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BACTERIAL BIOFILMS IN NATURE AND DISEASE

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INTRODUCTION

The growth of bacteria in pure cultures has been the mainstay of microbiological technique from the time of Pasteur to the present. Solid media techniques have allowed the isolation of individual species from complex natural populations. These pure isolates are intensively studied as they grow in batch cultures in nutrient-rich media. This experimental approach has served well in providing an increasingly accurate understanding of prokaryotic genetics and metabolism and in facilitating the isolation and identification of pathogens in a wide variety of diseases. Further, vaccines and antibiotics developed on the basis of in vitro data and tested on test-tube bacteria have provided a large measure of control of these pathogenic organisms.

During the last two decades microbial ecologists have developed a series of exciting new techniques for the examination of bacteria growing in vivo, and often in situ, in natural environments and in pathogenic relationships with tissues. The data suggest that these organisms differ profoundly from cells of the same species grown in vitro. Brown & Williams (12) have shown that bacteria growing in infected tissues produce cell surface components not found on cells grown in vitro and that a whole spectrum of cell wall structures may be produced in cells of the same species in response to variations in nutrient status, surface growth, and other environmental factors (67). We and others (28) have used direct ecological methods to examine bacterial cells growing in natural and pathogenic ecosystems, and we find that many important populations grow in adherent biofilms and structured consortia that are not seen in pure cultures growing in nutrient-rich media. In fact, it is difficult to imagine actual natural or pathogenic ecosystems in which the bacteria would be as well nourished and as well protected as they are in single-species batch cultures.

In this review we summarize and synthesize the data generated by the new direct methods of studying mixed natural bacterial populations in situ. Generally, morphological data give us a basic concept of community structure, direct biochemical techniques monitor metabolic processes at the whole-community level, and specific probes define cell surface structures in situ. Any in vitro techniques used in these ecological studies are selected to mimic the natural ecosystem as closely as possible. In our estimation, data from studies of bacteria growing in single-species batch cultures continue to be very valuable. However, these data represent a single, and perhaps unrepresentative, point in the broad spectrum of bacterial characteristics expressed in response to altered environmental factors. In retrospect, it may become apparent that the phenotypic plasticity of bacteria (12, 107) and their ability to form structured and cooperative consortia will prove to be their most remarkable characteristics.

STRUCTURE AND DYNAMICS OF BACTERIAL BIOFILMS

Bacteria in natural aquatic populations have a marked tendency to interact with surfaces (120). Recent work has demonstrated that many bacteria associate with surfaces in transient apposition, particularly in oligotrophic marine environments (75). Some of these bacteria adhere to these surfaces, initially in a reversible association and eventually in an irreversible adhesion (76), and initiate the development of adherent bacterial biofilms. We recognize the inaccuracy of simple physical models in which bacteria are represented as smooth 1-μm particles with various surface properties and are placed in computer-simulated association with similarly homogeneous substrate surfaces. The substrate surfaces of importance in natural and medical systems are invariably coated with adsorbed polymers, and the surfaces of bacteria growing in these environments are a forest of protruding linear macromolecules such as pili, lipopolysaccharide O antigen or teichoic acid, and exopolysaccharides (9). Thus, the initial association between bacterial and substrate surfaces in real aquatic environments is difficult to model, simply because it consists of the association of numerous linear polymers. However, Fletcher and associates (39), in a useful and very extensive series of empirical experiments, have shown that the rate of bacterial adhesion to a wide variety of surfaces is responsive to some physical characteristics such as hydrophobicity (40).

A bacterial cell initiates the process of irreversible adhesion by binding to the surface using exopolysaccharide glycocalyx polymers (Figure 1). Cell division then produces sister cells that are bound within the glycocalyx matrix, initiating the development of adherent microcolonies. The eventual production of a continous biofilm on the colonized surface is a function of cell

division within microcolonies (69) and new recruitment of bacteria from the planktonic phase (Figure 1). Consequently, the biofilm finally consists of single cells and microcolonies of sister cells all embedded in a highly hydrated, predominantly anionic matrix (110) of bacterial exopolymers and trapped extraneous macromolecules. As the bacterial biofilm gradually occludes the colonized surface, newly recruited bacteria adhere to the biofilm itself (Figure 1, *F*). Differences in the rates of colonization of various surfaces usually disappear in long-term colonization experiments.

PHYSIOLOGY OF BIOFILM BACTERIA

In their very comprehensive review (12), Brown & Williams have provided detailed experimental evidence for Smith's earlier conclusion (107) that the molecular composition of bacterial cell walls is essentially plastic and is remarkably responsive to the cell's growth environment. The iron deprivation inherent in growth within infected tissues profoundly changes the cell wall protein composition of gram-negative bacteria. Thus, the cell walls of bacteria recovered directly from the sputum of cystic fibrosis patients (3) or from infected urine (105) or burn fluids (4) exhibit iron depletion and differ radically from those of cells of the same organism grown in batch culture in vitro. Similar bacterial surface changes are seen in response to alterations in

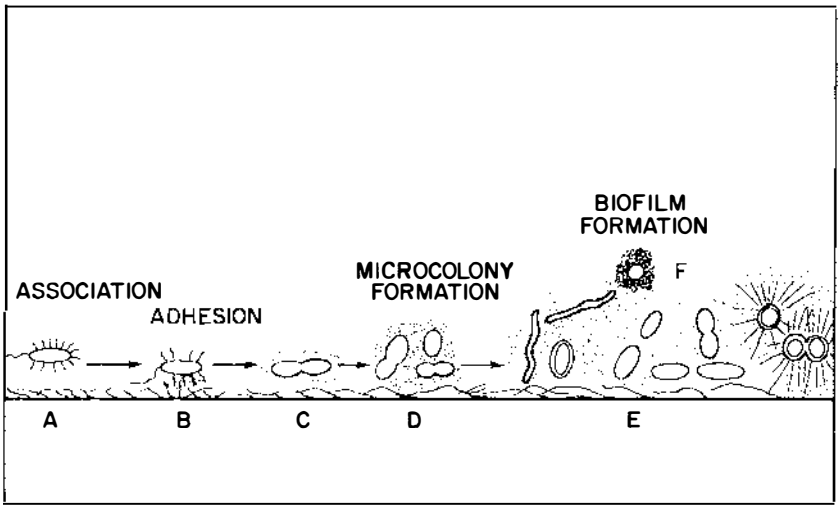

Figure 1 Bacteria may associate reversibly with a polymer-coated surface (*A*), or they may adhere irreversibly (*B*) and divide (*C*) to produce microcolonies (*D*) within an adherent multispecies biofilm (*E*). The biofilm grows by internal replication and by recruitment (*F*) from the bulk fluid phase.

growth rate (67, 118), exposure to subinhibitory concentrations of certain antibiotics (66), and growth on solid surfaces (67). Equally profound differences in enzyme activity have been noted between bacterial cells adherent to surfaces and planktonic cells of the same organism (C. S. Dow, R. Whittenbury & D. Kelly, unpublished). These data suggest that sessile cells have more active reproduction and general metabolism, while planktonic cells are phenotypically committed to motility and to the colonization of new surfaces.

In light of the remarkable phenotypic plasticity documented above and the fact that biofilm bacteria live in specific microenvironments, we must conclude that these cells are structurally and functionally very different from planktonic cells grown in rich media at high growth rates in batch culture. It seems unwise to extrapolate from batch-culture data conclusions about bacteria growing in biofilms in natural and pathogenic environments. Therefore, we have adapted existing methods for planktonic bacteria (60) to study the physiology and resistance to antimicrobial agents of biofilm bacteria in situ.

When microbial biofilms are developed on rock surfaces suspended in flowing streams, the overall metabolic activity of cells within the mixed adherent populations can be assessed by a modification of the heterotrophic potential technique (30, 61). Biofilm cells have a greater capacity to convert C^{14} glutamate to labcled cell components and to $C^{14}O_2$ than cells in the planktonic phase of the same stream or dispersed biofilm cells from equivalent surfaces. The increased metabolic activity of biofilm cells may result from phenotypic changes in response to sessile growth (C. S. Dow, R. Whittenbury & D. Kelly, unpublished). It may also result from nutrient trapping (Figure 2, *A*), which occurs when organic nutrients are bound to the biofilm matrix and are readily dissociated for use by the component organisms. High rates of biofilm development in oligotrophic environments (45) and in distilled water systems argue for the importance of this nutrient trapping strategy. Nutrients produced by component organisms (e.g. by photosynthesis) also enter the biofilm, and microcolonies of cells capable of primary production of nutrients are often surrounded by heterotrophic organisms that are stimulated by the exudates to grow and to produce adjacent microcolonies (Figure 2, *B*). The seasonal death and cell lysis of primary producers often radically stimulates biofilm growth (45), because biofilms tend to trap and recycle cellular components. When biofilms form on the surfaces of insoluble nutrients (e.g. cellulose), the initial events of adhesion (Figure 2, *C*) favor specific bacteria that can digest that substrate (e.g. cellulolytic bacteria). The primary colonizers in such a system produce cell-associated digestive enzymes that attack the insoluble substrate and produce soluble nutrients that stimulate the growth of adjacent heterotrophic organisms until a digestive consortium is formed (Figure 2, *C*). Electron

transfer may also take place in these consortia. The conformation and juxtaposition of the component organisms vis-a-vis their insoluble substrate is optimally maintained by the biofilm mode of growth. In modern techniques for the in situ study of biofilms an adherent population is treated almost like a multicellular tissue in matters of nutrient uptake, nutrient cycling, respiration, and overall growth.

Because of the matrix-enclosed mode of growth of biofilm bacteria, a substantial ion exchange matrix arises between the component cells and the liquid phase of their environment (Figure 2). Additionally, the gellike state of the predominantly polysaccharide biofilm matrix limits the access of antibacterial agents (Figure 2, *D*), such as antibodies (6), bacteriophage, and phagocytic eukaryotic cells, to its component bacteria. Therefore, biofilm bacteria are substantially protected from amebae, white blood cells (96, 104, 115), bacteriophage, surfactants (47), biocides (100), and antibiotics (90, 91).

DISTRIBUTION OF BIOFILMS IN NATURAL AND PATHOGENIC ENVIRONMENTS

The formation of biofilms on surfaces can be regarded as a universal bacterial strategy for survival and for optimum positioning with regard to available

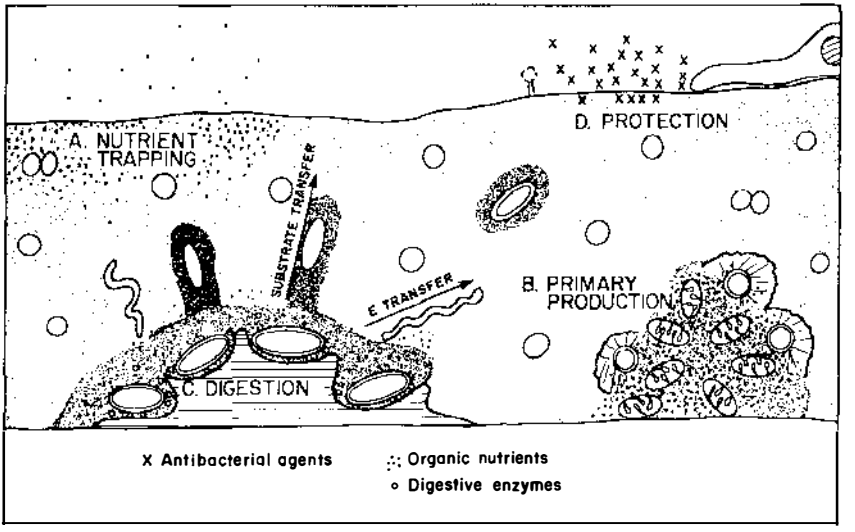

Figure 2 Modes of nutrient acquisition and protection of a bacterial biofilm. (*A*) Nutrient trapping. (*B*) Primary production. (*C*) Formation of a digestive consortium. (*D*) Exclusion of antibacterial substances.

nutrients. Bacterial populations living in this protected mode of growth produce planktonic cells, with much reduced chances of survival. These detached cells may colonize new surfaces or may burgeon to form large planktonic populations in those rare environments where nutrients are plentiful and bacterial antagonists are few.

Natural Aquatic Environments

In an exhaustive survey of the sessile and planktonic bacterial populations of 88 streams and rivers, the sessile population exceeded the planktonic population by 3–4 logarithm units in pristine alpine streams and by 200 fold in sewage effluents (65). A very widespread exception to the general trend occurs in the extremely oligotrophic environments of the deep ocean and deep groundwater, where bacteria are in an advanced stage of starvation (2). These cells alter their cell surfaces (56) and their patterns of peptide synthesis (51) in response to starvation and do not expend their scarce metabolic resources in exopolysaccharide synthesis unless they are revived by nutrient stimulation. Specific insoluble nutrient substrates (e.g. cellulose, solid hydrocarbons) within these aquatic environments are rapidly colonized by bacteria specialized for their digestion; the consistent presence of these materials in an aquatic system stimulates the development of large and vigorous populations of primary and secondary colonizers.

Industrial Aquatic Systems

The relatively high levels of nutrients and the high surface areas in many industrial aquatic systems predispose these systems to biofilm formation. Adherent populations bedevil most industrial systems (24) by plugging filters and injection faces, fouling products, and generating harmful metabolites (e.g. H_2S). Bacteria gradually colonize the water-cooled side of metal surfaces in heat exchangers, and the resultant biofilm insulates against heat exchange so effectively that exchange efficiency is gradually reduced to $<10\%$ of designed values. This costly problem may now be solved by a recently patented biofilm removal technique (23) in which slow freezing cycles are used to produce large ice crystals within the biofilm. Thawing of the crystals then leads to complete removal of the biofilm.

Bacterial corrosion of metals is an economically important consequence of bacterial biofilm formation that illustrates several fascinating aspects of the structure and physiology of these adherent bacterial populations. The bacteria most commonly associated with the corrosion of metals are the anaerobic sulfate-reducing bacteria (SRB), but other sulfur-cycle organisms are also important in this process (92). Geesey et al (44) have found that metal corrosion can occur, even in the absence of living bacterial cells, when two polymers with different metal-binding capacities (Figure 3) are adsorbed to

adjacent areas of a metallic surface. Little et al (64) have reported that a measurable corrosion potential is generated between an uncolonized metal surface and a metallic surface colonized by bacteria. Bacteria organized into structural consortia occupy developing corrosion pits (27), and local pH differences as great as 1.5 units can occur in the lower zones of biofilms growing on metallic surfaces. These data have allowed us to construct a conceptual model of a functioning bacterial "corrosion cell" (Figure 3) using established physicochemical concepts. The microcolony of sister cells at site *A* on the metallic surface would produce an exopolysaccharide matrix that would bind metal ions (+), and their metabolic activities would generate a local pH of 7.0. In an adjoining site (*B*) another bacterium (probably an SRB) would establish a consortium with other organisms, and the coordinated metabolic activity of this structured community would generate a lowered local pH (perhaps 5.5) and an exopolysaccharide matrix with a low natural affinity for soluble metal ions. The scenario described so far is hypothetical, but the physicochemical differences between sites *A* and *B* would, by immutable physicochemical laws, cause the mobilization of metal at site *B* and the formation of a corrosion pit. Thus, bacterial corrosion of metals is really an activity of structured bacterial biofilms in which physicochemical differences

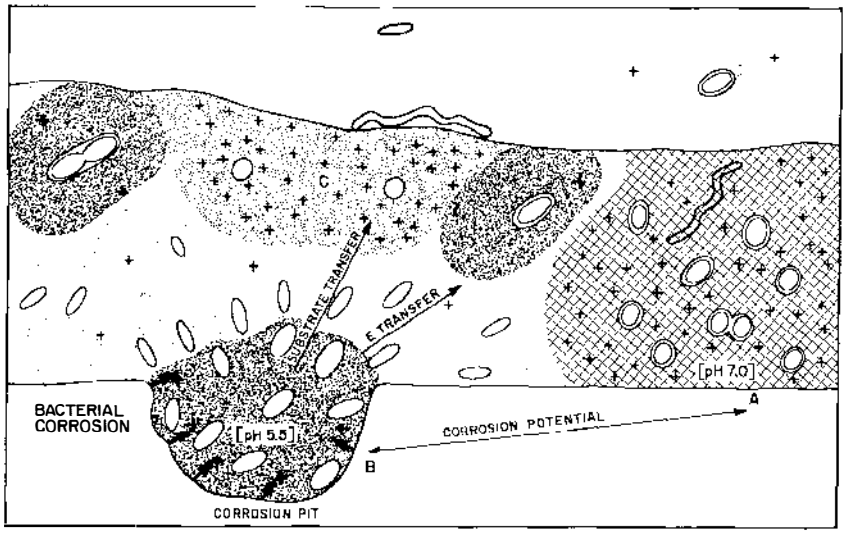

Figure 3 Bacteria within biofilms adherent to metal surfaces grow in the form of discrete microcolonies whose exopolymers may (*A* and *C*) or may not (*B*) bind certain metal ions. Metabolic activities in some microcolonies may produce local differences in pH, abetted by electron and substrate transfer in structured consortia (*B*). These local surface differences can produce an electrochemical "corrosion cell."

between adjacent loci on the metallic surface are created and maintained by differential metal-binding and metabolic activity until deep corrosion pits have been produced. Engineers have long noted that regular scraping (pigging) of pipelines prevents corrosion. We have now shown, in the field and in the laboratory, that pigging disturbs these highly structured bacterial corrosion cells; several days are required to reestablish their structure and activity (J. W. Costerton, unpublished data).

Medical Biomaterials

Medical biomaterials are the plastic, rubber, and metallic materials that are used to construct the myriad of medical devices and prostheses. With the remarkable modern advances in medicine and the concommitant increases in the numbers of elderly and immunocompromised patients, the use of these biomaterials has increased exponentially. An increasing number of bacterial infections is centered on implanted devices (31). These infections have the following unique characteristics. (*a*) They often have indolent pathogenic patterns with alternating quiescent and acute periods. (*b*) There may be an initial response to antibiotic therapy, but relapses are frequent because bacteria in the biofilms are protected from antibiotics and constitute uncontrolled foci that often necessitate the removal of the device. (*c*) While these infections are often polymicrobial, the predominant bacteria are either common members of the autochthonous skin or bowel flora or very common environmental organisms (e.g. *Pseudomonas*) that are often only pathogenic in immunocompromised patients. (*d*) Bacteria may be difficult to recover from adjacent fluids when the device is in place and from the device itself when it is removed.

All of these disease characteristics indicated that bacterial biofilms might be involved in biomaterial-related bacterial infections. Therefore, methods of direct observation and quantitative recovery developed in our environmental and industrial studies have been used to examine a large number of medical devices that were demonstrable foci of infections. Extensive bacterial biofilms were found on transparent dressings, sutures (50), wound drainage tubes, hemodialysis buttons, hemasite access devices, intraarterial and intravenous catheters (94), Hickman and silastic cardiac catheters (112), central venous lines, Swann-Ganz pulmonary artery catheters, dacron vessel repair sections, cardiac pacemakers (70, 72), bioprosthetic and mechanical heart valves (57), Tenckhoff peritoneal catheters (74), Foley urinary catheters (87) and urine collection systems, nephrostomy tubes, ureteral stents, biliary stents, penile prostheses (88), intrauterine contraceptive devices (IUDs) (71), endotracheal tubes, and prosthetic hip joints (48, 49). In all instances of bacterial colonization of medical biomaterials, extensive bacterial biofilms were seen by scanning and transmission electron microscopy (SEM and

TEM). Large numbers of living organisms were recovered by quantitative (scraping-vortex-sonication) recovery techniques (90), but not by routine methods used in clinical laboratories, including the otherwise very useful Maki technique (68). We have now implanted several of these medical devices (e.g. Foley catheters (85), cardiac catheters, Tenckhoff catheters, intrauterine contraceptive devices) in experimental animals to follow the time course of their bacterial colonization. We have noted that autochthonous and environmental organisms commonly form biofilms on accessible biomaterial surfaces. Further, these biofilms spread up to 100 cm along these colonized surfaces in as few as 3 days, in spite of active host defense systems (Figure 4, *A*) and prophylactic doses of antibiotics (Figure 4, *B*). These bacterial biofilms on the biomaterial surfaces do not usually cause overt infection or even detectable inflammation. However, they do constitute a nidus of infection when the host defense system fails to contain them. Consequently, they are able to give rise to disseminating planktonic cells that trigger acutely pathological sequellae (Figure 4, *C*). Conventional therapeutic doses of antibiotics often suffice to kill the disseminated bacteria (Figure 4, *B*) and to control the symptoms of infection, but the biofilm cells are not killed (90); thus, they constitute a continuing nidus for relapse of the infection.

Because it protects component cells from host defense mechanisms and

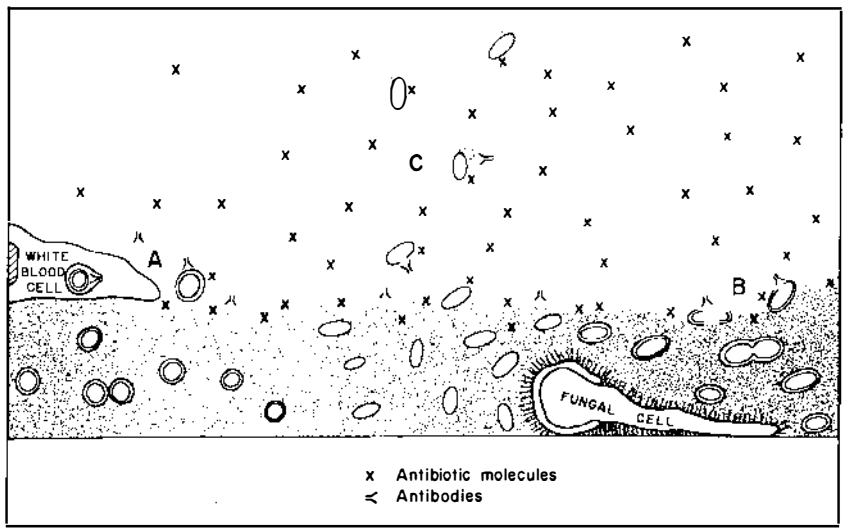

Figure 4 Microbial cells growing in adherent biofilms on medical biomaterials are generally protected from host defense mechanisms and antibacterial agents. Phagocytic white blood cells (*A*) and antibodies (*B*) can kill bacteria at the biofilm surface and planktonic bacteria (*C*) that have left the protection of the biofilm to initiate an acute disseminated infection, but usually cannot kill bacteria within the deeper layers of the biofilm.

from antibiotics, the biofilm mode of growth is of pivotal importance in the progressive colonization of biomaterials. Currently, the most effective strategy for the prevention of biomaterial-associated infections takes advantage of this fact. By following the progress of an auxotrophically marked uropathogenic strain of *Escherichia coli,* Nickel et al (85) established that biofilm development on the plastic and rubber surfaces of the luminal route through the Foley catheter and urine collection system is an important mechanism of bacterial access to the catheterized bladder. Accordingly, they have placed a plastic luminal sleeve impregnated with a powerful industrial biocide (26) in the drainage spout of a new urine collection system. Bacteria are attracted to this biocide-releasing surface, where they are killed; thus biofilm development is not initiated. This strategy delayed bladder colonization for 6–8 days in a rabbit model system (86). The sleeve device is currently being tested in patients.

Digestion and Biodeterioration

Biodeterioration of materials, including the digestion of insoluble nutrients by bacterial populations in the digestive tracts of higher animals, usually involves a focused enzymatic attack on particular loci at the material surface. This focused attack produces the pitting characteristic of the biodeterioration of a variety of insoluble substrates ranging from the digestion of cellulose to the corrosion of stainless steel. Physical attachment is necessary for active cellulose digestion, and the enzymes involved remain in particularly close association with bacterial cells growing in biofilms on the surface of cellulose fibers (Figure 5).

The colonization of cellulose fibers exposed to normal rumen flora is very rapid. Primary cellulose-degrading organisms can be heavily enriched by placing sterile cellulose fibers in rumen fluid, allowing 5 min for the adhesion of the cellulose degraders, and recovering the colonized fibers by centrifugation (83). Monocultures of the three main cellulose-degrading species from ruminants (*Bacteroides succinogenes, Ruminococcus albus,* and *Ruminococcus flavefaciens*) adhere avidly to cellulose fibers, but they degrade this insoluble substrate at a rate much slower than that in natural digestive systems. Cells of *B. succinogenes* are closely apposed to the surface of the cellulose (Figure 5, *A*), and small cell-derived vesicles are produced, which retain adhesive and cellulolytic properties (41). Conversely, cells of the gram-positive coccoid *Ruminococcus* species adhere to the cellulose surface with much greater separation (Figure 5, *B*) and do not usually detach cellulolytic vesicles.

The specific adhesion of bacteria to cellulose is the essential first step in ruminant digestion, but realistic rates of cellulose digestion are not achieved in the laboratory until the organisms are combined with other bacteria (58) or

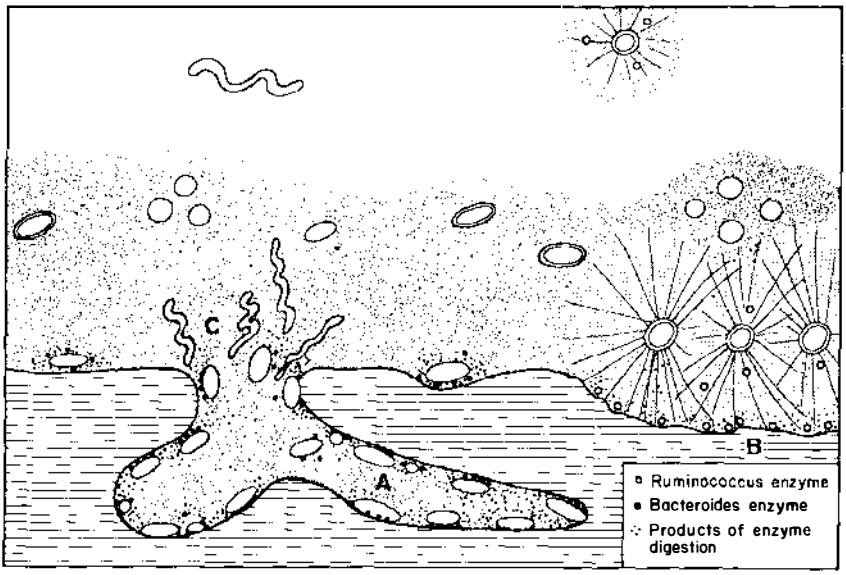

Figure 5 In the bacterial digestion of cellulose, *Ruminococcus* cells remain at some distance from the substrate surface while their enzymes initiate digestion (*B*). Other cellulolytic bacteria (e.g. *Bacteroides*) adhere intimately to the substrate (*A*) and detach vesicles and enzymes that cause a focal pitting attack. This attack is accelerated by the metabolic activity of noncellulolytic bacterial members (*C*) of structured consortia.

with fungi (116) to promote the development of functional microbial consortia (Figure 5). The products of cellulose digestion associate with the biofilm; these soluble nutrients are available to the cellulolytic bacteria themselves and to other heterotrophic organisms that may be attracted by chemotaxis and stimulated to divide and to form structured consortia. Cells of *Treponema bryantii* and *Butyrivibrio fibrisolvens* (Figure 5, *C*) are very commonly found in association with adherent cellulolytic cells of *Bacteroides succinogenes* (Figure 5, *A*); while these spirochaetes and curved rods have no cellulolytic enzymes, they enhance the cellulolytic activity of the *Bacteroides* species (>9%) when grown in mixed biofilms on cellulose fibers (58). These noncellulolytic bacteria may accelerate cellulose digestion by drawing products away from the consortium (Figure 5, *C*). Methane-producing bacteria combine with cellulolytic fungi to produce the most active in vitro cellulotytic consortium reported to date (116). Even though they are not intimately associated with the primary cellulolytic organisms in structured consortia, other heterotrophic bacteria growing in these mixed biofilms may subsist, directly or indirectly, on the soluble products of cellulose digestion and may contribute to the generation of the volatile fatty acids (VFAs) that are the main

vehicle of nutrient transfer to the host animal. When the cellulose fibers are completely digested, all of the bacterial components of this digestive biofilm become, perforce, planktonic organisms that await the provision of similar nutrient substrates that will be specifically colonized and rapidly digested by a reconstituted digestive biofilm. Structurally complicated forage materials undergo a complex and sequential bacterial attack (20). This ecological process gradually accelerates as the bacterial populations of the rumen become adapted to new feeds, as when forage-fed range cattle are transferred to feedlots and fed barley concentrates. Some chemical agents (e.g. methyl cellulose) cause the complete dissociation of adherent cells of *B. succinogenes* from cellulose fibers (59, 83), while other agents (e.g. 3-phenyl propanoic acid) enhance adhesion (110). The specificity of the bacterial degradation of cellulose suggests that some manipulation of this important microbial activity may be practicable in the near future.

Natural and Protective Associations with Tissue Surfaces

Direct obse

in the definition of bacterial populations on tissue surfaces. In a systematic examination of the bacterial populations at 25 sites in the digestive tracts of more than 100 cattle, Cheng and his colleagues (21) observed many bacterial morphotypes growing as adherent biofilms on food materials and tissue surfaces. Morphological "keys" (e.g. details of cell wall structure) have been used to equate many of these morphotypes to isolates obtained by homogenization and quantitative microbiological examination of the same food materials and tissues. Three distinct bacterial communities have been located in this organ system (19). Small numbers of bacteria live preferentially in the rumen fluid; the largest bacterial population is cellulolytic and is associated with food materials (see the preceding section); and perhaps the most unique population forms an adherent biofilm (Figure 6) on the surface of the stratified squamous epithelium of the rumen wall. This latter bacterial community, which develops during the first three days of life, contains many facultative organisms that consume the oxygen that diffuses from the animal tissue (Figure 6, *A*); it thus protects the fastidious anaerobes within the rumen. Proteolytic bacteria within this tissue surface biofilm (Figure 6, *B*) digest the dead distal cells of the stratified tissue and recycle their cellular components to benefit the host animal. Many of the bacteria in this tissue surface biofilm produce urease, as detected in cultures and as visualized in situ by a newly developed histochemical technique (81). Urease has a vital role in the digestive physiology of the host animal; it converts the urea that diffuses through the rumen wall (Figure 6, *C*) to ammonia, which constitutes an essential nitrogen source for the bacteria within the rumen. The epithelial tissue does not produce urease (19) and thus depends on bacterial

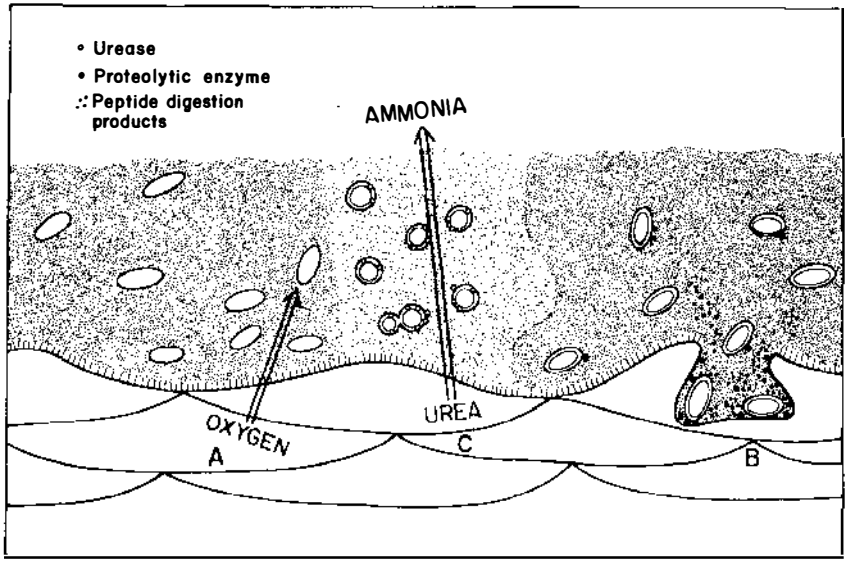

Figure 6 Component bacteria of the complex adherent biofilms found on the epithelial tissues of the bovine rumen facilitate the overall function of this organ by utilizing oxygen (*A*), digesting and recycling dead epithelial cells (*B*), and converting urea to ammonia (*C*).

cells within this specific tissue surface biofilm to produce an enzyme essential to the survival of the host animal. This is a clear demonstration of what we believe to be a general principle of microbial ecology: Stable tissue surface ecosystems attract and maintain adherent bacterial populations whose enzymatic activities are often integrated with those of the tissue itself.

Bacterial relationships with the surface of mucus-covered tissues such as the intestine are much more complex. Rozee et al (99), using new methods that allow the thick (~400 μm) mucus layer to be retained during preparation for electron microscopy, have visually confirmed Freter et al's (42) notion that most intestinal bacteria inhabit the mucus itself and that very few are actually attached to the tissue surface in normal animals. When the physical integrity of the mucus layer was disturbed by treatment with lectins (7) or by irradiation (114), autochthonous bacteria and protozoa that usually grow in this viscous phase proliferated and formed adherent biofilms on the tissue surface. Thus, mucus-covered tissues are not usually colonized by biofilms per se, but they are covered by dynamic viscous structures within which bacteria live and proliferate and only rarely associate directly with tissue surfaces. These vigorous mucus-associated microbial populations constitute a formidable ecological barrier that prevents the access of extraneous bacterial pathogens to their targets at the tissue surface (1). Direct observation and quantitative recovery have yielded very useful data regarding intestinal dis-

eases in which mobile pathogens (e.g. *Campylobacter jejeuni*) must traverse the mucus layer to invade specific gut cells (38) and regarding ecological diseases in which antibiotic stress leads to an overgrowth of toxin-producing bacteria (e.g. *Clostridium difficile*). These modern techniques are especially useful when tampon-induced changes in the human vagina stimulate the growth of autochthonous staphylococci (36) and the location of these toxin-producing cells, in relation to epithelial tissue, is more important than their total numbers within the affected organ.

The autochthonous bacterial populations of several tissue surfaces have been described by modern ecological methods (19). Well developed bacterial biofilms have been seen on the epithelia of the distal human female urethra (73), on the tissue surfaces of the rabbit vagina (55), and on the epithelia of the human vagina and cervix (8). These adherent organisms have been characterized following quantitative recovery. The same methods have been used to define the adherent bacterial populations on tissue surfaces in surgically constructed structures such as the ileal urinary conduits that connect the ureters to the body surface following surgical removal of the bladder. Cheng et al (21) have suggested that well established tissue surface biofilms composed of autochthonous bacteria act as ecological barriers to upstream colonization by pathogenic organisms (18, 101). This concept is supported by data on bladder infections that follow the disturbance of distal-urethra biofilm populations by broad-spectrum antibiotic therapy. Similarly, the physical bypassing of the urethral surface biofilm population by urinary catheters (87), of the adherent cervical population by IUDs (71), and of the bile duct population by biliary catheters (A. G. Speer, P. B. Cotton & J. W. Costerton, manuscript submitted) leads to rapid bacterial invasion of the upstream organ in each system. Recent successes in the prevention of bladder infections in rats by the colonization of their distal urinary tract tissues with autochthonous strains of *Lactobacillus* (98) encourage some hope that we may be able to reinforce and manipulate these ecological barrier populations to prevent upstream infections. We are presently examining the natural extent of these protective tissue surface biofilms in organ systems that are colonized at their distal extremity but consistently sterile in their proximal organ (e.g. urethra–bladder; cervix–uterus; duodenum–gall bladder). A more complete understanding of the nature of the sustained boundaries of autochthonous bacterial colonization will enable us to predict what procedures and treatments are likely to produce failure of these ecological barriers and consequent upstream colonization by pathogens.

Pathogenic Associations with Tissue Surfaces

Sustained interest in bacterial pathogenic mechanisms throughout the last three decades has resulted in detailed accounts of pili (111) and cell wall proteins that promote adhesion to target tissues, and of toxins (107) that

mediate pathogenic effects on host tissues. These elegant molecular mechanisms have appealed to our collective affection for order and simplicity; researchers have even developed model animal infections in which the genetic deletion of a single pathogenic mechanism renders the bacterial cell nonpathogenic. However, these attractive concepts have often fared poorly in the real world. A specific pathogenic mechanism, although real and fully operative, is frequently only one of many factors that facilitate bacterial pathogenicity (32, 84). Pathogenic bacteria often use more than one mechanism to mediate their attachment to target tissues (17), and most pathogens must persist and multiply in the infected system in order to exert deleterious effects on the host. The frequent encounters of healthy individuals with pathogenic bacteria actually lead to overt disease in a minute fraction of instances; the study of the entire etiological process from contact, through persistence, to infection reveals dozens of points (107) at which the pathogenic process may be aborted. In short, pathogenesis is an ecological process in which a particular bacterial species occasionally colonizes and persists in spite of all adverse environmental factors to produce a population sufficiently numerous, active, and well-located to exert a pathological effect upon the host.

Figure 7 is a hypothetical diagram that summarizes some of the postulated events of the early stages of the formation of pathogenic biofilms on tissue surfaces. The bacterial characteristic that is emphasized is the exceptional phenotypic plasticity that allows cells of a given species to change their cell surface characteristics radically in response to changing environmental factors (1, 12). Bacterial pili are avid and specific mediators of adhesion to receptors on target tissues (Figure 7, *A* and *C*). However, specific adhesion is not sufficient to establish colonization if the adherent cells lack the exopolysaccharide glycocalyx that affords protection from surfactants and other important host defense factors (Figure 7, *A*). Pathogenic bacteria often employ less specific and less avid exopolysaccharide-mediated adhesion mechanisms, which may act alone (Figure 7, *B*) or in concert with the pili (Figure 7, *C*). Exopolysaccharide-mediated adhesion is strong and resistant to shear forces (13), while pili are comparatively fragile structures (93). Although the occlusion of a target tissue surface by a confluent autochthonous bacterial biofilm (see the preceding section) would virtually preclude specific interactions between bacterial pili and tissue surface receptors, nonspecific glycocalyx-mediated adhesion could still be operative.

Once established on the tissue surface, the adherent pathogens must compete for iron with remarkably effective host siderophores. The expression of the genes controlling bacterial siderophore production (Figure 7, *D*) is a sine qua non of bacterial growth and persistence on the colonized tissue (108). Because phagocytic host cells are chemotactically attracted to complexes of

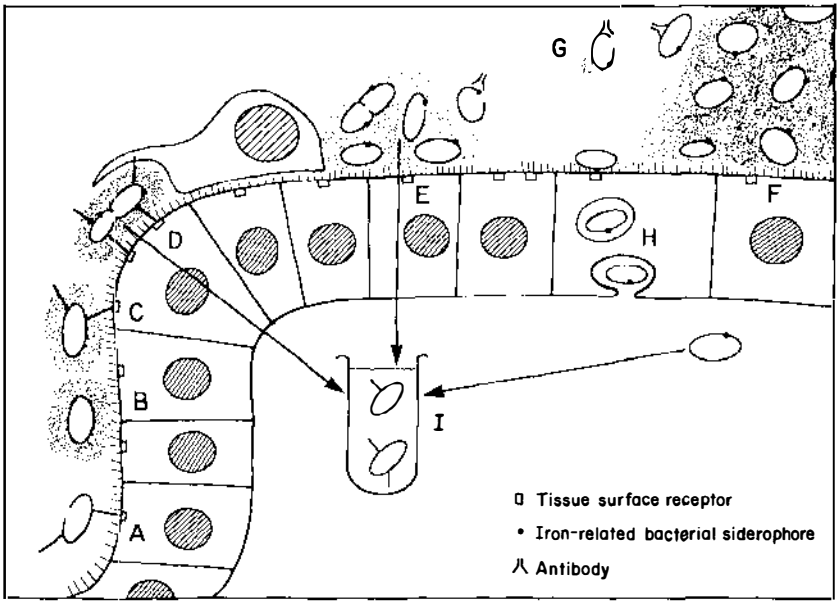

Figure 7 Diagrammatic representation of the variety of phenotypic forms bacteria may assume during the etiology *A-D* of acute (*E*, *G*, and *H*) or chronic (*F*) disease, contrasted with their phenotype when grown in nutrient-rich batch culture (*I*). See text for detailed explanation.

secretory IgA with the pili of many bacteria (1), bacterial survival may depend on the deletion of these structures from the bacterial surface (Figure 7, *E*). Development of glycocalyx-enclosed microcolonies (Figure 7, *E*) sufficiently large to withstand phagocytosis (52) would also contribute to bacterial persistence on the colonized tissue. We estimate that very few contacts with pathogenic bacteria proceed to this protected microcolony stage, but those organisms that do establish adherent "beachheads" can then proliferate to form a tissue surface biofilm (Figure 7, *F*). Bacteria within these protected tissue surface biofilms are not always overtly pathogenic (50), but their persistence and growth eventually result in massive unresolved bacterial masses resembling those seen in the lung in cystic fibrosis (62) and in the medullary cavity in osteomyelitis (78). Growth within protected glycocalyx-enclosed biofilms imposes several constraints on bacterial pathogenicity, in that bacterial cells and toxins are retained within the biofilm matrix or neutralized by antibodies or phagocytes at the biofilm surface; thus bacteremia and toxemia are rarely seen in these chronic diseases. Bacterial antigens at the biofilm surface stimulate the production of antibodies. These antibodies cannot penetrate the biofilm to resolve the infection, but they do form immune complexes at the biofilm surface (35) (Figure 7, *E* and *G*) and

thus often damage the colonized tissue (103). When bacterial biofilms form on vascular surfaces, such as the endothelium of heart valves, they accrete blood components such as fibrin and platelets (102). The resultant vegetations (82) often grow sufficiently large to interfere with the mechanical functions of these organs. When the metabolic activities of bacteria within tissue surface biofilms produce insoluble salts, the biofilm matrices trap the resultant crystals, and "infection stones" develop in the affected organs. Bacterial biofilms form the structural matrix of the struvite calculi that develop in the kidney when urease-producing bacteria produce ammonia that is deposited as $MgNH_4PO_4$ (89). Similarly, the production of deconjugated cholesterol or calcium bilirubinate can lead to the development of thick occlusive biofilms in the biliary tract (A. G. Speer, P. B. Cotton & J. W. Costerton, manuscript submitted). The pivotal role of bacterial biofilms in these occlusive diseases results from the tendency of their exopolysaccharide matrices to trap and accrete fine particles that would otherwise move harmlessly through the system.

Like biofilm-covered biomaterials (Figure 4), the tissue surface biofilms formed in chronic infections are foci of humoral and cellular inflammatory reactions that are usually sufficient to kill individual bacterial cells released at their surfaces (Figure 7, *G*). However, host defense mechanisms sometimes fail, especially in debilitated patients, and disseminated single cells of these pathogenic species may escape to exert their full toxic or invasive potential (Figure 7, *H*). Because of phenotypic plasticity (12), a totally different phenotype is produced when bacterial cells are recovered from any etiological stage of the infection and grown in an iron-rich medium (Figure 7, *I*) in the absence of host defense factors.

RESISTANCE OF BIOFILM BACTERIA TO ANTIMICROBIAL AGENTS

The functional environments of individual bacterial cells growing within biofilms differ radically from those of planktonic cells in the same ecosystem, and even more radically from those of planktonic cells in batch culture in nutrient media (Figures 1–7). We must now accept the unequivocal evidence (12) that bacteria respond to changes in their environment by profound phenotypic variations in enzymatic activity (C. S. Dow, R. Whittenbury, D. Kelly, unpublished), cell wall composition (105), and surface structure (4). These phenotypic changes involve the target molecules for biocides, antibiotics, antibodies, and phagocytes, and also involve the external structures that control the access of these agents to the targets. Therefore, we must expect that biofilm bacteria will show some alterations in susceptibility to these antibacterial agents. Further changes in susceptibility to antibacterial agents

are dictated by the structure of biofilms, within which the bacterial cells grow embedded in a thick, highly hydrated anionic matrix that conditions the environment of individual cells and constitutes a different solute phase from the bulk fluids of the system. Biofilms could therefore increase the concentration of a soluble antibacterial agent in the cellular environment by trapping and concentrating its molecules much as it traps and concentrates nutrients (Figure 2, *A*). On the other hand, the numerous charged binding sites on the matrix polymers and on the most distal cells in the biofilm may afford the innermost cells a large measure of protection from these agents. Peterson et al (95) have shown that aminoglycoside and peptide antibiotics are bound to the lipopolysaccharide molecules of *Pseudomonas aeruginosa* in a manner similar to that proposed here. We speculate that the dissociation constants of molecules bound within the biofilm are important because they dictate the concentrations of nutrients and of antibacterial agents available at the surface of individual cells.

An ecological examination of the whole aquatic system is a necessary first step in controlling bacterial problems in industry. Fouling, corrosion, and plugging all involve bacterial biofilms, but traditional monitoring procedures sample the much smaller planktonic populations that are intermittently shed from these adherent communities. Treatment with traditional concentrations of biocides kills these planktonic organisms and gives the appearance of success, but leaves the biofilm populations virtually unaffected (100). Thus millions of dollars have been wasted in ineffectual treatments. Costerton et al (27) and McCoy & Costerton (80) have developed a series of biofilm samplers that constitute parts of a pipe wall which are removable to yield an accurate biofilm sample. When biofilm sampling indicates an important problem, biocides are used at concentrations that completely kill the biofilm bacteria. These sampling and biocide procedures have been very useful in the oil recovery industry (27).

Direct sampling of biofilm populations is seldom possible in human disease, but the biofilms within the patient can be mimicked in vitro. When urine containing a uropathogenic strain of *P. aeruginosa* is passed through a modified Robbins device containing discs of catheter material (90, 91) the biomaterials develop a bacterial biofilm that closely resembles those observed on catheters recovered from patients infected by the same organisms (88). When planktonic cells within this system were treated with 50 μg ml^{-1} of tobramycin, 8 hr of contact was sufficient to yield a complete kill, but 12-hr contact with 1000 μg ml^{-1} of tobramycin did not kill the biofilm cells (90). When these resistant biofilms were washed free of tobramycin and dispersed to yield single cells, these essentially planktonic cells were susceptible to 50 μg ml^{-1} of the antibiotic. This study has now been expanded to include other antibiotics and other organisms (J. C. Nickel & J. W. Costerton, unpublished

data), and the inherent resistance of biofilm bacteria to antibiotics has been found to be a general phenomenon. A bacteremic patient with an endocardial pacemaker (70) presumed to have developed a *Staphylococcus aureus* biofilm on its biomaterial surfaces was treated for 6 wk with 12 g cloxacillin and 600 mg rifampin per day (72). When staphylococcal bacteremia recurred after antibiotic therapy was discontinued, the pacemaker was removed. It was covered by a thick biofilm (70) containing living cells of *S. aureus*. Chronic diseases in which the pathogenic bacteria grow in biofilms on tissue surfaces must be treated with high sustained doses of antibiotics to kill any of these protected organisms (77, 97). Complete resolution of these continuing bacterial foci is rarely achieved.

We conclude that bacteria within biofilms are generally much more resistant to biocides and antibiotics than their planktonic counterparts. We are presently examining this resistance to determine whether it derives from physiological changes in these sessile organisms or from the penetration barriers provided by the exopolysaccharide matrix and the distal cells of the biofilm itself. Recent successes in the prevention of biofilm development by the incorporation of biocides (86) or antibiotics (113) into biomaterials during polymerization have shown that antibacterial molecules leaching from a biomaterial surface can influence bacterial adhesion and subsequent biofilm development. While this preemptive strategy may be successful in preventing some of the infections associated with the short-term use of medical devices (86), control of infections during the long-term use of such devices will require an ecological understanding of the microbiology of the system and the judicious use of antibiotics in adequate doses.

When medical or industrial problems involve planktonic cells, as in acute bacteremic diseases or bacterial contamination in microchip production, antibacterial agents should be tested against planktonic cells in menstrua resembling those of the affected systems. Such tests routinely permit successful selection of antibiotics and biocides to solve these problems. Even when medical and industrial problems involve biofilm bacteria, tests against planktonic cells may be very useful; they indicate efficacy against planktonic cells that may detach from the biofilms, and negative planktonic results disqualify an agent for use against biofilm organisms. However, when the objective of treatment is the eradication of bacterial biofilms in either medical or industrial systems, antibacterial agents must be tested against biofilm bacteria. Tests against biofilm bacteria have now led to the identification of penetrating industrial biocides (100), but it is sobering that virtually all antibiotics in current use were selected for their efficacy against planktonic bacteria. The extension of biofilm test methods into the medical area is expected to identify penetrating antibiotics that may be useful in the treatment of the chronic biofilm-related diseases that are increasingly common in modern medical practice.

In vitro experiments have shown that encapsulated bacteria are less susceptible than their unprotected counterparts to the bactericidal and opsonic effects of specific antibodies (6) and to uptake by phagocytic cells (96, 104, 115). However, the most useful perceptions of the sensitivity of biofilm bacteria to antibodies and phagocytes are derived from studies of infections in animals and patients. Ward et al (K. Ward, M. R. W. Brown & J. W. Costerton, unpublished data) initiated growth of biofilm bacteria on biomaterial surfaces in the peritoneum of experimental animals and monitored the host animals' immune responses by crossed immunoelectrophoresis (XIE) and by immune blotting of SDS PAGE gels. They noted that large amounts of antibodies were produced against a small number of surface-located antigens, but that these antibodies failed to resolve the chronic infections. Similarly, large amounts of antibodies were produced against a limited number of bacterial antigens when cells of *P. aeruginosa* grew in biofilmlike masses in the lungs of rats in the chronic lung infection model (15) and in the lungs of cystic fibrosis patients (33). These antibodies did not control the further growth of biofilm bacteria within the lung, and they contributed to the formation of immune complexes (53), which profoundly damaged the infected tissue. Direct examination of biofilm-colonized peritoneal biomaterials (34) and of lung tissue in chronic *Pseudomonas* infections (15) showed that the phagocytic cells characteristic of the inflammatory response are mobilized and activated in response to the presence of these biofilm bacteria. However, the very prolonged course of the chronic infections (>20 yr in many cases) indicates a failure of the combined humoral and cellular defense mechanisms of infected hosts. Thus, while the molecular basis of the resistance of biofilm bacteria to clearance by antibodies and phagocytes remains obscure, it is unequivocally a general phenomenon that biofilms persist in spite of the vigorous immunological and inflammatory reactions of the infected host.

IMPACT OF THE BIOFILM CONCEPT ON EXPERIMENTAL DESIGN

The development of methods for the quantitative recovery of bacteria from biofilm populations was an important first step in the genesis of the biofilm concept (45). Because these biofilms are coherent and continuous structures, they can be removed by scraping with a sterile scalpel blade. Additional sessile cells can then be removed from the colonized surface by irrigation using a Pasteur pipette. The Robbins device (79, 90) provides disclike sample studs whose sides and back are sterile. The scraped material, the irrigation fluid, and the stud itself can be processed together by vortex mixing and gentle sonication (45, 90). Plating of the dispersed bacterial cells from fully disrupted natural biofilms usually yields a count about one logarithmic unit lower than the acridine orange direct count (AODC) of the same preparation

(43). We attribute this consistent discrepancy to the incomplete dispersal of biofilm fragments into single cells and to the presence of some dead bacteria that were either dead within the biofilm or killed during recovery and processing. Some bacteria remain on the colonized surface and some may be killed during dispersal by vortex mixing and sonication, so quantitative recovery data should be expressed as the minimum number of organisms present in the biofilm on each square centimeter of the colonized surface. New methods are now available for determining the viability [2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) technique (119)] and the metabolic activity [sessile heterotrophic potential (30, 60)] of bacteria within biofilms. The surfaces within a given system should be examined by several of these techniques to determine whether the majority of living cells within the biofilm are recovered by the quantitative recovery techniques used. Recovery media must often be adjusted to obtain isolates corresponding to the important morphological types (e.g. spirochetes) and physiological activities (e.g. urease activity) seen by direct observation of the biofilm in situ.

A preliminary ecological examination of an entire aquatic system is a necessary step in the design of a sampling program that will yield reliable and useful data. In environmental, industrial, and medical systems a simple preliminary statement of the approximate number and type (e.g. gram-positive rods) of bacterial cells on surfaces and in fluids throughout the system allows us to design a rational sampling program. If planktonic bacterial cells constitute the problem, planktonic samples are of paramount importance, and well designed sessile samples may permit detection of biofilms that periodically shed these free-floating cells. Conventional planktonic minimal inhibitory concentration (MIC) data will predict the concentration of antibiotics or biocides necessary to control these planktonic populations, and sessile MIC data (90) will predict the amount of these agents necessary to kill bacteria within the biofilms where they may have originated.

When a problem demonstrably involves biofilm bacteria, it is important that biofilm samples be obtained and that sessile MIC data be used to design biocide and antibiotic treatment strategies. When sessile samples cannot be obtained, planktonic bacteria from the affected system may be induced to produce biofilms outside the system, and potential control agents may be tested against these sessile organisms (90). The most important principle in these basically ecological studies of whole systems is that many different types of samples and many different types of data are useful, but we must not extrapolate from test tube–grown cells to individual planktonic cells or to biofilm cells in determining activities or sensitivity to antibacterial agents.

Because we acknowledge the phenomenal phenotypic plasticity of bacteria, we place an especially high value on direct observations or measurements of the activity of bacterial cells growing in the system of interest. Modern

monoclonal antibody probes (16) allow us to detect the bacterial production of specific adhesins (17) or toxins on the infected tissue and thus to assess their role, if any, in the etiology of particular diseases. Alternatively, the immune system of an infected animal or patient constitutes a remarkably sensitive monitor of bacteria growing in affected tissues. When bacteria grow within protected biofilms in chronic bacterial infections, the host's immune system recognizes and produces antibodies against only a limited number of bacterial antigens (54, 63). Detailed XIE (63) or immunoblotting (4) analyses of sera from infected patients reveal which bacterial surface structures are produced in situ and sufficiently accessible to the immune system to stimulate the production of specific antibodies. The emergence of planktonic bacteria from protected biofilm foci can be detected by a sharp rise in the number of different antibodies produced (54), and these instances of bacterial dissemination can often be effectively treated with antibiotics. Special immunological methods can detect both the formation of immune complexes (35, 53, 117) and their resolution by natural increases in leukocyte elastase activity (37) or by therapy with immune suppressants (5). When the formation of immune complexes can be accurately monitored in individual patients, this devastating sequel of chronic bacterial infections due to the biofilm mode of growth may be effectively controlled by selective immune suppression (5). In many chronic biofilm diseases the causative organisms are difficult to recover because of sampling and microbiological problems, but experience leads us to expect the involvement of certain pathogenic bacteria (46). The use of XIE and immunoblotting techniques may permit immunologic identification of these pathogens by the detection of rising titers of antibodies against specific marker antigens characteristic of certain species (e.g. *Bacteroides fragilis*).

The biofilm concept has prompted the development of several new techniques, but perhaps its greatest impact in the area of methodology has been to force us to examine conventional sampling and analytical techniques to determine exactly what each can tell us about the coordinated functioning of whole microbial ecosystems in nature and in disease.

EPILOG

Because the breadth of this review has necessitated some generalizations, it may be useful to summarize major points that are supported by unequivocal evidence. Using methods of light and electron microscopy that can cause their loss but not their acquisition, bacterial biofilms have been found adhered to most surfaces in all except the most oligotrophic environments (22). Detailed examination of biofilms in several of these environments has shown a secondary level of organization in which bacteria form structured consortia with cells of other species (106) or with cells of host tissues (19). Within these

consortia, instances of physiological cooperativity have been detected (19, 106). Biofilms have been found on the surfaces of biomaterials (31, 48, 70) and tissues (62, 78, 82) in chronic bacterial diseases characterized by their resistance to antibiotic chemotherapy and clearance by humoral or cellular host defense mechanisms (24). Bacterial biofilms constitute a hydrated viscous phase composed of cells and their exopolysaccharide matrices, within which molecules and ions may occur in concentrations different from those in the bulk fluid phase (29). Bacterial cells respond to changes in their immediate environments by a remarkable phenotypic plasticity (12) involving changes in their physiology (60; C. S. Dow, R. Whittenbury & D. Kelly, unpublished), their cell surface structure (10), and their resistance to antimicrobial agents (11, 90). Compelling evidence shows that bacteria growing in diseased tissue (3, 4, 105) differ from test tube–grown cells of the same organism in several important parameters, and extrapolation from in vitro data to actual bacterial ecosystems is now even more strongly contraindicated.

Techniques have been developed for the direct observation of biofilm bacteria (30) on inert surfaces and tissues, including mucus-covered epithelia (99), and for their quantitative recovery from these colonized surfaces (45, 90). New in situ techniques allow the detection of specific cell surface structures (14, 16), the measurement of general (30) and specific (81) metabolic activities, and the assessment of antibiotic sensitivity (90) in intact biofilms. The demonstrated ubiquity of bacterial biofilms, their established importance in many industrial and medical problems, and this new availability of methods for their detection and analysis should herald an exciting era in which microbiologists will come to recognize and perhaps counteract this basic bacterial strategy for growth and survival in nature and disease.

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