\partial \hat{\mathbf{n}}} - \frac{\partial V^*}{\partial \hat{\mathbf{n}}} \right) \right] \quad (16)$$

$$\frac{d\ell^*}{dt^*} = \frac{\tau_t}{\tau_g} \ell^* \ln(2) \quad (17)$$

where superscript $*$ indicates a dimensionless quantity and use has been made of the following definitions and ratios $\mathbf{x}^* = \mathbf{x}/r$, $\ell^* = \ell/r$, $t^* = t/\tau_t$, $V^* = V/\epsilon_0$ and $U_{bdy}^* = U_{bdy}/\epsilon_0$. Note that τ_t can be interpreted as the translational relaxation time, i.e. a time scale of how long it takes for a bacterium to reach an equilibrium configuration from the cell-cell interaction potential. If $z_o^* > 0$, the dimensionless

boundary potential is $U_{bdy}^* = U_{bdy}/\epsilon_0 = \epsilon_r \exp(z_o^*/\sigma_{bdy}^*)$ where $\epsilon_r = \epsilon_{bdy}/\epsilon_0$, $z_o^* = z_o/r$, and $\sigma_{bdy}^* = \sigma_{bdy}/r$.

A custom, highly parallelized individual cell-based code employing graphics processing units (GPUs) was developed to perform the simulations. At each time step, we calculate cell-cell interactions using the all-pairs approach¹⁸ such that all pair-wise interactions are evaluated. We use a standard explicit Euler scheme to numerically integrate the dimensionless translational and orientational dynamics, Eq. (15) and (16) and growth law Eq. (17).

The key simulation parameters used for the simulations are shown in Supplementary Table 3.

Parameter	Value	Unit	Description
r	0.2775	μm	Average half-width of the bacteria from experimental measurements.
τ_g	6130	s	Growth time constant (average cell division time of biofilm-associated cells obtained from experiments).
μ_m	1	$\text{Pa}\cdot\text{s}$	Estimate of the dynamic viscosity of EPS matrix at room temperature ¹⁹ .
γ_m	5.23	$\text{pN}\cdot\text{s}\cdot\mu\text{m}^{-1}$	Typical drag coefficient for Stokes' drag in EPS matrix ($\gamma_m = 6\pi\mu_m r$).
ϵ_r	10		Ratio comparing the strength of the bacteria-boundary interaction to the strength of the bacteria-bacteria interaction $\epsilon_r = \epsilon_{bdy}/\epsilon_0$.
σ_{bdy}^*	1		Non-dimensional boundary potential length scale parameter.
τ_t	8.05	s	Translational time scale due to repulsion in matrix (typical time needed for daughter cells in matrix to reach their equilibrium configurations due to repulsion after cell division).
l_{add}^*	3.65		Non-dimensional length added to bacteria after division.
$\sigma_{birth\ size}^*$	0.07		Non-dimensional standard deviation of the normal distribution for the daughter bacteria birth size.
κ	100		Concentration parameter for the von Mises-Fisher distribution for the daughter bacteria division orientation.
ϵ_0	$5\cdot 10^{-20}$	J	Strength of the repulsive part of the cell-cell potential.
λ_r	1.65		Width of the repulsive part of the cell-cell potential (corresponds to $1.16\ \mu\text{m}$ at a typical overlap factor of $\sigma = 0.7\ \mu\text{m}$, which is the value it would take for a sphere with the typical mean cell volume of $0.4\ \mu\text{m}^3$).
ν	0.13 (WT*)		Strength of the attractive part of the cell-cell potential (corresponds to $0.65\cdot 10^{-20}\ \text{J}$).
λ_a	0.16 (WT*)		Well-width of the attractive part of the cell-cell potential (corresponds to $0.11\ \mu\text{m}$ at a typical overlap factor of $\sigma = 0.7\ \mu\text{m}$).
ρ_a	2.93 (WT*)		Position of the attractive part of the cell-cell potential (corresponds to $2.0\ \mu\text{m}$ at a typical overlap factor of $\sigma = 0.7\ \mu\text{m}$).

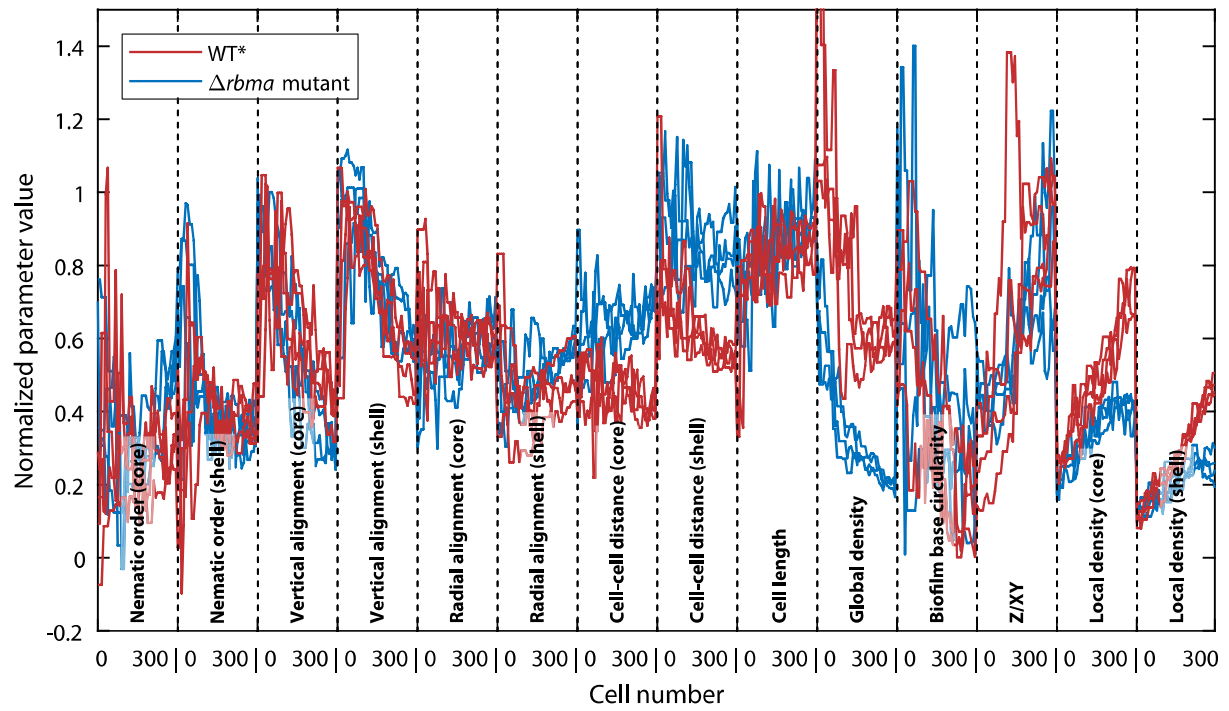
Supplementary Table 3 – Simulation parameters. All parameters are measured directly from experimental biofilms, except for the potential parameters ν , λ_a , ρ_a , ϵ_0 , λ_r , which were determined by fitting as described in the main text.

3.2. Comparing simulations with experimental data

For the purpose of comparing simulated and experimental biofilms, a set of parameters was chosen to represent the phenotype and architecture as fully and accurately as possible. These parameters include averaged single cell parameters, i.e. local order, vertical alignment, radial alignment, cell-cell distance, local density, cell length, and the following global biofilm parameters: global density, biofilm aspect ratio, and biofilm base circularity (see section on “Biofilm features”). The single cell parameters were determined for the biofilm core ($d_{centre} < \max(d_{centre})/2$) and the biofilm shell ($\max(d_{centre})/2 < d_{centre} < \max(d_{centre})$). The time-evolution of these parameters was compared for biofilms of cell numbers ranging from 10 to 300. To account for logarithmic growth, biofilms were sampled at 40 intermediate logarithmically spaced cell numbers yielding a characteristic biofilm feature vector shown in Supplementary Fig. 14. In addition, each parameter was normalized to a typical range as indicated in Supplementary Table 4. These feature vectors therefore capture biofilm architectural properties (via the different architectural parameters), as well as the temporal biofilm development (via the measurement of these parameters at different cell numbers). The similarity between a vector characterizing a simulation and an experimental biofilm was assessed in terms of the mean square distance (*MSD*) between the two feature vectors. The different parameters were weighted differently to account for their relative importance in representing biofilm phenotypes, as summarized in Supplementary Table 4.

Parameter	Normalization range [min max]	Weight
Local order	0 – 0.8	10
Vertical alignment	0.2 – 1.37	10
Radial alignment	0.2 – 1.37	3
Cell-cell distance	0.8 – 2	20
Local density	0 – 0.2	10
Cell length	1 – 3	1
Global density	0 – 0.15	5
Biofilm aspect ratio (<i>Z/XY</i>)	0 – 0.8	1
Biofilm base circularity	0 – 0.8	1

Supplementary Table 4 – Biofilm parameters and corresponding normalization ranges and weights.



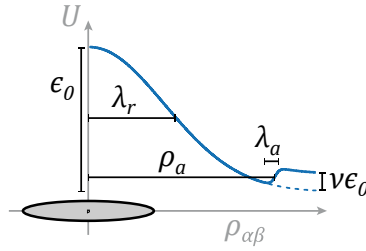
Supplementary Figure 14 – Characteristic biofilm feature vector for several biofilms of the WT* (red) and the $\Delta rbmA$ -mutant (blue). The parameters correspond to the ones listed in Supplementary Table 4, whereby relevant ones are spatially resolved (core and shell of the biofilm).

3.3. Cell-cell interaction potential

In our model, cells are subject to forces by neighbouring cells due to purely mechanical effects, including steric repulsion and the repulsion mediated by the effective osmotic pressure in the intercellular space, as well as the interaction with secreted matrix components (such as RbmA). While steric interactions and the osmotic pressure in the intercellular space are expected to result in net repulsive forces, RbmA is thought to directly link the cells together, thereby causing net cell-cell attraction^{20,21}, which we quantitatively confirmed (Fig. 2a). To account for these effects we suggest the cell-cell interaction potential between two particular cells, cell α and cell β , described by equation (18).

$$U = \epsilon_0 \epsilon_1 \left(e^{-\frac{\rho^2}{\lambda_r^2}} + \frac{\nu}{1 + e^{\left(\frac{\rho_a - \rho}{\lambda_a}\right)}} \right) \quad (18)$$

The first term in the interaction potential corresponds to cell-cell repulsion, and the second term corresponds to cell-cell attraction. The vector $\mathbf{r}_{\alpha\beta} = r_{\alpha\beta} \hat{\mathbf{r}}_{\alpha\beta}$ joins the cell centres and is directed from cell α to cell β , ϵ_0 describes the energy of the cell-cell interaction, ϵ_1 is a strength parameter accounting for the cell orientation configuration, $\rho = r_{\alpha\beta}/\sigma$ is the cell-cell distance normalized by the overlap shape-factor σ , λ_r is the repulsion width, ν is the attraction strength, ρ_a is the attraction shift and λ_a is the attraction width, as summarized in Supplementary Table 3 and Supplementary Fig. 15.



Supplementary Figure 15 – Effects of the cell-cell interaction potential parameters. ϵ_0 : Strength of repulsion due to combined effects of steric and matrix repulsion. λ_r : Range of repulsion in cell diameters. ν : Relative strength of RbmA-mediated attraction. λ_a : Effective attractive range of RbmA in cell diameters. ρ_a : Effective distance of the attractive potential well.

The above strength and range parameters lead to different length scales of the cell-cell interaction forces depending on the orientation of the cells (see Fig. 2b,c, Supplementary Fig. 18, and Supplementary Fig. 19, respectively). The generalized forms of the strength and range parameters for non-identical ellipsoids are given by Cleaver *et al.*²² and are reproduced below for convenience

$$\epsilon_1 = \left[1 - \chi^2 (\hat{\mathbf{n}}_\alpha \cdot \hat{\mathbf{n}}_\beta)^2 \right]^{-1/2} \quad (19)$$

$$\sigma = \sigma_0 \left[1 - \chi \left(\frac{\xi (\hat{\mathbf{n}}_\alpha \cdot \hat{\mathbf{r}}_{\alpha\beta})^2 + \xi^{-1} (\hat{\mathbf{n}}_\beta \cdot \hat{\mathbf{r}}_{\alpha\beta})^2 - 2\chi (\hat{\mathbf{n}}_\alpha \cdot \hat{\mathbf{r}}_{\alpha\beta}) (\hat{\mathbf{n}}_\beta \cdot \hat{\mathbf{r}}_{\alpha\beta}) (\hat{\mathbf{n}}_\alpha \cdot \hat{\mathbf{n}}_\beta)}{1 - \chi^2 (\hat{\mathbf{n}}_\alpha \cdot \hat{\mathbf{n}}_\beta)^2} \right) \right]^{-1/2} \quad (20)$$

where

$$\sigma_0 = \sqrt{r_\alpha^2 + r_\beta^2}, \quad (21)$$

$$\chi = \left[\frac{(\ell_\alpha^2 - r_\alpha^2)(\ell_\beta^2 - r_\beta^2)}{(\ell_\alpha^2 + r_\beta^2)(\ell_\beta^2 + r_\alpha^2)} \right]^{1/2}, \quad (22)$$

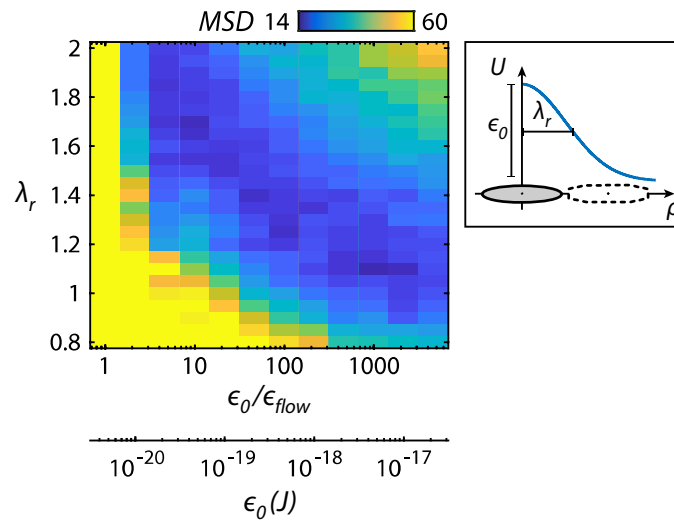
and

$$\xi = \left[\frac{(\ell_\alpha^2 - r_\alpha^2)(\ell_\beta^2 + r_\alpha^2)}{(\ell_\beta^2 - r_\beta^2)(\ell_\alpha^2 + r_\beta^2)} \right]^{1/2}. \quad (23)$$

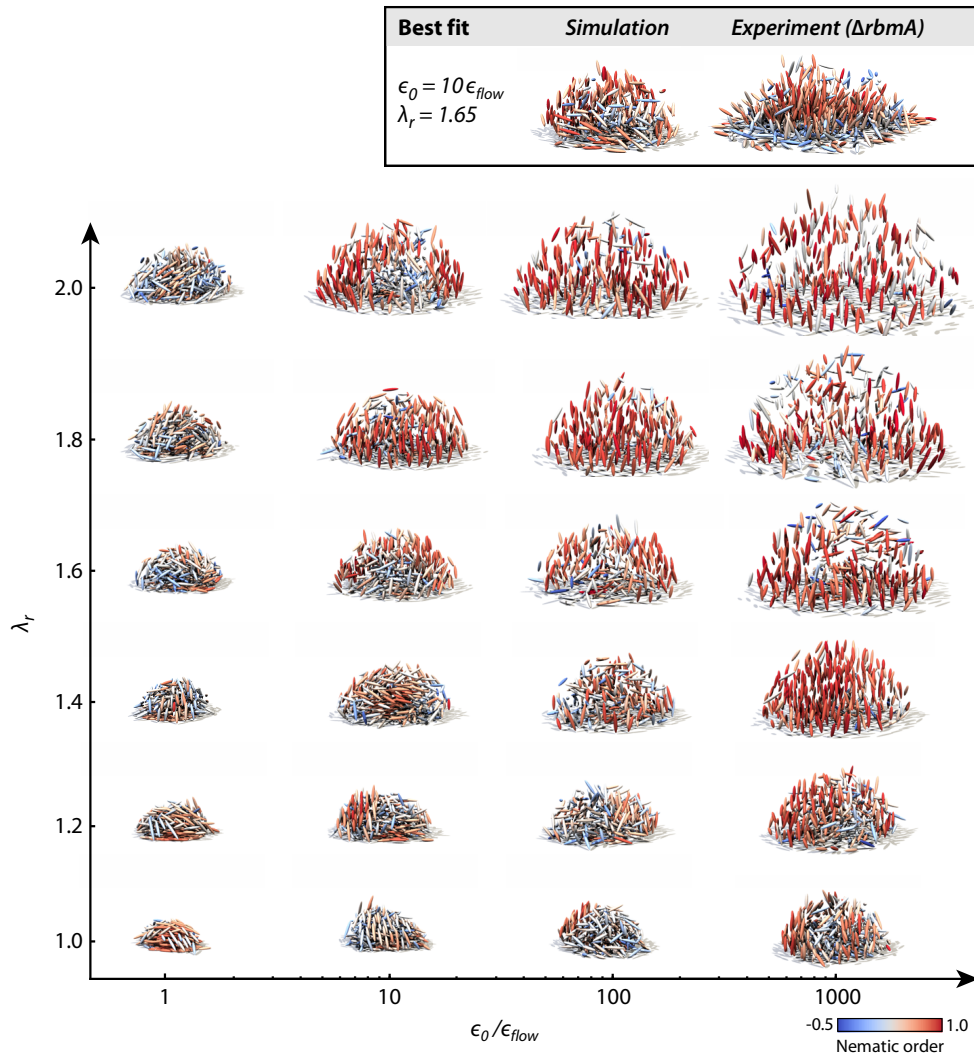
For two ellipsoids α and β , $\hat{\mathbf{n}}_\alpha, \hat{\mathbf{n}}_\beta$ are their axial unit vectors, ℓ_α, ℓ_β are their half lengths, and r_α, r_β are their half widths.

Influence of cell-cell repulsion on biofilm architecture

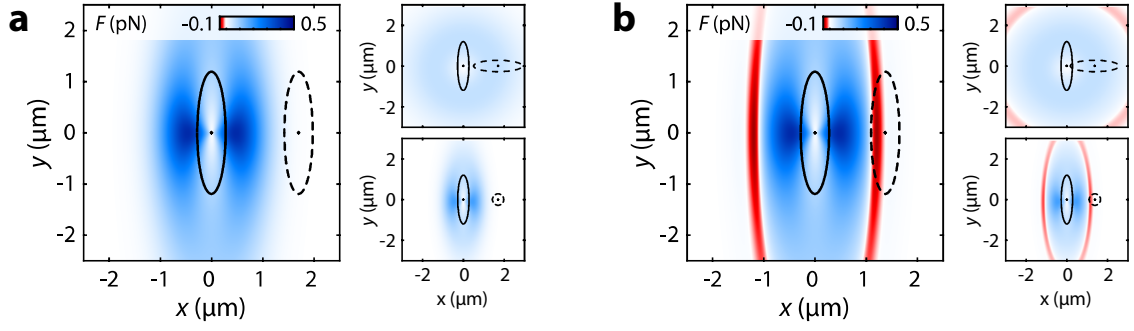
Biofilms grown from $\Delta rbmA$ cells, which lack RbmA proteins, were simulated by setting the strength of the attractive part of the cell-cell potential to zero ($\nu = 0$), as cell-cell attraction is primarily due to the levels of RbmA. Before computing systematic parameter scans for obtaining the repulsion-parameters ϵ_0 and λ_r of the interaction potential U , we estimated the parameters using the physical considerations: The energy scale of the cell-cell interactions was estimated to be within several orders of magnitude of the energy scale of interactions between the cells and the flow. Therefore, we simulated biofilms for values of ϵ_0 ranging from $1 \cdot \epsilon_{flow}$ to $10^4 \cdot \epsilon_{flow}$, where the cell-flow interaction energy $\epsilon_{flow} = 0.005 \text{ pN} \cdot \mu\text{m}$ was calculated by considering Stokes flow (with flow rate $0.1 \mu\text{L}/\text{min}$ in a channel with the dimensions used in the experiment) past a sphere with the typical cell volume of $0.4 \mu\text{m}^3$, being locating $2.4 \mu\text{m}$ above a no-slip boundary (as is typical for cells in the outer shells of biofilms). A typical cell-cell distance in the core of biofilms, where cell-cell repulsion dominates, is around $1 \mu\text{m}$, which corresponds to $\lambda_r = 1.4$ (for a typical overlap shape-factor of $\sigma = 0.7 \mu\text{m}$, which is the value it would take for a sphere with the typical mean cell volume of $0.4 \mu\text{m}^3$). Therefore, we used values of λ_r from 0.8 to 2. The resulting *MSD* values upon a systematic variation of the cell-cell interaction energy ϵ_0 and repulsion width λ_r are visualized in Supplementary Fig. 16. Corresponding biofilm architecture phenotypes are shown in Supplementary Fig. 17. The identified parameters for the best fit between simulations and the experimentally observed $\Delta rbmA$ -phenotype are $\epsilon_0 = 10 \cdot \epsilon_{flow}$ ($5 \cdot 10^{-20} \text{ J}$) and $\lambda_r = 1.65$. The resulting translational and rotational forces are visualized in Fig. 2b, Supplementary Fig. 18a, and Supplementary Fig. 19a, respectively.



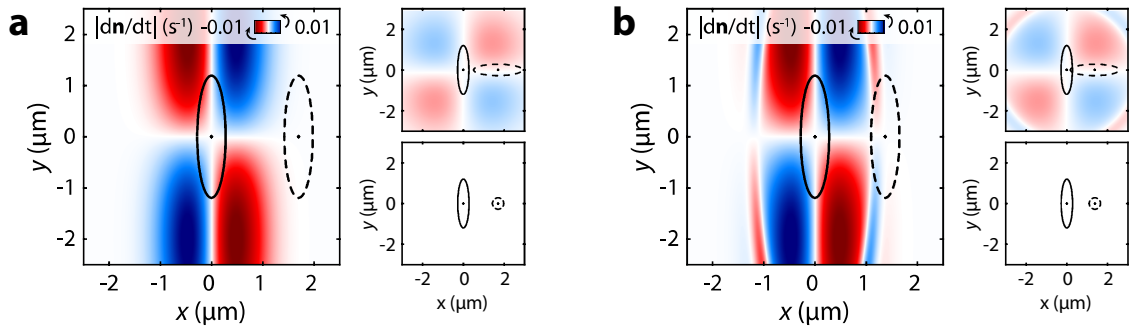
Supplementary Figure 16 – Parameter screen to test the influence of cell-cell repulsion on biofilm architecture phenotype. a, Mean *MSD* values between feature vectors of $\Delta rbmA$ mutant biofilm simulations and experiments (see section on “Comparing simulations with experimental data”) upon variation of cell-cell interaction strength ϵ_0 and repulsion range λ_r . Inset: effect of parameter variation on the cell-cell interaction potential.



Supplementary Figure 17 – Resulting simulated biofilms for different levels of cell-cell repulsion. The figure shows a subset of biofilm renderings corresponding to a range of different values for ϵ_0 and λ_r (cf. Fig. 2b and Supplementary Fig. 16). The colour of each cell corresponds to the nematic order.



Supplementary Figure 18 – Translational cell-cell interaction forces. **a**, Translational forces for $\epsilon_0 = 10 \cdot \epsilon_{flow}$ ($5 \cdot 10^{-20}$ J), $\lambda_r = 1.65$, and $\nu = 0$ (corresponding to the $\Delta rbmA$ -mutant, cf. Fig. 2b) for different orientations. **b**, Translational forces for $\epsilon_0 = 10 \cdot \epsilon_{flow}$ ($5 \cdot 10^{-20}$ J), $\lambda_r = 1.65$, $\nu = 0.13$, $\lambda_a = 0.164$, and $\rho_a = 2.93$ (corresponding to an arabinose concentration of 0.5%, cf. Fig. 2c) for different orientations.



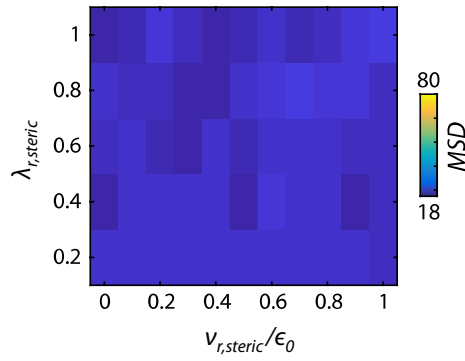
Supplementary Figure 19 – Rotational cell-cell interaction dynamics. $|dn/dt| = |(I - \hat{n}_\beta \hat{n}_\beta)(-\Omega^{-1} \partial U / \partial \hat{n}_\beta)|$ acting on two neighbouring cells. **a**, Rotational dynamics for $\epsilon_0 = 10 \cdot \epsilon_{flow}$ ($5 \cdot 10^{-20}$ J), $\lambda_r = 1.65$, and $\nu = 0$ (corresponding to the $\Delta rbmA$ -mutant, cf. Fig. 2b) for different orientations. **b**, Rotational dynamics for $\epsilon_0 = 10 \cdot \epsilon_{flow}$ ($5 \cdot 10^{-20}$ J), $\lambda_r = 1.65$, $\nu = 0.13$, $\lambda_a = 0.164$, and $\rho_a = 2.93$ (corresponding to an arabinose concentration of 0.5%, cf. Fig. 2c) for different orientations. A positive speed (indicated in red) results in a clockwise rotation.

Influence of steric and osmotic pressure-mediated cell-cell repulsion on biofilm phenotype

The joint effects of steric cell-cell repulsion and osmotic pressure-mediated cell-cell repulsion were modelled with a relatively soft Gaussian function, cf. Eq. (18). To test if this model is appropriate, we performed the following analysis: To check the effect of both contributions to cell-cell repulsion separately, we embedded a second, short-ranged and very strongly repulsive part, representing steric repulsion, into the existing potential by adding another Gaussian function peaked at $\rho = 0$ characterized by v_{steric} and $\lambda_{r,steric} < \lambda_r$, see Eq. (24). Here, ϵ_0 and λ_r are fixed to the optimal values obtained by comparison with the experimental values for the $\Delta rbmA$ -mutant.

$$U = \epsilon_0 \epsilon_1 \left(e^{-\frac{\rho^2}{\lambda_r^2}} + v_{steric} \cdot e^{-\frac{\rho^2}{\lambda_{r,steric}^2}} + \frac{v}{1 + e^{-\frac{\rho_a - \rho}{\lambda_a}}} \right) \quad (24)$$

The exploration of the parameter space is shown in Supplementary Fig. 20. The additional term for hard-steric repulsion in Eq. (24) does not have any influence on the biofilm phenotype as the MSD values show no variance irrespective of interaction strength v_{steric} and range $\lambda_{r,steric}$. This justifies modelling the effects of hard steric and soft osmotic pressure-mediated repulsion in a combined manner, as cells are unlikely to interact exclusively via hard and very short-ranged steric repulsion (the matrix prevents them from getting close enough together).

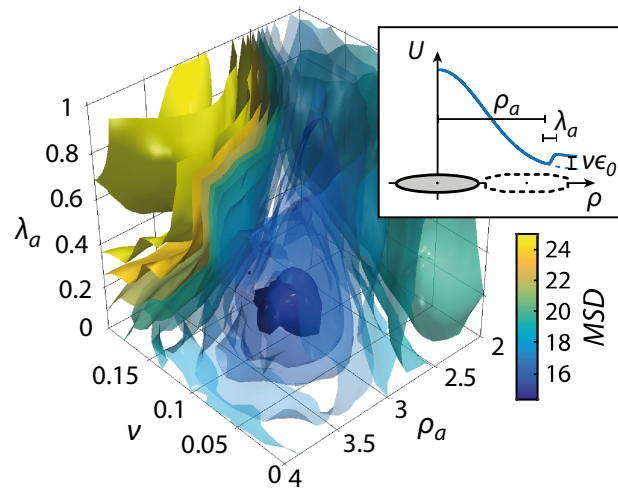


Supplementary Figure 20 – Parameter screen to test the influence of hard steric cell-cell repulsion on biofilm phenotype. The figure shows MSD values upon variation of v_{steric} and $\lambda_{r,steric}$. Owing to its longer length scale, soft, osmotic pressure-mediated repulsion prevents cells from getting close enough to interact directly through hard steric repulsion.

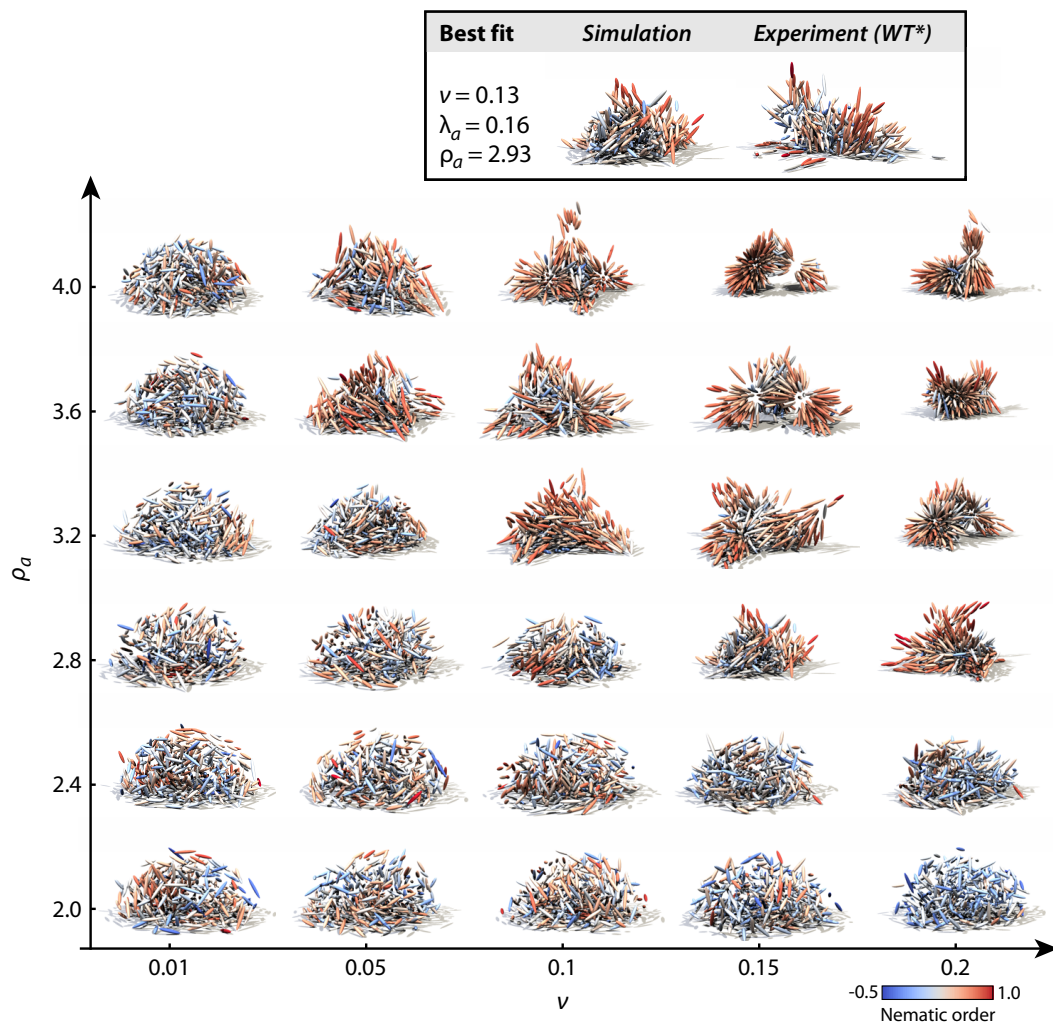
Influence of RbmA-mediated cell-cell attraction on biofilm phenotype

The attractive part in the cell-cell potential is thought to be governed by RbmA. Having fixed the cell-cell repulsion parameters ϵ_0 and λ_r for the $\Delta rbmA$ phenotype, the remaining parameters influencing cell-cell attraction (namely ν , ρ_a , and λ_a) were determined by finding the best fits when comparing the simulations with biofilms of different RbmA concentrations (by varying the expression of the *rbmA* gene using the arabinose-inducible P_{BAD} promoter). Prior to systematic parameter scans with simulations, we estimated the parameters using the following considerations: The attraction shift ρ_a was estimated for each arabinose concentration by considering the typical cell-cell distance at the edge of biofilms, where attraction dominates. These estimates suggested using values of ρ_a between 2 and 4 (again using $\sigma = 0.7 \mu\text{m}$). The attraction width λ_a was estimated by considering the standard deviation of cell-cell distances near the edge of biofilms, which was found to be approximately $0.3 \mu\text{m}$ for all biofilms; therefore, we expected λ_a to be around 0.4 (again using $\sigma = 0.7 \mu\text{m}$).

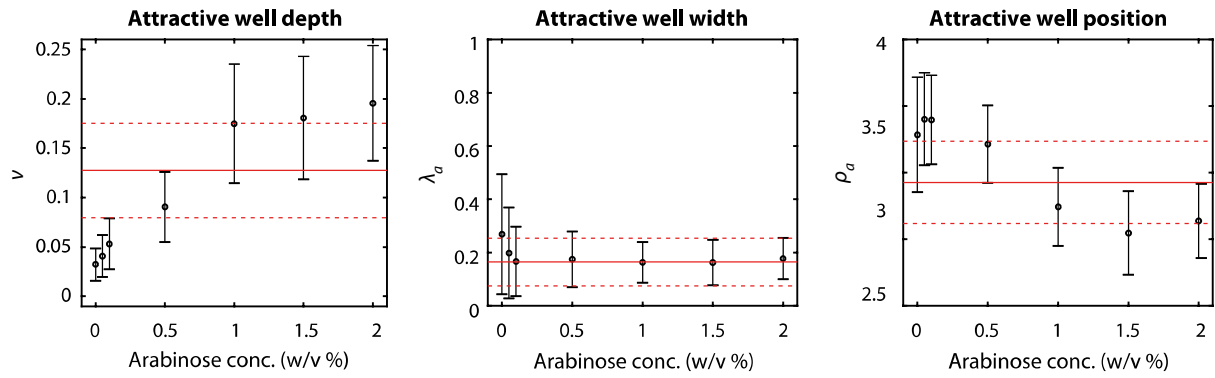
The resulting MSD values resulting from simulations conducted with a systematic variation of ν , λ_a and ρ_a are shown in Supplementary Fig. 21. The resulting translational and rotational forces for selected conditions are visualized in Fig. 2c, Supplementary Fig. 18b, and Supplementary Fig. 19b, respectively. Corresponding biofilm phenotypes are shown in Supplementary Fig. 22. The 5% best matching simulations (5% lowest MSD values) for a given condition were chosen and the mean of the corresponding values for ν , λ_a and ρ_a was defined as the best fit. In Supplementary Fig. 23 the obtained mean values \pm standard deviation are displayed. The best-fit parameters for the experimentally observed WT*-phenotype were $\nu = 0.13$, $\lambda_a = 0.16$ and $\rho_a = 2.93$.



Supplementary Figure 21 – Parameter screen to test the influence of RbmA-mediated cell-cell attraction on biofilm phenotype. *MSD* values for feature vectors of biofilms with arabinose-inducible *rbmA* expression grown at 0.5% arabinose (w/v) and simulations (see section “Comparing simulations with experimental data) upon variation of cell-cell interaction strength v , and well width λ_a and well position ρ_a . Inset: effect of parameter variation on the cell-cell interaction potential.



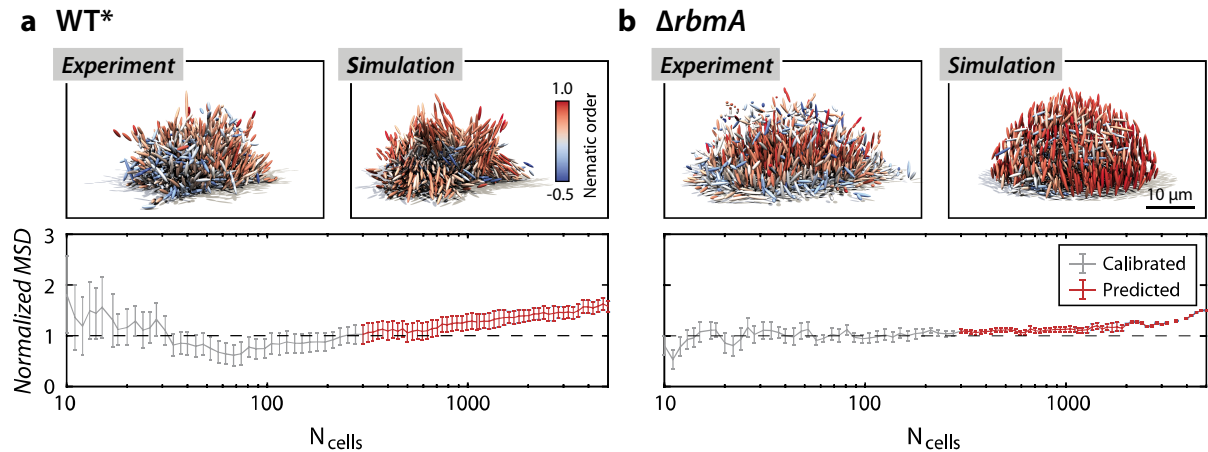
Supplementary Figure 22 – Resulting simulated biofilms for different levels of RbmA-mediated cell-cell attraction. The figure shows biofilm renderings corresponding to selected values of v and ρ_a while keeping λ_a constant at $\lambda_a = 0.16$ (cf. Fig. 2e). Each cell is coloured according to the nematic order.



Supplementary Figure 23 – Fitting of the attractive potential parameters. The graphs show the approximate position of the *MSD* minimum in $(\nu, \lambda_a$ and $\rho_a)$ -space of the 5% best-matching simulations at increasing arabinose concentrations, corresponding to increasing amounts of secreted RbmA. Each data point refers to the median position of the minimum and the error bars indicate the lower and upper quartile. The position of the WT* is shown in red (solid lines: median values, dashed lines: lower/upper quartiles).

3.4. Predicting the structure of larger biofilms

The cell-cell interaction potential was calibrated based on biofilms with up to 300 cells (section “Comparing simulations with experimental data”). To test the ability of the calibrated simulations to predict the structure of biofilms with more than 300 cells, a feature vector containing the same parameters as the ones used in section 3.2 was used. The *MSD* between the experimental and simulation feature vectors was calculated for biofilms sampled at 40 intermediate logarithmically spaced cell numbers from 10 to 300 and from 300 to 1000, separately for each chosen number of cells (in contrast to section 3.2 where a single *MSD* between temporal feature vectors was calculated), and was normalized by the average value of the *MSD* between 10 and 300 cells. For cell numbers between 300 and 1000, the *MSD* at each cell number was found to be close to the average value of the *MSD* between 10 and 300 cells for WT* and $\Delta rbmA$ biofilms, and strong qualitative agreement was observed between simulated and experimental biofilms with up to 1000 cells (Supplementary Fig. 24, Supplementary Movie 5). These results indicate the remarkable ability of the potential-based simulations calibrated on biofilm development up to 300 cells to predict the development of larger biofilms.



Supplementary Figure 24 – Predicting phenotypes of larger biofilms. **a**, Rendered WT* experimental (left) and simulated biofilm (right) for approximately 1000 cells. Bottom: normalized *MSD* of feature vectors for experimental ($n = 7$) and simulated biofilms ($n = 3$). These data are also shown in Fig. 2g,h. **b**, Rendered $\Delta rbmA$ experimental (left) and simulated biofilm (right) for approximately 1000 cells. Bottom: normalized *MSD* of feature vectors for experimental ($n = 4$) and simulated biofilms ($n = 3$). Error bars were calculated by Gaussian error propagation of the standard deviations of the individual measurements.

4. Continuum Model

4.1. Mathematical model of a growing biofilm in shear flow

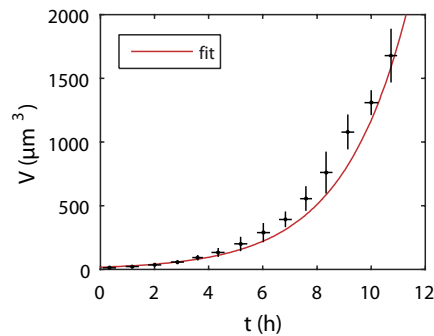
For this model, we assume that the biofilm colony is located in the centre of a rectangular channel. Fluid flows through the channel in the x -direction with a prescribed flow rate. Flow is modelled by the Stokes equations

$$\nabla \cdot \mathbf{u} = 0, \quad \mu \nabla^2 \mathbf{u} = \nabla p \quad (25)$$

with a no-slip condition ($\mathbf{u} = \mathbf{0}$) applied on the channel walls and on the surface of the biofilm. The biofilm surface $z = S(x, y, t)$ grows or shrinks in the direction of its outer unit normal \mathbf{n} , with velocity

$$\begin{aligned} \mathbf{U} &= \alpha(\mathbf{x}, t)\mathbf{n} - \beta |(\mathbb{I} - \mathbf{nn})\boldsymbol{\sigma} \cdot \mathbf{n}| \mathbf{n} \\ &= \alpha(\mathbf{x}, t)\mathbf{n} - \beta \boldsymbol{\tau} \mathbf{n} \end{aligned} \quad (26)$$

where the first term on the right hand side represents movement owing to cell growth and division, and the second term represents erosion proportional to the shear stress $\boldsymbol{\tau}$ imposed on the surface by the fluid; here $\boldsymbol{\sigma}$ is the stress tensor of the fluid. For a hemispherical biofilm growing uniformly in the radial direction at a rate r_0 , with a base centred at the origin, $\alpha(\mathbf{x}, t) = r_0 r$, where $r = |\mathbf{x}|$. This leads to exponential volumetric growth in time, $V(t) = V_0 \exp(3r_0 t)$, where V_0 is the volume of the biofilm at $t = 0$. The growth rate r_0 is found by fitting an exponential curve to experimental data of volume versus time for a $\Delta rbmA$ -mutant in a channel with an average flow velocity of $\langle v \rangle = 0.03$ mm/min (Supplementary Fig. 25).



Supplementary Figure 25 – Volume of $\Delta rbmA$ -mutant (black) growing at a very low flow speed ($\langle v \rangle = 0.03$ mm/min). Exponential fit (red), error bars correspond to the standard deviation ($n = 6$ biofilms).

4.2. Numerical method

The surface of the biofilm is represented by the zero level set of a level set function ϕ , which satisfies the Hamilton-Jacobi equation²³

$$\phi_t + a(\mathbf{x}, t)|\nabla\phi| = 0. \quad (27)$$

Here $a = |\mathbf{U}|$ is the normal speed of the surface.

Equation (27) is solved using the Level Set Toolbox²⁴ in Matlab, which approximates the time derivative with an explicit total variation diminishing Runge-Kutta integration scheme. The spatial grid is uniform in each direction, with a smaller step size in the z -direction. A symmetry condition ($\nabla\phi \cdot \mathbf{n} = 0$) is applied along the xz -plane through the centre of the biofilm. At all other boundaries, ghost nodes are added and filled with data linearly extrapolated from the computational boundary²⁴.

At each timestep, the steady Stokes equations (25) are solved using the finite-element package deal.II²⁵, with the surface height $z = S(x, y, t)$ used as input to denote the grid points at which the no-slip condition should be applied. The finite-element mesh is locally refined in all directions around the biofilm surface. A uniform velocity value is imposed at the inflow boundary, and the flow is allowed to develop in an entrance region. A symmetry condition ($\mathbf{u} \cdot \mathbf{n} = 0$) is applied on the xz -plane through the centre of the biofilm. The stress tensor σ at the biofilm surface is output for use in equation (26) at the next timestep. The value of σ is interpolated to the level set spatial grid. The total drag force in the direction of the flow on a hemisphere is checked to agree with the value calculated by Pozrikidis *et al.*²⁶.

The shear stress applied to real biofilms is calculated by incorporating experimental data from wild-type biofilms into the continuum model. The convex hull of the set of cell centroids at a specific time point is extracted, after some outlying cells and cells not descended from the biofilm population have been removed. The surface is smoothed by binarizing the volumes inside and outside the convex hull, then applying a Gaussian filter. The flow and the biofilm surface are assumed to be symmetric about the mid-plane of the biofilm, as before (i.e. flow is only simulated on one half of the experimental domain, chosen arbitrarily).

5. Supplementary Data

Strain	Genotype/Relevant features	Reference
<i>E. coli</i>		
S17-1 λpir	$\Delta lacU169$ ($\Phi lacZ\Delta M15$), <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i> , λpir	De Lorenzo <i>et al.</i> ²⁷
TOP10	<i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) $\Phi 80 lacZ\Delta M15\Delta lacX74 deoRecA1$ <i>araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
<i>V. cholerae</i>		
KDV101	wild type strain N16961 (O1 El Tor, Sm ^R)	Meibom <i>et al.</i> ²⁸
KDV103	N16961 <i>lacZ:P_{tac}-mKOKappa</i>	Drescher ¹
KDV115	N16961 <i>vpvC^{W240R} lacZ:P_{tac}-mKOKappa</i>	Drescher ¹
KDV148	N16961 <i>vpvC^{W240R}</i> (matrix hyperproducer phenotype)	Drescher ¹
KDV383	N16961 $\Delta rbmA$, <i>lacZ:P_{tac}-mKOKappa</i>	Drescher lab
KDV611	N16961 <i>vpvC^{W240R}</i> , $\Delta crvA$	This work
KDV613	N16961 <i>vpvC^{W240R}</i> , $\Delta crvA$ contains plasmid pNUT542	This work
KDV692	N16961 <i>vpvC^{W240R}</i> , $\Delta rbmA$ contains plasmid pNUT542	This work
KDV698	N16961 <i>vpvC^{W240R}</i> , $\Delta crvA$, $\Delta rbmA$	This work
KDV815	N16961 <i>vpvC^{W240R}</i> , $\Delta rbmA$, $\Delta crvA$ contains plasmid pNUT542	This work
KDV829	N16961 <i>vpvC^{W240R}</i> , <i>rbmA::3xFLAG</i>	This work
KDV835	N16961 <i>vpvC^{W240R}</i> , <i>rbmA::3xFLAG</i> contains plasmid pNUT542	This work
KDV1026	N16961 <i>vpvC^{W240R}</i> , $\Delta crvA$, <i>rbmA:mRuby3</i>	This work
KDV1027	N16961 <i>vpvC^{W240R}</i> , $\Delta crvA$, <i>rbmA:mRuby3</i> and plasmid pNUT542	This work
KDV1082	N16961 <i>vpvC^{W240R}</i> , $\Delta rbmA$, $\Delta crvA$ contains plasmid pNUT1519	This work
KDV1218	N16961 <i>vpvC^{W240R}</i> , $\Delta crvA$ contains plasmid pNUT844	This work
KDV1228	N16961 <i>vpvC^{W240R}</i> , $\Delta crvA$ contains plasmid pNUT1734	This Work
KDV1231	N16961 <i>vpvC^{W240R} lacZ:P_{tac}-mKOKappa</i> and plasmid pNUT1734	This Work

Supplementary Table 5 – Bacterial strains used in this study.

Plasmids	Origin, Marker	Comments	References
pNUT015	pR6K, Amp ^R	pKAS32	Skorupski <i>et al.</i> ²⁹
pNUT144	pR6K, Amp ^R , Kan ^R	pKAS32 with Kan ^R	Drescher <i>et al.</i> ³⁰
pNUT336	pR6K, Amp ^R	pKAS32 $\Delta rbmA$ (<i>vc0928</i>)	Nadell <i>et al.</i> ³¹
pNUT462	pR6K, Amp ^R	pNUT144 <i>rbmA::3xFLAG</i> (translational fusion)	Drescher lab stock
pNUT542	pSC101*, Gent ^R	<i>P_{tac}-sfgfp</i>	Singh <i>et al.</i> ³²
pNUT844	p15a, Gent ^R	<i>P_{tac}-sfgfp</i> , <i>P_{vpst}-mRuby2</i>	Drescher lab stock
pNUT961	pR6K, Amp ^R , Kan ^R	pNUT144 $\Delta crvA$ (<i>vca1075</i>)	This work
pNUT1268	pSC101*, Gent ^R	pNUT542 with <i>P_{BAD}-rbmA</i> to replace <i>P_{tac}-sfgfp</i>	This work
pNUT1401	pR6K, Amp ^R , Kan ^R	pNUT144 <i>rbmA:mRuby3</i> (transcrip- tional fusion)	This work
pNUT1519	pSC101*, Gent ^R	pNUT1268 with <i>P_{tac}-sfgfp</i>	This work
pNUT1734	pSC101*, Gent ^R	pNUT1268 with <i>sfgfp</i> to replace <i>rbmA</i>	This work

Supplementary Table 6 – Plasmids used in this study.

Name	Sequence	Function
KDO1182	GGGTCTAGAGCTCGATATAGCGATCCACCACTTC	Plasmid pNUT961 construction
KDO1183	GAGTTTTACCCACACCGTCCCTG- CAACATATTTATGTTTAGCC	Plasmid pNUT961 construction
KDO1184	GGGACGGTGTGGGTAAAACCTCAGACCAAACAAAG	Plasmid pNUT961 construction
KDO1185	TTTGCGGCCGCCGCATGCCGTCATAGAAACCAG	Plasmid pNUT961 construction
KDO1433	TTTAAAGTTAGACAACGCAATATATCCTAG- TTATAAAAAATTTAACGCCA	Plasmid pNUT1268 construction
KDO1434	CTAGGATATATTGCGTT- GTCTAACTTTAAAGGATCTATCATGAACAAA	Plasmid pNUT1268 construction
KDO1435	TTATTTTTTTTACCACTGTCATTGACTGTTC	Plasmid pNUT1268 construction
KDO1436	ATGCATAAATAACAAAAACTGCTAAACGTT	Plasmid pNUT1268 construction
KDO1667	TTGCGGCCGCGTTACAAGAACCCGGAAGAATGTG	Plasmid pNUT1401 construction
KDO1716	CCTAGTGGCTTATTTTTTTTACCACTGTCATTGACTGTTCC	Plasmid pNUT1401 construction
KDO1717	CAGTGGTAAAAAATAAGCCACTAGGAGGTGGTTGATGGTG	Plasmid pNUT1401 construction
KDO1718	GAAGTGTATAAATAAATTTACCTAGTCACTTAGTCG- TATGTATAAAAAACCGC	Plasmid pNUT1401 construction
KDO1719	CTAAGTGACTAGGTAATTTATTTATACAG- TTCATCCATACCACCACCCAG	Plasmid pNUT1401 construction
KDO1720	GGTTGCTAGCGTCTACCTTGGCAGCACTAAAGGTACC	Plasmid pNUT1401 construction
KDO389	GCTTGCTCAATCAATCACCGGATCC	Plasmid pNUT1519 construction
KDO578	TTCACACCTCCTGTACGCGCGGC	Plasmid pNUT1519 construction
KDO2162	ATAACTAGGATATATTGCGATGAGCAAAGGTGAA- GAAGTGTTC	Plasmid pNUT1734 construction
KDO2163	GCTCATCGCAATATATCCTAGTTATAAAAAATTTAAC- GCCAG	Plasmid pNUT1734 construction
KDO2164	GAGCTCTACAAGTAAATGCATAAAA- TAACAAAAACTGCTAAACGTTATTC	Plasmid pNUT1734 construction
KDO2165	GTTATTTATGCATTTACTT- GTAGAGCTCGTCCATGCCGTGAG	Plasmid pNUT1734 construction

Supplementary Table 7 – DNA oligonucleotides used in this study. Sequences are given in the 5' → 3' direction.

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