Zooming in to see the bigger picture: Microfluidic and nanofabrication tools to study bacteria

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REVIEW SUMMARY

MICROBE ANALYSIS

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BACKGROUND: Nanotechnology and bacteriology at first sight may seem like two disparate worlds, but a rapidly moving field of research has formed at the interface of these disciplines in the past decade. Bacteria experience spatial structure at many scales: Individual bacteria interact with nanoscale surface features, whereas bacterial communities are shaped by landscape structure down to the microscale. Nanofabrication and microfluidics are ideally suited to define and control the environment at those scales, allowing us to zoom in on the peculiarities of individual cells and to broaden our understanding of the processes that shape multi-species communities. Recently developed nanotools provide unprecedented control over the bacterial microenvironment and have been key to the discovery of new phenomena in bacteriology.

ADVANCES: Nanofabrication and microfluidics have expanded our view on a myriad of bacterial phenomena. Microfluidics provides ways to study individual bacteria in dynamic and well-defined environments and has been used to address long-standing questions concerning bacterial aging and antibiotic persistence. Biological insights have been gained by exploring bacterial growth and movement in nanofabricated constrictions and revealed that bacteria can penetrate constrictions as narrow as only half their width. Furthermore, nanofabrication has been used to discriminate between competing hypotheses regarding the mechanisms that underlie intercellular electron transport. Confinement of single bacteria in tiny volumes has provided an individualistic perspective on collective phenotypes and demonstrated that density-dependent behaviors can even be exhibited by individuals. Bacteria growing in nanofabricated chambers adopt predefined shapes and have been used to study the geometry dependence of intracellular processes. Microfluidics and nanofabrication have been combined to create synthetic ecosystems in which the spatial eco-evolutionary dynamics of bacterial communities can be explored. Various approaches to mimic the intricate spatial structure of natural bacterial habitats now contribute to our understanding of competition and cooperation within bacterial populations. Microfluidic platforms have boosted research on unculturable environmental species by eliminating the need for pre-analysis culturing. On-chip whole-genome amplification of environmental isolates has recently provided a first genotypic glimpse on this "dark matter of biology."

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OUTLOOK: Looking ahead, it is clear that the doors that nanofabrication and microfluidics have opened will continue to make important contributions to basic bacteriology research. A comprehensive investigation of the uncultured majority with microfluidic technologies, for instance, may uncover the vast potential of currently unknown species. Practical applications such as microbial fuel cells or antibacterial surfaces will benefit from the understanding of bacterial behavior at the nanoscale. Microfluidic devices are now beginning to be commonly used in microbiology labs because of a demand for precise measurements in complex environments that can be controlled at the microscale. This trend will undoubtedly continue as scientists delve deeper into the complex lives of bacteria. ■

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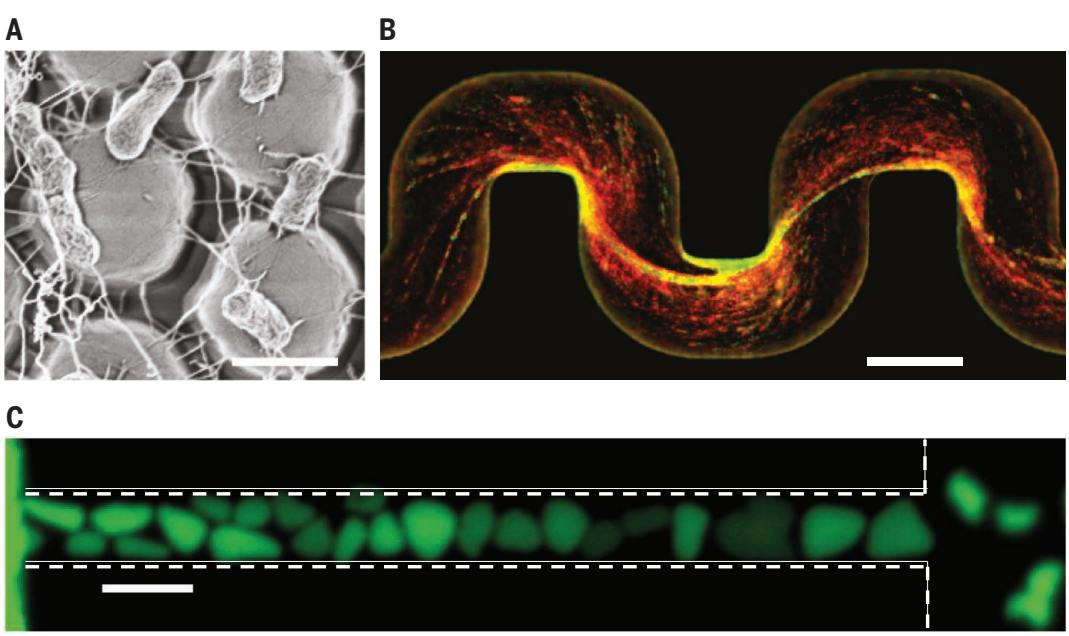

Studying bacteria using nanofabrication and microfluidics. (**A**) *Escherichia coli* bacteria use their flagella to exploit submicrometer crevices for surface attachment [Reprinted with permission from (*5*) (reference list of full paper online)]. (**B**) Biofilm streamers form in a meandering flow channel (*Pseudomonas aeruginosa*, red; extracellular polymeric substances, green) [Reprinted with permission from (*93*)]. (**C**) *E. coli* undergo a shape transition when squeezing into a nanofabricated channel as shallow as half their width [Reprinted with permission from (*26*)]. Scale bars, (A) 2 μm; (B) 200 μm; and (C) 5 μm.

REVIEW

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Zooming in to see the bigger picture: Microfluidic and nanofabrication tools to study bacteria

Felix J. H. Hol and Cees Dekker*

The spatial structure of natural habitats strongly affects bacterial life, ranging from nanoscale structural features that individual cells exploit for surface attachment, to micro- and millimeter-scale chemical gradients that drive population-level processes. Nanofabrication and microfluidics are ideally suited to manipulate the environment at those scales and have emerged as powerful tools with which to study bacteria. Here, we review the new scientific insights gained by using a diverse set of nanofabrication and microfluidic techniques to study individual bacteria and multispecies communities. This toolbox is beginning to elucidate disparate bacterial phenomena—including aging, electron transport, and quorum sensing—and enables the dissection of environmental communities through single-cell genomics. A more intimate integration of microfluidics, nanofabrication, and microbiology will enable further exploration of bacterial life at the smallest scales.

At first glance, nanotechnology and bacteriology may seem like two disparate worlds. However, in the past decade a dynamic and rapidly expanding field of research has formed at the interface of these two disciplines. Many "nanotools" have been developed to study individual bacteria as well as multispecies communities in complex yet well-defined environments. In addition to a host of exciting new techniques, the confluence of nanotechnology and bacteriology has yielded new biological insights that would have been inaccessible without the merging of these disciplines.

Natural bacterial habitats, ranging from volcanic soil to our gastrointestinal tract, are spatially structured at multiple scales. The soil matrix, for instance, consists of a three-dimensional (3D) network of micrometer-sized patches (*1*, *2*), and habitats ranging from the gut to the ocean contain chemical gradients down to the microscale (*3*, *4*). At even smaller scales, bacteria can squeeze through submicrometer constrictions and exploit nanosized crevices for surface attachment.

In these spatially structured habitats, multispecies consortia form organized communities. Understanding how these bacterial communities assemble and function is a grand challenge that requires an interdisciplinary approach. The fundamental units of communities are cells, and the typical dimensions of a bacterial cell match well with the scales at which micro- and nanotechnology can shape and manipulate the environment (Fig. 1A). Traditional methods to culture bacteria—test tubes and petri dishes—are not compatible with long-term monitoring of an individual cell or the precise manipulation of its microenvironment. As a result, seemingly simple questions such as "How long can a bacterium live?" or "What is the smallest constriction a bacterium can pass through?" remained unanswered until recently. Answers to such long-standing questions are now emerging at the interface of microbiology and microfluidics.

Nanofabrication provides unprecedented control over the local environment of cells and allows researchers to visualize the mechanical role of flagella in surface attachment (*5*) or to investigate individual bacteria donating electrons to electrodes (*6*). To study population-level processes, microfluidics and nanofabrication can create well-defined on-chip ecosystems that mimic the complexity of the natural environment. Experiments with such synthetic ecosystems are revealing how the microscopic spatial structure of bacterial habitats affects the cooperation, competition, and evolution of bacteria (*7*–*11*).

Here, we review the development and use of novel nanofabrication and microfluidics tools that provide detailed insight into the complex lives of bacteria, and we highlight some of the exciting microbiological discoveries that have been made with those techniques. We first review several approaches to study bacterial growth and shape at the single-cell level. Subsequently, we discuss how single-cell studies of bacterial electron transport and quorum-sensing provide insight into population-level manifestations of those phenomena. We then discuss efforts to understand bacterial community dynamics by using synthetic ecosystems, and to dissect natural communities by using microfluidics. We finish with a brief outlook on new opportunities at the interface of bacteriology and nanotechnology.

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Bacterial growth and shape

Microfluidics and nanofabrication provide powerful tools to control, shape, and manipulate the environment of individual bacteria. In this section, we describe microfluidic approaches that address long-standing questions concerning bacterial growth, and we discuss how nanofabrication opens doors to studying bacterial growth and shape.

Mysteries of bacterial growth solved with microfluidics

Monitoring an individual cell for extended periods in a well-defined environment is crucial to answer fundamental growth and aging-related questions. However, long-term imaging of a single cell in a constant environment is challenging; because of exponential growth rates and resource consumption, a bacterial colony creates internal chemical gradients and reaches an unmanageable size within a few generations, precluding the tracking of a single cell for long periods. One model species for bacterial aging, *Caulobacter crescentus*, helps to solve this problem by only dividing as long as it is attached to a surface. While the dividing mother cell is stuck to the bottom of a channel, the planktonic progeny can get flushed out (*12*). In many other species, however, growth and dispersal are not mutually exclusive, hence their aging process remained a mystery until recent microfluidic innovations allowed the long-term (in principle, indefinite) monitoring of an individual bacterium in a constant environment. To accomplish this, Wang *et al.* immobilized bacteria in dead-end channels having a width and height matched to that of the cell body (*13*). The device is designed to flush out the offspring after division (Fig. 1B). Using this device (dubbed "the mother machine"), single *Escherichia coli* bacteria were monitored for more than 200 cell divisions, constituting a marked improvement compared with earlier work in which aging *E. coli* could typically be followed up to seven generations (*14*, *15*). Long-term monitoring revealed that the division rate of *E. coli* remained constant for more than 200 generations, but the death rate increased after 50 divisions. These results suggest that growth and death in *E. coli* are uncoupled processes—a property not previously found in model organisms used to study aging.

Norman *et al.* recently used a similar microfluidic device to investigate cell-fate switching between an individual planktonic state and a sessile multicellular state by individual *Bacillus subtilis* bacteria and characterized the underlying regulatory circuit (*16*). In order to observe single *B. subtilis* differentiate, Norman *et al.* fabricated long dead-end channels connected to 500-nm-shallow medium reservoirs. These shallow reservoirs prevent gradients from developing along the length of the channel and ensure a constant environment over the long time scales associated with cell-fate switching (Fig. 1C). Tracking

of individual cells revealed that switching from the motile state is "memoryless"—the probability to switch to the multicellular state is independent of how long a cell has been planktonic. Switching back to the motile state, however, is tightly controlled, resulting in a low variability of the lifetime of multicellular growth. Differences in lifetime regulation of the two states likely reflect the necessity to coordinate behavior over multiple generations in the multicellular state and the absence of such coordination in the motile state.

The phenomenon of bacterial persistence presents another long-standing question of bacterial growth physiology that was addressed by using microfluidics. More than seven decades ago, it was observed that a small fraction of a clonal nonresistant population of bacteria can survive antibiotic treatment—an observation of obvious clinical relevance (*17*). Upon regrowth, these persistent bacteria ("persisters") give rise to a population that remains susceptible to the antibiotic used, showing that resistance is not genetically acquired.

The mechanism that allows a fraction of the susceptible population to remain refractory to antibiotic treatment remained obscure until the phenomenon was investigated at the single-cell level (*18*), which revealed that a heterogeneity in growth rates is key. *E. coli* growing in open-ended channels [a single-cell chemostat (Fig. 1B)] showed the presence of two subpopulations: The vast majority of cells grew at a rate identical to the growth rate in batch culture, whereas a small fraction of the population was dormant. The use of a microfluidic device permitted tracking of the growth and death of individual *E. coli* bacteria upon transient exposure to ampicillin (medium and antibiotics were applied from above through a membrane covering the bacterial channels) and revealed that the cells that were dormant before antibiotic exposure survived the treatment. On-chip observations of single persister cells furthermore revealed stochastic growth-resumption of persisters when antibiotic-free medium was supplied after exposure. Later studies showed that persisters may transiently exhibit metabolic activity, resulting in a short-lived state of antibiotic susceptibility (*19*).

A recent microfluidics study reported a different mechanism underlying persistence in populations of *Mycobacterium smegmatis* exposed to the antibiotic isoniazid. Here, persistence was found to relate to the stochastic expression of KatG, an enzyme necessary for activation of the antibiotic. Cells that infrequently expressed *katG* continued to grow and divide, whereas cells that were frequently pulsing *katG* were prone to die. This balanced growth and death rates and resulted in a population of "dynamic persisters" (*20*). A thorough characterization of the various mechanisms underlying persistence is key to identifying the weaknesses of persister cells that may facilitate their clinical management.

Although arranging individual bacterial cells in single file is conceptually simple, the ability to monitor the same cell for unlimited periods of time while having accurate temporal control over its chemical environment is a powerful new tool in microbiology (*21*–*24*). In particular, studies of phenotypes that exhibit temporal

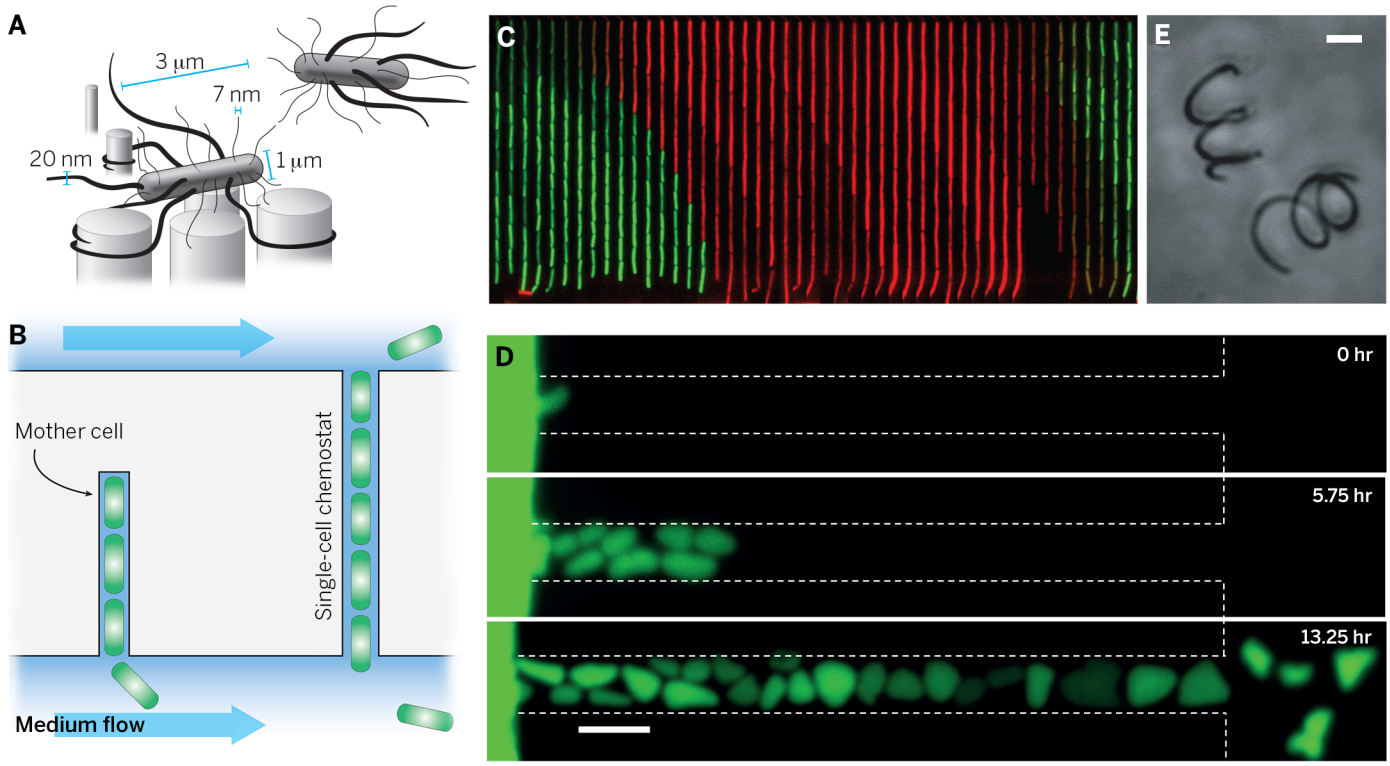

Fig. 1. Bacterial growth and shape in microstructures. (**A**) The scales at which nanofabrication and microfluidics can shape and control the environment matches well to the characteristic size scales of a bacterium (0.3 to 10 μm) and its subcellular components (for example, flagella and pili are ~20 and ~7 nm wide, respectively). (**B**) A cell immobilized in a dead-end channel (left) pushes its offspring out through growth and division. The mother cell remains in the same location and can be observed indefinitely. A linear colony growing in a channel open at both ends (right) is a continuous culture technique (chemostat) that allows single-cell imaging. In both designs, the growth channel abuts a larger flow channel that washes out offspring, supplies fresh nutrients, and can be used to change medium conditions. (**C**) Kymograph of *B. subtilis* in a dead-end channel. Switching from motile growth (green) to chained growth (red) by an individual cell can be seen; frames are taken 10 min apart and stacked horizontally. [Reprinted by permission from Macmillan Publishers, *Nature* (*16*) copyright (2013)] (**D**) *E. coli* squeezing through a 300-nm-shallow nanochannel (50 μm long and 5 μm wide) connecting two deep chambers. Dashed lines mark the lateral edges of the channel. Scale bar, 5 μm. Cells that squeeze into the nanochannel undergo a shape transition and become flat and aberrantly shaped upon confined growth and division. The population of squeezing cells traverses the nanochannel through growth and division. Once cells enter the deep chamber on the right, they adopt various globular shapes (bottom right). [Reprinted with permission from (*26*)] (**E**) A filamentous *E. coli* cell adopted a spiral shape after growth in a circular channel. Scale bar, 10 μm. [Reprinted with permission from (*28*) copyright (2005) American Chemical Society]

dynamics spanning many generations, such as cell-fate switching (*16*) and circadian oscillations (*25*), benefit from this innovation.

Shape-shifting bacteria

In their natural habitats, bacteria often encounter submicrometer holes and crevices that are smaller than the cell body. Indeed, in clinical and industrial settings submicrometer-pore filters are readily used to sterilize liquids. In order to investigate whether constrictions narrower than a cell indeed rigorously prevent bacteria from penetrating, Männik *et al.* fabricated nanochannels of a range of widths and determined the narrowest constriction through which bacteria could pass. Surprisingly, *E. coli*—which have a diameter of 800 to 900 nm—could penetrate channels as narrow as only half their width (*26*). Shown in Fig. 1D are *E. coli* cells that squeeze into a 300-nm-shallow channel, which demonstrates that a population of squeezing cells can traverse a shallow nanochannel of several hundred micrometers in length by means of growth and division. Cells popping out on the other end are aberrantly shaped but readily give rise to a population of normal rod-shaped cells. In contrast, the Gram-positive bacterium *B. subtilis* could not penetrate channels of a width narrower than its cell body, suggesting that cell-wall rigidity and turgor-pressure differences between Gram-negative (such as *E. coli*) and Gram-positive bacteria may have implications regarding their ability to penetrate submicrometer constrictions.

In a follow-up study, the squeezing *E. coli* were used as a model system with which to study the shape-dependent functioning of *E. coli*'s cell-division machinery (*27*). Despite their irregular shape, squeezed *E. coli* produce equally sized daughter cells with a high division accuracy that is nearly identical to normal rod-shaped cells. Visualizing various components of the cell-division machinery in these squeezed cells demonstrated the importance of the nucleoid in positioning the divisome (the contractile ring that divides the cell) and showed that nucleoid occlusion provides a robust mechanism for division-site selection in aberrantly shaped cells.

Shaping bacteria into diverse morphologies can enhance our understanding of the spatial organization and geometry dependence of intracellular processes. The squeezed cells shown in Fig. 1D, however, adopt irregular and undefined shapes and as such are not ideal to systematically study the shape-dependence of intracellular processes. Bacteria confined in nanofabricated chambers with a specified geometry can adopt a predefined shape through growth. An example of a cell that adopted a spiral shape after being cultivated in a circular chamber is shown in Fig. 1E (*28*). A similar approach has been used to manipulate the local curvature of cell membranes, showing that curvature has a regulatory role in localizing cardiolipin and various membrane-associated proteins (*28*–*30*). We anticipate that new nanofabrication approaches to grow bacteria into predefined shapes will prove to be valuable in appreciating the adaptive virtue of cellular shapes and enhance our understanding of the geometry dependence of intracellular processes (*31*).

Hydrodynamic flow presents another method to deform bacterial cells. Two studies have recently investigated the bending dynamics of filamentous bacteria protruding from growth channels by using flow perpendicular to the growth direction of the cells (*32*, *33*). These studies showed that rapid mechanical perturbations lead to elastic bending, whereas constantly applied forces lead to growth-induced plastic deformations of the cell body. When the bending force is removed, plastically deformed cells eventually recover their rod shape through dislocation-mediated cell-wall growth.

Motility and adhesion: Bacteria-surface interactions

In contrast to their laboratory counterparts in a test tube or a chemostat, bacteria living in natural habitats are often in close contact with a surface. The surfaces on which bacteria grow and move are structured at the nanoscale, which has implications for surface adhesion and motility. In this section, we discuss how visualizing the interactions of bacteria with nanofabricated structures is revealing the mechanisms that bacteria use to migrate and settle into a biofilm lifestyle.

Settling down

Surface-attached growth is a common lifestyle among bacteria, and genetic analyses have suggested important roles for flagella and pili in the initial stages of biofilm formation (*34*). Mechanical insight into the interaction of bacteria with nanoscale features, however, was lacking. Recently, Friedlander *et al.* (*5*) visualized the interaction of *E. coli* with nanofabricated structures and elegantly showed how *E. coli* use their flagella to "reach" into submicrometer crevices to enhance surface attachment (Fig. 2, A and B) (*5*). Imaging this process demonstrated that bacterial flagella do not only function in bacterial motility but also facilitate surface attachment by allowing bacteria to grasp submicrometer objects. A further characterization of the structural mechanisms underlying bacterial attachment to nanoscale surface

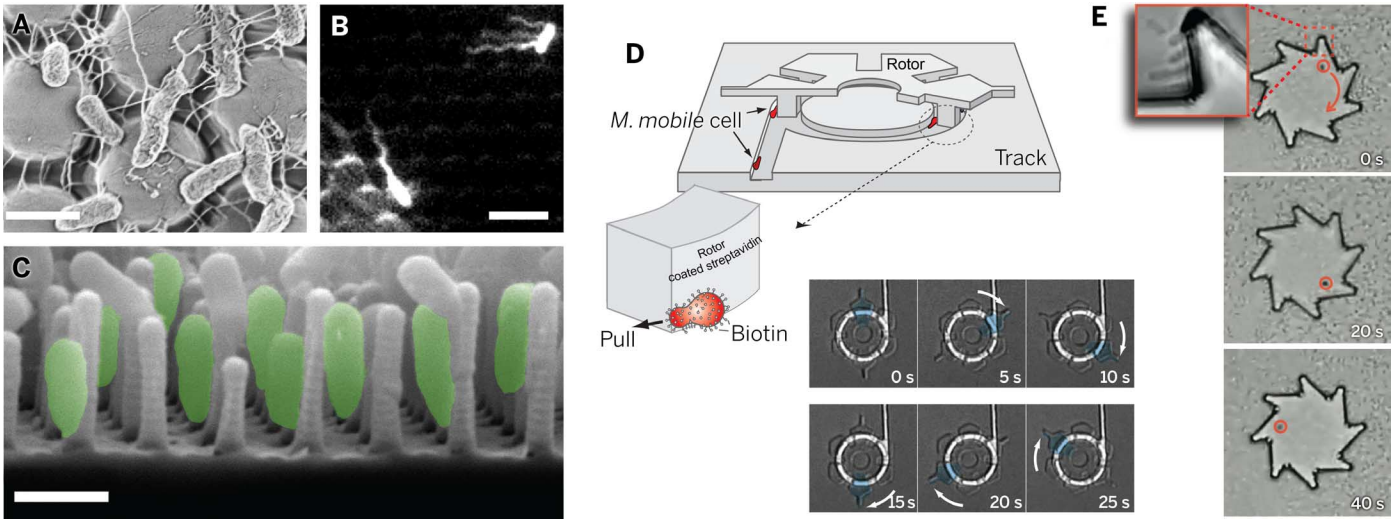

Fig. 2. Bacterial attachment and motility probed with nanofabricated structures. (**A** and **B**) Flagella "grasp" submicrometer crevices to improve surface attachment. (A) Scanning electron microscopy image (scale bar, 2 μm) and (B) fluorescence microscopy image (scale bar, 5 μm) of several *E. coli* on an array of hexagonal posts separated by 440-nm-wide trenches. Flagella nestled in the trenches facilitate surface attachment. [Reprinted with permission from (*5*)] (**C**) *P. aeruginosa* cells attach to 300-nm-diameter posts. An array of nanoposts induces the self-organization of an ordered colony in which the majority of cells stands upright between the posts. Cells are false-colored in green to enhance visibility. Scale bar, 1 μm. [Reprinted with permission from (*42*) copyright (2010) American Chemical Society] (**D**) Schematic of a microrotor powered by *M. mobile*. Gliding cells attach to the 20-μm-diameter rotor and rotate it, as can be seen from microcopy images (bottom right). [Reprinted with permission from (*58*) copyright (2006) National Academy of Sciences, USA] (**E**) Swimming bacteria rotate a gear at 1 rpm (48 μm in diameter) by bumping into its asymmetric teeth. [Reprinted with permission from (*59*)]

features is necessary to elucidate how bacteria lay the foundation for biofilm growth.

A better understanding of bacterial surface attachment at the nanoscale is relevant to prevent biofouling. At the scale of 10 to 100 nm, surface roughness can promote the adhesion of certain species while inhibiting attachment of others. Such species-specific interactions are suggested to depend on the size and shape of a cell (spherical or rodlike). For example, depending on how their shapes matched nanofabricated surface features, spherical *Staphylococcus aureus* cells attached to nanostructured surfaces, whereas rod-shaped *Pseudomonas aeruginosa* did not, and vice versa (*35*). The systematic characterization of bacterial surface-attachment at the nanoscale has attracted strong interest, and general principles relating surface characteristics to adhesion are currently emerging (*36*–*38*). Addressing the many questions that remain, however, is of clear importance for designing next-generation antibacterial surfaces (*39*, *40*).

Posts measuring 300 nm in width guide swimming motility and have been suggested to induce preferential attachment to the posts instead of to the flat surface that supports them (*41*). *P. aeruginosa* cells standing upright between posts are shown in Fig. 2C. Remarkably, periodic arrays of such nanoposts can facilitate the self-organization of bacterial populations into spatially patterned colonies (*42*). Arrays of nanostructures can be used to form highly ordered colonies in which cells assume different orientations, depending on the periodic post spacing. This behavior is exhibited by several species and does not depend on the action of flagella or pili because defective mutants show similar behavior.

On the move

Larger fabricated structures (an order of magnitude larger than the nanofeatures involved in surface attachment) can have an intriguing effect on bacterial motility. When swimming near surfaces, bacteria become hydrodynamically trapped and continue swimming along the surface (*43*, *44*). This effect can have interesting consequences when bacteria navigate microfluidic channels and can be used to sort or direct cells. Playing with the hydrodynamic properties of the floor and ceiling of a microfluidic channel, DiLuzio *et al.* made *E. coli* "swim on the right-hand side" and take a right turn when approaching intersecting channels (*45*). In contrast, the traffic rules are reversed when *E. coli* swims near a liquid-air interface, where it takes left turns (*46*). In addition to steering swimming bacteria, the hydrodynamic effects of bacteria swimming near surfaces can be exploited to concentrate randomly swimming bacteria by using nanofabricated funnels (*47*, *48*) or sort them according to their size by using microfluidic ratchets (*49*).

Bacterial chemotaxis, the ability of motile cells to sense and navigate chemical gradients, has been studied extensively by using microfluidics to create defined chemical gradients (*50*). The opportunity to track individual cells in arbitrarily shaped gradients that are continuous or time-variant has provided insight into the coherence of chemotactic populations (*51*), the remarkable sensitivity of chemotaxis (*52*), and the various strategies that marine bacteria adopt to optimize their foraging (*53*, *54*). The use of microfluidic platforms to study chemotaxis has been reviewed extensively in (*55*) and (*56*).

An interesting potential of motile bacteria that interact with microstructures is the possibility to extract useful work from them. Besides motility, bacteria have many capabilities—such as self-reproduction and sensing of the environment—that make them interesting candidates to propel or transport micrometer-sized objects (*57*). Several studies have provided proof-of-concept experiments that bacterial motility may be used to power microscale rotors (*58*–*60*). A design by Hiratsuka *et al.* is shown in Fig. 2D, in which gliding *Mycoplasma mobile* cells attach to a 20-μm-diameter rotor running on a silicon track (*58*). In this approach, *M. mobile* cells collectively managed to rotate the motor at 2 rotations per min (rpm). Recent studies demonstrated conceptually simpler designs in which asymmetric rotors suspended in a bacterial population were rotated by swimming bacteria that bumped into the asymmetric teeth (Fig. 2E) (*59*, *60*). Asymmetric environments have also been used to let randomly swimming bacteria that bump into colloids deliver the particles to the center of a structure consisting of asymmetric barriers (*61*). Although the prospect of using nanofabricated structures to harness the power of bacteria is still remote, these studies illustrate the potential of hybrid micromachines.

Zooming in on single cells to learn about population-level phenomena

In nature, cells rarely live in isolation. Microbial communities are often composed of many interacting cells from various species. The functions of bacterial interactions are as stunningly diverse as the communities in which they occur. For example, interactions serve to assess whether it is beneficial to induce costly collective behaviors, or give rise to peculiar phenomena such as long-range electron transport through biofilms. Below, we discuss two examples—electron transport and quorum-sensing—that show how zooming in on the building blocks of populations (individual cells) enhances our understanding of population-level phenomena.

Bacterial electricity

To facilitate respiration, various bacterial species exchange electrons with their external environment. The mechanisms that underlie bacterial electron transport are not well understood. A detailed understanding of these processes is, however, imperative to exploit the potential of electricity-generating bacterial populations in the development of microbial fuel cells or the processing of organic waste (*62*–*64*). In theory, bacteria can donate electrons to extracellular insoluble acceptors (such as electrodes) in various ways: Soluble redox-active molecules can transport electrons by means of diffusion, redox-active molecules on the outer cell surface may transfer electrons upon contact, and biofilms perhaps may facilitate long-range electron transport through contacts formed by conductive pili (*65*). It remains a matter of debate which of these mechanisms bacteria use to transport electrons (*66*, *67*).

To distinguish between the three modes of electron transport mentioned above, Jiang *et al.* fabricated electrodes patterned with an insulating layer (*68*). The insulating layer was designed to prevent direct cell-electrode contact while allowing diffusing molecules to reach the electrode through nanoscale holes. Using this approach, it was shown that the cell body of *Shewanella oneidensis* does not need to be in direct contact with an electrode in order for it to generate a detectable current, suggesting that electron transport takes place through pili or diffusing mediators. Pili of *S. oneidensis* were previously demonstrated to be radially conductive (*69*) and later also suggested to transport electrons along their long axis (*70*). Two observations by Jiang *et al.*, however, argue against an important role for pili in mediating electron transfer: Simultaneous optical imaging and current recording showed that a current can be detected before cells are in the proximity of the electrodes. Furthermore, the removal of diffusible redox mediators after a layer of cells had formed on the electrode abolished the current. These observations suggest that *S. oneidensis* mainly relies on diffusible molecules for electron transport and that pili may only have a minor role (*68*).

Experiments on *Geobacter sulfurreducens* suggested that it uses a different mechanism to transport electrons (*6*). Simultaneous imaging and current detection of single *G. sulfurreducens* in contact with a nanofabricated electrode is shown in Fig. 3A. Stepwise current increases can be observed when individual cells come into contact with the electrodes, and in contrast to *S. oneidensis*, the removal of diffusible redox mediators did not reduce the current produced by *G. sulfurreducens*. These observations suggest that *G. sulfurreducens* exchanges electrons through redox-active molecules on its cell surface or along conductive pili.

The spatial scale over which bacterial electron transport occurs becomes vastly larger when multiple cells form a biofilm (*71*) or in cables composed of filamentous bacteria (*72*). Such multicellular assemblies have been shown to transport electrons over centimeter distances. Where studies at the level of single cells suggest a minor role for pilus-mediated transport in *S. oneidensis* and focused on cell-body contact for *G. sulfurreducens*, pili were reported to be crucial for the conductivity in biofilms of *G. sulfurreducens* (*71*, *73*), which were claimed to exhibit a high conductivity, approaching that of synthetic metals (*71*).

The above studies illustrate that the use of nanofabrication is valuable for enhancing our understanding of electron transfer at the single-cell and biofilm levels. However, many open questions remain, and a thorough investigation of the diverse mechanisms that single bacteria use to transport electrons is warranted to estimate the

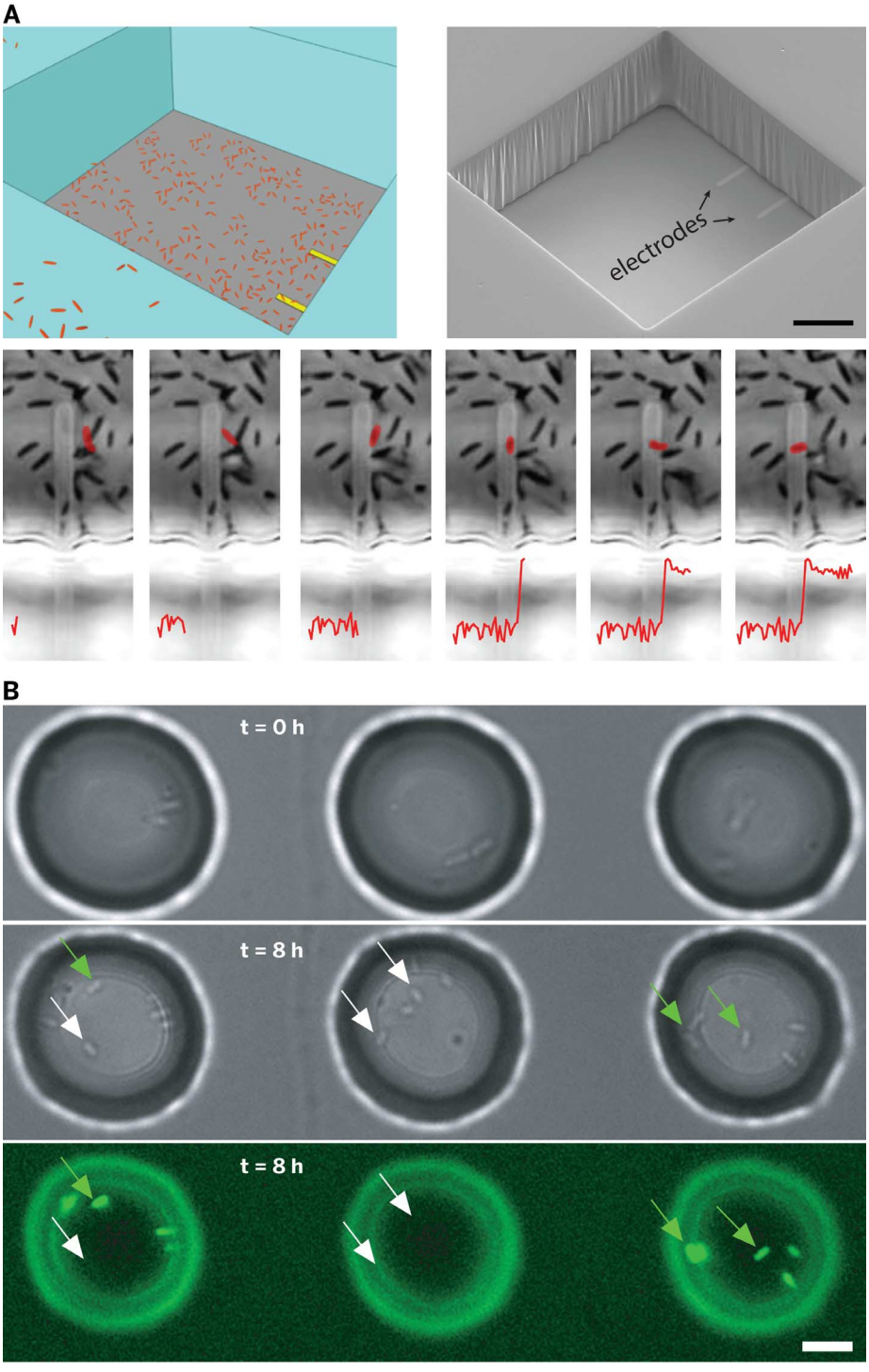

Fig. 3. Single-cell measurements of electron transport and quorum-sensing. (**A**) Schematic (top left) and scanning electron microscopy image (top right) of a well containing two electrodes. Scale bar, 20 μm. A time sequence of *G. sulfurreducens* bacteria interacting with an electrode is shown at bottom. When the cell labeled in red comes in contact with the electrode, a stepwise current increase is measured (current is plotted in red). [Reprinted by permission from Macmillan Publishers, *Nature Communications* (6) copyright (2013)] (**B**) Small groups of *P. aeruginosa* are confined in 100-fl droplets. (Top and middle) Bright-field images demonstrating growth of cells. Scale bar, 5 μm. Not all cells in the same droplet initiate quorum sensing, showing that quorum sensing initiation is heterogeneous (green arrows point to cells that induced quorum-sensing; white arrows point to cells that did not). [Reprinted with permission from (79) copyright (2009) Wiley]

full potential of bacteria populations as tools to turn waste into energy.

Lonely quorums

Quorum-sensing is a phenomenon in which bacteria sense secreted molecules and undergo genetic reprogramming when these molecules reach threshold densities (74). For example, this form of density-dependent signaling allows bacterial populations to induce "expensive" collective behaviors when a critical population density (a quorum) makes those worthwhile. Quorum-sensing has mainly been investigated in traditional assays by using well-mixed cultures grown in flasks. However, the mediation of quorum-sensing by diffusible molecules makes it evident that the geometry of an environment and the spatial arrangement of cells are key to these processes (75). A lively debate (76–78) on what bacteria are actually sensing—such as population density or medium diffusibility—spurred basic questions regarding the induction of quorum-sensing.

Two parallel efforts focused on the question of whether a single bacterium can quorum-sense—whether it can induce density-dependent pathways in response to a self-secreted signal. This can be probed by confining individuals or small groups of cells in a femtoliter- to picoliter-sized droplet (Fig. 3B). Boedicker *et al.* developed a microfluidic device in which *P. aeruginosa* were encapsulated in droplets created in microstructured wells (79), whereas Carnes *et al.* used a self-assembly process in which lipid/silica nanostructures encapsulated *S. aureus* bacteria (80–82). The small droplets provide the opportunity to have individual bacteria at a "density" that in essence is similar to a population approaching stationary phase in a test tube (~10^9 cells/mL). Both studies confirmed that individual cells could indeed induce quorum-sensing pathways that were previously only observed in large, high-density populations. The observations revealed that the initiation of quorum-sensing is heterogeneous; cells in identical droplets (equal cell density) did not necessarily exhibit similar quorum-sensing induction kinetics. The induction of quorum-sensing pathways substantially increased the viability of wild-type *S. aureus* compared with their non–quorum-sensing counterparts.

Recent efforts that encapsulate *E. coli* bacteria in 2D arrays of microdroplets open doors to investigate the effect of the spatial arrangement of cells on quorum-sensing (83). Arrays of droplets may similarly be used to spatially organize multispecies consortia in an effort to unravel the various inter- and intraspecies languages that bacteria speak (74).

Unraveling bacterial communities

Natural bacterial habitats have in common that they are spatially structured and chemically heterogeneous environments. Bacteria usually live in multispecies communities of which the majority is unculturable (84). Two distinct approaches aimed at understanding the complexity of bacterial communities are currently gaining ground. First, synthetic ecosystems are used in a "bottom-up"

approach in which the ecology of bacterial populations is explored in well-controlled on-chip ecosystems. This approach allows researchers to build a laboratory landscape from scratch and populate it with a defined multispecies consortium. Secondly, "top-down" approaches to dissect naturally occurring bacterial communities are facilitated by technological advancements that allow the isolation and characterization of unculturable bacteria directly isolated from environmental samples.

Building up bacterial communities

Approximately a decade ago, researchers first created on-chip ecosystems to study the spatial dimension of bacterial community dynamics in detail. Nanofabrication and microfluidics have the potential to transform the way bacterial ecology is studied by providing the tools to create ecosystems that mimic characteristics of natural environments. In the absence of external mixing (which occurs in shaken flasks), dispersal, chemotaxis, and aggregation become key drivers of bacterial community dynamics. As a result of these factors, the population expansion of bacteria that colonize a structured habitat does not obey the statistics of an ideal gas; instead, high-density demographic clusters form in close proximity to unoccupied territory (*7*, *85*–*88*). Keymer *et al.* demonstrated that these processes allow *E. coli* to organize into a structured metapopulation (a population of populations) when colonizing an array of coupled habitat patches (*7*).

The spatial structure of a community affects the competitive interactions between different cell types (*8*). The microscale diffusion of nutrients (*53*, *54*), secreted public goods (*10*, *89*), and toxins, for example, set the spatial scale at which cooperative or spiteful interactions emerge. Synthetic ecosystems (Fig. 4) provide the opportunity to study the interaction between competing bacteria in different ecological scenarios (such as varying geometry and medium composition) and track the resulting community dynamics at high spatiotemporal resolution. Such approaches provide insight into the eco-evolutionary interaction of bacteria with their environment and have shed light on the strategies bacteria use to solve social dilemmas concerning resource management (*9*, *90*) and the exploitation of public goods (*10*). A two-strain community is shown in Fig. 4A consisting of cooperator and cheater *E. coli* that inhabit a microfabricated habitat. These two strains do not stably coexist when cocultured in a well-mixed flask where resource competition (due to mixing) is global. However, in a habitat that is spatially structured by microfabrication, cooperator and cheater bacteria only compete with their local neighbors and self-organize into a structured community in which cooperators and cheaters coexist (*90*).

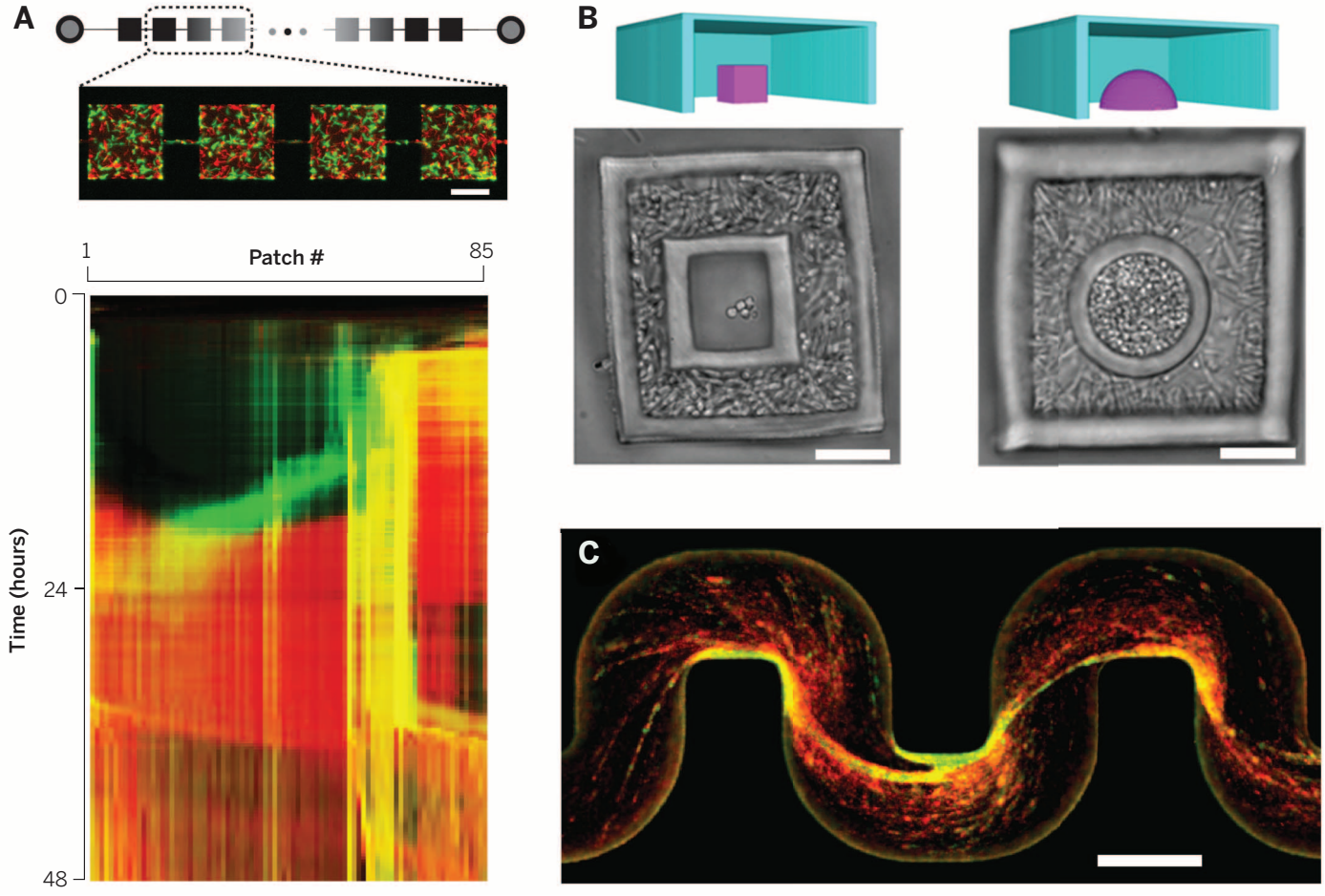

Fig. 4. Bacterial communities in synthetic ecosystems. (**A**) Kymograph of cooperator and cheater *E. coli* colonizing a microfabricated habitat consisting of 85 habitat patches (100 by 100 by 10 μm) connected by corridors. Space is depicted horizontally, and time is depicted vertically. Each pixel represents a habitat patch that is color-coded according to its cooperator (green) and cheater (red) occupancy; yellow indicates coexistence. Lateral population shifts occur readily, and the community remains dynamic for days. A schematic of the microhabitat, and a zoom-in of fluorescently labeled bacteria inhabiting four patches, are shown above the kymograph. Scale bar, 50 μm. [Adapted from (*90*)] (**B**) Two examples of nested communities in 3D-printed ecosystems. *S. aureus* populations confined in rectangular (left) and hemispherical (right) structures are surrounded by *P. aeruginosa*. Scale bars, 10 μm. The 3D-printed structures are porous. [Reprinted with permission from (*95*)] (**C**) *P. aeruginosa* (labeled in red) form biofilm streamers in a microfluidic flow channel. A sievelike network consisting of bacteria and extracellular polymeric substances (labeled in green) can be discerned. Over time, the build-up of cells and EPS forms a network that eventually clogs the flow channel. Scale bar, 200 μm. [Reprinted with permission from (*93*)]

An important aspect of bacterial life is the formation of biofilms. Microfluidics have been used to investigate how bacteria form biofilm streamers to enhance their survival in porous habitats (*91*–*93*). Streamers formed by *P. aeruginosa* growing in a meandering flow channel are shown in Fig. 4C. Biofilms that initially grow on the channel walls secrete extracellular polymeric substances (EPS) that form a sievelike network spanning the full width of the channel. As the biofilm constantly sheds bacteria, those planktonic cells get "caught" in the EPS network. The gradual build-up of biofilm streamers eventually leads to a catastrophic clogging event and the abrupt cessation of flow. Such an extraordinary ability of bacterial communities to self-structure, organize, and modify their environment is a central theme that emerges in various studies that use synthetic ecosystems.

A promising recent innovation is provided by the 3D printing of bacterial ecosystems by use of multiphoton lithography (*94*, *95*). Connell *et al.* confined defined bacterial populations within 3D-printed containers consisting of photo-crosslinked gelatin and protein. These ecosystems can have arbitrary 3D shapes, while the gelatin matrix allows diffusion through the walls, facilitating chemical interactions between confined populations. A population of *S. aureus* is shown in Fig. 4B contained in a 3D-printed structure embedded within a population of *P. aeruginosa*. This technique can potentially be used to reproduce the spatial structure and species-composition of complex natural communities in an experimentally accessible manner.

Studies such as (*93*, *95*, *96*) are examples of the move toward laboratory experiments that strive to be a realistic representation of the natural environment while retaining a high degree of experimental control and permitting visualization at high spatiotemporal resolution. This development will not only continue to enhance our understanding of the intricacies of bacterial communities but will also prove to be beneficial for the development of new approaches to manage bacteria in clinical and industrial settings.

Breaking down bacterial communities

Microfluidic platforms enable researchers not only to perform controlled ecological experiments in well-defined ecosystems but also facilitate the characterization of naturally occurring communities. It is estimated that less than 1% of all existing bacterial species is culturable (*84*). The vast majority of Earth's bacterial diversity—the so-called "dark matter of biology"—can thus currently not be analyzed from pure culture and hence remains mysterious (*97*). In addition to satisfying our curiosity about the natural world, analyzing and culturing these species may unlock a vast repertoire of useful, but currently unknown, chemical compounds.

Microfluidic techniques are currently unveiling various secrets of the unculturable majority by enabling the isolation and analysis of individual bacteria from environmental samples. The ability to confine individual cells in microfluidic chambers eliminates the need for pre-analysis culturing and provides the opportunity to do single-cell genomics on cells that are obtained directly from natural communities. Target genes of interest, or the presence of phage, are identified from multiplex digital polymerase chain reaction (PCR) on individual cells. This approach has proven to be useful for characterizing environmental communities by allowing researchers to link specific species to specific community functions or identify new host-page interactions (*98*, *99*).

Owing to recent advances in on-chip, single-cell, whole-genome amplification, DNA can be obtained directly from individual cells isolated on-chip. The use of nano- or picoliter volumes in such platforms overcomes challenges posed by the amplification of contaminants when working with larger volumes (*100*–*102*). The obtained DNA can subsequently be used for off-chip analyses such as genome sequencing. Such approaches have resulted in the genome sequence of various unculturable species coming from habitats including the human mouth (*97*) and various aquatic ecosystems (*103*, *104*). In earlier work using microfluidic devices to isolate bacteria, single cells were usually obtained probabilistically by dispersing cells over many isolation chambers. The integration of micro- and optofluidics, however, now facilitates targeted isolation of single cells and allows for additional selection criteria such as morphology or size.

Apart from enabling the analysis of single isolated bacteria, microfluidic techniques may also be used to develop new platforms that permit the culturing of the uncultured (*105*, *106*). A bottleneck that is prohibiting the culturing of certain species is the dependence on factors secreted by other microbes that are part of their natural ecology (*107*). Microfluidic approaches are ideally suited to physically separate cells or small populations while allowing chemical communication to take place between them. Approaches that allow several species to collectively define the chemical complexity of the medium while retaining the contributing cells in pure culture could, for instance, be implemented by using the on-chip ecosystems or 3D-printing technique described above. Other techniques that establish the physical segregation of populations while allowing chemical interactions rely on coupling separate culturing wells through a porous membrane (*8*) or hydrogel (*108*) or use shallow slits that prevent cellular migration from one compartment to the other (*87*, *109*). We expect that these and other (*110*) approaches will prove to be valuable tools to unravel the complexity of natural bacterial communities.

Looking ahead

Microfluidic devices are beginning to be common in microbiology laboratories. This trend undoubtedly will continue because microbiologists have a need for tools that allow precise measurements in environments that can be manipulated at the microscale. Reproducible and controlled experiments are needed to enhance our understanding of bacterial individuality and to unravel complex processes that rely on the coordinated action of many cells, such as biofilm formation. In addition to the techniques reviewed above, imaging mass spectrometry approaches [for example, reviewed in (*56*) and (*111*)] have reached subcellular resolution. Although the required sample preparation currently complicates the integration of such approaches with microfluidic platforms, the possibility to add high-resolution spatial information about the chemical composition of a sample to the phenotypes observed on-chip is promising.

Another emerging trend is the use of bacterial communities inhabiting engineered landscapes as model systems for sociomicrobiology (*9*, *10*, *90*, *112*). The ability to track large numbers of individual cells competing in complex landscapes makes synthetic ecosystems ideally suited to test ecological predictions concerning the environmental conditions that favor cooperation or the implications of habitat structure for biodiversity. The opportunity to probe the same community in diverse ecological scenarios undoubtedly will be valuable to elucidate bacterial social behaviors. Single-cell genomics approaches using novel microfluidic platforms will likely be key to uncover the dark matter of biology. Efforts aimed at dissecting natural bacterial communities are currently only scratching the surface of the vast repertoire of useful substances that presumably are produced by unculturable species. Among those, new classes of antibiotics may, for example, be identified. Other areas of research in which nanotechnology-driven basic research may find important practical applications are the development of microbial fuel cells and the production of nanostructured antibacterial surfaces.

It has been approximately a decade since nanotechnology and microfluidics gained a foothold in microbiology laboratories as new tools with which to study bacteria. This endeavor led to the development of powerful new tools and yielded fundamental biological insights. However, many open questions remain, and exciting times lie ahead at the crossroads of microbiology, microfluidics, and nanofabrication. A further integration of these disciplines will facilitate a deeper understanding of bacterial life by focusing on the details of single cells and broadening our perspective on complex multispecies communities.

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