Three-dimensional architecture of *Vibrio cholera* biofilms

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Microbes That Are out of Place

Inspired by Lord Chesterfield, anthropologist Mary Douglas noted that what is classified as dirt in a given society is matter that is considered out of place. From this premise, Douglas went on to an analysis of ritual and religion, interrelations between perceptions of purity, contamination, defilement, and danger, and our anthropological need for symbolic boundary maintenance (1). (Judaic kosher dietary laws are well-known examples of her analysis.) Douglas probably did not anticipate that these ideas may have an unexpected valence in the way we think about microbial communities. What happens when a microbe is "out of place" and what are the associated dangers? Because microbial communities are found in diverse terrestrial environments, from the earth to the ocean to the atmosphere to environmental niches in hosts, such as humans and other animals, they impact, transform, and sustain life as we know it on the planet. However, this ubiquity also gives plenty of opportunities for microbes to be found out of place, to adapt and precipitate diverse unexpected consequences. *Staphylococcus epidermidis* is normally an innocuous commensal microbe found on human skin, but it has also become the most common source of infections on implanted biomedical devices, such as vascular catheters (2). There is even evidence that its more virulent cousin, *Staphylococcus aureus*, became the "superbug" known as methicillin-resistant *Staphyloccus aureus* (MRSA) by acquiring the methicillin-resistance cassettes from *S. epidermidis* (2). Other examples of misplaced microbes that become dangerous or even lethal include *Clostridium difficile* infections in the gut, *Pseudomonas aeruginosa* infections in cystic fibrosis airways, and *Vibrio cholera*, the aquatic microbe that sometimes finds its way into a human host, and the subject of the present study by Drescher et al. in PNAS (3).

Being out of place is not always bad: the commensal microbes in our gastro-intestinal tracts "train" our immune systems and allow us to digest vegetables. Engineered environmental microbes can degrade plant cell walls for generating biofuels. In current thinking, strategic management of microbial communities has the potential to impact diverse medical conditions, including asthma, diabetes, obesity, psychiatric illnesses, and (of course) infectious diseases (4, 5). Microbiomes can be targets for new drugs, but they can also be sources of new drugs (6), given the constant competition among community microbes in diverse environments. It is humbling that despite our long acquaintance, we have recognized only relatively recently how narrow our knowledge of microbial communities is. This knowledge gap is all the more keenly felt given the importance of microbes to current concerns in health, energy, the environment, and agriculture. In fact, the recently proposed Unified Microbiome Initiative seeks to address this need to understand microbiomes in a broad range of contexts (7, 8). We often do not know the precise compositions, roles, structures, interactions, and dynamics of microbial communities. One of the significant obstacles to progress is the general lack of appropriate tools. We need to have measurement technologies that can enable and speed up basic science discovery and translation to applications (9). Drescher et al. make an impressive contribution in this area, by visualizing 3D biofilms of *V. cholera*, and their intricate processes of formation (3).

Vibrio cholera Biofilms and their 3D Structure

V. cholera is a Gram-negative bacterium that lives in coastal and brackish waters. Pathogenic strains of *V. cholera* are the causative agents for cholera, the acute diarrheal disease that can kill within 12 h of the first symptoms (10, 11). The biofilm mode of bacterial organization has been shown to be important for the survival, dissemination, and transmission of *V. cholera* (12–14), and provides enhanced protection from different types of environmental stresses, such as nutrient limitation, predation stress (protozoan grazing, host immune system), and attack by viruses (bacteriophages) (15, 16). Studies have shown that *V. cholera* are connoisseurs of surfaces. They prefer to form biofilms on surfaces of phytoplankton and zooplankton (17, 18). The chitin found on the exoskeletons of the latter can

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provide them with a carbon source and induce natural competence (19). Recent work even suggests that *V. cholera* have adapted near-surface motility strategies to allow them to assay a surface before attachment to start the biofilm program of development (20). Despite the importance of biofilm organization to the *V. cholera* life cycle, we do not really know what a mature, *V. cholera* biofilm looks like at single-cell resolution. The majority of biofilm studies involve simple microscopy experiments where bacteria are fluorescently labeled so that biofilms appear as large blobs of biomass.

In a technical tour de force, Drescher et al. (3) combine sophisticated segmentation and image analysis algorithms with a custom-built spinning-disk confocal microscope to image the evolving 3D architecture of *V. cholera* biofilms on glass, ranging from communities that contain a few cells to those that contain thousands of cells. The authors reconstructed the evolution of *V. cholera* biofilms by using ensemble averages of biofilm structure at various stages of development, and discovered four basic phases of growth. For communities of ~1–6 cells, the growth was predominately 1D, where communities elongate along one direction; for ~20–100 cells, the growth evolved to 2D growth, and for ~200–1,000 cells, the growth became fully 3D. Interestingly, as the community increased in size to more than 2,000 cells, domains with an increase in the orientational order began to emerge. A key factor in the success of these experiments was the use of high information density-measurement techniques that allow converging lines of evidence. Drescher et al. (3) measured the evolution of cell sizes and cell aspect ratios, and found that the average size of individual cells decreases with the stage of growth, from about 2.4 μm for communities with fewer than 100 cells to about 1.8 μm for communities with more than 100 cells. However, the authors found that the average interbacterial distances also decrease with increasing cell number, so that the number concentration of cells increases in a subtle manner. What is the point of these biometrics, and how do they impact biofilm development?

Lars Onsager was a Norwegian-American chemical engineer who had a rocky time as an academic, including repeated dismissals from various universities, and having his doctorate grudgingly granted by the chemistry department only after repeated interventions and strong endorsement by the math department at Yale. He eventually went on to win the Nobel Prize in chemistry in 1968. Onsager's contributions were many, and included the eponymous Onsager reciprocal relations in thermodynamics, the Onsager solution to the 2D Ising problem, and the Onsager criterion in liquid crystals. This last contribution can potentially inform our thinking on *V. cholera* biofilm development, as Drescher et al. (3) show.

Liquid crystals are states of matter with properties intermediate between those of solid crystals and those of liquids. For example, liquid crystals can flow like a liquid but maintain some degree of solid-like order, in a manner reminiscent of crystals. Because of their hybrid nature, these materials form the basis of our liquid crystal display technology commonly used on computer screens, such as the one on which this Commentary is read. The simplest example of a liquid crystal is a collection of anisotropic rod-like molecules or colloids. These objects can form a nematic phase, which is characterized by orientational order (solid-like behavior where rods point along the same direction) but no positional order (liquid-like behavior where rods are not positioned on a regular lattice). Onsager showed in a pioneering paper that an ensemble of rods with length L and diameter D can undergo a phase transition from an isotropic phase of randomly oriented rods to a nematic phase, where the long axes of the rods are aligned along a common direction, but only if the concentration of rods were high enough (more precisely, if the volume fraction of rods is greater than $\sim 4\,D/L$, which also places a limit on the minimal aspect ratio required for a nematic) (21). These ideas can provide qualitative insight to guide

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us, but the results of the Drescher et al. (3) study are more complex than the idealized Onsager model. For example, in the largest biofilms observed in their paper, oriented cells "radiate" from the center of the biofilm's basal plane. This example shows how microbiology can significantly enrich and transform the physics of simple model systems such as the one originally described by Onsager. *V. cholera* cells in biofilms do not only interact with one another as rods, they also interact with and are organized by a matrix of complex polymers called *Vibrio* polysaccharides (VPS), which in turn interact with matrix proteins with specific spatial relations to the cells and the VPS.

Using both conventional and superresolution light microscopy, Berk et al. (22) showed how four essential matrix proteins work together with VPS to organize biofilm architecture: the protein RbmA provided cell–cell cohesion; Bap1 provided cell-surface adhesion; mixtures of VPS, RbmC, and Bap1 formed envelopes that surround clusters of cells. In a series of deletion mutant studies, Drescher et al. (3) found that of all of the *V. cholera* matrix proteins assayed, only RbmA significantly perturbed cellular orientations and the resultant biofilm architecture. In contrast to wild-type cells, the majority of cells in the RbmA mutant were oriented vertically and exhibited a high degree of orientational order at smaller biofilm sizes. Interactions between cells and a polymeric network with well-defined architecture-modifying components are expected to strongly modify idealized Onsager behavior, and we therefore need a more inclusive theoretical description than what we currently have. Here, it can be seen that the report by Drescher et al. (3) exemplifies a new type of biofilm inquiry made possible not only by novel experimental tools but also by concepts from other fields that are not often used in microbiology. Perhaps to meet the emerging challenges of the microbiome and engage microbes that are often out of place and wind up where they are not supposed to be, the field may benefit from contributions from multidisciplinary scientists who are also out of place, and thereby not circumscribed by boundaries between fields: biologists and physicists willing to work together with the right mentality. I imagine Onsager would probably agree.

1 Douglas M (2002) *Purity and Danger: An Analysis of Concept of Pollution and Taboo. Routledge Classics* (Routledge, London).
2 Otto M (2009) *Staphylococcus epidermidis*—The 'accidental' pathogen. *Nat Rev Microbiol* 7(8):555–567.
3 Drescher K, et al. (2016) Architectural transitions in *Vibrio cholerae* biofilms at single-cell resolution. *Proc Natl Acad Sci USA*, 10.1073/pnas.1601702113.
4 Cox LM, Blaser MJ (2015) Antibiotics in early life and obesity. *Nat Rev Endocrinol* 11(3):182–190.

5 Hsiao EY, et al. (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155(7):1451–1463.

6 Garber K (2015) Drugging the gut microbiome. *Nat Biotechnol* 33(3):228–231.

7 Alivisatos AP, et al.; Unified Microbiome Initiative Consortium (2015) MICROBIOME. A unified initiative to harness Earth's microbiomes. *Science* 350(6260):507–508.

8 Biteen JS, et al. (2016) Tools for the microbiome: Nano and beyond. *ACS Nano* 10(1):6–37.

9 Wong GCL, O'Toole GA (2011) All together now: Integrating biofilm research across disciplines. *MRS Bull* 36(5):339–342.

10 Charles RC, Ryan ET (2011) Cholera in the 21st century. *Curr Opin Infect Dis* 24(5):472–477.

11 Teschler JK, et al. (2015) Living in the matrix: Assembly and control of *Vibrio cholerae* biofilms. *Nat Rev Microbiol* 13(5):255–268.

12 Alam M, et al. (2007) Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. *Proc Natl Acad Sci USA* 104(45):17801–17806.

13 Faruque SM, et al. (2006) Transmissibility of cholera: In vivo-formed biofilms and their relationship to infectivity and persistence in the environment. *Proc Natl Acad Sci USA* 103(16):6350–6355.

14 Islam MS, et al. (2007) Biofilm acts as a microenvironment for plankton-associated *Vibrio cholerae* in the aquatic environment of Bangladesh. *Microbiol Immunol* 51(4):369–379.

15 Beyhan S, Yildiz FH (2007) Smooth to rugose phase variation in *Vibrio cholerae* can be mediated by a single nucleotide change that targets c-di-GMP signalling pathway. *Mol Microbiol* 63(4):995–1007.

16 Matz C, et al. (2005) Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *Proc Natl Acad Sci USA* 102(46):16819–16824.

17 Rawlings TK, Ruiz GM, Colwell RR (2007) Association of *Vibrio cholerae* O1 El Tor and O139 Bengal with the copepods *Acartia tonsa* and *Eurytemora affinis*. *Appl Environ Microbiol* 73(24):7926–7933.

18 Tamplin ML, Gauzens AL, Huq A, Sack DA, Colwell RR (1990) Attachment of *Vibrio cholerae* serogroup O1 to zooplankton and phytoplankton of Bangladesh waters. *Appl Environ Microbiol* 56(6):1977–1980.

19 Meibom KL, et al. (2004) The *Vibrio cholerae* chitin utilization program. *Proc Natl Acad Sci USA* 101(8):2524–2529.

20 Utada AS, et al. (2014) *Vibrio cholerae* use pili and flagella synergistically to effect motility switching and conditional surface attachment. *Nat Commun* 5:4913.

21 Onsager L (1949) The effects of shape on the interaction of colloidal particles. *Ann N Y Acad Sci* 51(4):627–659.

22 Berk V, et al. (2012) Molecular architecture and assembly principles of *Vibrio cholerae* biofilms. *Science* 337(6091):236–239.