good guide. Arnold Steinhardt, the violinist, recounts that after the inaugural performance by his string quartet (the Guarnari) they were visited backstage by Rudolf Serkin. The pianist said, "The last time I heard Mozart played like that was in Vienna — and that is why I left."

And perhaps the hardest lesson to accept is that, unless it's a thesis exam or something of the sort, your audience really doesn't care how hard you worked to get to your answer. Frank Stahl once said to me that most experiments are just forays to teach you how to do the right one. One good experiment is worth ten messy ones. So if you have something to say, say it simply and directly — you'll make a better impression than if you feel obligated to say everything and bits of nothing all at one time.

"Science strives to make the new intelligible in terms of the familiar" — Nietszche. But there is no *lingua franca*, and everyday terms mean one thing to some of us, and something else to others. Writing is even harder than speaking — unless you are a master, there are no rhythms, inflections, and half sentences to steer the reader along. There is the constant tension between being communicative and being strictly correct — we sometimes have to 'lie the truth'. This does not mean that the best of us speak or write the same way. I think of two inspirations of opposite styles: Francois Jacob and Jacques Monod on one hand, and Al Hershey on the other. Jacob and Monod, Cartesians at heart, seemed to invent the world before they stooped to discover it; whereas Hershey took us through the nitty gritty — such as the drama of DNA folding and unfolding — as though it were happening before our very eyes, and thereby revealed a world. I would read both with exhilaration, thinking that what I wanted was to do some experiment that would enable me to find a voice so that I could write — speak — *like that*.

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Primer

Microbial biofilms: e pluribus unum

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A biofilm is a surface-associated population of microbes embedded in a matrix of extracellular polymers. Biofilms can be made up of a single species or, often, of multiple species of fungi and bacteria. Existence in a biofilm is a common natural growth state of microorganisms wherever moisture and sufficient nutrients are available. Although the canonical definition of a biofilm includes its association with a solid surface and the presence of extracellular matrix, there are biofilm-like multicellular structures that float at liquid–air interfaces or lack abundant matrix material.

Some notable examples of biofilms include the sludge that blocks our pipes and water systems, the slime that we find on rocks near bodies of water, the glowing light organ in the marine bobtail squid, and the grime that grows in our toilet bowls and showers. In addition, biofilms have a major impact on human health. For example, the coating of plaque that grows on our teeth, the chronic colonization of airways of cystic fibrosis patients, and the layer of microorganisms that grows on our mucosal surfaces in the form of thrush in babies or the very common yeast infection in women are all biofilms. Ironically, many advances in medical technology have provided new and perilous niches for biofilm formation. Implanted medical devices, such as catheters and artificial heart valves, provide surfaces for biofilm growth, thus sustaining a reserve of infecting microbes.

Existence in a biofilm is different from that in the planktonic or free-living state: biofilms encompass an array of different microenvironments that yield an intrinsically heterogeneous population. Although phenotypic heterogeneity may be observed among planktonic cells, they are typically studied in culture conditions that yield a homogeneous environment. There are planktonic growth conditions that model features of biofilms — notably, the nutrient depletion and high cell density of stationary-phase cells has been used to model biofilm cell properties — but there is nonetheless evidence for unique biofilm-specific features, revealed by phenotypic analysis as well as gene-expression profiling.

Biofilm formation may be viewed as a developmental process consisting of four common attributes: attachment and aggregation; extracellular matrix production; coordinated behavior and communication; and generation of heterogeneity. The mechanisms underlying these attributes are vastly different from species to species, but they converge to produce very similar outcomes.

Coming together — cell–surface and cell–cell adherence

The first step in biofilm formation is the initial attachment or colonization of a new surface by the first cells to find the surface — these cells are referred to as 'pioneer cells' (Figure 1). Attachment is followed by cell proliferation and aggregation to yield a basal layer of anchoring microcolonies (Figure 1). For motile species, this process often requires a transition to non-motile surface-associated cells. In the bacteria *Pseudomonas aeruginosa* and *Pseudomonas putida*, the initial attachment of cells is mediated by cell motility, as flagellar motility mutants are defective in surface binding. Subsequent colonization and microcolony formation depends upon a distinct structure called a type IV pilus that is dispensable for initial surface binding. Thus,

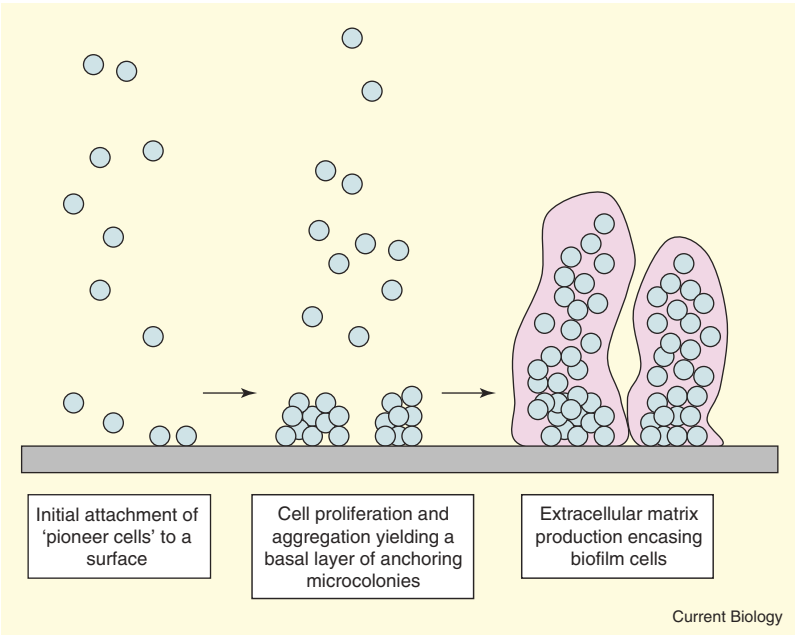

Figure 1. Stages of biofilm formation (see text for details).

flagellar motility is important for the cell–surface interaction, while type IV pili are important for the cell–cell interaction. In contrast, the bacterium *Staphylococcus epidermidis* lacks flagella, so it must use a different cell–surface attachment mechanism (which is unfortunately not understood). In this organism, cell–cell aggregation requires a polysaccharide intercellular adhesin (PIA) and an autolysin/adhesin (Aae). Distinct mechanisms and molecules may therefore be used to accomplish each type of adherence, both of which are critical for biofilm formation.

The fungal biofilm-former *Candida albicans* also appears to use distinct mechanisms for cell–surface and cell–cell adherence, based on *in vitro* studies. Several biofilm-defective mutants have been characterized *in vitro*, and all retain some capacity for cell–surface attachment. Thus it appears that, as for *S. epidermidis*, the cell–surface attachment mechanism has yet to be elucidated. Many *C. albicans* biofilm-defective mutations affect regulatory genes encoding transcription factors, but genetic manipulations indicate that cell-surface proteins Hwp1 and Als3 are critical targets of these regulators. The well-established roles of Hwp1 and Als3 as adhesins in other contexts suggest that they function in biofilms to promote cell–cell adherence.

Analysis of *in vivo* biofilm models for *C. albicans*, relevant to the major problem of catheter-based infections, has raised the question of whether the neat partitioning of cell–cell and cell–surface adherence mechanisms is universally relevant. The *C. albicans bcr1* transcription factor mutant, which forms a rudimentary biofilm *in vitro*, is unable to adhere to a catheter surface *in vivo*. Interestingly, increased expression of either of the surface proteins Hwp1 or Als3 restores cell–surface adherence to the *bcr1* mutant *in vivo*. Hence these surface proteins may function as cell–surface adhesins *in vivo*, perhaps in addition to a role in cell–cell adherence.

Surface structures called curli are important for both cell–surface and cell–cell aggregation in the enteric bacteria *Escherichia coli* and *Salmonella enterica*. Curli are amyloid protein fibers, that is, they have an intrinsic propensity to form self-propagating aggregates. Interestingly, the *C. albicans* adhesin Als5, a close relative of the biofilm adhesin Als3, also forms amyloid-like structures. Perhaps amyloid structure formation will emerge as a common feature of diverse biofilm adhesins. Additionally, the presence of curli or amyloid-like fibers may be a means to promote cross-species adherence.

The extracellular matrix, an environmental buffer

Once a sufficient number of cells have aggregated to form microcolonies, these cells begin to produce an extracellular matrix, which is also known as an exopolymeric substance (Figure 1). Matrix is broadly defined as an extracellular material maintained within a biofilm and may derive from directed synthesis and secretion as well as lysis of a fraction of the biofilm cells. In the setting of a biofilm infection, matrix constituents may derive from the host as well. Thus, as one can imagine, the components of the matrix can vary widely from biofilm to biofilm depending on the species and the local environment.

Biofilm matrix functions are as diverse as their structures, but the common theme that has emerged is that the matrix may play roles in adhesion and protection. The adhesive function involves maintaining cell–cell and cell–surface interactions, thus preserving the architectural integrity of the biofilm. The protective function includes binding to or disabling of antimicrobial agents and evasion of the host immune response.

For most biofilms, the matrix consists predominantly of extracellular polysaccharides. Cellulose is a critical component of *Salmonella typhimurium*, *Salmonella enteritidis*, and *E. coli* biofilm matrices, where it enhances biofilm antimicrobial resistance. In *Acetobacter xylinum* and *Sarcina ventriculi*, the presence of extracellular cellulose may protect these microbes from the local environment and host defenses. Other matrix polysaccharides,

such as PIA in *Staphylococcus* species, contribute to the adhesive function of the matrix. In addition to adhesive function, PIA also has protective function, as loss of surface PIA increases killing of some bacteria by host immune cells. In *P. aeruginosa* biofilms, the polysaccharide polymers PEL and PSL contribute to the matrix of these biofilms. And, although matrix structure is much less explored in fungi than in bacteria, polysaccharides are a major component of the *C. albicans* biofilm matrix as well. For both *P. aeruginosa* and *C. albicans* biofilms, matrix polysaccharide functions to bind some antimicrobial agents and thus contributes to the intrinsic resistance of the biofilm.

Another key matrix component, though generally less predominant than polysaccharides, is protein. Both surface and secreted proteins contribute to matrix. For example, curli are cell-surface protein fibers that contribute to the adhesive function of the matrix. In fungal biofilms, many cell-surface proteins, such as Hwp1 and Als3 in *C. albicans* improve adhesion and may contribute to the adhesive function of the matrix.

Matrix formation depends upon other types of molecules in some species. For example, fatty acids called mycolic acids are required for *Mycobacterium smegmatis* biofilm formation, as mutants defective in GroEl1, a chaperone protein that controls mycolic acid synthesis in biofilms, do not develop into mature biofilms. It has been speculated that secreted mycolic acids may be the major matrix component of these biofilms and probably contribute to the adhesive function of the matrix. Another surprising matrix component of the *P. aeruginosa* matrix is extracellular DNA. This DNA may be secreted or be generated by cell lysis, and it acts to support the integrity of *P. aeruginosa* biofilms. In fact, DNase treatment inhibits biofilm formation but not cell growth. This finding may help to explain the efficacy of DNase treatment for cystic fibrosis patients, whose lungs are colonized by *P. aeruginosa* biofilms. Thus, the presence of extracellular DNA as a matrix component may contribute to biofilm integrity.

Working together — coordinating behavior through communication

After surface colonization, microbes talk to each other and coordinate their behavior through a language termed quorum sensing, so named because it was discovered as a mechanism underlying cell-density-dependent behaviors. This communication results from secreted signaling molecules that are detected by other cells in the environment. Cells respond to these signaling molecules as a population through global changes in behavior and gene expression.

Diverse molecules are used for cell–cell communication. The molecule autoinducer-2 (AI-2) may be used for interspecies quorum sensing, including communication between Gram-negative and Gram-positive bacteria. Other molecules can be used for either interspecies or intraspecies signaling, depending on their specific modifications. Such molecules include acyl homoserine lactones (AHLs), which are used by Gram-negative bacteria, and short-chain peptides, which are used by Gram-positive bacteria. Additional classes include γ-butyrolactones of *Streptomyces*, which are structural analogs of AHLs, and 2-heptyl-3-hydroxy-4-quinolone (PQS) of *Pseudomonas*. In fungi, both alcohols and peptide mating pheromones are known to function as cell–cell signaling molecules. The alcohols, including farnesol and tyrosol, may be used for interspecies signaling, while peptide mating pheromones appear to be species specific.

As biofilm formation is a complex developmental process involving much coordinated behavior, it is not surprising that quorum sensing is critical for biofilm construction. For example, biofilm architecture of *P. aeruginosa* depends upon a specific AHL; a defect in the AHL results in an aberrant mat-like biofilm structure. In *Bacillus subtilis*, the ComX peptide pheromone regulates production of surfactants that permit formation of a biofilm-like structure on the surface of water. Quorum-sensing molecules can also inhibit biofilm formation, however, as illustrated by the inhibition of *C. albicans* biofilm formation by farnesol. Farnesol levels increase during the later stages of biofilm formation, in keeping with the general hypothesis that programmed biofilm disruption may represent a cell-dispersal strategy.

Quorum-sensing mechanisms may also coordinate specialized biofilm functions. For example, in *Vibrio fischeri*, the bioluminescent bacterium that colonizes and forms a biofilm in the epithelium-lined light organ of the squid *Euprymna scolopes*, a specific AHL coordinates luminescence of the biofilm that ultimately protects the squid from predators.

Within the context of a biofilm, quorum-sensing molecules may also play a role in the exchange of genetic material. For example, the ability to take up DNA from the environment is known to require quorum-sensing signals in *V. cholerae*. For *C. albicans*, presence of mating pheromones accelerates biofilm formation, and the biofilm structure assists in relaying mating signals between disperse mating-competent cells. An interesting possibility is that improvement of genetic exchange may be a selective force that maintains biofilm formation ability in diverse species.

Heterogeneity in a biofilm — genetic diversity and persister cells

Biofilm cells are diverse and immensely heterogeneous. Cellular diversity reflects in part the differences in the environment throughout the biofilm. Because different areas within a biofilm have

differing levels of nutrients and other environmental factors, microniches tend to develop. But, in addition to responses to environmental gradients, biofilms harbor populations that are heterogeneous due to both epigenetic and genetic phenomena. The clearest and most worrisome epigenetic heterogeneity is the production of persister cells, the small fraction of the population that is tolerant to a lethal stress, such as antimicrobial treatment. These cells are dormant (or nearly so) and thus survive challenges that are lethal to growing cells. Many bacteria produce persister cells in biofilms as well as in stationary-phase planktonic culture, which is depleted of nutrients. In contrast, the fungus *C. albicans* produces persister cells much more efficiently in biofilms than in planktonic culture. The presence of persister cells helps to explain the overall resistance of biofilm-based infections to antimicrobial therapy.

Biofilms can also support the generation of genetic heterogeneity. One mechanism, mentioned above, is through encouragement of genetic exchange. Because of their communal structure, biofilms may provide a safe harbor for genetic variation to arise. The biofilm environment promotes generation of a broad range of phenotypic variants for the bacterium *P. aeruginosa*. The presence of variants provides greater capability for adaptation to environmental changes. The variant phenotypes are stable over numerous generations and may arise from genetic alteration.

Polymicrobial biofilms — a microbial melting pot

Although most studies have focused on biofilms of individual microbial species, it is clear that polymicrobial communities are the natural state of existence for many microbial species. For example, there are over 500 species of microorganisms detected in a single layer of plaque (half of which are non-culturable in the laboratory!). We are just beginning to understand the complex interactions that may lead to stable mixed-species biofilms. Similarly, we are beginning to understand the strategies that one microbe may use to suppress the growth of its competitors to favor a single-species biofilm.

Because of its complexity and accessibility, dental plaque is an outstanding system for analysis of interspecies interaction. Not surprisingly, both cooperation and competition have been observed. Cooperation is illustrated by the interaction between *Streptococcus gordonii,* which ferments sugars, and *Veillonella atypica*, which uses not sugars but fermentation products. Their interaction may be supported by interspecies communication, because *V. atypica* induces expression of a sugar-liberating alpha-amylase gene in neighboring *S. gordonii* cells. Plaque offers numerous illustrations of competition, notably the antagonism between *Streptococcus sanguinis*, a relatively benign oral inhabitant, and *Streptococcus mutans*, whose overgrowth causes dental caries. Elucidation of such interaction mechanisms may point toward novel antimicrobial therapies as well as probiotic interaction strategies.

Undoubtedly many interspecies interactions in biofilms are mediated by quorum-sensing molecules and the subterfuge of quorum sensing through diverse 'quorum-quenching' interactions. However, the analysis of mixed-species biofilms has also revealed unanticipated interaction mechanisms. For example, in a model mixed-species biofilm, *P. aeruginosa* outcompetes *Agrobacterium tumefaciens*, and this relationship depends upon *P. aeruginosa* type IV pili, which mediate surface attachment. These pili have no effect on competition between the two species in planktonic culture, thus pointing to their biofilm-specific function in this context. A novel competitive mechanism affecting biofilm formation has been revealed through analysis of diverse *E. coli* isolates. Several *E. coli* strains produce a soluble polysaccharide capsule that prevents biofilm formation by both Gram-positive and Gram-negative bacteria. Surface treatment with the polysaccharide is sufficient to inhibit subsequent biofilm formation by many different species. This fascinating finding invites broader strategies to identify anti-adhesion molecules.

Bacteria have also trained their offensive weapons on fungal biofilm competitors. Trans-kingdom competition has been recognized for some time; for example, use of broad-spectrum antibiotics is a major risk factor for fungal infections. In addition, many drugs with antifungal properties are derived from bacteria. Recent work has begun to unravel the mechanisms by which *P. aeruginosa* suppresses competition by *C. albicans*: the *P. aeruginosa* AHL quorum-sensing molecule inhibits formation by *C. albicans* of long, tubular cell chains called 'hyphae'. Formation of hyphae is critical for *C. albicans* biofilm formation and numerous adherence phenomena. There is good evidence that AHL acts as a mimic of *C. albicans*' own quorum-sensing molecule farnesol. This bacterium may therefore use a fungal language in order to spread misinformation!

Perspective

There are many critical areas for future biofilm research. Besides the biology that we have focused on in this primer, there are diverse industrial applications that await development. However, we highlight three kinds of study in the area of biofilm biology that we look forward to seeing. First, there are many unanswered mechanistic questions concerning biofilm construction and communication for important species, and we believe that well-studied biofilm-formers such as *P. aeruginosa* may provide insight

into the specific questions that must be addressed. Second, there remains the pressing question of how to define biofilm-specific phenotypes and their genetic and epigenetic origins. And finally, we see the interactions between species in biofilms as a key area for future exploration, providing insight into evolution and ecology, while yielding therapeutic and probiotic strategies.

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Interactions between circadian rhythm and immunity in *Drosophila melanogaster*

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In mammals, disrupted circadian rhythm is often correlated with infection and disease [1] and immunity can be specifically affected by circadian rhythm [2]. The molecular underpinnings of these interactions are unclear. *Drosophila* is a proven system for the study of both circadian rhythm [3] and innate immunity [4]. Microarray analyses of the fly have shown that the transcription of several immunity genes [5–8] is regulated in a circadian manner, but the significance of this regulation is not known. Here we demonstrate a functional, bidirectional relationship between circadian rhythm and innate immunity in *Drosophila melanogaster*. We show that fruit flies infected with the pathogenic bacteria *Streptococcus pneumoniae* or *Listeria monocytogenes* lose circadian regulation of locomotor activity several days before death and that circadian mutant flies (lacking either *timeless* or *period*, two central clock proteins in *Drosophila*) are highly sensitive to infection with these bacteria.

We first found that flies infected with *S. pneumoniae* (Figure 1) exhibit disrupted circadian rhythm. Healthy flies (media-injected) entrained by a circadian light–dark cycle have oscillatory rest–activity patterns in the dark (Figure 1A); they alternate between approximately twelve hours of high activity ('day') and low activity ('night'), as measured by the number of movements per five-minute interval. In contrast, sick flies move constantly and lose circadian regulation of locomotion (Figure 1B). By chi-squared periodogram analysis, 81.25% of healthy flies were rhythmic whereas 0% of infected flies were rhythmic.

Specifically, we found that sick flies do not sleep well. Sick flies do not lose circadian rhythm due to excessive lethargy or hyperactivity; when we quantify total number of movements per day, sick flies do not move significantly more or less than healthy flies ($p = 0.5003$, Mann-Whitney test). Instead, sick flies have fewer sleep bouts (defined as a five-minute interval without activity) than healthy

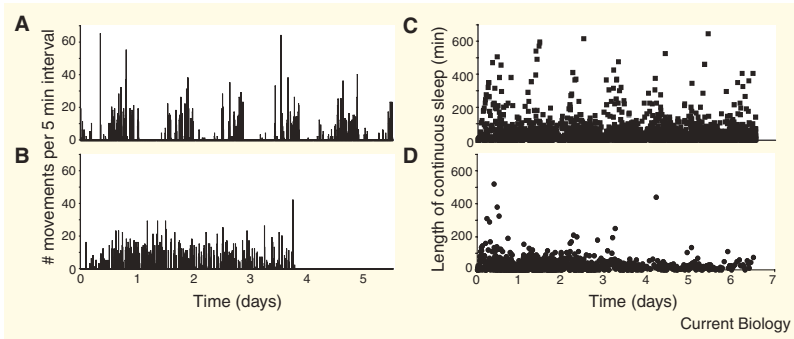

Figure 1. Flies lose circadian rhythm when infected with a lethal dose of *S. pneumoniae*. Shown here are examples of locomotor patterns for wild-type (Canton S) flies injected with (A) PBS or (B) *S. pneumoniae*. Sick flies do not sleep as well as healthy flies; these graphs show the lengths of continuous sleep sessions for flies injected with (C) PBS or (D) *S. pneumoniae*. Sick flies sleep for significantly shorter lengths of time than healthy flies ($p < 0.0001$ by Mann-Whitney test, n = 16 healthy flies and 13 sick flies, using 1881 x five-minute interval recordings from each fly).