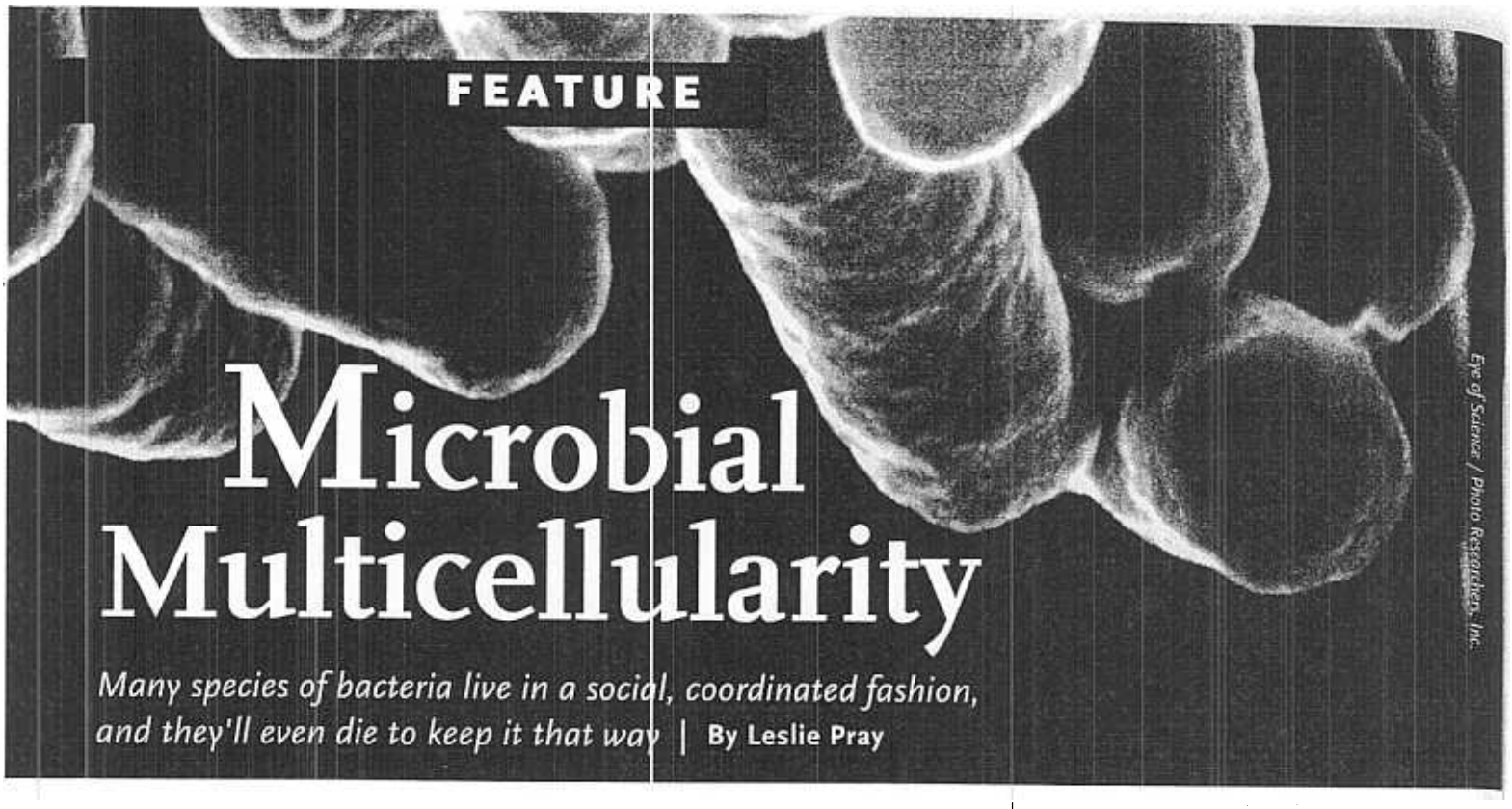


FEATURE

Microbial Multicellularity

Many species of bacteria live in a social, coordinated fashion, and they'll even die to keep it that way | By Leslie Pray

Eye of Science / Photo Researchers, Inc.

"The general character and structure of the rod-like individuals, together with their vegetative multiplication by fission, renders their schizomycetous nature as individuals a matter hardly to be doubted: but, on the other hand, the question may fairly be asked whether the remarkable phenomena may not indicate a possible relationship in other directions."

—Roland Thaxter, 1892

While walking through the New England woods one day in the late 19th century, Harvard microbiologist Roland Thaxter came across a bright orange, fungi-like growth unlike any organism he had ever seen. He took some of the mysterious organic matter back to his laboratory. Over the next two years, Thaxter collected and cultivated several more samples of this peculiar new organism, which he named *Myxobacteriaceae*. Characterized by an unusually complex life history for a bacterium, involving the formation of an elaborate, macroscopic fruiting body, Thaxter considered his find an "altogether so unique" exception to the unicellular rule.[1]

Myxobacteria, as they are commonly known, may not be so unusual after all. "The kind of behavior that myxobacteria exemplify is widely present, perhaps even universally present, among bacteria," says Martin Dworkin, a bacteriologist at the University of Minnesota, Minneapolis. James Shapiro of the University of Chicago concurs: "Even very standard bacteria, like *Escherichia coli*, do things in a multicellular, coordinated fashion."

It wasn't until nearly a century after Thaxter's discovery, when Shapiro shocked his colleagues with descriptions of regulated colony growth and pattern formation in *E. coli*, that the notion of microbial multicellularity attracted serious attention. At about the same time, Bill Costerton at Montana State University, Bozeman, coined the term "biofilm" to refer to highly structured, matrix-encased communities of bacterial cells living cooperatively. Now, microbiologists generally agree with Costerton that, in nature, most bacteria live as biofilms, with the exception of planktonic marine microbes in the open ocean.

SOCIAL CREATURES Biofilms, myxobacterial fruiting bodies, and *E. coli* pattern formations are remarkable, even beautiful, sculpture-like ingenuities of nature, but there's more to bacterial multicellularity than morphology. Some scientists, such as evolutionary biologist Gregory Velicer, Max Planck Institute for Developmental Biology, Tübingen, Germany, consider bacterial cells social creatures. This social capacity can rapidly degrade, he says, if they live in an asocial environment, like a liquid batch culture, where the bacteria don't need to develop fruiting bodies or behave like multicellular organisms to survive.

In the last decade, researchers discovered that population density alters gene expression in bacterial cells, a mechanism known as quorum sensing. Originally thought to be unique to the marine bacterium *Vibrio fischeri*, in which it controls bioluminescence, quorum sensing is now known to control a diverse range of behaviors, such as sporulation, genetic competence, and virulence factor production, in more than 100 species of bacteria. Scientists study the links between quorum sensing and a range of multicellular bacterial behaviors, including biofilm formation and swarming motility. In the latter, cells assemble into flagellar-driven rafts that can scoot over surfaces that individual cells wouldn't otherwise be able to cross.

The basic principle of quorum sensing is the same for all bacteria, though the signaling pathways and molecules that regulate them, known as autoinducers, vary between species.[2] In Gram-positive bacteria such as pneumococci, autoinducing peptides move into the environment until they reach a certain concentration at which sensors on the cell surface can recognize them. The sensors then initiate a phosphorylation cascade, which activates a DNA-binding protein that modifies gene expression.

In Gram-negative species such as *Pseudomonas aeruginosa*, where peptides could potentially get stuck in the cell membrane, acyl-homoserine lactones (AHLs) do the job. AHLs, synthesized by LuxI-type proteins in the cytoplasm, diffuse across the cell membrane until the AHL concentration is the same inside and outside each cell. When the concentration reaches a certain level, AHLs form complexes with LuxR-type proteins that bind promoters of target genes, thus activating transcription. Bacteria may have one or more AHLs, depending on the number of their genes controlled by quorum sensing. "Some bacteria control hundreds of genes [with quorum sensing], while others only control a few genes," says microbiologist Peter Greenberg, University of Iowa, Iowa City.

CROSS TALK As AHLs are species-specific, other nearby bacteria cannot intercept their signals; however, researchers have

implicated mechanisms similar to those controlling intraspecies communication in interspecies signaling.[3] The first evidence of cross talk came from *Vibrio harveyi*, which uses unique autoinducer molecules: AI-1 serves as its intraspecies signal, while AI-2 allows communication with others. Princeton University's Bonnie Bassler says that AI-2 is a universal signal that allows bacteria to detect the presence of other species in a community, but that other, yet unknown molecules probably control behavioral responses. Says Bassler: "AI-2 regulates a fundamental collection of genes that all bacteria have, but layered on top of that will be niche-specific controls .... If AI-2 is the generic language, then you can start to think about making a drug that affects all bacteria."

AI-2 production depends on an enzyme called LuxS, while its detection requires LuxP and LuxQ. AI-2/LuxS signaling controls behaviors as varied as motility in *Campylobacter jejuni*, and virulence in *Streptococcus pneumoniae* and *Vibrio cholerae*. Genes encoding LuxS have already been found in more than 40 species of Gram-negative and Gram-positive bacteria.[2]

However, communicating with other species of bacteria can be a risky business, because "you don't know what the other critter is going to do," says Greenberg. *Staphylococcus aureus* autoinducers upregulate their own virulence factor production cascade and inhibit the cascade in nearby colonies—a form of competition between quorum-sensing systems. *Pseudomonas aureofaciens*, a plant bacterium, uses AHLs to regulate its own antibiotic production and detect antibiotic molecules of nearby species, so they can kill them.

Soil bacteria play especially dirty. *Bacillus subtilis* can recognize the presence of its neighbor *Erwinia carotovora* and render it avirulent, while *Variovorax paradoxus* consumes the AHLs of other species for their carbon and nitrogen.[2]

BIOFILMS In some species, such as *P. aeruginosa*, biofilms can form without quorum-sensing signals, although AHLs are needed for the metabolic diversity and development of complex structures within them.[4] AI-2/LuxS signals are known to coordinate biofilm formation in *Streptococcus* and *Salmonella* species. In the human oral cavity, LuxS is required for the formation of mixed-species biofilms.

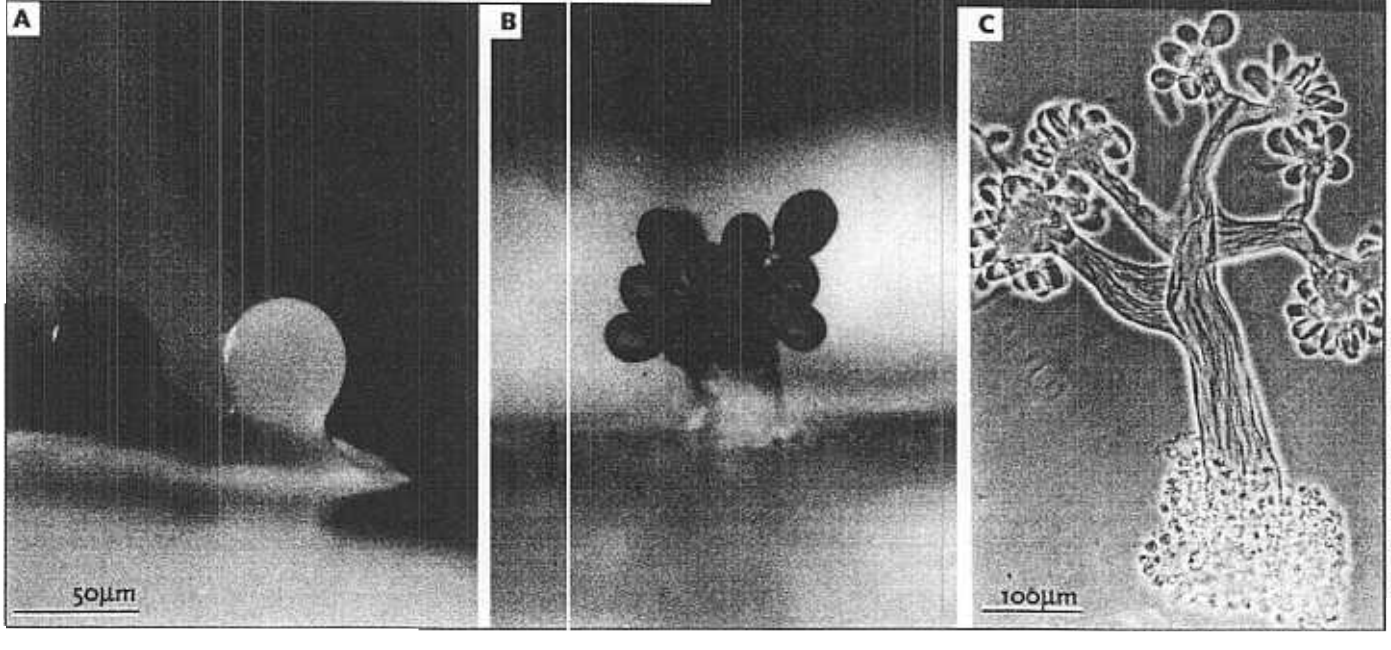
With swarming motility, certain vegetative cells can elongate, grow extra lateral flagellae, and differentiate into swarmer cells, which can then leave the colony as a group to search for more food, explains microbiologist Rasika Harshey, University of Texas, Austin. This type of swarming motility, responsible for the spread of *E. coli* in urinary tract infections, occurs only on surfaces, not in broths, and requires the making of sugary slime. The slime's polysaccharides may be inversely related to those needed for biofilm formation: motility is movement, while biofilms involve "hunkering down," says Harshey. She adds that while quorum sensing may help swarming motility by controlling slime production, there is no evidence yet that quorum sensing has any direct control over swarming motility.

A DATE WITH DEATH That bacteria are social is perhaps nowhere more evident than in how they kill themselves and each other, through a process known as programmed cell death (PCD), which is mediated by an intracellular death program. Northeastern University microbiologist Kim Lewis explains PCD as the self-elimination of cells that have made informed decisions to commit suicide.

PCD plays a critical role in eukaryotic development. In humans, for example, apoptosis eliminates cells that would otherwise develop into webbing between fingers and toes, like that seen in frogs and ducks. But the term apoptosis cannot properly be used to describe PCD in bacteria; apoptosis involves complex processes such as organelle disassembly, which are unique to eukaryotes. Even the term "programmed cell death" is debated as to whether it properly references microbes. When he talks about bacterial PCD, says Ken Bayles, University of Idaho, Moscow, Idaho, "People ask me, where is the "programmed" in programmed cell death?"

Bacteriologists aren't trained to think of bacteria (except myxobacteria) as having developmental cycles worthy of the kind of intercellular coordination and communication that PCD requires. When *Myxococcus xanthus*, a commonly studied myxobacterium, forms fruiting bodies, for example, anywhere from 20% to 90% of the cells die. That, says Lewis, is an obvious, nearly trivial example of cell suicide in bacteria. Lewis and Bayles both suspect that PCD

FRUITS OF THEIR LABORS: *Three fruiting bodies: (A)* Myxococcus fulvus; *(B)* Stigmatella aurantiaca; *and (C)* Chondromyces crocatus. *S. aurantiaca stands at about 150 microns tall. (From M. Dworkin,* Microbiol Rev, *60:70-102, 1996)*



is widespread among microbes and may even have something to do with bacteria succumbing to and ultimately overcoming antibiotic assaults. "We know that antibiotics kill bacteria," says Bayles, "but we don't call it programmed cell death—yet."

THE EVIDENCE FOR BACTERIAL PCD Strong evidence supporting the existence of eukaryotic-like bacterial PCD comes from Staffan Kjelleberg, University of New South Wales, Australia, and collaborators. In August, Kjelleberg and his Australian and Danish colleagues reported the first known case of PCD in biofilm development, in *P. aeruginosa*, a pathogen associated with cystic fibrosis.[5] The patterns of cell death that they observed during development of *P. aeruginosa* colonies were, they reported, "strikingly similar" to the autoinduced killing events that characterize myxobacterial sporulation. The team also identified a phage infection-mediated mechanism that likely plays a role in the process.

More recently, Kjelleberg and colleagues identified another, similar pattern of cell death during biofilm development of the newly described marine bacterium *Pseudoalteromonas tunicata*. As Kjelleberg explains, *P. tunicata* typically inhabits living marine surfaces, such as tunicate surfaces (hence its name), algae, and marine plants. Because of its sessile lifestyle, Kjelleberg wondered if, as a way of regulating its growth and preventing overcrowding, the bacteria practiced some form of PCD. Indeed, they do. His team observed that *P. tunicata* biofilms reach a point during their development at which cells start to die, hollowing out the biofilm structure, causing it to burst open and detach from its substrate. Afterward, only single cells remain attached to the host surface. "It happens like a clock," says Kjelleberg. The various stages of cell death and detachment occur at predictable intervals, indicating that some sort of intracellular programming is likely. But in this case, the mediator is not a prophage but a novel autotoxic protein, AlpP. Mutant *P. tunicata* with defective AlpP-associated activity either can't kill themselves, or they take much longer to go through the development-detachment process.

Kjelleberg says that one of the reasons this death phenomenon is only now being recognized is because it occurs relatively late in biofilm development. He likens the study of bacterial PCD to the study of aging. Most organism research stops when an individual reaches adulthood or reproductive maturity; few investigators wait around to see what happens afterwards. Meanwhile, Kjelleberg says he has unpublished, preliminary evidence for PCD in a cholera species.

ANTIBIOTICS: SUICIDE INDUCER Apoptosis in eukaryotes is largely mediated by signaling from proteins in the Bcl-2 family. Bayles, who studies Cid/Lrg-mediated killing in *S. aureus*, suspects that the conserved proteins Cid and Lrg are functional, if not molecular, analogs to Bcl-2 proteins. The connection is only speculative at this point, he says, but he notes that the work is indicative of changing views on the mechanisms of bacterial cell death: "Programmed cell death isn't just more evidence for multicellularity; it makes sense because of multicellularity."

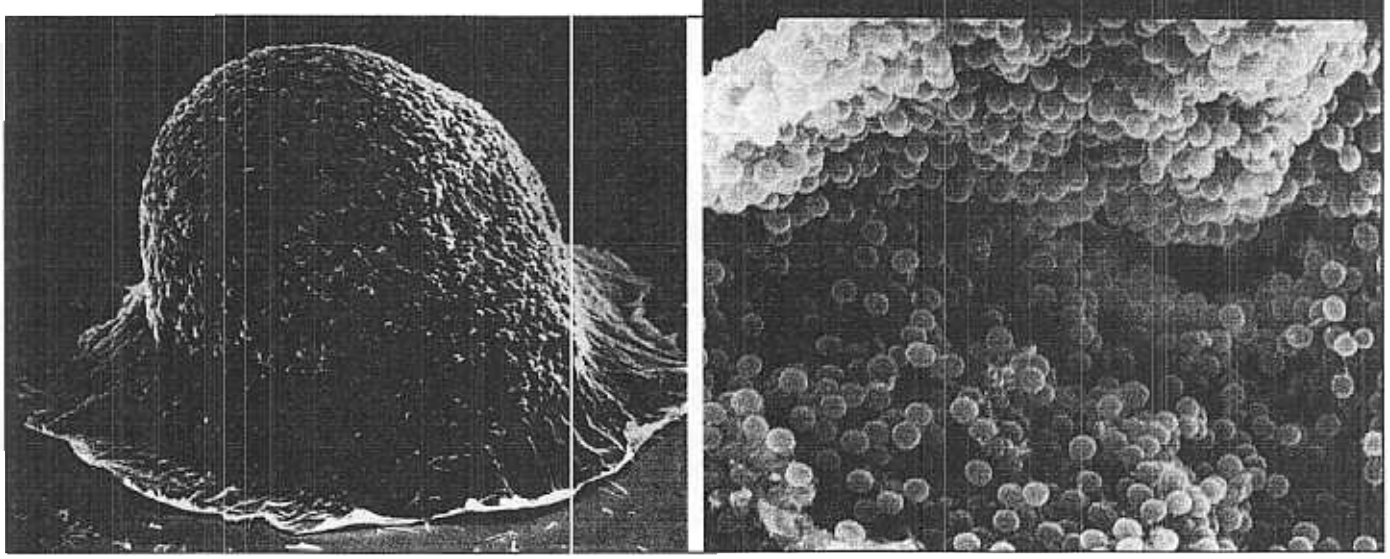
The big unsolved question regarding PCD isn't whether it happens, says Lewis, but whether defective bacterial cells, including those damaged by antibiotics, commit suicide. Along with its developmental role, PCD in eukaryotes helps eliminate damaged cells that would otherwise burden their neighbors. Lewis has proposed that PCD may function similarly in bacterial colonies in the face of bactericidal attack. He suspects that cells damaged irreparably by penicillin or other antibiotics kill themselves so that they don't become a burden to their remaining bacterial brethren.

The argument makes evolutionary sense in a group selectionist way.[6] A suicidal cell may not appear to be doing itself any good, but the act may benefit the population as a whole. Moreover, if other members of the population are related to the suicidal cell (and thus contain some of its DNA), that cell might increase its chances of passing its genes to the next generation. By killing themselves, not only would the damaged cells no longer burden the bacterial population, but their lysed bodies also would provide needed nutrients for their kin and neighbors.

Although many evolutionary biologists accept this altruistic logic, those in the microbiology community have yet to take a strong hold of the notion. "Everybody agrees that mother-cell lysis is programmed cell death, but that is also rather trivial," says Lewis. "What people will not necessarily accept is the notion that defective cells commit suicide."

Lewis suspects that, at a certain point during an antibiotic attack, the suicidal programming shuts off in at least some of the damaged cells. Otherwise, strong antibiotics could potentially force all the cells to kill themselves, which, from a bacterial point of view, doesn't do anybody any good. He calls these deprogrammed cells "persistor cells," which he says are genetically identical to their suicidal kin but with unexpressed PCD genes. If his predictions are

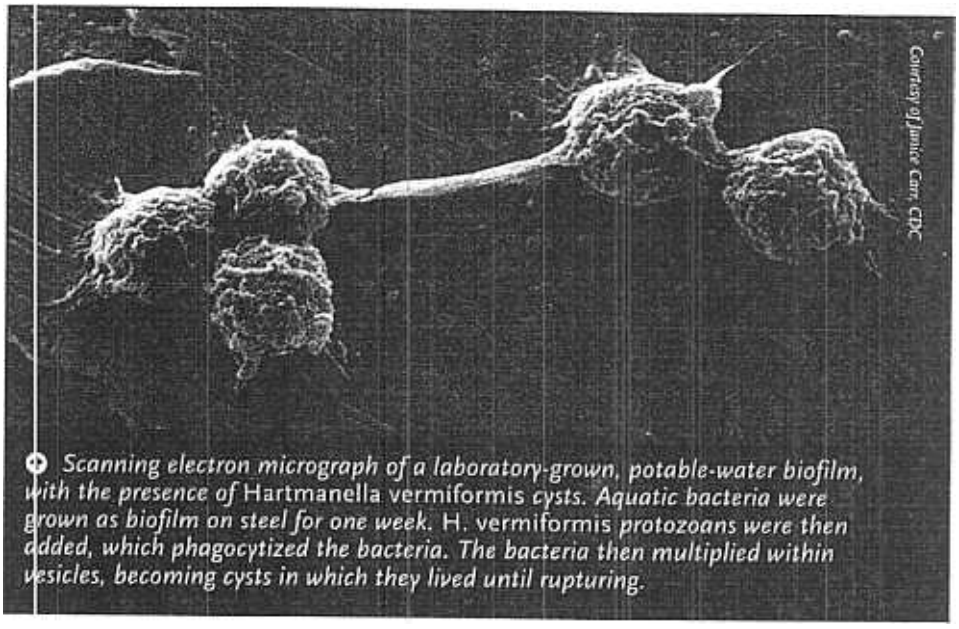
STAVING OFF HUNGER: *A fruiting body of* Myxococcus xanthus *asssembles in response to starvation from about 100,000 cells (right). A fruiting body that is cracked open to reveal the spherical pores inside (left).*



Courtesy of Jerry Kaiser and Dale Kaiser, Stanford University

correct, understanding PCD in bacteria could have enormous implications for fighting infection. "We don't have drugs against persistor cells," he says, so finding a drug that activates the gene(s) responsible for programmed cell death would be a good strategy.

CANNIBALISM: A NEW TYPE OF BACTERIAL PCD Although PCD connotes self-inflicted death, Rick Losick and colleagues from Harvard University and the National Institute of Aerospace Technique, Huelva, Spain, recently found that colonies of *B. subtilis*, a close cousin to *Bacillus anthracis* (the etiological agent of anthrax), undergo a newly discovered nonsuicidal, cannibalistic PCD.[7] Two years ago, Losick and collaborators discovered that spore formation in *B. subtilis* isn't the simple, single-celled process that microbiologists had long believed. Instead, when faced with impending starvation, motile cells align themselves to form multicellular aerial structures that resemble, but are not nearly as grandiose, as the spatially complex myxobacterial fruiting bodies. As with the myxobacteria, cells inside the fruiting body eventually develop into inert spores, which can remain dormant for hundreds of years, says Losick.



Scanning electron micrograph of a laboratory-grown, potable-water biofilm, with the presence of Hartmanella vermiformis *cysts. Aquatic bacteria were grown as biofilm on steel for one week.* H. vermiformis *protozoans were then added, which phagocytized the bacteria. The bacteria then multiplied within vesicles, becoming cysts in which they lived until rupturing.*

Entering sporulation is not an easy decision for a cell to make, Losick explains. It is a time-consuming (s x to seven hours), energy-intensive (especially for a hungry cell) process that could leave a cell unable to reproduce for a very long time. Cells are better off delaying sporulation for as long as possible, scavenging in the meanwhile, in case things take a turn for the better. So they wait until they are absolutely, irrevers bly starved for food before they enter sporulation.

Cells can resist sporulation altogether by killing their kin and feeding on the released nutrients. The cannibalistic act occurs when, in some cells, the regulatory protein SpoOA switches on two operons, skf (sporulating killing factor) and sdp (sporulating delay protein). Skf produces a killing factor which, when exported from the cell, lyses nearby cells that have inactive SpoOA. Sdp makes the victims hypersensitive to the killing agent, probably by repressing a gene that would otherwise protect the SpoOA-inactive cells from being lysed. "The target cells get a double whammy," says Losick.

Although cannibalism seems like a destructive behavior, Losick says that it happens "for the good of the species, so to speak." As with cell suicide, the act is ultimately advantageous for the population as a whole, since it delays and, if all goes well, prevents sporulation.

MULTICELLULARITY: THE NEW MANTRA *B. subtilis* has been considered a paradigm of unicellularity for a long time. Researchers' discernment that this model organism exhibits such remarkable multicellular behaviors illustrates just how far microbiology has come over the last century. "It's insulting to microbiologists like myself, who have been barking up the wrong tree for the past 150 years," says Costerton.

Losick says that he would not have detected any of these behaviors in *B. subtilis* if he had chosen to study laboratory strains that originated 100 years ago. Instead, he went *au naturel*. "The interesting point is that we can only see this when we study wild strains," he says. Velicer has conducted experiments demonstrating that, when grown isolated in liquid culture, even *M. xanthus* loses its multicellular, social capacities.

The pure-culture concept is "an incredibly powerful tool that served microbiology very well for 100 years," says Dworkin. Medical microbiologists have used pure cultures of isolated, single-cell organisms to demonstrate the causes of many blood-borne infectious diseases, from typhoid to bubonic plague. By studying microbes in isolation, scientists also have gathered tremendous information about how individual cells function.

But, as modern microbiologists have found, bacteria in natural environments, even pathogens in the human body, are not the single-minded opportunistic creatures that Robert Koch's paradigm has led microbiologists to believe. Koch, a Nobel laureate, one of the founders of bacteriology, and Thaxter's peer, devised what are known as Koch's postulates: empirical steps required to identify the etiological cause of infectious disease. The postulates rely on the notion that microbial agents are single-celled organisms that can be isolated readily in pure culture.

Not that Koch was wrong. "Koch was right on," says Losick. "But, nonetheless, a single cell can grow up into a colony and that colony can behave in a multicellular manner. In the early years, people were so interested in what a cell was and how it worked, the idea that cells were interacting with each other wasn't on the horizon."

But now, says Costerton, "By only studying planktonic cells, because that's what we're equipped to study, it's like studying our sperm and eggs without ever getting to the liver, let alone the genes that are expressed in the liver." Says Shapiro, "In the future, we will come to appreciate [that] much of what bacteria do in nature, in all their different roles, involves interaction and communication with other cells." ⊗

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