Extracellular-matrix-mediated osmotic pressure drives *Vibrio cholerae* biofilm expansion and cheater exclusion

Jing Yan1,2, Carey D. Nadell1,3, Howard A. Stone 2, Ned S. Wingreen1 & Bonnie L. Bassler 1,4

Biofilms, surface-attached communities of bacteria encased in an extracellular matrix, are a major mode of bacterial life. How the material properties of the matrix contribute to biofilm growth and robustness is largely unexplored, in particular in response to environmental perturbations such as changes in osmotic pressure. Here, using *Vibrio cholerae* as our model organism, we show that during active cell growth, matrix production enables biofilm-dwelling bacterial cells to establish an osmotic pressure difference between the biofilm and the external environment. This pressure difference promotes biofilm expansion on nutritious surfaces by physically swelling the colony, which enhances nutrient uptake, and enables matrix-producing cells to outcompete non-matrix-producing cheaters via physical exclusion. Osmotic pressure together with crosslinking of the matrix also controls the growth of submerged biofilms and their susceptibility to invasion by planktonic cells. As the basic physicochemical principles of matrix crosslinking and osmotic swelling are universal, our findings may have implications for other biofilm-forming bacterial species.
Bacteria survive over a remarkable range of osmotic pressures\(^1\). Indeed, some bacteria can transition between fresh water and seawater, withstanding a change in osmotic pressure up to 50 atm (~2 Osm)\(^2\). Adaptation to extremes in osmolality depends on active and passive mechanisms that maintain constant osmotic pressure differentials between individual cells and the environment\(^1\)\(^2\). However, how bacteria respond collectively to osmotic pressure changes is not clear, particularly in spatially structured communities such as biofilms\(^3\)\(^4\), surface-attached bacterial collectives embedded in a secreted polymeric matrix\(^5\). Biofilms are an underlying source of chronic infection\(^6\), and clog networks and filters in industry\(^7\), but they are also useful in contexts such as waste-water treatment\(^8\) and microbial fuel cells\(^9\).

The biofilm matrix protects the embedded cells against environmental insults such as mechanical shear, predation, invasion, and antibiotics\(^5\)\(^10\). Major components of the typical matrix are extracellular polysaccharides (EPS), which function in conjunction with accessory proteins and, in some cases, extracellular DNA\(^11\). Intensive research has focused on defining the functions of the matrix components and the regulatory mechanisms driving matrix production\(^12\). Much less well studied are the physical nature and material properties of matrix networks\(^11\)\(^13\). In biofilms, the high local concentration of polymer molecules surrounding the cells necessarily produces an osmotic pressure difference between the matrix and the external environment\(^14\). This pressure differential is likely an important environmental parameter that varies depending on context, for example, from hypotonic fresh water to saline ocean water to hyper-saline sludge environments. The influence of osmotic pressure gradients on the growth characteristics of biofilms and the fitness of the bacteria residing in them remains under-explored beyond a few seminal studies\(^14\)\(^15\)\(^16\)\(^17\).

Pioneering work by Seminara et al. analyzing *Bacillus subtilis* colonies on agar plates suggested a crucial role for EPS-generated osmotic pressure differences in facilitating nutrient uptake\(^14\). Specifically, matrix-secreting colonies of *B. subtilis* expanded more rapidly than colonies of non-matrix-secreting cells, leading Seminara et al. to develop a theory for water transport into biofilms in which the biofilms were modeled as a viscous fluid with secreted EPS modeled as an extracellular osmolyte. Taking this precedent as motivation, we investigate the generality of osmotic-pressure-driven expansion of biofilms on air-solid interfaces as well as on submerged surfaces, using a different model bacterial biofilm producer, *Vibrio cholerae*\(^18\), the causal agent of the pandemic disease cholera. We systematically validate

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**Fig. 1** Osmotic pressure drives *V. cholerae* colony expansion. **a** Representative images of colony biofilms of the rugose (Rg_M) and EPS\(^-\) (ΔvpsL) *V. cholerae* strains grown for 2 days on LB medium containing the designated percentages of agar. Scale bar: 0.5 cm. **b** Colony biofilm diameter as a function of agar concentration (rugose (Rg_M: red) and EPS\(^-\) (ΔvpsL: cyan)). To mimic the swelling effect that occurs in colonies exposed to osmotic pressure changes, 20 μL liquid droplets of LB containing 15% dextran (~2000 kDa) were placed on semipermeable membranes on top of LB medium solidified with the designated concentrations of agar. Increases in the droplet weight were measured over time. **d** Diameters of 4-day-old colony biofilms of Rg_M (red) and ΔvpsL (cyan) strains grown on semipermeable membranes placed on LB medium solidified with the specified percentages of agar. Unpaired t-tests with Welch’s correction were performed for statistical analyses. **e** To differentiate the contribution of growth from that of passive swelling during colony expansion, Rg_M (red) and ΔvpsL (cyan) colonies were initially grown for 4 days on semipermeable membranes on top of LB medium solidified by 1.5% agar at 37 °C (denoted Original). The membranes containing the colonies were subsequently floated on LB liquid medium overnight at 4 or at 37 °C. To control for growth, a separate set of plates containing membranes with colonies that had been grown for 4 days were held at 4 °C overnight (denoted Control). In all cases, the diameters of the colonies were measured after 20 h. Paired t-tests were performed for statistical analyses. NS denotes not significant; * denotes P < 0.05, ** denotes P < 0.01, *** denotes P < 0.001, and **** denotes P < 0.0001. Error bars correspond to standard deviations with n = 3 in c and n = 4 in other panels.
the importance of the osmotic pressure gradient in driving colony expansion. We also examine the differences in biofilm growth and expansion that arise due to the hydrogel-like elastic properties of the *V. cholerae* biofilm matrix. In particular, we assess the consequences of osmotic pressure differentials on colony morphologies and we characterize the individual and combined contributions of particular extracellular matrix protein components to osmotic expansion. Finally, we explore the ecological consequences of osmotic pressure on biofilm-producing cells.

**Results**

**Osmotic pressure changes drive colony biofilm expansion.** To explore the physical principles connecting osmotic pressure differentials to colony biofilm growth, we studied a commonly used *V. cholerae* constitutive biofilm-forming strain 10. This strain has a missense mutation (vpsL<sup>CW240R</sup>) that causes elevated levels of the second messenger molecule c-di-GMP which, in turn, drives hypersecretion of the biofilm matrix 19. This strain is denoted R<sub>G</sub> for "rugose" and was used as the parent strain in all of the submerged biofilm analyses. To avoid the confounding effects of *V. cholerae* swarming on semi-solid media, we deleted the flagellar motor gene *pomA* 22 in the vpsL<sup>CW240R</sup> mutant background. The resulting strain, which is denoted R<sub>G</sub>_M, is the parent strain used for all of the colony biofilm analyses. All additional mutations and fluorescent reporter fusions were introduced into these two parent strains.

Figure 1a shows representative transmission microscopy images of 2-day-old *V. cholerae* R<sub>G</sub>_M colony biofilms grown at 37 °C on Luria-Bertani (LB) medium solidified with different percentages of agar. The R<sub>G</sub>_M parent (top row) shows a dramatic increase in colony size as a function of decreasing agar concentration, as quantified in Fig. 1b. For example, the colony diameter on a 0.6% agar plate is ~3 times larger than that on a 1.5% agar plate. In contrast, the sizes of colonies of an EPS- mutant (ΔvpsL; a gene required for synthesis of the key matrix polysaccharide) 23 do not depend on the agar concentration. These findings demonstrate that EPS production is essential for *V. cholerae* colony biofilms to expand differentially in response to changes in agar concentration.

We hypothesize that the differences in colony size on media containing various concentrations of agar stem from the different osmotic pressure contrasts across the colony-agar interfaces during active cell growth. This inference mirrors that of Seminara et al. 14 from their studies of the Gram-positive biofilm former *B. subtilis*. The internal osmotic pressure of the biofilms is primarily generated by the EPS, a macromolecular osmolyte secreted and maintained by the cells. The resulting interfacial osmotic pressure contrast could boost colony biofilm expansion by drawing nutrient-carrying flow through the interface, thereby promoting growth and at the same time physically swelling the entire colony. To test this physical picture, we devised an experiment to mimic features of the process occurring in Fig. 1a. We placed liquid droplets containing the macromolecular osmolyte dextran (15%: molecular weight ~2000 kDa) on semipermeable membranes on top of agar plates containing different percentages of agar (Fig. 1c and Supplementary Fig. 1). The semipermeable membranes prevent the dextran polymers from diffusing into the agar, simulating the EPS surrounding and attached to biofilm cells. However, liquid can pass through the membrane, and the direction and speed of the flow will depend on the osmotic pressure difference. Indeed, uptake of water from the agar plate across the membrane and into the dextran solution occurred on the time scale of a few hours. Moreover, lower percentages of agar generated higher osmotic pressure differences across the interfaces, and hence, increased water uptake by the droplet. The results from this model experiment parallel the colony biofilm size measurements (Fig. 1a, b). Hence, we find that biofilm matrix-producing cells could employ the same physical driving force, i.e., the osmotic pressure contrast, to expand their colony biofilms.

An alternative or additional mechanism that could contribute to the observed dependence of biofilm colony size on agar concentration concerns the change in agar stiffness that occurs as a function of agar concentration 24. To separate mechanical contributions stemming from agar stiffness from contributions arising from osmotic pressure on colony growth, we grew *V. cholerae* colony biofilms on semipermeable membranes atop different percentage agar plates (Fig. 1d). In this arrangement, because the bacteria are not in direct contact with the agar, the only differential interaction they experience is the osmotic-contrast driving force that depends on the underlying agar concentration. Colony biofilm size continued to be negatively correlated with the underlying agar concentration in the R<sub>G</sub>_M strain, verifying that the osmotic pressure contrast plays a role in determining colony biofilm size. We also note that the overall colonies expanded much slower when on membranes than when grown directly on the agar, presumably due to the increased friction colony biofilms experience on the semipermeable membrane surface compared to on agar. Notably, colony biofilm growth of the ΔvpsL strain slowed to a much larger extent than did growth of the R<sub>G</sub>_M strain on the membrane compared to on agar: the ΔvpsL colony biofilms were two–three times smaller in diameter than the R<sub>G</sub>_M colony biofilms, irrespective of the agar concentration. We hypothesize that the spreading of the droplet-like colonies of the ΔvpsL strain was retarded more on the semipermeable membrane than on agar because of the membrane’s reduced wettability (shown by its larger contact angle in Supplementary Fig. 2) 16. We can compare these results to those in Fig. 1b, in which the size of the R<sub>G</sub>_M colony biofilm is larger than that of the ΔvpsL strain on low-concentration agar but smaller than the ΔvpsL strain on high-concentration agar, with a crossover agar concentration between 0.8 and 1.0%. On agar plates, the presence of the polymeric matrix hampers the expansion of the R<sub>G</sub>_M colony biofilm when the osmotic differential is low. With decreasing agar concentration, increased R<sub>G</sub>_M cell growth and biofilm expansion occur because the osmotic differential dominates the growth/expansion process. We note that such a crossover in relative size is not observed in the case of *B. subtilis* biofilms 14, in which the matrix-producing colonies are consistently larger than non-producing colonies, suggesting a fundamental difference in matrix properties between the two species, which we investigate further in later sections.

The above results demonstrate a role for osmotic pressure in assisting *V. cholerae* colony biofilm expansion, and suggest two interdependent mechanisms underlying expansion: (1) physical swelling of the entire colony biofilm and (2) enhanced cell division due to increased influx of nutrients. To prove that both phenomena are relevant and to quantify the respective contributions of the two mechanisms, we first grew *V. cholerae* R<sub>G</sub>_M colony biofilms on semipermeable membranes on top of 1.5% agar plates. Subsequently, each membrane containing a colony biofilm was transferred to the surface of liquid LB medium, which generates an even larger osmotic pressure imbalance than does the agar plate. We compared the colony biofilm sizes before and after overnight flotation on the liquid medium. We performed the experiment at both 4 and 37 °C to control for the cell growth rate (Fig. 1e). Control experiments show that cell doublings at 4 °C were negligible. Nonetheless, at 4 °C, the R<sub>G</sub>_M colony biofilm diameter expanded by 16% following exposure to the increased osmotic pressure contrast.
Therefore, physical swelling does occur. Moreover, the colony biofilm diameter increased by a total of 38% if growth was allowed (37 °C, Fig. 1e). Finally, neither of these colony biofilm expansion mechanisms occurred in colonies lacking EPS (Fig. 1e). We conclude that both passive physical swelling of the colony matrix and enhanced nutrient uptake leading to increased cell doubling contribute to \textit{V. cholerae} colony biofilm expansion, and both processes are only possible if the cells produce EPS, which functions as an extracellular osmolyte.

**Osmotic pressure drives biofilm spatial architecture.** Bacteria form distinct colony architectures on agar plates, reflecting different underlying growth and patterning programs\cite{25,26}. We therefore investigated what changes occur in the colony biofilm architecture in response to osmotic-pressure-driven expansion. \textit{V. cholerae} Rg\_M colony biofilms contain three identifiable regions (Fig. 2a). Region I is located at the center and possesses randomly oriented ridges. Region II is a transition zone of decreasing biofilm thickness. The most interesting area is region III, at the outer-most zone, featuring occasional, radially orientated ridges extending to the periphery of the colony biofilm. In between the ridges, the colony is essentially flat (i.e., two dimensional), as judged from the homogeneous intensity in the transmission image (Fig. 1a). Region I shows no size dependence on agar concentration, whereas both regions II and III show strong size dependence (Fig. 2b). \textit{ΔpsiL} mutant colonies lack these visible architectural features and appear as homogeneous droplets (Fig. 1a).

We propose a model, based on the above data, to account for these three regions (Fig. 2c). For bacterial cells that are unable to produce EPS, expansion occurs exclusively through cell division\cite{14}, and nutrients enter the colony biofilm via diffusion (Fig. 2c, top)\cite{27}. Over time, the cells at the core become depleted of nutrients, and therefore only cells at the edge of the community grow\cite{28}. In colony biofilms that possess non-crosslinked or weakly crosslinked EPS (Fig. 2c, middle and discussed further in the next section), an osmotic pressure difference across the biofilm-substratum interface enhances nutrient uptake; however, the overall colony biofilm remains featureless, with a smooth 3D contour. By contrast, the \textit{V. cholerae} Rg\_M colony biofilm expands in 2D, coupled with ridge formation characteristic of a thin elastic material\cite{29,30}. These observations are consistent with the reported elastic properties of \textit{V. cholerae} biofilms\cite{31}. We therefore suggest that the \textit{V. cholerae} biofilm matrix is similar to a crosslinked hydrogel (Fig. 2c, bottom)\cite{13,32}. Within this context, we can rationalize how the morphological features of the \textit{V. cholerae} Rg\_M colony biofilm arise. Region I is the nutrient-depleted region, which is characterized by higher cell death relative to other regions in the colony biofilm (Fig. 2d)\cite{33}. In
regions II and III, the EPS hydrogel network swells and takes up nutrients, which increases cell growth. The colony biofilm first extends in a 3D fashion (region II), but subsequently, transitions to a 2D thin sheet (region III) attached to and expanding on top of the agar. This 3D-to-2D transition is possible only for colony biofilms possessing sufficiently strong internal stiffness and adhesion to the substrate, both of which are provided by the V. cholerae matrix. This 2D growth mode contrasts with the 3D droplet growth mode proposed to underpin the expansion of B. subtilis biofilms. The characteristic radial ridges in the 2D growth mode, we believe, are formed by localized delamination of the biofilm from the substrate, similar to a mechanical blister.

**Osmotic expansion is influenced by matrix proteins.** To provide further support for the model in Fig. 2c, we altered the physical properties of the biofilm matrix to reduce elasticity and/or surface adhesion using mutants in biofilm protein components. The Yildiz group identified three proteins required for the assembly of the V. cholerae matrix and proper biofilm formation. The RbmA protein links mother-daughter cells together, and the RbmC and Bap1 proteins play partially redundant roles, with RbmC being primarily responsible for interaction with the EPS and Bap1 being primarily responsible for surface adhesion. To investigate the contributions of these proteins to osmotic-pressure-driven colony biofilm expansion, we generated ΔrbmA, ΔrbmC, and Δbap1 single, double, and triple mutants in the Rg_M background, and we repeated the colony expansion experiment from Fig. 1a, b. In terms of colony size, the ΔrbmA mutant does not differ significantly from the parent (Fig. 3a). By contrast, if both bap1 and rbmC are deleted, in either the Rg_M or the ΔrbmA background, the pattern of decreasing colony size with increasing agar concentration is significantly attenuated. We hypothesize that in order for the colony biofilm to expand as a thin sheet, rather than cells simply piling up, strong adhesion to the substratum is required, and this function is provided by Bap1 and RbmC. Indeed, consistent with the primacy of Bap1 in surface adhesion, the single Δbap1 mutant exhibits a more severe phenotype than the single ΔrbmC mutant (Supplementary Fig. 3).

The mutant colony biofilm morphologies are also qualitatively different, most notably on the agar plates containing 0.6% agar (Fig. 3b). Specifically, the ΔrbmA mutant colony biofilms form much smaller ridges in region III than the Rg_M parent, consistent with the reduced mechanical strength of ΔrbmA biofilms. The Δbap1ΔrbmC double-mutant colony biofilms exhibit a curious star-shaped pattern: each delaminated ridge expands into a wedge shape rather than a straight line, consistent with reduced adhesion of the colony biofilm to the surface. The ΔrbmAΔbap1ΔrbmC triple-mutant colony biofilm is completely smooth. We propose that, similar to the early stage of B. subtilis biofilm development and the mucoid phenotype of Pseudomonas aeruginosa, in the absence of the matrix protein components, the remaining polysaccharide EPS network is still capable of generating an osmotic pressure contrast, albeit reduced compared to the wild type (Fig. 3a, yellow bars). However, the polysaccharide component of the matrix alone does not possess sufficient elasticity or surface adhesion to support 2D expansion. In summary, the protein and polysaccharide components of the extracellular matrix combine to foster the characteristic two-dimensional, osmotic-pressure-driven expansion of V. cholerae colony biofilms on nourishing surfaces.

**Osmotic expansion defends biofilms against cheaters.** The enhanced nutrient uptake enabled by osmotic pressure contrast could be considered a mechanism that supplies public goods, benefiting all the cells embedded in the matrix. If so, an interesting question arises concerning whether “cheater” cells—i.e., those which do not produce matrix components—can take advantage of this public good without paying the cost of matrix production. To probe this possibility, we used the ΔvpsL strain as the putative cheater (Supplementary Fig. 4). We
co-inoculated the Rg_M strain with the ΔvpsL strain at a 1:1 ratio and performed competition experiments on LB medium containing different agar percentages for 2 days at 37 °C (Fig. 4a).

On 1.5% agar, the Rg_M strain was overgrown by the ΔvpsL mutant, restricting the Rg_M strain to the nutrient-depleted region at the center of the colony biofilm. Upon decreasing the agar percentage, the Rg_M strain expanded in size, and, strikingly, physically displaced the ΔvpsL strain, relegating it to the periphery of the expanding colony biofilm.

We analyzed the composition of the colony biofilms to quantify the competition results. Increased growth of the Rg_M strain compared to the ΔvpsL mutant occurred with decreasing agar percentage (Fig. 4b). Specifically, in competition on 1.5% agar, the absolute frequency of the Rg_M strain decreased by 0.25, whereas on 0.6% agar, its absolute frequency increased by 0.21. Consistent with the cell counts, Fig. 4c shows the quantitation of the exclusion effect, demonstrating that the sizes of the regions occupied by the ΔvpsL cells decrease as the Rg_M colonies increase their osmotic-pressure-driven expansion. Figure 4d shows a schematic representation of the proposed physical exclusion process. Red and cyan cells denote the matrix producer and non-producer, respectively. Red wavy lines depict matrix polymers.
Response of submerged biofilms to osmotic pressure differences. In addition to colony biofilms on surfaces, *V. cholerae* also frequently exists in submerged biofilms. The osmotic pressure contrast effects we observed above for *V. cholerae* colony biofilms at air-solid or air-liquid interfaces could also be relevant for submerged biofilms. Directional cell proliferation and tight cell packing are the main processes underlying the compact development of 3D submerged *V. cholerae* Rg biofilms. By contrast, the ΔrbmA strain forms larger, loosely packed submerged biofilms with increased cell-to-cell distances.

We have suggested previously that in the ΔrbmA biofilm, increased expansion of the EPS fills the spaces between cells. Our present results lead us to hypothesize that the enhanced polymer expansion in the ΔrbmA submerged biofilm is osmotic in origin: the presence of a high local concentration of polymers generates an osmotic pressure differential that draws water into the biofilm, forcing the cells apart. By contrast, polymer expansion in the submerged Rg biofilms is restricted by the rigid scaffold formed by RbmA-mediated cell-to-cell linkages.

To prove that osmotic-pressure-driven polymer expansion controls the Rg and ΔrbmA submerged biofilm architectures, we measured their responses to osmotic pressure contrast changes using confocal microscopy. Addition of 15% dextran to the growth medium increases the osmolarity of the medium by ~50 mOsm as measured by vapor pressure osmometry (Supplementary Fig. 1). Such an addition causes a hyperosmotic shock to an existing submerged biofilm. This hyperosmotic shock caused minimal change to the sizes of submerged Rg biofilms, whereas the same shock caused submerged ΔrbmA biofilms to dramatically shrink (Fig. 5b). Shrinkage must be a physical response of the polymer network to the osmotic pressure change because the experimental time scale (~5 min) was too short for biological responses such as an alteration in matrix production.

We measured the change in the probability distribution $P(r)$ of the cell-to-cell distance $r$ in the different submerged *V. cholerae* biofilms. Rg biofilms have an average $r$ around 1.9 μm that changes minimally (<2%) upon hyperosmotic shock. The peak $P(r)$ of the ΔrbmA submerged biofilm upon hyperosmotic shock shifts from 3.0 to 2.6 μm, corresponding to a volume decrease of ~32% (Fig. 5c and Supplementary Fig. 5). The behavior of the Δbap1ΔrbmA submerged biofilm is similar to that of the Rg strain: the peak position (2.0 μm) changes minimally upon hyperosmotic shock (<5%). The effect of hyperosmotic shock on the ΔrbmAΔbap1ΔrbmA submerged biofilm is the largest with a peak $r$ of 4.4 μm that shifts to 3.2 μm corresponding to a 60% decrease in volume (Fig. 5c and Supplementary Fig. 5).

We interpret the above results starting with the case of the ΔrbmAΔbap1ΔrbmA triple mutant, which contains the least crosslinked EPS network. Upon hyperosmotic shock, the increasing osmotic pressure in the external environment drives water out of the biofilm, causing the matrix to shrink. With respect to the ΔrbmA submerged biofilm, in this case, RbmC and Bap1 are present and are known to interact with the polysaccharide component of the matrix. We suggest that these interactions can be conceptualized as providing crosslinks within the EPS network and, in so doing, they limit the swelling of the EPS network during biofilm formation and thus attenuate its response to hyperosmotic shock. With respect to the Δbap1ΔrbmA mutant and the Rg parent strain, in these cases, RbmA is present and it connects the cells to one another. We suggest that RbmA creates a dense scaffold of cells to which the EPS adheres. This stiff cell-based scaffold strongly restricts the expansion of the EPS in the Rg and Δbap1ΔrbmA strains during submerged biofilm growth, and consequently, limits shrinkage of the EPS upon hyperosmotic shock. The absence of this cell-based scaffold underlies the differences in the submerged biofilm architectures, with the Rg architecture driven primarily by extension of the cellular scaffold due to directional cell division and the ΔrbmA architecture driven primarily by osmotic-pressure-mediated enlargement of the EPS network.

We can estimate the osmotic pressure $Π$ acting on the submerged biofilms by calculating the total polymer volume...
fraction \( \phi \) in the biofilm, which is roughly 0.04 for the Rg strain (Supplementary Discussion). According to a scaling law for a semi-dilute polymer solution of ideal chains\(^{33, 52} \), we estimate \( \Pi_{\text{EPS}} \sim kTb^2 \times \phi^3 \), in which \( kT \) is the thermal energy, \( b \) is roughly the linear size of a sugar monomer (~0.5 nm), and the \( \phi^3 \) dependence arises from polymer-polymer excluded volume. This treatment yields a value \( \Pi_{\text{EPS}} \sim 2 \text{kPa} \), which falls well within the range measured for biofilm elastic moduli (10–10,000 Pa)\(^{33, 54} \). The strong (cubic) dependence of \( \Pi_{\text{EPS}} \) on \( \phi \) means that the elastic modulus of the \( \Delta rbmA \) strain, for which \( \phi \sim 0.01 \), will be \~64 times lower than that of the Rg strain, which explains the much reduced mechanical strength of the \( \Delta rbmA \) biofilm\(^{20, 31} \).

Osmotic pressure modulates biofilm resistance to invaders. In nature, biofilms are challenged by invader cells that can take advantage of public goods produced by the cells residing in the existing biofilm community\(^{35, 36} \). Invader cells can be distinguished from cheater cells because invaders encounter surfaces occupied by existing biofilms. A key determinant underlying resistance to invasion is biofilm compactness\(^{35} \). In the context of our current work, the reduction in cell-to-cell distance that occurs in the \( \Delta rbmA \) submerged biofilms upon hyperosmotic shock suggested to us that vulnerability to invader cells could also be controlled by osmotic pressure. To test this hypothesis, we performed an invasion assay using motile \( \Delta vpsL \) cells as the invaders and the \( \Delta rbmA \) strain or the Rg strain as the resident submerged biofilm. Consistent with previous results\(^{35} \), invader cells could not penetrate the submerged Rg biofilms but could invade the submerged \( \Delta rbmA \) biofilms (Fig. 6a, b). However, in the presence of 15% dextran, the \( \Delta rbmA \) resident biofilms became compact and, importantly, resistant to invasion (Fig. 6c). To quantify these results, we analyzed the distribution of invader cells in the resident biofilms as a function of penetration depth (Fig. 6d). The Rg curve has a sharp decay: the number of invader cells declines by more than a factor of three by a depth of 3 \( \mu \)m into the submerged biofilm. By contrast, the \( \Delta rbmA \) curve shows a much slower decay. Indeed, invader cells are present deep within the biofilm (>30 \( \mu \)m). Upon the addition of 15% dextran, however, the \( \Delta rbmA \) invasion curve shows a sharper decay, approaching that of the Rg submerged biofilm. This observation is consistent with the data in Fig. 5c: the cell-to-cell distance decreases in the submerged \( \Delta rbmA \) biofilm in the presence of dextran, approaching the cell-to-cell distance in the Rg submerged biofilm. We note that the increased viscosity of the osmolyte solution could further reduce invasion by slowing the swimming speed of the invaders\(^{35} \). However, our control experiment shows that the presence of dextran does not affect invasion of the Rg-resident biofilms under our experimental conditions, suggesting that swimming speed is not the limiting factor in invasion (Supplementary Fig. 6).

Discussion

We have demonstrated here the importance of osmotic pressure generated by the hydrogel-like \( V. \) *cholerae* EPS matrix to the growth and surface coverage of \( V. \) *cholerae* biofilms residing at air-solid interfaces and submerged in liquids. The physical principles underlying the behavior of this biological hydrogel should be applicable to other biofilm matrices, although the exact expansion/shrinkage characteristics due to changes in osmotic pressure will vary depending on the specific biochemistry of the different matrix polysaccharides and the functional roles of the participating proteins. The success of cheater and invader cells is strongly affected by osmotic pressure, which could provide an explanation for reports that submerged biofilms can exclude matrix non-producers\(^{35, 57, 58} \). Our results, furthermore, underscore the importance of understanding the physical forces that together with biochemical and genetic features drive biofilm ecology. The physical responses of the biofilm matrix to osmotic pressure offer possibilities to use external osmolytes to control the accessibility of small molecules such as antibiotics and signaling compounds\(^{39} \) to biofilm-dwelling cells. Such manipulation could be used to remove harmful biofilms or to enhance the robustness of beneficial biofilms by osmotically weakening or strengthening the biofilm structures, respectively.

With respect to the relevance of our findings to the natural ecology of \( V. \) *cholerae*, this bacterium resides in fluctuating environments that undergo extreme changes in osmotic pressure\(^{60, 61} \). Indeed, \( V. \) *cholerae* cycles between the ocean and fresh water aquatic environments and it resides in the human intestine during infection\(^{62} \). \( V. \) *cholerae* forms robust biofilms in each of these niches\(^{33, 63, 64} \). We suggest that a scaffold is formed by RbmA-mediated interconnections among the cells, and this structure plays an overarching role in restricting the osmotic expansion of the underlying polysaccharide network. Recently, a new intracellular niche was discovered for \( V. \) *cholerae* in the marine amoeba \( Acanthamoeba castellani\)\(^{65} \). Interestingly, \( V. \) *cholerae* cells survive and replicate in this host’s contractile vacuole, a dynamic organelle involved in the osmoregulation of the amoeba. Following intracellular replication, wild-type \( V. \) *cholerae*, but not EPS mutants, can destroy this organ and disperse from the host. We speculate that the capacity of the wild type to successfully disseminate from the vacuole could be related
to the osmotic pressure response of the biofilm matrix shown here. Similar osmotic effects could also influence *V. cholerae* colonization of the mucus layer in the human intestine, since mucus is a bio-hydrogel that expands and contracts in response to polymer concentrations in the gut.17

### Methods

#### Strains and media. All *V. cholerae* strains used in this study are derivatives of the wild-type *V. cholerae* O1 biowar El Tor strain C676/66, harboring a missense mutation in the *opxA* gene (*VpvC W240R*). Additional mutations were engineered into this strain using *Escherichia coli* S17 λpir carrying pKAS32.26. All strains were grown in LB medium at 37 °C with shaking. When designated, fresh LB medium solidified with different percentages of agar was used. Submerged biofilms were grown in M9 minimal medium, supplemented with 2 mM MgSO₄, 100 μM MnCl₂, and 0.5% glucose as the carbon source. To generate hypertonic shock to submerged biofilms, 15% dextran (1500–2800 kDa, Sigma-Aldrich) solutions were prepared by adding dextran powder to M9 medium or LB medium followed by shaking at 30 °C for at least 3 h. A detailed strain list is provided in Supplementary Table 1.

**Statistical analysis.** Throughout the text, error bars correspond to standard deviations of the mean ± 1 S.D. for each condition and for each time point, and each replicate contained four biological replicates. The data presented in Fig. 5c were obtained by combining all neighboring cell pairs in each biofilm from different experiments.

**Confocal microscopy.** Images were acquired with a Yokogawa CSU-X1 confocal spinning disk unit mounted on a Nikon Ti-E inverted microscope, using a ×60 water objective with a numerical aperture of 1.2 and an Andor iXon 897 EMCCD camera. For each experiment, a ×43 laser (Omega DPSS) was used to excite cells expressing mKùO, and a ×1.5 post-magnification lens was used to obtain sufficient magnification for automated cell segmentation. The magnification was 166 nm/pixel in the xy plane with a 200 nm step size in the z direction. The point spread function of the system was measured under identical conditions (with 50 nm z-step size) using 200 nm fluorescent polystyrene beads (ex540/em561 Life Technologies). For the invasion experiment, two diode lasers (543 and 592 nm) were used sequentially along with customized dichroic filters (Chroma) for spectral separation. For live-dead staining, 488 and 592 nm lasers were used combined with the appropriate filter sets. All experimental images in this work are raw images rendered by Nikon Element software.

**Colony biofilm growth on agar plates.** *V. cholerae* strains were streaked on LB plates containing 1.5% agar and grown at 30 or 37 °C overnight. Individual colonies were selected and inoculated into 3 mL of LB liquid medium containing glass beads, and the cultures were grown with shaking at 37 °C to mid-exponential phase (5–6 h). Subsequently, the cells in the cultures were mixed by vortex, OD₆₀₀ was measured, and the cultures were back-diluted to an OD₆₀₀ of 0.5. An aliquot of 1 μL of this inoculum was spotted onto pre-warmed agar plates solidified with different percentages of agar. The plates were inverted and incubated at 37 °C for 2 days. Four colonies were grown per agar plate. After 2 days of growth at 37 °C, the entire plate was imaged with an ImageQuant LAS-4000 gel imager (GE Healthcare) in transmission mode. Nikon Element software was used to quantify the diameters of the colony biofilms. When semipermeable membranes were included, the procedure was identical except the inocula were applied to EMD Millipore, VSWP002500 semipermeable membranes that had been placed on top of the agar. Also, growth was extended to 4 days, and imaging was performed in the reflection mode since the semipermeable membranes are not transparent. Two technical replicates were performed for each biological replicate, and four biological replicates were performed. In the experiments in Fig. 1e, agar plates were transferred to 4 °C (denoted Control) and the semipermeable membranes harboring the colony biofilms were floated in 100 mm petri dishes containing 25 mL of liquid LB overnight at 4 or 37 °C. The semipermeable membranes were subsequently transferred to a plastic tray and imaged with the gel imager. One technical replicate was performed for each biological replicate and four biological replicates were performed.

**Quantification of cell death in the colony biofilms.** *V. cholerae* colony biofilms were grown for 2 days at 37 °C as described above. Different regions of the colonies were isolated using a pipette tip and the cells were resuspended in M9 medium lacking a carbon source but containing a 1:1000 dilution of the LIVE/DEAD BacLight bacterial viability kit components (Thermo Fisher). The suspension was centrifuged, resuspended in wash, 425 μL, and incubated at 37 °C for 5 min, and subsequently, 5 μL of the suspension was placed between the cube of the solution was placed onto a clean LB plate. The contact angle measurements were performed. The contact angle measurements were performed with a goniometer with a numerical aperture of 1.4. The number of cells in ten 100 × 100 μm sections of each region from two colonies was quantified for each biological replicate. For quantification of cell death in the colony biofilms, it was not technically possible to isolate region II from region III because they are continuous. Automatic cell finding codes were written in Matlab to track the number of live (green) and dead (red) cells in these regions and their frequencies were subsequently calculated.

**Competition experiments between matrix-producing cells and matrix-non-producing cheater cells.** Mid-exponential phase cultures of *V. cholerae* strains or *E. coli* were used as described above. Cultures were transferred to 2 mL glass vials containing glass beads (Acid-washed, 425–500 μm, Sigma), and vigorously mixed by vortex. These steps were necessary to disassemble cell clusters that had formed in the liquid cultures enabling accurate OD₆₀₀ measurement and equalization. An inoculum containing a total number of cells equal to OD₆₀₀ = 0.5 was prepared with 1:1 mixture of the two competing strains. An aliquot of 1 μL of this inoculum was spotted onto pre-warmed agar plates solidified with different percentages of agar. The initial inoculum was temporarily frozen after mixing with 80% glycerol, and the exact frequency f₀,Rgm in the inoculum was measured at a later point. After 2 days of colony growth at 37 °C, the entire colony was carefully harvested using the pipette tip and suspended in 1 mL of M9 medium lacking a carbon source, mixed using glass beads for 30 min at 37 °C, serially diluted in M9 medium lacking a carbon source, and subsequently, plated on LB plates containing 1.5% agar.

### Hypersmotic shock experiment

Submerged biofilms were grown as described above, and imaged with confocal microscopy. Immediately after imaging, the medium was replaced with fresh M9 medium lacking glucose but containing 15% dextran, and the wells were imaged again. Whenever possible, the same biofilms were imaged prior to and after hypersmotic shock. As noted in the legend of Supplementary Fig. 5, this was not always possible with the ΔstrAΔmkbC and ΔrenAΔskpAΔmkbC biofilms because they adhere only poorly to the substratum. In these cases, different biofilms were imaged pre- and post-hypersmotic shock.

Automatic cell segmentation steps were performed as described. After segmentation, the position of each cell was recorded. 3D Delaunay triangulation was performed to identify neighboring cell pairs, with a threshold distance of 6 μm. Due to surface attachment, cells close to the substratum pack more densely than those in the bulk, and this effect is most prominent in ΔmkbC biofilms. To avoid this substratum effect, we only included cells more than 6 μm away from the surface in our analyses; the exact threshold, however, does not significantly affect our conclusions. For each biofilm cluster, a Gaussian fit to the probability distribution of the cell-to-cell distance r was applied to obtain the average r. The average cell volume was obtained by integrating the volume function and after hypersmotic shock. Three clusters were analyzed for each biological replicate, and four biological replicates were performed for each strain. The data presented in Fig. 5c were obtained by combining all neighboring cell pairs in each biofilm from different experiments.

**Swelling of an osmolyte solution on an agar plate.** Semipermeable membranes (EMD Millipore, VSWP00300) were placed on LB plates containing different agar concentrations for at least 30 min to allow them to equilibrate to room temperature. Each plate was placed on a balance, and a 20 μL aliquot of dextran solution in LB was applied to the top of the semipermeable membrane, and its weight recorded. Swelling was allowed to proceed for the designated times at room temperature, and subsequently, the membrane along with the solution was transferred onto a balance. The total weight of the droplet plus the membrane was measured, and the weight of the membrane was calibrated separately. Using this method, the instantaneous weights of the droplets were obtained. Three replicates were performed for each condition and for each time point, and each replicate contained two technical replicates.
In separate experiments, the 2-day-old colonies containing the mixtures of competing strains were directly imaged with the gel imager and the appropriate filter sets (Cy2 and Cy5). The sizes of the regions occupied by the different strains were quantified using Nikon element software. All competition experiments were repeated three times using Rg M cells harboring mKate2 and Δpsl cells harboring mTFP1. The experiment was repeated three times with the fluorescent marker exchanged. Each biological replicate in the competition experiment contained two technical replicates. Images in Fig. 4a were obtained using a Leica stereoscope with CFP and mCherry filter sets or with no filter (reflection mode).

**Invasion of resident biofilms.** Confluent resident biofilms were grown according to the above procedure. Invader cells were grown overnight at 37 °C in liquid LB medium with shaking, back-diluted 30-fold, and grown for an additional 3 h with shaking in LB medium until mid-exponential phase (OD600 = 0.5–1.0). Cultures of known OD600 were transferred to Eppendorf tubes, pelleted, and washed once with PBS. Fresh M9 medium (no glucose) with or without 15% dextran was added to make the final OD600 of 1.0. The Eppendorf tubes were subjected to vigorous vortex for 10 min, and subsequently, the cell suspension was taken out and the resident biofilms were washed three times prior to imaging. Six different locations each with a total size of 250 × 250 μm with a 2-mm interval of 3 μm were imaged in each biological replicate. The total imaging height was 60 μm if ΔrbmA cells were the resident biofilm or 36 μm if the Rg cells were the resident biofilm. Each invasion condition was repeated twice with resident biofilm harboring mKate2 and invading cells harboring mKO. The experiment was performed twice with the fluorescent markers reversed. Invader cell numbers were analyzed with custom-written Matlab codes.

**Data availability.** The data supporting the findings of this study are available from the corresponding authors on request. Custom-written Matlab scripts used in this study are available at https://github.com/yanjing32/Osmotic-Pressure-Project.

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**Author contributions**

J.Y. and B.L.B. initiated this work. J.Y. performed the experiments. J.Y., C.D.N., H.A.S., N.S.W., and B.L.B. analyzed the data. All authors contributed to writing the paper.

**Additional information**

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