Phenotypic plasticity in bacterial biofilms as it affects issues of viability and culturability

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CC-017

Nonculturable Microorganisms in the Environment
Edited by R. R. Colwell and D. J. Grimes

Chapter 9

Phenotypic Plasticity in Bacterial Biofilms as It Affects Issues of Viability and Culturability

J. William Costerton

Microbiology is approaching a critical intellectual challenge, which is brought into focus by the discovery of viable but nonculturable organisms in nature. In a continuous operational sequence, beginning with Louis Pasteur one and a half centuries ago, we have been preoccupied by the planktonic bacterial cells that grow so rapidly and readily in our cunningly formulated media in vitro. Until very recently a bacterial cell was not considered to exist, if it could not make the very rapid transition from its phenotype in its natural environment to this stylized entity in the test tube. Modern direct observations of natural populations have shown hundreds of morphotypes of bacterial cells that yield no corresponding planktonic cells on culture, and even more modern nucleotide analyses have shown the presence of many organisms that have never yielded culturable cells. Microbiology is seen to have concentrated on the minority of bacteria that can be cultured from nature, partly because of our understandable preoccupation with organisms that cause specific problems such as acute diseases of ourselves or of our domestic plants and animals. For these special organisms we have always developed suitable media and culture methods, and many of these media and methods actually discourage the growth of "environmental" species. This approach has produced the vaccines and antibiotics that still control many bacterial diseases, but the science of microbiology has committed a grave intellectual error, and we know very little of the neglected organisms that didn't happen to like to grow in our specialized media.

While each cell of a multicellular eukaryotic plant or animal is totipotent in that it contains all the genetic information of the species, we know that a leaf cell and a liver cell cannot readily replicate the entire organism in culture because each has assumed a specialized phenotype. Some of these individual cells of multicellular organisms can readily reverse their phenotypic specialization and grow in culture, but most cannot. We have heretofore assumed that bacterial cells exist in a single planktonic phenotype, but a collective sinking feeling steals over us as we realize

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that even stationary-phase planktonic cells have a distinct phenotype and that starvation survival involves the assumption of a profoundly different phenotype. In recent months it has become evident that sessile bacterial cells growing in biofilms also express a phenotype that is equally different from planktonic cells, and this revelation is devastating because the majority of the bacteria in the biosphere exist in either the starved or the biofilm phenotype. We must therefore begin to come to terms with the growing realization that not only have we concentrated almost all our attention on only one of many bacterial phenotypes, but that we have chosen the rare planktonic phenotype that exists in very small numbers in nature. The blind spot in microbiology can be blamed largely on the anthrocentric mindset of our very practical and very useful science.

BACTERIAL BIOFILMS AS A DIFFERENTIATED MULTICELLULAR COMMUNITY

While microbiology had its beginnings in the direct microscopic observations of such pioneers as Antonie van Leuvenhoek and Claude Zobell, the ennui induced by watching planktonic cells swimming in culture fluids soon converted most microbiologists into molecular biologists. Bacterial biofilms were examined by scanning and transmission electron microscopy (SEM and TEM, respectively) of dehydrated specimens, when they were examined at all, and their complex architecture was largely lost in drying and embedding artifacts. Only the use of confocal scanning laser microscopy (CSLM), with its ability to resolve three-dimensional detail in living, fully hydrated biofilms, has revealed the complexity and sophistication of the biofilms that are the epitome of the plasticity of phenotypic expression in bacteria. Figure 1 is a diagrammatic representation of the structure of a wide range of in vitro-grown and natural multispecies biofilms, as seen by CSLM and compiled by image analysis, and this basic pattern of microcolonies and water channels has been confirmed in many ecosystems (1). The sessile bacterial cells actually grow in microcolonies of a wide variety of shapes and sizes, in which they are embedded in an exopolysaccharide matrix material that comprises about 85% of the volume of each of these structural units of the biofilm. The microcolonies are separated by water channels, even in very thick biofilms, and these channels carry water and nutrients throughout the sessile community by convective flow (12). These direct observations of living biofilms, many of which were made and reported by biofilm engineers, clearly show two features heretofore held to characterize multicellular eukaryotes. These features are the formation of primitive but structurally organized "tissues" of accumulated cells and the presence of a primitive but functional circulatory system.

Whenever cells are immobilized in a structured mass, like a matrix-enclosed microcolony, the laws of chemistry and physics dictate that the local microenvironments of individual cells will differ. Peripheral cells will see nutrients, including oxygen, more readily than deeply embedded cells, and the phenotype of each sessile cell will be dictated by the vagaries of its own microniche (2) within the structural whole. Biofilm engineers have made direct measurements of such parameters as dissolved oxygen and pH at many specific locations within biofilms, and huge

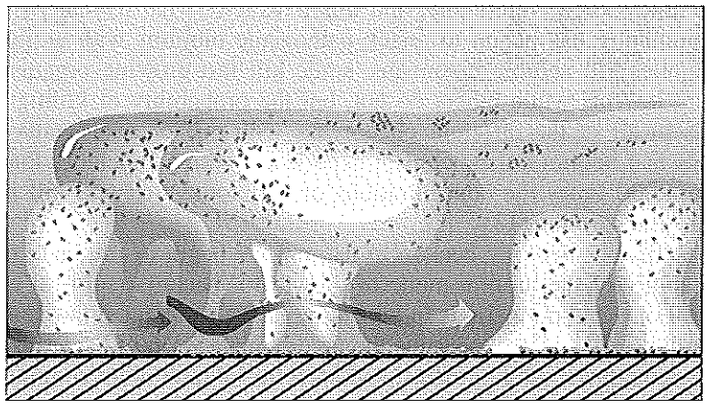

Figure 1. Diagrammatic representation of the structure of bacterial biofilms based on the systematic analysis of CSLM images of natural and laboratory biofilms. Note that the biofilm community is composed of microcolonies, in which sessile cells grow in matrix material, and that the microcolonies are separated by open water channels where convective flow occurs. Microcolonies are frequently seen to detach from these complex sessile communities.

variations have been found (6). The physiological diversity of sessile cells in a biofilm, in which some cells grow aerobically just tens of microns from where some other cells grow anaerobically (Fig. 2), can be interpreted as a form of cell specialization and recorded as another characteristic usually attributed to multicellular eukaryotes. If we add the diversity of sessile cells, some of which express the *rpo S* controlled genes of the stationary-growth phase (Pulcini, Stewart, and Camper, unpublished data) while others grow very rapidly, we begin to see the biofilm as a mosaic of differing phenotypes which are functionally integrated in a multicellular community. It is axiomatic that phenotypic diversity confers a measure

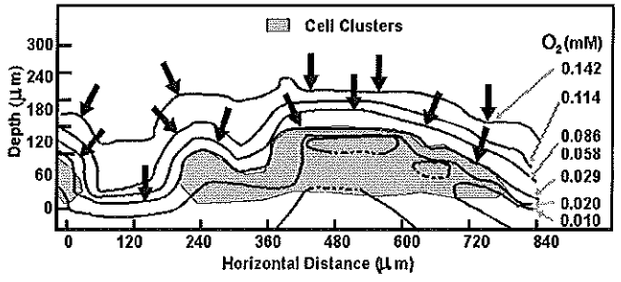

Figure 2. Diagrammatic representation of CSLM data locating a microcolony within a biofilm, on which dissolved oxygen data obtained by systematic analysis using a dissolved oxygen microelectrode has been superimposed to create a dissolved oxygen "map." Note that the center of this microcolony of matrix-enclosed cells of *P. aeruginosa*, growing in ambient air in a flow cell, is functionally anaerobic.

of survival fitness on a community, and this is exactly what has happened as the medical community has begun to attack pathogenic biofilms with antibiotics (3). Some antibiotics kill aerobic organisms, some kill fast-growing organisms, some work well at low pH while others do not, but the probability of killing all of the sessile cells in all of the different phenotypes with one or two agents is indeed remote. This fact is born out by clinical experience, which concludes that biofilms resist up to 1,000 times the concentrations of antibiotics that will kill planktonic cells of the same species (13).

The pleasures of teleology beckon to us, and we begin to ruminate on the exquisite suitability of the biofilm mode of growth for bacterial survival and proliferation, in all of the many periods in which they have constituted the dominant life form on earth. Sessile growth remains the predominant form of bacterial growth in all nutrient-rich aquatic environments, and bacterial/algal biofilm communities still predominate in the coastal ecosystems. Spatial juxtaposition to primary producers provides nutrients, as does location at a surface, and this situation is especially serendipitous if the surface that is colonized is also a nutrient, as in the case of cellulose (Fig. 3). The matrix of the biofilm concentrates nutrients, and sessile cells within the biofilm community cooperate in prodigious feats of metabolism (11) in which they digest complex substrates at speeds hundreds of times faster than those seen in pure planktonic cultures. Because the matrix material that comprises most of the volume of each microcolony is highly elastic, these structural units of the biofilms respond to shear stress by elongating and oscillating in the turbulent flow zone (14), which further enhances nutrient trapping. Thus the bacterial biofilm

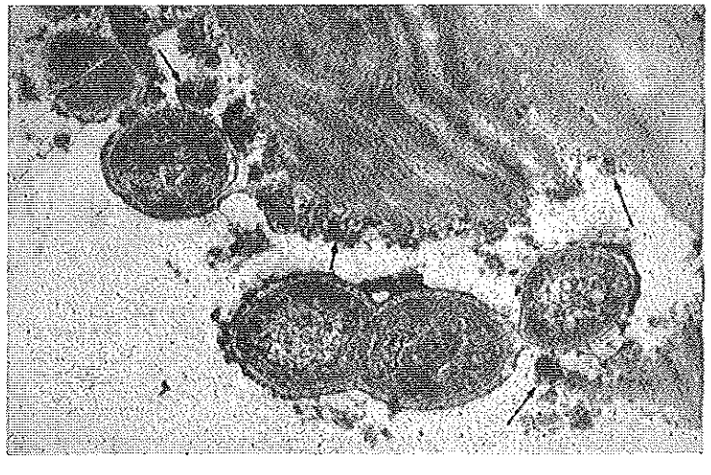

Figure 3. TEM of a section of a stained preparation of cellulose that had been incubated with cells of *Fibrobacter succinogenes*, showing the colonization of the insoluble cellulose by these cells, which have formed a biofilm on this nutrient surface. Some bacterial cells have produced a cavity in the surface of the cellulose, and many small fragments of the bacterial cell surface (arrows) have detached and are carrying out a focused enzymatic attack on this substrate.

seems to be the optimal mode of growth when these organisms had the earth pretty much to themselves, but this community structure also provided protection against two antibacterial challenges that surely occurred early in the evolutionary process. The matrix of the microcolonies excludes most bacteriophage and other viruses, and it makes sessile bacteria virtually completely resistant to uptake by amoeba and by modern day phagocytes (10).

THE BIOFILM PHENOTYPE

Direct observation of gene expression, using a reporter construct whose expression can be visualized by light microscopy, has allowed Center for Biofilm Engineering (CBE) scientists (4) to monitor the up regulation of a gene which must *a priori* be associated with biofilm formation. In *Pseudomonas aeruginosa*, the synthesis of the matrix polymer alginate is down regulated in planktonic cells, but direct observations of living cells show that it is up regulated shortly after the adhesion of these planktonic cells to a surface prior to biofilm formation (Fig. 4). The fusion of a *lac Z* reporter gene downstream from the promoter of the *alg C* gene, which codes for the phosphomannomutase enzyme of the alginate synthesis cascade, produces a reporter system that reveals up regulation of this gene. The use of this reporter strain, under a light microscope, shows that this gene is up regulated within 15 min of the adhesion of planktonic cells of this species to a surface (Fig. 5), and non-microscopic studies (Hongwei Yu, PhD thesis, University of Calgary, 1992) indicate that the *algD* gene is similarly regulated. Because both

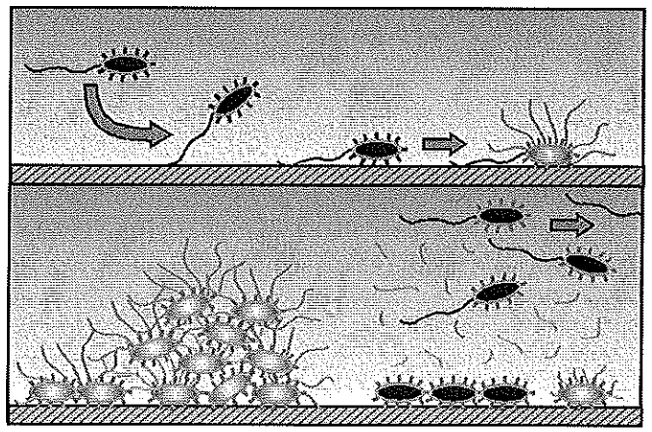

Figure 4. Cartoon summarizing some features of biofilm formation. Planktonic cells adhere to a surface, sometimes using their flagella as adhesins, and they adopt the biofilm phenotype and begin to produce exopolysaccharide matrix material soon after adhering. Sessile cells in the biofilm phenotype build highly structured biofilms (lower panel) composed of microcolonies within which some cells are programmed to return to the planktonic phenotype, digest the matrix material, and swim away to establish new biofilms.

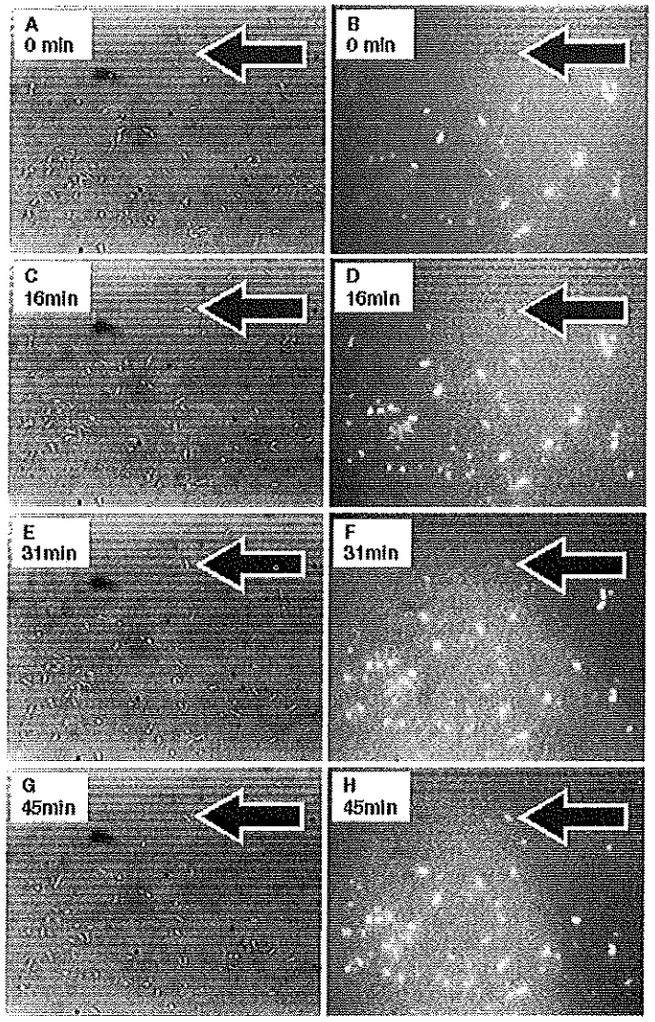

Figure 5. Light micrograph series in which a reporter construct has been cloned into cells of *P. aeruginosa*, by inserting a *lacZ* cassette downstream from the promoter controlling the expression of the *algC* gene of the alginate synthesis cascade. A planktonic cell adheres to the glass surface, as seen by phase contrast microscopy (left panel), and remains adherent. Within 45 min of its adhesion to this surface, the cell up regulates the *algC* gene, as indicated by the *lacZ*-mediated color reaction seen by epifluorescence microscopy (right panel).

algC and *algD* are regulated by a sigma factor coded by *algU* (7), we deduce that this regulatory gene must be activated by adhesion to a surface. The comparison of the gene products (proteins) present in both planktonic and sessile cells of *P. aeruginosa*, by two-dimensional electrophoresis, shows that the *rpoS* gene is up

regulated in biofilms and in planktonic cells in the stationary phase of growth. These observations were expected, especially in the case of the alginate synthesis genes, but nothing prepared us for the revelation that even the outer membrane proteins (OMPs) of sessile cells of *P. aeruginosa* differ very profoundly from those of planktonic cells of the same species (Fig. 6). Because the OMPs of *P. aeruginosa* have been implicated in everything from adhesion to outer membrane permeability, these profound differences suggest that sessile cells in a biofilm comprise a phenotype that is as different from the planktonic phenotype as a seed is from a leaf.

To put the biofilm phenotype in context, we should crystallize our contention that this sessile phenotype is triggered soon after adhesion and as a precondition of biofilm formation since this process requires alginate synthesis. The sessile cells need alginate synthesis to form the matrices of the microcolonies in which they will live, and a very large number of secondary phenotypic changes occur thereafter in response to the conditions in each individual microniche occupied by a sessile cell. We should not assume that sessile bacterial cells respond to environmental stimuli, such as oxygen deprivation, in the same way as planktonic cells respond, because the basic phenotypes of the sessile cells are different. A muscle cell does not respond to oxygen deprivation in the same way as a lung cell. However, unlike most of the phenotypic differentiations of eukaryotic cells, the biofilm-planktonic variation in bacteria is reversible, and biofilms regularly undergo programmed detachment events (Fig. 4) in which cells growing in the biofilm phenotype revert to the planktonic phenotype and leave the sessile community.

THE REGULATION OF PHENOTYPIC VARIATION BY CHEMICAL SIGNALS

The very complex pattern of phenotypic variation in bacteria, in which both the planktonic and biofilm phenotypes can respond to environmental stimuli in sophis-

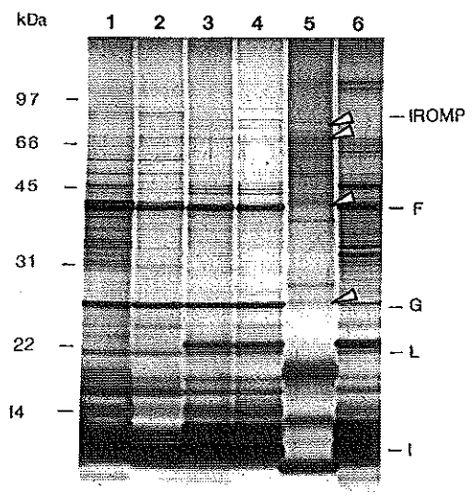

Figure 6. Polyacrylamide gel showing the OMPs extracted from the cell walls of planktonic and biofilm cells of *P. aeruginosa*. The OMPs seen in lanes 1 through 4 and 6 are those of planktonic cells grown in various iron concentrations, while those seen in lane 5 are those of biofilm cells of the same strain grown in the same medium as the planktonic cells whose OMPs are seen in lane 6. The OMPs of the planktonic phenotype are seen to differ very radically from those of the biofilm phenotype.

ticated ways, is under the control of simple chemical signals that resemble both hormones and pheromones in their functions. Biofilm formation in *P. aeruginosa* is controlled (5) by oxy-dodecanoyl homoserine lactone (ODdHL), coded for by the *lasI* gene, which may in turn be controlled by the polyphosphokinase (PPK) regulatory system (Kornberg, Rashid, and Davies, unpublished data). Reversion to the planktonic phenotype of this organism may be controlled by another quorum-sensing molecule called butryl homoserine lactone (BHL), and we expect that both biofilm formation and detachment will prove to be controlled by similar types of chemical signals in other gram-negative bacteria. Peptide signals seem to control the same activities in gram-positive organisms (8). These universal chemical signals were discovered because they control quorum sensing in planktonic cells (9), but we contend that their primary role in bacterial evolution may have been in their control of the formation and detachment of biofilms.

The search for biofilm control signals was initiated because we reasoned that the elaborate shapes of microcolonies and the open spaces that serve as water channels must be formed and maintained by a system of hormone-like signals that control cell replication and matrix production. Without such a system and in the absence of some form of control of cell growth and slime production, the biofilm would degenerate into a random and disorganized accretion of bacterial cells on a surface. This line of reasoning predicts, a priori, that many more phenotypes will be found in biofilms. An example would be a cell at the edge of a microcolony that had open access to nutrients and space to replicate, but that was constrained in replication by a signal molecule whose role it was to maintain open spaces in the biofilm. The uncontrolled replication of cells in a multicellular eukaryote constitutes cancer, and bacterial biofilms throughout the natural world resemble controlled tissue growth much more than they resemble cancer (Fig. 1). Perhaps the greatest damage done to microbiology by our unfortunate and exclusive emphasis on the planktonic phenotype has been the tragic delay in finding and describing these complex biofilm communities that clearly represent the apogee of prokaryotic development.

THE RECOVERY OF CULTURABLE BACTERIA FROM NATURAL ECOSYSTEMS

When bacteria are being enumerated by recovery and plating methods, enumeration is most accurate when planktonic cells are recovered from a fluid culture medium and spread on the surface of a chemically identical agar medium to determine how many colonies develop. Even in these idealized and totally unnatural systems, the cell count is often only one-tenth of the microscopic count, because planktonic cells clump, and clumps of cells yield only one colony. This is like counting plant seeds with a germination assay, because the planktonic cells in question are adapted to the liquid medium in which they are growing, and they grow well on the same medium with agar added. However, like plant seeds, some planktonic bacteria may fail to grow, because they have entered a dormant phase of growth or because local concentrations of a growth control signal may inhibit their replication. When we attempt to recover and enumerate planktonic bacte-

rial cells from a mixed-species natural ecosystem in which they have entered the starvation-survival mode of growth, only a very small number of these cells may be able to grow on the agar chosen for the study. Some cells may be too far committed to the dormant mode of growth that results from starvation, and others may not find the nutrients offered in the medium suitable for rapid growth. We might liken this recovery operation to a germination assay of a very old sample of mixed plant seeds, some of which are too senescent to germinate, and some of which cannot germinate in the soil provided.

If we then consider the recovery and enumeration of sessile cells from well-established mixed-species natural biofilms, using the traditional "scrape and plate" methods, we see even more obstacles to success. Biofilms contain sessile cells in a very wide variety of different phenotypes that are modifications of the basic biofilm phenotype formed immediately after the adhesion of the initial planktonic cells to a surface (Fig. 1 and 4). In any biofilm some sessile cells will be found in the anaerobic phenotype, while others will have expressed the *rpoS* gene and entered senescence, and some will be in locations in which growth is inhibited by high concentrations of growth control signals. Some of these cells may fail to grow on the agar plate because they are in the wrong phenotype, and others may fail to grow because they require different nutrients. Because of their matrix-enclosed mode of growth (Fig. 1), many of the sessile cells will be recovered in coherent clumps (Fig. 7) that will yield only one colony, but some planktonic cells will also be recovered because biofilms shed these free-living cells continuously. Because they are in a phenotype that is well suited to growth on the recovery agar, the planktonic cells that are freshly shed from biofilms always dominate the recovered population, and these methods recover only a very small fraction of the sessile cells from the biofilm itself. In the analogy of the plant germination assay, this procedure is like cutting mature plants from a given area of mature growth and shaking a mixture of stems, leaves, and seeds onto a soil. The seeds will germinate if the soil is suitable, and some leaves may act as propagules, but the major part of the plant biomass will not be registered in the assay. The "scrape and plate" recovery and enumeration techniques yield little of value in ecological studies of biofilms, except some limited data on species composition and some indirect data on the rates at which planktonic cells of certain species are detaching from the biofilm.

Direct observations, during the past 9 years, have shown that biofilms are highly organized multicellular communities of bacteria, with many of the characteristics previously thought to be the exclusive properties of eukaryotic organisms. These communities have primitive circulatory systems, and their component cells show a measure of physiological diversity and functional specialization. The initiation and maintenance of these complex structures are controlled by a sophisticated system of chemical signals whose function is analogous to the hormones and pheromones of eukaryotic organisms, and we now know that biofilms are not simply random accretions of bacterial cells in an unstructured matrix. The distribution of biofilms is accomplished by the programmed detachment of planktonic cells whose function is similar to that of plant seeds, and we can regard these creatures with suitable

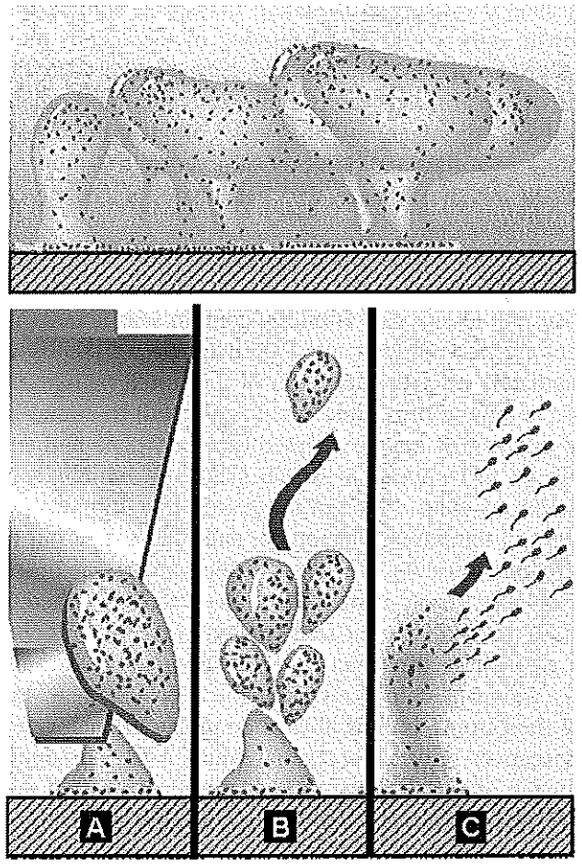

Figure 7. Cartoon showing the process of scraping to remove biofilms from surfaces for the enumeration of their component bacterial cells. The sterile scalpel blade removes some large microcolonies intact, fragments other microcolonies, and encounters some microcolonies that are in the process of shedding planktonic cells as a programmed activity. Each large fragment of a microcolony will yield a colony on plating, independent of its size or of the number of cells it contains, as will each planktonic cell harvested in this procedure. For this reason, shedding biofilms yield huge counts in scrape and plate assays, while nonshedding biofilms yield very low numbers, even though they contain millions of actively growing cells.

awe because this most ancient of all biological communities still dominates the biosphere.

A MODEST PROPOSAL FOR A BETTER SYSTEM FOR STUDYING MICROBIAL ECOLOGY

The excuse most commonly offered for our misplaced emphasis on planktonic cells in culture is that the subjects of our arcane scientific speciality are microscopic and that we could not see how they really grow in nature. This is simply not true. We can see bacterial biofilms on our teeth, on the surfaces of recovered medical devices, and on the coral reefs that delight us on tropical vacations. High school science projects have described the development of mixed bacterial/algal biofilms on the walls of tropical fish tanks, and the surfaces of the rocks in any neighborhood stream provide a biofilm that is both visible and slippery to the touch. Microscopes capable of resolving bacterial biofilms have been available since the mid-1700s,

and the modern confocal scanning laser microscope has been used since the 1960s to image subcellular structures deep within plant and animal cells. Modern confocal microscopes are now virtually ubiquitous, entirely capable of providing a three-dimensional image of living microbial communities at a resolution of at least 0.6 microns and capable of imaging these communities on opaque surfaces.

Even organisms that cannot be cultured can be identified by PCR, and fluorescent in situ hybridization (FISH) oligonucleotide probes keyed to signature sequences in their genomes can be used to identify cells of that species in biofilms. These FISH probes cannot always be used to identify individual species in living biofilms because of penetration problems, but their use on cryosectioned biofilm samples has yielded excellent data (Fig. 8). Fluorescent antibodies can be used to identify cells of a particular species, or to locate antigenic cell products such as toxins and enzymes. The armamentarium of modern probes is virtually limitless, and some probes detect respiratory activity (Fig. 9), while others detect the viability of individual cells on the basis of membrane integrity (Fig. 10). Perhaps the most sophisticated in situ probes are reporter constructs in which reporter sequences (e.g. green fluorescent protein [GFP]) are inserted downstream from the promoter of interest, and the reporter system is turned on when the gene is up regulated (Fig. 5). Biofilms are complex communities in which the location of individual cells in any one of a vast spectrum of different microniches determines the phenotype that

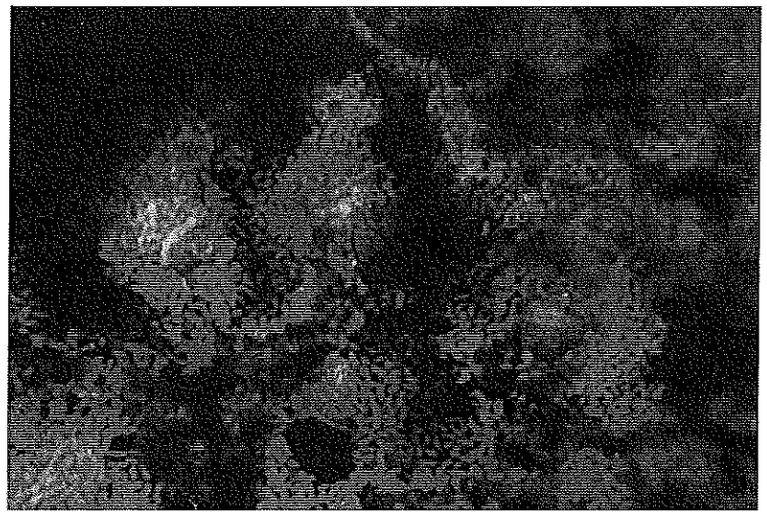

Figure 8. CSLM micrograph of a mixed biofilm containing cells of both *P. aeruginosa* and *Klebsiella pneumoniae*. In this x-z optical section, perpendicular to the colonized surface, the pseudomonas cells stain red and the klebsiella cells stain green with the probes used in this study. Note that the klebsiella cells tend to occupy the interior space of mushroom-like microcolonies formed by the pseudomonas cells, indicating some form of "tissue-like" organization.

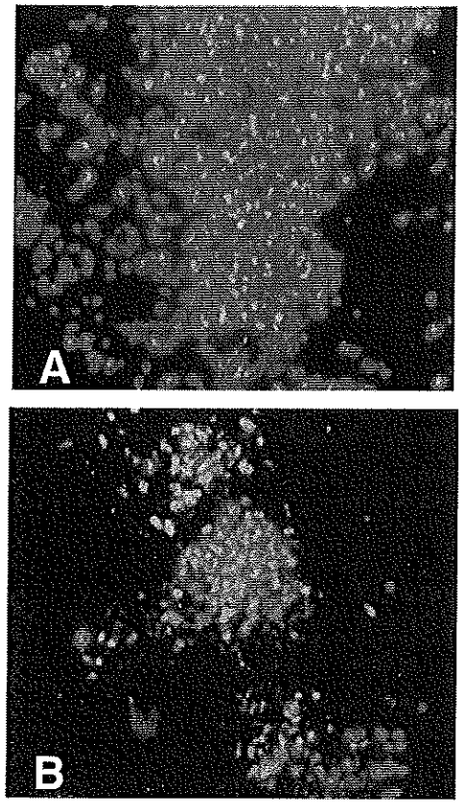

Figure 9. CSLM micrograph of frozen sections of biofilms of cells of *P. aeruginosa* that have been stained with CTC, which is a tetrazolium compound that is reduced to an orange-colored formazan by living bacteria capable of respiration. This x-z section, perpendicular to the colonized surface, shows a majority of living cells in the untreated biofilm (A), while only few living cells remain, in clumps within discrete microcolonies, in the biofilm (B) that has been treated with a biocide. This direct microscopic method, especially when combined with whole biofilm respirometry, gives very accurate data in the study of the efficacy of antibacterial agents in killing sessile bacteria.

the sessile cell will adopt, so morphological examinations of individual cells are really de rigeur in the study of these sessile populations.

The twin weaknesses of morphological methods are the fact that they are forced to concentrate on a limited area of the colonized surface and the fact that they can give only limited chemical information on integrated physiological processes. Biofilm engineers have developed a series of very accurate physical probes (microelectrodes) that can measure chemical parameters (e.g., dissolved oxygen concentration) with a resolution of <15 μm and can therefore map these parameters (Fig. 2). These maps of chemical parameters can be related to images showing the locations of microcolonies, and physiological conclusions such as the essential anaerobiasis of the centers of microcolonies can be supported by data generated in living biofilms. However, the optimal methods for the study of the cooperative physiology of biofilm populations are respirometry and other direct techniques that measure the chemical changes wrought by biofilms growing over large areas of colonized surfaces. If microscopy of selected areas of a colonized medical device showed only nonrespiring cells and cells whose membranes were no longer intact but respirometry of the whole device showed the consumption of oxygen and the

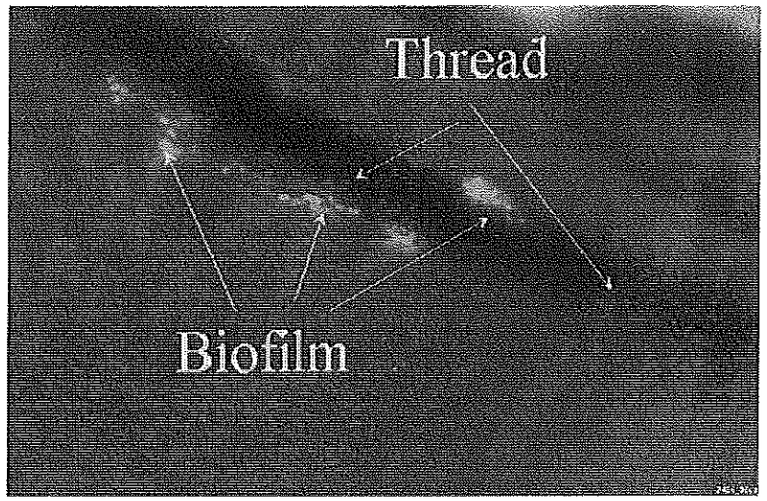

Figure 10. CSLM micrograph of a thread from the sewing cuff of a mechanical cardiac valve that had been incubated with cells of *Staphylococcus epidermidis* to assess its putative resistance to bacterial colonization and biofilm formation. The bacterial cells have adhered to the material and have formed a biofilm that is clearly visible in this living fully hydrated preparation. The viability of most of the sessile cells in this nascent biofilm is attested by the green color formed because the BacLite "live-dead" probe is excluded by their intact membranes. However, one cell is red because its damaged membrane failed to exclude this probe, and two more cells are orange or yellow because their membranes are compromised. Direct counts of living bacterial cells on any surface of interest can readily be made by this method.

generation of CO_2, we would conclude that some areas of the biofilm remained alive. The study of large biofilm populations by respirometry allows us to detect the final gaseous products of cooperative metabolic activities, such as respiration or methane generation, and the values obtained are the averages of hundreds or thousands of microcolonies over a very large area (e.g., 2 in^2).

VALETE

Never in the recent history of any biological science has one of these sciences been faced with such an urgent necessity of updating its concepts and methods, in order to correct a seriously mistaken emphasis and to accommodate radically new data. Microbiologists have discovered that the planktonic phenotype that they have studied so assiduously for >150 years is only one of many phenotypes adopted by bacteria in nature, and that this favored phenotype is neither the most prevalent nor the most interesting of these phenotypes. The starvation-survival phenotype is certainly the most prevalent form adopted by bacteria in the biosphere, and the biofilm phenotype is clearly the most complex mode of bacterial growth and the form that is more commonly encountered in the environments in which we live. The plank-

tonic phenotype received disproportionate attention because it is most commonly found in acute human disease and in vitro cultures, but the starvation-survival phenotype and the biofilm phenotype are of much greater importance in microbial ecology.

This flood of new perceptions concerning bacterial phenotypes causes current practitioners of the important science of microbial ecology to question the traditional recovery and culture methods of studying bacteria and to rely increasingly on direct observations of living populations. These direct methods may be as simple-minded as confocal microscopy of living biofilms, as powerful as physiological studies of living mixed-species communities, or as sophisticated as PCR analysis of the nucleic acids extracted from mixed-species populations growing in natural environments. Their appeal, and their common thread, is that they are direct studies of whole populations without the intermediate selection and culture of the rare phenotypes and the rare species that happen to grow on selected agar surfaces when they are ripped from their natural habitats and deposited on recovery plates. Because the bacterial diseases that affect humans in the developed world have a major ecological component, in that biofilm-forming bacteria from natural ecosystems are invading our hospitals (3), the considerable resources of medical institutions (e.g., National Institutes of Health) will also now be thrown into this new activity. As in all systems in which a major error is corrected, we can now look forward to a renaissance in microbial ecology that will surpass even the phenomenal bursts of activity in bacterial physiology and in bacterial genetics that have excited microbiologists during the past century.

Acknowledgment. This work was supported by cooperative agreement ECD-8907039 from the National Science Foundation.

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