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FEATURES

Perspective

Bacterial Biofilms, Other Structures Seen as Mainstream Concepts

Get used to it: bacterial microcolonies form regular shapes, such as nanowires or honeycomb-like structures

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As the notion that some bacteria live in structurally complex, multicellular communities gains momentum, let us pause to collect our thoughts. Many microbiologists consign organisms with complex structures or behaviors as "weird" and outside the mainstream. Thus, microbiologists who focus on *Escherichia coli* K-12 acknowledge the complexities of *Myxobacteria* and *Beggiatoa* but may not spend much time thinking about them!

When we see a particular structure or behavior in one organism, we really should look for this structure or behavior throughout the domain. Woody Hastings and Ken Nealson described signal-controlled luminescence in marine vibrios in 1977. However, another two decades elapsed before Peter Greenberg, now at the University of Washington in Seattle, and Barbara Iglewski at the University of Rochester in Rochester, N.Y., established that cell-cell signaling is critical throughout the bacterial domain, and even longer for our group to recognize that such signals help to control bacterial community development. Now we can search for genetic homologies "in silico," enabling us to search more efficiently for common molecular mechanisms anywhere within microbiology.

Summary

- Microbial species reproducibly form regular structures, including "honeycombs" and "veils" that can grow to macroscopic sizes.
- These structures, which are not artifacts, occur both in cultures and ecosystems, and they constitute a genetically determined, heretofore unrecognized structural component of many microbial communities.
- These structures are associated with large numbers of bacterial cells when they are first formed, but may be devoid of cells once the structures mature.
- The structures are not composed of a single extracellular constituent, but appear to contain many components of the cells that form them.

Structured Microbial Communities Come in Many Forms

When individual cells of a single species such as *Myxobacteria* aggregate, they may produce macroscopic communities (Microbe, January 2007, p. 18). However, many natural biofilms typically are featureless until viewed with light microscopes. These magnified views reveal microcolonies in an English garden of topiary

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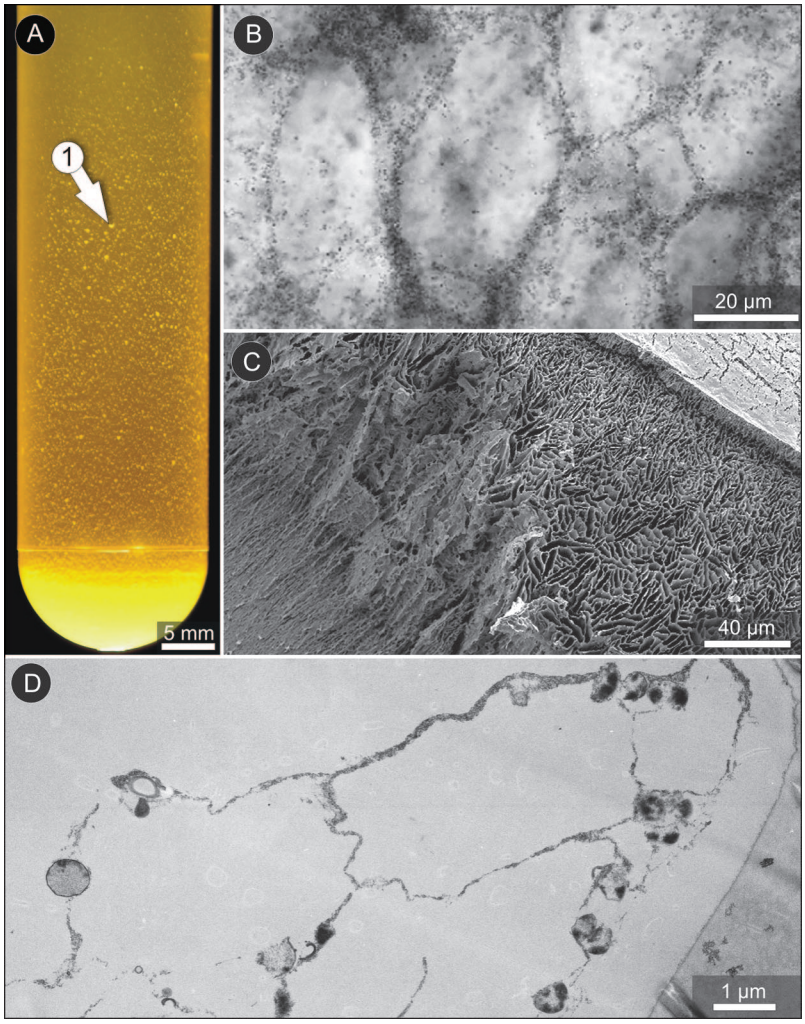
FIGURE 1



(A) Unmagnified view of a 3-day liquid culture of the MH strain of *S. epidermidis* showing the white "nodes" that are suspended in a network that fills the whole test tube and gradually forms a dense white pellet. (B) Confocal micrograph of hexagonal honeycomb structures in a living liquid culture of the MH strain of *S. epidermidis*. (C) The extensive honeycomb-like structures formed, as the result of eutectic formation, when concentrated protein solutions are frozen by the liquid propane method. (D) TEM of the cells and honeycomb elements of a honeycomb produced by the # 355547 ATCC strain of *S. epidermidis*, in a preparation that had been frozen at high pressure to preclude eutectic formation.

delights, taking shapes that resemble mushrooms, towers, and arboreal structures that sometimes bear spores at their apices.

From viewing an extensive variety of such structures, we conclude that phenotypically distinct sessile bacterial cells surround themselves with extracellular polymeric substances (EPS) to form microcolonies whose shape and structure are determined by cell-cell signals and influenced by environmental conditions. Perhaps the epitome of community organization is found in the very extensive (more than 10 cm^2) "veil" communities formed by *Thiovulum* on the surfaces of marine sulfide deposits, with some cells retaining their flagella with which they "ventilate" the whole community while suspended from a common scaffolding.

We can see these highly structured bacterial communities change shape under microscopes. Indeed, we now realize that matrix components form the structures and carry out many of those behaviors. Because self-assembling protein structures are well defined and because activities such as motility and conjugation are measureable, the pili in the intracellular spaces of biofilms can be deduced and in some cases detected. Moreover, nanowires have been discovered, helping to explain how energy is shared among bacteria in a community, how individual cells can be brought together to conjugate, how cells and whole communities can move by twitching or gliding, and perhaps how particular cells can be "placed" within a biofilm.

Are we ready to think of pathogenic bacteria in periodontitis or in the lungs of patients with cystic fibrosis (CF) as members of highly organized communities? If so, we can begin to develop strategies for disrupting those communities by jamming their signals or draining their energy supplies.

Discoveries of Nanowires and Honeycomb-Like Structures at First Prompted Skepticism

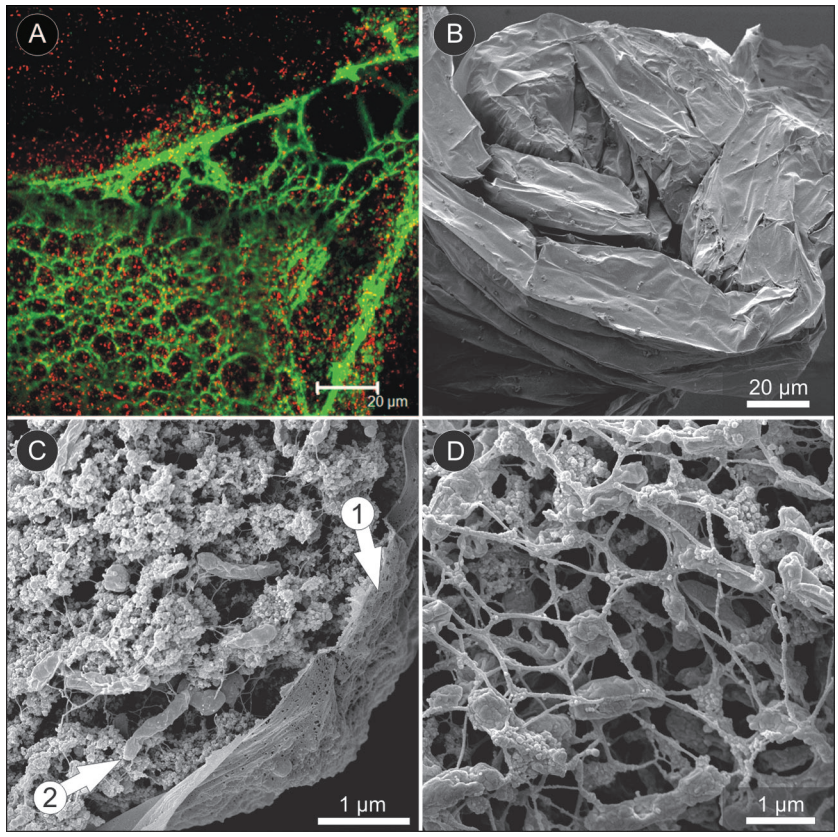
Describing microbial communities as producing nanowires and honeycomb-like structures is to assert a major leap in their complexity. Because both those discoveries came through scanning electron microscopy (SEM), they were met with skepticism. SEM is a useful, high-resolution imaging method. However, specimens for SEM must be dehydrated, either in air or by solvents, which leaves dissolved organic components behind.

These "sticky" residues coat the surfaces of solid components such as cells and pili, sometimes generating bogus "overcoats" and bridges. Simple freezing also removes water and leaves dissolved organic molecules behind, in this case in an equally sticky "eutectic" that can appear as artifacts of baroque complexity when the eutectic freezes. Hence, to rule out artifacts, microscopists insist that novel structures be seen using at least two independent methods before they are considered credible.

For instance, after Yuri Gorby of the Pacific Northwest National Laboratory in Richland, Washington, described nanowires, skeptics told him that they might be artifacts consisting of simple eutectic bridges. These arguments led him to use transmission electron microscopy (TEM) and other methods to prove that nanowires are genuine structures that carry electrical currents for hundreds of microns through microbial communities, laying such doubts to rest.

Meanwhile, we visualized the honeycomb-like structures formed by the MH strain of *Staphylococcus epidermidis* by direct observation (Fig. 1A) and confocal microscopy (Fig. 1B) of living, fully hydrated preparations. However, when we examined the honeycomb-like structures by SEM (Fig. 3 and Fig. 4), we suspected an artifact because Paul Webster could produce a honeycomb-like image by freezing concentrated protein solutions to form a eutectic (Fig 1C). When he next used high-pressure freezing to prepare sections of the MH community for TEM, this more reliable means for preparing specimens showed that the cells produce an extensive honeycomb-like structure (Fig. 1D). Because the honeycomb-like structures of the MH strain are seen in unfixed fully hydrated preparations and by using the rapid high-pressure freezing method, we are confident in using SEM to study them.

FIGURE 2



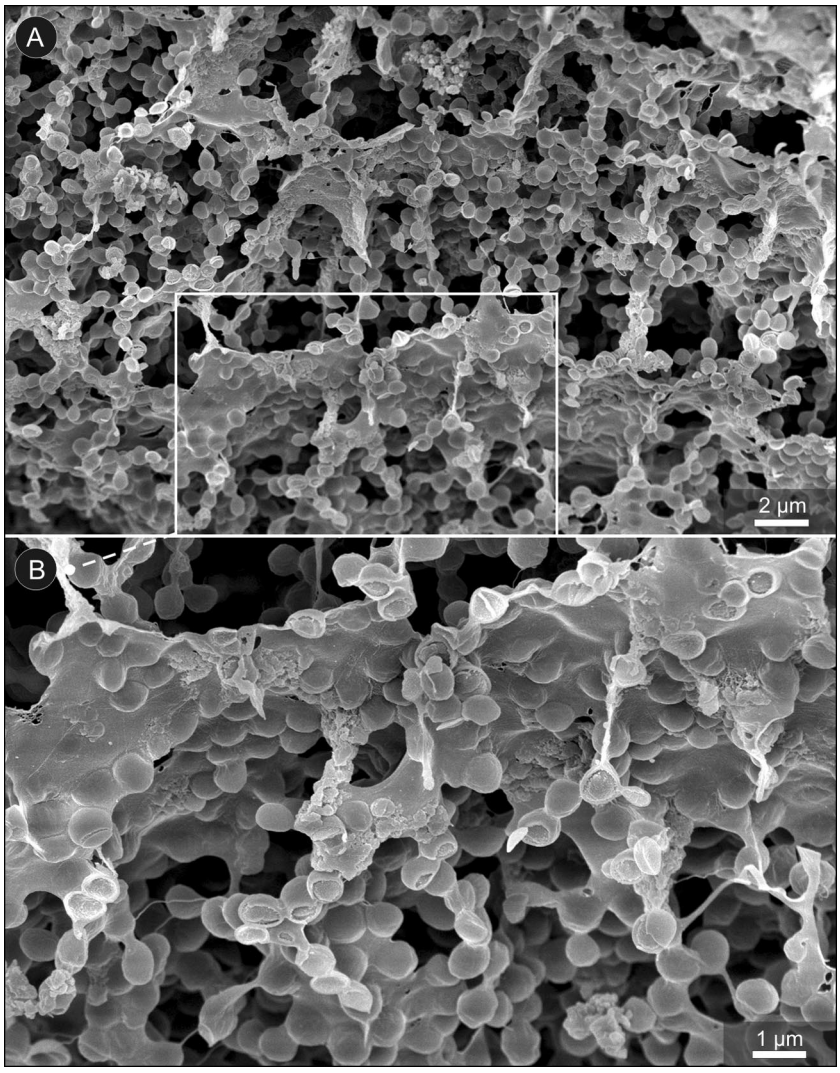
(A) Confocal micrograph of the honeycomb (green) formed by the cells (red) in a 3-day liquid culture of the PAO 1 strain of *P. aeruginosa*. (B) SEM of a collapsed and folded streamer formed in a shaken liquid culture of the EvS4-B1 strain of *Pseudomonas* sp. *TM7_1*. (C) Detail of the membrane (arrow 1) of a streamer which is cleaved to show the distribution of cells (arrow 2) in an amorphous matrix inside this structure. (D) Detail of the interior of another area of the same streamer in which the bacterial cells are integrated into a honeycomb structure with a very fine periodicity of <1 µm.

Biofilms and Other Structures Can Be Regular and Reproducible

Because EPS generally lacks tensile strength, we at first assumed that the characteristic shapes of microcolonies in biofilms result from the rigidity of the cells and the equally rigid pili and nanowires. EPS was thought to account for the overall viscoelastic properties of biofilms and for the elastic deformations seen when these communities are subjected to shear forces.

However, Doug Robinson helped to refine this sense of what underlies the size and complexity of microbial communities when he described the honeycomb-like structures that can fill test tubes in liquid cultures of the MH strain of *S. epidermidis* (Fig. 1A). Similarly, Paul Stoodley and Marc Baum noted that their PAO1 and EvS4-B1 strains of *Pseudomonas aeruginosa* and *Pseudomonas* sp. *TM7_1* form comparable macroscopic networks (Fig. 2). These

FIGURE 3



SEMs of the honeycomb structures produced by the MH strain of *S. epidermidis* showing (A) the development of plate-like structures that extend for as far as 100 microns through the liquid culture, and (B) the alignment of the plates at intervals of +/− 8 µm and the development of partitions at similar intervals. Note that the coccoid bacterial cells are aligned with the plates and partitions, and appear to be intimately associated with these honeycomb structures.

large networks can be lifted from test tubes and laid on microscope slides. Once magnified, the cells are seen as associated with a flexible, three-dimensional, honeycomb-like structure.

Many strains of *S. epidermidis* from dogs with lymphomas produce these huge honeycomb-like structures for one or two serial transfers, and then lose this ability. However, the MH strain retains this capability, and several ATCC strains of *S. epidermidis* and the PAO 1 and EvS4-B1 *Pseudomonas* strains also retain this community-building capacity through many transfers. The EvS4-B1 strain of *Pseudomonas* sp. *TM7_1* that Marc Baum's group isolated from soil forms very extensive honeycomb-like structures when cultivated in a shaken fluid culture (Fig. 2B-D), while the PAO 1 strain of *P. aeruginosa* forms similar structures (Fig. 2A) when cultivated in flowing liquid culture with periodic nutrient replacement.

We speculate that these network structures help biofilms survive when they are subject to fluid forces. A rigid sheet of honeycomb-like structures may provide mechanical stability that could serve as an important virulence factor, helping to wall off host defenses. The elastic honeycomb sheet may allow deformation in response to stress applied along any one of the six axes of symmetry. Flexible networks could deform yet allow the biofilm to return to its original structure once a stress is removed.

A honeycomb arrangement may also provide a "rip-stop" function, limiting tears by distributing the force over six vertices. This attribute would be a useful when a biofilm is exposed to a multidirectional flow field, such as those of streambeds and ocean sediments. The fluttering and stretching observed when these structures are subjected to shear forces supports this hypothesis. In addition, honeycomb-like structures may lower the energy costs of individual cells faced with limited nutrients. Moreover, the tertiary structure of honeycombs may maximize the surface area available for absorbing nutrients.

Regularly Structured Matrices Can Form within Microbial Communities

In flowing systems, the PAO 1 strain of *P. aeruginosa* makes honeycomb-like structures (Fig. 2A) that form discrete membrane-enclosed streamers very similar to those that the EvS4-B1 strain forms in shaken cultures (Fig. 2B). After 3

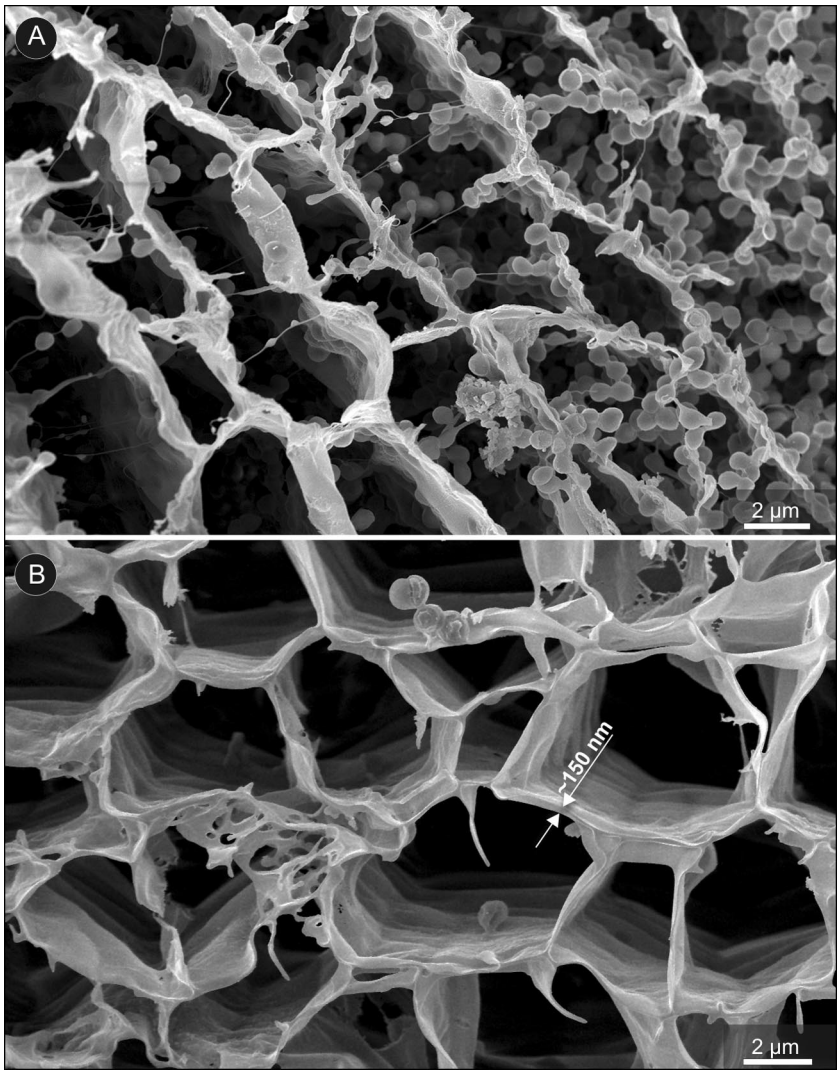
days, the flow-based PAO1 culture is composed of a mixture of hexagonal sheets (Fig. 2A) and discrete streamers, while the shaken culture of the EvRS-B1 strain is composed predominantly (Fig. 2B) of streamers (at 7 days). Those streamers contain numerous rod-shaped cells encased in a structured matrix material (Fig. 2C, arrow 2, and Fig. 2D).

In both strains the streamers containing the bacterial cells are surrounded by a coherent membrane (Fig. 2B and Fig. 2C, arrow 1) that encloses the cells in a manner that would preclude their escape into the fluid. In some areas within the streamers the cells of EvS4-B1 strain of *Pseudomonas* sp. *TM7_1* are embedded in an amorphous material (Fig. 2C). However, elsewhere, they are enmeshed in a honeycomb-like structure with a very fine periodicity of less than 1 μm (Fig. 2D).

Liquid cultures of the MH strain of *S. epidermidis* grow as a suspension of individual planktonic cells until day 2, when white macroscopic "nodes" begin to form. With SEM, we can reconstruct the process of honeycomb building (Fig. 3 and 4). Simple plate-like structures continue to appear in the culture until thin planar "walls" extend for more than 100 μm (Fig. 3A). In other locations the walls align at distances of about 8 μm, and individual cells intimately associate with these planar structures, forming sites that appear clear and smooth in some places, while others are studded with coccoid bacteria.

When the walls are structurally coherent and almost devoid of adherent cells, cells gather into rows at approximately 8-μm intervals on the wall surfaces. These aggregates of coccoid cells appear to form "partitions" joining the walls (Fig. 3B). Ultimately, mature honeycomb-like structures have walls of about 150 nm (Fig. 4B, arrows) and partitions of about 100 nm. These honeycomb-like structures are highly organized and no longer are associated with individual cells (Fig. 4B and cover).

FIGURE 4



SEMs of mature honeycomb structures produced by the MH strain of *S. epidermidis* in which (A) some cells are still associated with the walls and partitions and (B) in which some areas of these very regular structures are devoid of the bacterial cells that formed them. The very regular dimensions (arrows) of the walls (+/− 150 nm) and partitions (+/− 100 nm) can be seen, where they are cross-fractured, and each element of these complex structures is seen to be very deep (>30 μm).

Ruminations on the Ramifications of These Microbial Ramparts

These honeycomb-like structures in liquid cultures of the MH and other strains of *S. epidermidis* and of the PAO 1 and EvS4-B1 *Pseudomonas* strains are made from pure cultures of bacteria, all of which were transferred by standard techniques in serial cycles. They contain no eukaryotic cells or extraneous DNA.

These honeycomb-like structures fill the greater part of culture vessels, and each has an architecture peculiar to the organism and the genome concerned, indicating that their tertiary structures are firmly under genetic control. The developmental cycle of each community is repeated when the culture is transferred, meaning the ontogeny of the honeycomb-like structures is as reproducible as the embryology of higher organisms. Thus, we can formulate several "embryological" questions for these bacteria. How do the cells consistently produce a plate shape, not a blob or star? How do the plates align at such regular 8-μm intervals? How do these bacteria construct partitions at 8-μm intervals on the face of each wall? What stops the bacteria when a wall or a partition reaches its "programmed" thickness? Perhaps the networks function in other than structural capacities. For example, they might serve as communication routes for chemical signals, "roadways" along which bacteria glide, or, extending the nanowire concept, electrical conduits for solid-phase electron acceptors.

It is striking to us that the tertiary honeycomb-producing microorganisms include *S. epidermidis*, a human skin commensal species that is ubiquitous in our environment, and *P. aeruginosa*, the predominant aquatic organism on earth. These are not rare or unusual bacteria. Further, the ability to construct honeycomb-like structures is widespread among ATCC strains of *S. epidermidis*, and it is retained through multiple serial transfers. For instance, the EvS4-B1 strain of *Pseudomonas* sp. *TM7_1*, which was isolated from soil at Sulphur Mountain in Ventura County, Calif., has retained its ability to construct wall-enclosed honeycomb-like structures through at least 20 serial transfers, while the PAO 1 strain has been carried in various labs for 30 years. Meanwhile, the common occurrence of microbial veils on the surfaces of sulfidic deposits in marine environments indicates that *Thiovulum* species form complex biofilms, with some cells retaining their flagella while others adopt the biofilm phenotype.

These recent discoveries embarrass some of us for having overlooked them for so long. Thus, 3-day-old cultures of *S. epidermidis* typically contain visible white foci and complex honeycomb-like structures. Some of them likely sat unnoticed in test tubes on lab benches since the 1860s. But it is time to set such regrets aside. We now know the genomic sequences of both *S. epidermidis* and *P. aeruginosa*, and we can introduce mutations that will impair the ability of both these organisms to produce or control the detailed structures of their respective honeycomb architectures to determine what genes are responsible.

As we fill blank spaces in the genomes of bacteria, we will identify the genes that control biofilm formation, interspecies interactions, and the architecture of structures that constitute multispecies communities in which most bacteria live. We will also discover the genes that control the acquisition and structure of commensal biofilms on which much of human health depends, enabling us to cultivate our microbial friends and confound our microbiological enemies. Perhaps, microbial endocrinologists will develop signals that will make lactobacilli grow faster and *Streptococcus pyogenes* grow more slowly, while microbial neurologists will learn to short-circuit nanowires running within mixed-species communities in periodontal pockets. The borders between eukaryotic and prokaryotic biology are blurring, making microbiology even more exciting as we begin to apply general biological concepts to bacteria.

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SUGGESTED READING

Beveridge, T. J. 2006. Visualizing bacterial cell walls and biofilms. Microbe **1:**279–284.

Costerton, J. W. 2007. The biofilm primer. Springer, in press.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.

Gorby, Y. A., S. Yanina, J. S. McLean, K. M. Russo, D. Moyles, A. Dohnalkova, T. J. Beveridge, I. S. Chang, B. H. Kim, K. S. Kim, D. E. Culley, S. B. Reed, M. F. Romine, D. A. Saffarini, E. A. Hill, L. Shi, D. A. Elias, D. W. Kennedy, G. Pinchuck, K.

Watanabe, S. I. Iishi, B. Logan, K. H. Nealson, and J. K. Fredrickson. 2006. Electrically conductive bacterial nanowires produced by *Shewanella oneidensis* strain MR-1 and other microorganisms. Proc. Natl. Acad. Sci. USA **103:**11358–11363.
Moscoso, M., E. Garcia, and R. Lopez. 2006. Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. J. Bacteriol. **188:**7785–7795.
Robinson, D. H. 2005. Pleomorphic mammalian tumor-derived bacteria self-organize as multicellular mammalian eukaryotic-like organisms : morphogenetic properties in vitro, possible origins, and possible roles in mammalian 'tumor ecologies.' Med. Hypotheses **64:**177–185.
Stoodley, P., K. Sauer, D. G. Davies, and J. W. Costerton. 2002. Biofilms as complex differentiated communities. Annu. Rev. Microbiol. **56:**187–209.
Thar, R., and M. Kühl. 2002. Conspicuous veils formed by vibroid bacteria in sulfidic mine sediment. Appl. Environ. Microbiol. **68:**6310–6320.
Tolker-Nielsen, T., U. C. Brinch, P. C. Ragas, J. B. Andersen, C. S. Jacobsen, and S. Molin. 2000. Development and dynamics of *Pseudomonas* sp. biofilms. J. Bacteriol. **182:**6482–6489.
Webster, P., S. Wu, S. Webster, K. A. Rich, and K. McDonald. 2004. Ultrastructural preservation of biofilms formed by non-typeable *Haemophilus influenzae*. Biofilms **1:**165–182.