MICROBIOLOGY

One for All and All for One

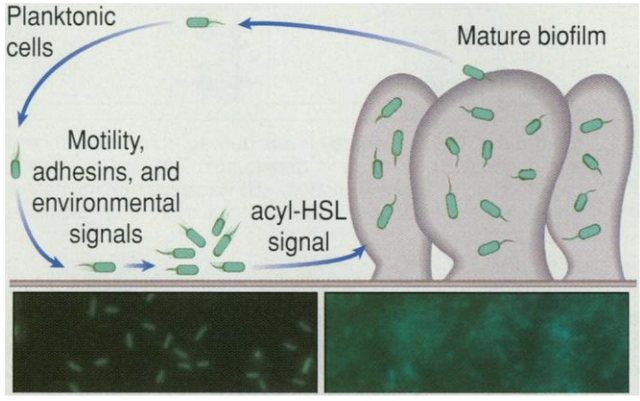
Roberto Kolter and Richard Losick

Communities form by interactions amongst individuals. In the beginning, a few wandering souls find an appropriate location in which to settle. As the population numbers increase at this spot, individuals must communicate closely to ensure adequate distribution of food and removal of wastes. If successful in these early stages, new communities can flourish and stabilize and their members can enjoy the shelter afforded by living in a protective environment.

These words could describe the growth of a village, but the same description can also be applied to community formation by the simplest of all organisms, the bacteria. Despite the widely held view of bacteria as primitive, unicellular organisms that struggle for individual survival, it is becoming clear that bacteria seldom behave as isolated organisms (*1*). Rather, the apparent simplicity of bacteria belies their extraordinary sophistication in communicating with one another and sometimes with higher organisms as well. Informed by chemical communication, motile cells of the myxobacteria and filamentous cells of the streptomycetes organize themselves into conspicuous multicellular structures that carry out specialized tasks in spore formation and dispersal (*2*). Furthermore, most bacteria have evolved elaborate mechanisms for adhering to solid surfaces and thereby establishing complex communities referred to as biofilms. But it has been mysterious how bacteria in these biofilm communities communicate with each other to coordinate their behavior. In a report appearing on page 295 of this issue, Davies *et al.* shed new light on cell-to-cell signaling during the development of a bacterial biofilm (*3*).

Bacterial biofilms are ubiquitous and play a multitude of important roles in different environments. In many instances they provide beneficial effects to other organisms. Such is the case for biofilms of *Pseudomonas fluorescens* that form on the surface of plant roots, thereby preventing the growth of fungal pathogens. In other situations, bacterial biofilms can have a deadly effect. In a clinical setting biofilms can wreak havoc when they form on catheters or on medical implants. Such infections are extremely difficult to control because biofilm bacteria, for reasons that remain largely unknown, are extremely resistant to the action of antimicrobial agents.

Even though most microorganisms grow as biofilms and the physical structure of different biofilms is well characterized, until recently, this important life form had not been studied with molecular genetic approaches. This is rapidly changing, and many bacterial mutants are now being analyzed for their effects on biofilm development (*3*, *4*). A good example of the productive results of this approach is the work of Davies *et al.*, who used specific mutants of *P. aeruginosa* to demonstrate that a signal molecule provides a form of cell-to-cell communication that is an essential component for the normal development of biofilms.



Construction of a biofilm. Free (planktonic) bacteria assemble on a surface (**left**). Cell-to-cell communication then induces the formation of multicellular pillars and columns (**right**).

The type of molecule responsible for the cell-to-cell communication in *P. aeruginosa* biofilms—an acylated homoserine lactone (acyl-HSL)—is already familiar to many microbiologists. Acyl-HSLs act as extracellular signaling molecules that control a plethora of phenomena, among them bioluminescence, exoenzyme synthesis, and virulence factor production (*5*). The acyl-HLSs (as well as many peptides) are secreted by bacteria such that they accumulate in the medium in proportion to the total number of cells. In this way, they provide an index of population densities, earning them the name of quorum sensors. With the new work, it now becomes clear that within a sessile community such signals are critical for the multicellular life of microorganisms.

The formation of biofilms can be considered as a developmental cycle (see the figure). It begins when free-swimming (planktonic) bacteria recognize a surface and firmly attach. For many bacteria, this process requires flagella or surface adhesins and depends on nutritional signals from the environment (*4*, *6*). Subsequently, the attached cells grow and divide and at the same time recruit additional planktonic cells that attach to the cells already on the surface. But left unchecked, simple growth of the bacteria on the surface would eventually lead to extreme crowding, possibly starving many cells that might not be able to obtain nutrients. At the same time, toxic metabolic wastes could accumulate among the densely packed cells. The solution to these problems is to create space between loosely packed clusters of cells. Attached bacteria migrate slightly from the surface as they excrete extracellular polysaccharides that serve as the matrix for the biofilms. As the biofilm architecture develops, cells cluster in pillar- and mushroom-like structures, with water channels between them through which nutrients can flow in and waste products out, functioning very much like a primitive circulatory system (*6*). Mature biofilms thus have a specialized architecture that ensures the well-being of the individual cells that compose it. The sloughing-off of individual cells from the biofilm completes the developmental cycle.

Davies *et al.* have made the important observation that one of two acyl-HSLs synthesized by *P. aeruginosa* [*N*-(3-oxododecanoyl)-L-homoserine lactone) is a key signaling molecule in the development of biofilm architecture (*3*). Mutant cells unable to synthesize this acyl-HSL were still able to initiate biofilm formation by attaching to the surface and multiplying there. But the cells failed to create space between them and failed to form the elaborate architecture of mature biofilms, despite the fact that they produced the same amount of extracellular polysaccharide. Addition of synthetic signal molecule restored the architecture to the biofilm, suggesting that gradients of the molecule per se did not generate the

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structures characteristic of normal biofilms. Rather, it appears that the presence of the acyl-HSL normally initiates a process of differentiation that eventually leads to the maturation of the biofilm. Clearly, communication amongst cells by extracellular signaling molecules is a key step in the normal development of biofilms.

The work of Davies *et al.* has only scratched the surface of biofilm development, and these exciting results now open the way for much additional investigation.

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UPDATE: IMMUNOLOGY

The Numbers Game for Virus-Specific CD8$^+$ T Cells

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A viral infection is a race. For the infected organism to survive, cell-mediated immunity has to develop faster than the spread of the pathogen. The outcome depends on how many essential cells are compromised by the time the protective immune cells [cytotoxic T lymphocyte effectors (eCTLs)] enter the site of infection. In the end, it doesn't matter whether it's the virus or the eCTLs that destroy the infected cells—too much damage leads to death or severe impairment. What are the numbers that underlie this precarious balancing act? Precise methods for virus titration have been available for more than 50 years. Measuring the other half of the equation, the clonal expansion of the virus-specific T cell response and the size of the eCTL population, has proven to be much more elusive.

The best estimates of virus-specific CD8$^+$ T cell numbers have been derived from limiting dilution analysis (LDA), a microculture technique in which lymphocytes undergo at least 10 cycles of replication before eCTL function is assayed. The LDA method is extremely tedious, technically demanding, and notoriously variable. Even worse, the assay clearly fails to measure the size of the eCTL population in sites of virus-induced pathology (*1*), probably because further stimulation of these highly activated lymphocytes induces apoptotic cell death in the LDA cultures. Nevertheless, LDA provides a reasonable measure of the size of the memory T cell (mCTL) pool, the greatly expanded virus-specific CD8$^+$ set that persists for the life of a laboratory mouse and is readily recalled to defensive eCTL activity after a secondary exposure to a pathogen.

The laboratories of Bevan (*2*) and of Altman and Ahmed (*3*) have triggered a revolution in our understanding of the virus-specific eCTL and mCTL responses by finally developing accurate methods for measuring eCTL responses. These researchers analyzed CD8$^+$ T cell–mediated immunity to murine lymphocytic choriomeningitis virus (LCMV) by using one or more of three recent technical developments. Two of the methods measure interferon-γ (IFN-γ) production after stimulation with viral peptide. T cell numbers are determined either by measuring secreted IFN-γ with a 24-hour ELISA spot assay or by staining cytoplasmic IFN-γ in fixed cells after stimulating for 6 hours in the presence of brefeldin A (which prevents secretion of the IFN-γ). The third method quantitates antigen-reactive T cells by direct staining of the virus-specific CD8$^+$ set with tetrameric complexes of major histocompatibility complex (MHC) class I glycoprotein plus peptide. This latter protocol to determine virus-specific CD8$^+$ numbers in the blood of people and monkeys infected with HIV and SIV (*4*). Tetramer staining looks set to be the gold standard for quantifying virus-specific CD8$^+$ T cells. The numbers are almost identical to those determined by the alternative flow cytometric technique involving short-term peptide stimulation and staining for IFN-γ.

The basic message from the LCMV experiments is that the size of the eCTL population is 10 to 50 times that suggested by previous LDA studies (*5*). As many as 70% of the activated CD8$^+$ T cells in the spleen of an LCMV-infected mouse (the virus grows in this site) are specific for one or another LCMV peptide presented by MHC class I glycoproteins; this represents an expansion of more than four orders of magnitude over a period of 7 days. Though the finding is dramatic, it was not totally unexpected, as the amount of eCTL activity is extremely high in this experimental system. More than 20 years after the discovery of MHC class I restriction with LCMV eCTLs (*6*), we finally know how many players are involved!

Even more surprising is that mCTL numbers are some 10 times those determined by LDA. Though a recent report indicates that elements of LCMV can be copied back into the mouse genome (*7*), most evidence contradicts the idea that mCTL survival depends on viral persistence (*5*). The tetramer experiments with HIV and SIV also detected very high numbers of peptide-specific CD8$^+$ T cells in blood (*4*). These viruses are never eliminated, raising the question of the relative balance between the eCTL and mCTL components in such ongoing confrontations.

Although the total numbers in the eCTL and mCTL compartments have been greatly underestimated, the kinetics and duration of the virus-specific CD8$^+$ T cell response derived from LDA are essentially correct. As exemplified by application of the tetramer technology to analyze immunity to an intracellular bacterium *Listeria monocytogenes* (*8*), a spectrum of secondary explosions is likely to occur in this field as these new approaches are applied to other pathogens and the tetramers are used to sort antigen-specific T cells for functional characterization.

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