Chapter 11
The Functional Resistance of Bacterial Biofilms

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1 Pathogenic Bacterial Communities

There is intellectual coherence when a physician must tell patients that the bacteria causing their infection have tested resistant to the empiric antibiotic therapy, and that an alternative drug must be used. In this chapter, we will concern ourselves with the growing number of bacterial infections in which antibiograms of the causative organism show sensitivity to standard antibiotics in readily attainable concentrations, but the infection fails to be cleared. This discrepancy is troubling and frustrating for patients, physicians, and diagnostic laboratories alike, but it can now be resolved by concepts that have become widely accepted in microbial ecology.

Microscopic observations of natural ecosystems have shown that more than 99.9% of bacteria grow in slime-enclosed, surface-adherent biofilms, while only a minority exists as the free-floating planktonic cells we grow in laboratory broth cultures (1). Most cells within sessile biofilm communities show reduced metabolic rates and radically (more than 50%) different protein expression patterns compared to planktonic cells (2). One of the many consequences of these phenotypic alterations is their tolerance to almost all of the adverse factors (dehydration, antibiotic exposure, and the predation by amoebae) that readily kill their planktonic counterparts (1).

Using the same microscopic technologies, biofilm communities have been discovered in device-related and chronic infections. A pivotal report in 1982 documented large numbers of sessile, slime-embedded *S. aureus* on a pacemaker lead, which caused a systemic infection (Fig. 1a) (3). The biofilm had formed as a result of bacteremia secondary to an olecranon bursitis, and it drew considerable clinical attention because it resisted weeks of high-dose antibiotic therapy. Since then, biofilms have been revealed in an increasing variety of diseases (Table 1, Fig. 1a–f). As many as 60% of bacterial infections currently treated by physicians in the developed world are considered to be related to biofilm formation (4).

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Biofilm infections are especially frequent in the presence of foreign-body materials. Biofilms on intracorporeal devices mostly originate from perioperative contaminants; transcutaneous catheters become colonized by exponents of the skin flora within days after catheter insertion (5). A fragile balance between colonization and infection is often maintained for months. Host defenses control the shedding of planktonic bacteria and toxins and thereby prevent clinical symptoms, but they are unable to clear the biofilm. Episodes of acute inflammation, caused by the breakthrough of planktonic cells, can be successfully treated with antibiotics. Because short-term therapies usually fail to sterilize biofilms, however, flare-ups after treatment termination are frequent.

2 Stealthy Infections: Flying Below Our Radar

The diagnosis of biofilm infections is difficult. The biofilm mode of growth can delay overt symptoms for months or years. Diagnostic aspirates or swabs are often falsely negative, possibly because the microorganisms persistently adhere to a surface, but not in planktonic form. Individual biofilm fragments with hundreds of slime-enclosed cells may yield only a single colony when plated on agar, or may fail to grow at all because of the dormant state (as explained below) of the embedded bacteria. Consistently, the sonication of removed implants and PCR amplification techniques have shown increased sensitivity in the detection of bacteria sequestered in biofilms (6). Furthermore, many biofilm pathogens are skin organisms that may be dismissed as contaminants.

Culture-independent diagnostic techniques have revealed that several diseases associated with a presumably sterile inflammatory process are indeed bacterial infections that escape culture because of their biofilm mode of growth. For both culture-negative chronic otitis media with effusion (7, 8) and chronic prostatitis (9), a bacterial etiology has been evidenced by the detection of bacterial DNA and mRNA, as well as by electron and confocal scanning laser microscopy

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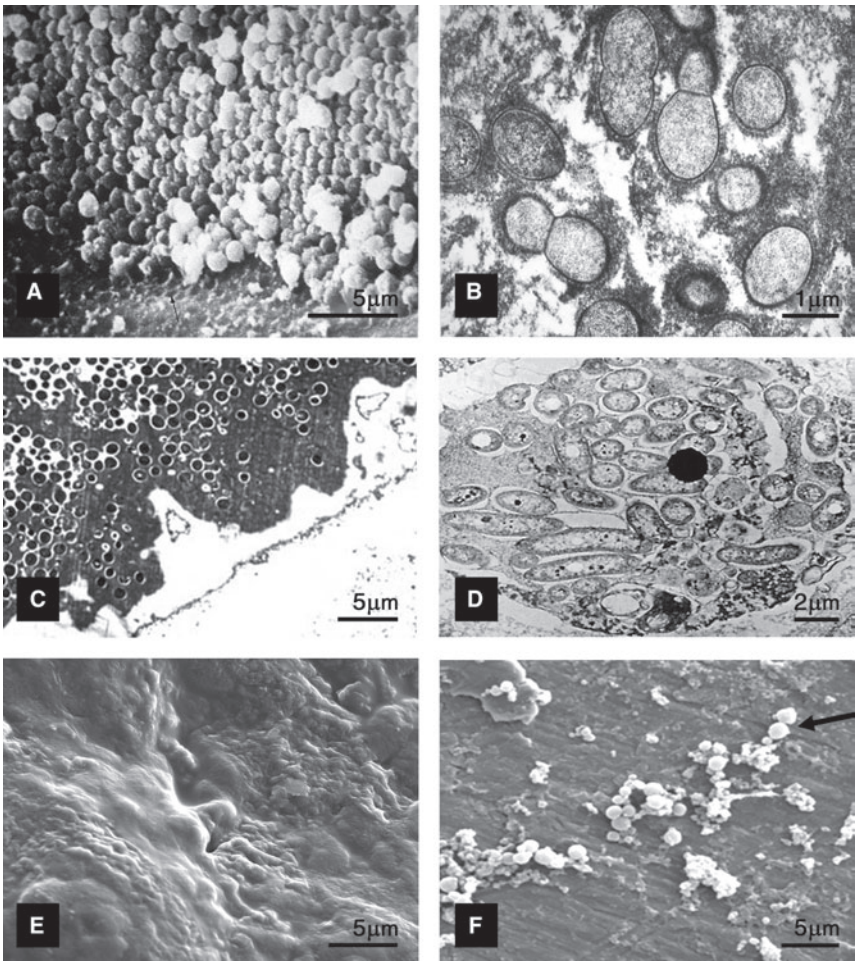

Fig. 1 Biofilms are increasingly recognized as a cause of chronic and device-related infections. Electron microscopy has documented surface-adherent bacteria embedded in extracellular slime on pacemaker leads (**a**), in endocarditis vegetations (**b**), on bone sequestrae in osteomyelitis (**c**), or chronic pneumonia in patients with cystic fibrosis (**d**). The microscopic detection of biofilms in culture-sterile samples of chronic otitis media with effusion (**e**) and aseptic prosthesis loosening (**f**) suggests an infectious etiology in these inflammatory states. The biofilm matrix in these images is reduced due to the dehydration process necessary for electron microscopy

Table 1 Partial list of human infections involving biofilms (adapted from (4))

Infection or disease	Common bacterial species involved
Dental caries	Acidogenic Gram-positive cocci (*Streptococcus sp.*)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypeable *Haemophilus influenzae*
Chronic tonsillitis	Various species
Cystic fibrosis pneumonia	*Pseudomonas aeruginosa, Burkholderia cepacia*
Endocarditis	Viridans group streptococci, staphylococci
Necrotizing fasciitis	Group A streptococci
Musculoskeletal infections	Gram-positive cocci
Osteomyelitis	Various species
Biliary tract infection	Enteric bacteria
Infectious kidney stones	Gram-negative rods
Bacterial prostatitis	*Escherichia coli* and other Gram-negative bacteria
Infections associated with foreign body material	
Contact lens	*P. aeruginosa*, Gram-positive cocci
Sutures	Staphylococci
Ventilation-associated pneumonia	Gram-negative rods
Mechanical heart valves	Staphylococci
Vascular grafts	Gram-positive cocci
Arteriovenous shunts	Staphylococci
Endovascular catheter infections	Staphylococci
Peritoneal dialysis (CAPD) peritonitis	Various species
Urinary catheter infections	*E. coli*, Gram-negative rods
IUDs	*Actinomyces israelii* and others
Penile prostheses	Staphylococci
Orthopedic prosthesis	Staphylococci

(Fig. 1e). We are currently investigating acetabular cup prostheses that had been removed because of "aseptic loosening" (Maale, Costerton et al., unpublished data). Preoperative synovial fluid aspirations and conventional cultures of the explanted prostheses had all yielded negative results. Direct microscopy and fluorescent in situ hybridization (FISH), however, have revealed extensive *Staphylococcus epidermidis* biofilms in eight out of ten samples tested to date (Fig. 1f).

3 Biofilm Structure and Physiology

Biofilm formation is a sequential process of microbial attachment to a surface, cell proliferation, matrix production, and detachment (2). This process involves a coordinated series of molecular events, which are partially controlled by quorum sensing, an interbacterial communication mechanism dependent on population density (10). As schematized in Fig. 2, mature biofilms demonstrate a complex 3-dimensional structure containing functionally heterogeneous bacterial communities. Embedded bacteria occupy numerous microenvironments differing in respect of osmolarity, nutritional supply, and cell density. This heterogeneity produces a variety of phenotypes within one biofilm – a single specific "biofilm phenotype" does not exist.

Biofilm-imaging using microsensors, fluorescent probes, and reporter gene technologies have allowed the correlation of the spatial distribution of nutrients with metabolic activity (Fig. 3) (11, 12). Both oxygen and glucose were completely consumed in the surface layers of the biofilms, leading to anaerobic, nutrition-depleted niches in the depths (13). Areas of active protein synthesis were restricted to surface layers with sufficient oxygen and nutrient availability (12, 14).

4 Resisting Host Defense

The biofilm mode of growth provides a variety of defense strategies against the host immune system. Phagocytes have a reduced efficacy in ingesting sessile bacteria and biofilm clumps. Biofilm fragments of eight to ten cells survived pulmonary host defenses, even when deposited into the lungs of healthy animals (15). Furthermore, large amounts of extracellular polymeric slime are believed to hinder the penetration of leucocytes into biofilms (16).

Perhaps the most invidious characteristic of biofilm infections, however, is not their resistance, but the induction of deleterious immune responses. Biofilms stimulate the production of antibodies and cytokines (16). Ensuing immune-complex deposits and the oxidative burst of macrophages, however, cause greater collateral damage to the host than to the slime-embedded biofilm (17). The destruction of heart valves in bacterial endocarditis, the de-ossification adjacent to infected joint prostheses, and the progressive fibrosis in cystic fibrosis lungs provide proof of these deleterious effects. In cystic fibrosis, progression toward chronic pneumonia has been associated with an immunologic shift toward a Th2 response (18).

5 Why Antimicrobials Fail: Learning from Planktonic Cultures

The minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) assess the effect of antibiotics against planktonic organisms in the exponential phase of growth. The physiology of these cells resembles that of rapidly dividing planktonic bacteria in acute infections such as septicemia. It is therefore no surprise that antibiotic efficacy against acute infections in vivo can be predicted from MIC and MBC measurements in vitro. On the other hand, MBCs of the same bacteria grown as a biofilm may be three orders of magnitude higher (19–21). What makes strains that are susceptible in exponential planktonic cultures turn highly tolerant to the very same antibiotic when grown as a biofilm? Nutritional depletion? High bacterial density? Both hypotheses can be tested by comparing exponential planktonic cultures with stationary phase planktonic cultures, because the latter contain high concentrations of starved bacteria.

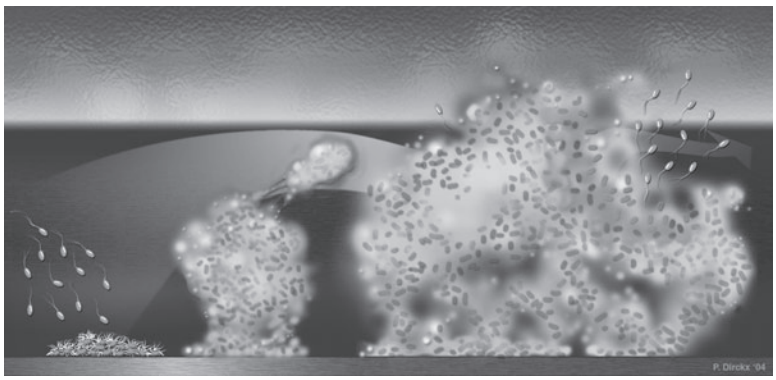

Fig. 2 The structural heterogeneity of biofilms is the product of continuous growth and detachment. This cartoon illustrates the various mechanisms involved in this process. P. Dirckx, Center for Biofilm Engineering

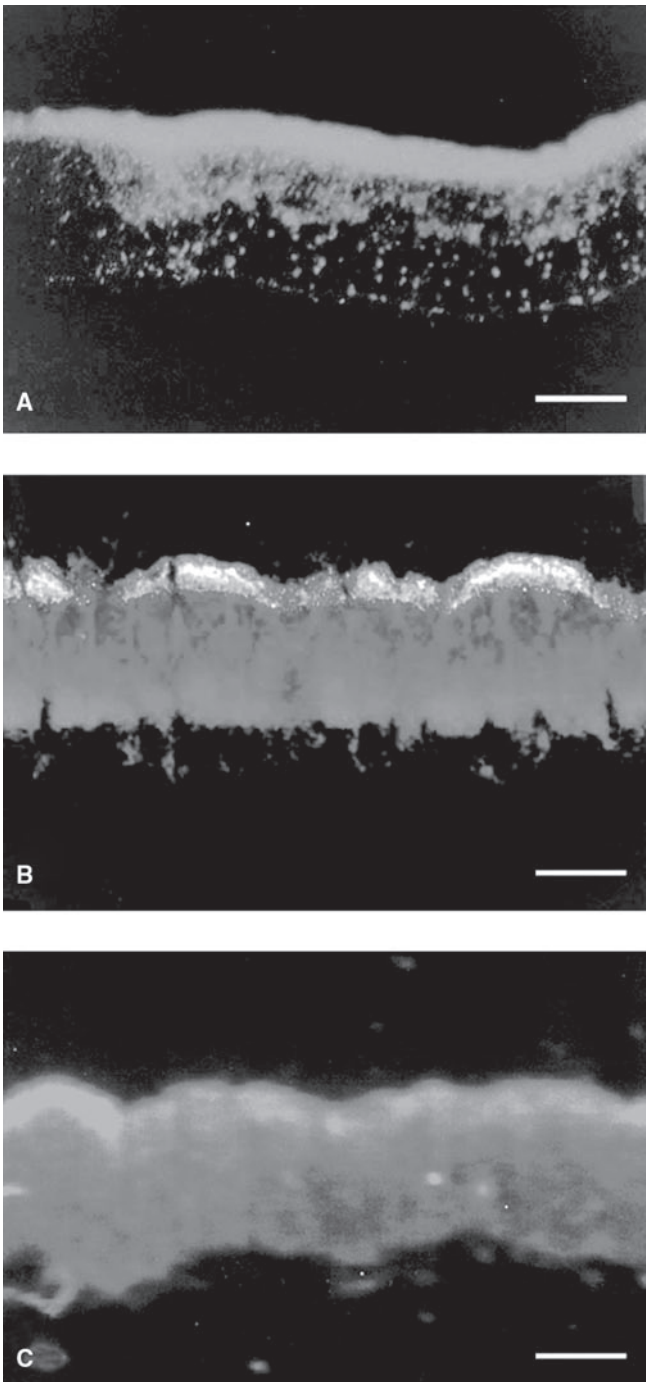

Fig. 3 Visualization of the spatial heterogeneity of respiratory activity, protein synthesis, and bacterial growth by epifluorescent microscopy. A *P. aeruginosa* biofilm was grown on a surface (*bottom*) covered by bulk fluid containing nutrients. (**a**) CTC-staining (*bright*) indicating respiratory activity. (**b**) Fluorescent staining of alkaline phosphatase (*white*) showing de novo protein synthesis under phosphate starvation; counterstaining of alkaline phosphatase-negative cells with propidium iodide (*grey*). (**c**) Biofilm section hybridized with a eubacterial oligonucleotide probe. The more intense staining near the bulk fluid suggests a higher rRNA content, and thus a more rapid growth rate than in the interior of the biofilm. Bar 50 µm. Adapted from (11) with permission of the publisher

Antimicrobials are more effective in killing rapidly growing cells rather than in stationary cells (22). While some antibiotic classes such as fluoroquinolones may kill non-growing cells, beta-lactams have an absolute requirement for cell growth in order to kill (23). Consistently, the rate of survivors in planktonic bacterial cultures challenged with antibiotics increased exponentially during logarithmic growth, to plateau in the stationary phase, with 100% survivors for a betalactam and 0.1–1% for quinolones (23, 24). Repeated re-inoculation of a culture to maintain it in the early exponential state eliminated any survivors, suggesting that antibiotic tolerance does not arise in the early logarithmic phase, but depends on the few cells remaining in the phenotype they expressed in the stationary phase (24). These survivors – alternatively known as "persisters" – were tolerant to immediate challenge with any of the antibiotic agents tested, but regained full antibiotic susceptibility after dilution in fresh medium (25). This observation suggests that persistence reflects an expressed phenotype rather than individual resistant clones, and that this phenotype can be overcome by nutritional stimulation and dilution. Interestingly, bacteria in high density (10^9–10^{11} CFU/mL as compared to 10^5 CFU/mL) remained tolerant to antibiotics despite transfer to fresh medium (23). Similar findings in rhizobium, where a greater percentage of cells survived in the stationary phase if cells were starved at high density (26), support the hypothesis that quorum sensing influences the proportion of survivors.

Based on elegant batch culture assays, Gilbert and coworkers directly related antibiotic tolerance to growth rate (25). They reduced bacterial growth rates by starvation, to the extent that bacteria were not susceptible to antimicrobials while still replicating fast enough to compensate for washout in a continuous culture system. Thus, bacteria did not need to be totally dormant in order to become persisters. Furthermore, they demonstrated that growth rates within a planktonic culture were strikingly heterogeneous. Mean doubling times of individual clones derived from late logarithmic culture varied between 500 and 45 min. For any time point between the lag phase and the stationary phase, a specific proportion of clones with maximum growth rates beneath the levels required for antibiotic susceptibility, i.e., survivors, could be determined. Any sample – irrespective of its proportion of replicating and dormant cells – repeated the general distribution pattern of active and inactive cells when diluted and re-grown in fresh medium. This again suggests that the distribution in active and susceptible versus inactive and tolerant cells is merely functional, and is an effect of alterations in growth medium and cell density.

The physiology of stationary phase planktonic bacteria is similar to biofilm-embedded cells. Both are affected by nutrient limitation and high cell densities. Both express similar degrees of antibiotic tolerance (Fig. 4) (21, 23). Like their planktonic counterparts, biofilm cells rapidly regained their

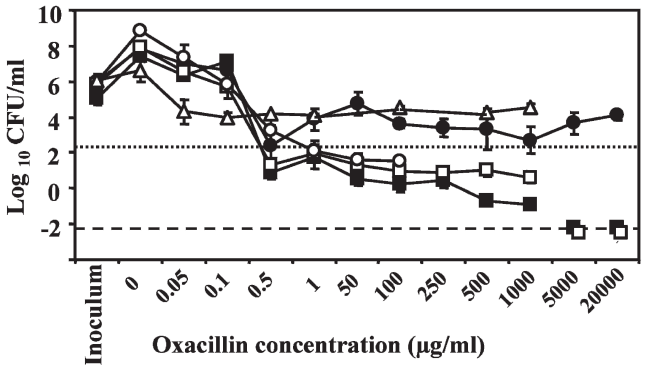

Fig. 4 Log reduction of viable cells in response to increasing oxacillin concentrations. The dotted line marks a 3-log reduction in CFU, and therefore indicates the minimal bactericidal concentration (MBC). Intact biofilm clumps tested in fresh medium (*filled circle*) and stationary phase planktonic cultures tested in spent medium (*open triangle*) were highly tolerant to antibiotics. Mechanically disrupted large clumps (o) regained their antibiotic susceptibility. Exponential phase planktonic cultures (*open square*) and stationary phase planktonic cultures in fresh medium (*filled square*) showed a conventional MBC of 0.5 µg/mL. The detection limit is represented by the *dashed line*. Error bars = 1 SD. Reprint from (21) with permission of the publisher

antibiotic susceptibility after mechanical disruption of the biofilm architecture and dilution in fresh medium (Fig. 4) (13, 21, 27). Disruption of the biofilm may provide cells, previously starving in deep layers, with new access to nutrients, which brings them back to the susceptible state of exponential growth. Alternatively, loss of tolerance may be explained by the dilution of protective cell signals – just as had been suggested for high-density planktonic cultures. The exponential increase in persister cells in planktonic cultures over time may mirror the increase in the number of dormant cells as we progress from the biofilm surface into its depths. Persisters in planktonic cultures may represent the viable but non-culturable bacteria found in many biofilm infections.

Furthermore, the patchy distribution of growth rates within any culture at any growth phase could explain why small pockets of surviving cells can be detected on the periphery of biofilms, where exposure to antibiotics and nutrients is unrestricted (25). As the availability of nutrients decreases into the depths of a thick biofilm, the density of bacteria growing at less than the critical growth rate necessary for antibiotic-mediated killing would increase.

How persisters survive. Persisters resist killing while remaining fully susceptible to growth inhibition (i.e., without changes in MIC) (24). Their phenotype is generally explained by reduced metabolic activity or even a dormant state. In addition, a variety of stress response systems are turned on once bacteria reach stationary growth phase, especially when stimulated by environmental stresses (such as alterations in nutritional quality, temperature, pH, or osmolarity) (28–30). Stress response genes protect bacteria from killing by antibiotics, the host immune system, and environmental toxins (29). Improved survival may be explained by an altered reaction to cell damage. For example, the SOS DNA-repair system, though not specifically reported in biofilms, is induced in ageing colonies on agar plates (31).

Stress response genes are regulated by a network of interacting signals, such as quorum-sensing, (p)ppGpp, or poly P kinase (PPK). In *E. coli*, expression of the *hipA* gene increased tolerance, probably by inducing (p)ppGpp synthesis, which potentiates the transition to a dormant state upon application of stress (32). Knock-out mutants for *hipA* contained 10–10,000 times more persisters during exponential growth than the wild-type (24). A *P. aeruginosa* PPK mutant showed inhibited quorum sensing, and failed to form thick, differentiated biofilms (33). Similar mutants of *E. coli* were unable to adapt to nutritional stringencies and environmental stress, which was attributed in part to their failure to express *rpoS* (34).

Sigma factors are key elements in general stress response. Bacteria lacking the sigma factor S had an increased susceptibility to oxidative stress during the stationary phase (30). *RpoS*, a sigma factor expressed in Gram-negative bacteria during the stationary phase, has been detected in *P. aeruginosa* biofilms in vitro (35) as well as in the sputa of CF patients (36). Whereas *rpoS* mutant *Escherichia coli* were dramatically impaired in biofilm growth (37), *rpoS* mutant *P. aeruginosa* grew thicker biofilms and showed higher antimicrobial tolerance (38, 39). Therefore, the role of *rpoS* in biofilm formation remains unclear, but may depend on strain-specific cofactors and specific growth conditions.

6 Biofilm-Specific Resistance

Metabolic dormancy and general stress responses are of crucial importance for phenotypic antimicrobial tolerance, both in planktonic and biofilm-grown bacteria. In addition, several biofilm-specific mechanisms of tolerance have been evaluated. They range from preventing antibiotics from reaching their site of action to reducing the susceptibility of embedded bacteria as a result of their biofilm mode of growth.

Impenetrable biofilms. The diffusion of antibiotics through biofilms has been assessed by concentration measurements and the visualization of bactericidal effects in the depths of in vitro biofilms (13, 19). While most studies have documented unimpaired antimicrobial penetration (19, 40), three exceptions have been noted: In a betalactamase-positive *Klebsiella pneumoniae* biofilm, betalactam antibiotics were deactivated in the surface layers more rapidly than they diffused (13). Second, biofilm penetration of positively charged aminogylcosides is retarded by binding to negatively charged matrices, such as the alginate in *P. aeruginosa* biofilms (14, 41). This retardation may allow more time for bacteria to implement adaptive stress responses. Third, extracellular slime derived from coagulase-negative

staphylococci reduced the effect of glycopeptide antibiotics, even in planktonic bacterial cultures (42, 43).

Once the antibiotic has successfully reached the bacterium, it may be inhibited from penetrating or may be shifted back out again by efflux pumps. A recent study identified a mutant of *P. aeruginosa* that formed biofilms in characteristic architecture, but did not develop tolerance to three different classes of antibiotics (44). As the mutant lacked periplasmic glucans, which were shown to bind tobramycin, tolerance was attributed to the sequestration of antimicrobial agents in the periplasm. Efflux pumps provide resistance to several antibiotic classes, including tetracyclines, macrolides, beta-lactams, and fluoroquinolones (45). Therefore, their upregulation seemed to be an attractive hypothesis to explain the class-independent tolerance of biofilms. However, current evidence cannot relate reduced biofilm susceptibility to an increased expression of these pumps. Temporal and spatial analyses in a developing *P. aeruginosa* biofilm revealed that the four multidrug efflux pumps decreased over time, with maximal expression occurring at the biofilm–substratum interface (46). Interestingly, quorum-sensing molecules are an alternative substrate for efflux pumps, and have been shown to accumulate when pumps are inactivated (47). In this context, a reduced pump activity within mature biofilms might contribute to biofilm tolerance through mechanisms related to cell density rather than to drug efflux per se.

Phase variation. While the transcription control of most bacterial genes permits a gradual response, phase variation constitutes an "all-or-none" mechanism. The high-frequency ON-OFF switching of phenotype expression is basically random, but modulated by environmental conditions (48). Phase variation has been discovered in a variety of bacterial species (48, 49). In *P. aeruginosa*, phenotypic variation to small colony variants occurred under the influence of antibiotics, both in vitro and in the lungs of patients with cystic fibrosis (48). Remarkably, small colony variants exhibited increased biofilm formation and antimicrobial tolerance. This first report certainly needs confirmation, but suggests therapeutic initiatives. The specific gene product that modulates the phenotypic "switch" from small colony variants back to the susceptible phenotype, for example, presents a promising target (48).

Quorum sensing. Many bacteria communicate via the production and sensing of autoinducer "pheromones" in order to control the expression of specific genes in response to population density. This so-called quorum-sensing (QS) coordinates gene expression within and among species (50). Given the tremendous changes associated with the switch from planktonic growth to growth within a mature biofilm community, it seems reasonable that cell–cell signaling regulates biofilm formation. As a matter of fact, planktonic *P. aeruginosa* depended on QS signals to form a differentiated, 3-dimensional biofilm structure under static conditions (10). Under flow conditions, however, biofilms of QS mutants and wild-type bacteria were exactly alike (51), indicating that, although important, QS is not indispensable. Many Gram-negative bacteria utilize *N*-acyl homoserine lactone (AHL)-dependent QS systems. These signals are involved in virulence gene expression and biofilm formation (10, 52). In vivo, AHLs have been detected in the urine of patients with catheter infections (53) and in the lungs of patients with cystic fibrosis, thereby coinciding with the development of respiratory biofilms (54). Two recent studies used microarray analysis to identify QS controlled genes in *P. aeruginosa* (55, 56). The QS regulated genes represented 6% (55) and more than 10% (56) of the genome, respectively.

The seaweed *Delisea pulchra* utilizes halogenated furanones to discourage bacterial colonization by blocking bacterial cell–cell communication (57). In vitro, similar compounds affected the architecture and enhanced the detachment of a *P. aeruginosa* biofilm (58), but also inhibited growth, motility, and biofilm formation of *Bacillus subtilis* (59). Possible strategies to influence QS were extensively reviewed by Camara and coworkers (50). Although promising, manipulation of QS is still a long way from clinical practice.

The "biofilm gene". Several studies have documented antimicrobial tolerance in biofilms too thin to pose a barrier to the diffusion of metabolic substrates (60, 61), thus arguing against starvation-induced dormancy as the only reason for antimicrobial tolerance of biofilms. This observation led to the hypothesis of a genetically controlled, biofilm-specific phenotype. Expression of a "biofilm gene" would lead to the cooperative development of a characteristic architecture, and to the expression of specific antimicrobial tolerance. This concept is of particular interest, as the control of key biofilm genes would offer excellent options to overcome tolerance. Biofilm-specific epitopes could further be used for diagnostic tests and vaccinations.

When assessed by DNA microarrays, gene expression in biofilms differed from planktonic cultures by 6% in *B. subtilis* (as assessed after 24 h) and 1% in *P. aeruginosa* (assessed after five days of culture) (39, 62). In *B. subtilis*, the transition from a planktonic to a biofilm state involved several transcription factors (62). Most were maximally active after eight hours of culture, when only 7% of the bacteria grew as a biofilm. Their increased activity under anaerobiosis, starvation, and high cell density suggest that these growth conditions stimulate biofilm formation. On the other hand, biofilm formation was inhibited by high glucose concentrations through the accumulation of an inhibitory catabolite in a phenomenon known as catabolite repression (62).

Staphylococcal biofilm formation is mediated by the polysaccharide intercellular adhesin PIA, a product of the *ica*ADBC gene cluster (63, 64). Ziebuhr et al. detected the *ica* locus in 85% of coagulase-negative staphylococci causing invasive infections, but only 6% of contaminating strains, and proposed targeting the *ica*-locus as a diagnostic marker for pathogenicity in staphylococci (65). This power to discriminate between invasive and non-invasive coagulase-negative staphylococci, however, could not be

confirmed (66). Knobloch and coworkers reported that virtually all *S. aureus* strains contain the *ica* gene cluster, but do not necessarily produce biofilms, thus stressing the importance of the control of gene expression (67). In 44% of the tested strains, biofilm formation was only seen in certain media. In addition, PIA synthesis was altered by subinhibitory antibiotic concentrations (68), phase variation (69), quorum sensing (70), or *icaR* (71), a transcriptional repressor of *ica* expression under environmental control (72). Despite the apparent relevance of the *ica* gene cluster and PIA for biofilm formation, no diagnostic or therapeutic targets have been found so far, the search being complicated by the vast number of co-variables.

The remainder of the differentially expressed genes and proteins identified so far in biofilms are involved in (mainly anaerobic) metabolism, the regulation of osmolarity, the production of extracellular polymeric slime, cell–cell signaling, and motility (2, 39, 73–75). Finelli et al. described five "indispensable" genes for *P. aeruginosa* biofilm formation (74). They include genes for aerobic and anaerobic metabolism, osmoregulation, a putative porin, and a gene thought to be involved in carbon metabolism, the production of virulence factors, and the response to environmental stresses. In *S. aureus* biofilms, five genes were identified as being upregulated compared to planktonic cultures, encoding enzymes needed for glycolysis, fermentation, and amino acid metabolism, as well as a general stress protein (73). Yet, none of these differentially expressed genes and proteins were irreplaceable in their function or reproducibly found among various species, and therefore do not promise diagnostic or therapeutic potential.

7 Trading Posts for Resistance Genes

Besides providing antimicrobial tolerance for embedded cells, biofilms promote the propagation of antibiotic resistance and virulence genes among the bacterial community by horizontal gene transfer. Competence factors and plasmids are key players not only in horizontal gene transfer, but also in biofilm formation. In *Streptococcus mutans*, a quorum-sensing system was found to propagate structural biofilm differentiation and genetic competence (76). Its activation altered biofilm architecture, and increased transformation frequencies in biofilm-grown bacteria by 10–600 times compared to planktonic cells.

The capacity of *E. coli* K12 to form biofilms dramatically improved upon the acquisition of a plasmid (77). The expression of conjugative pili thereby seemed to boost the formation of a 3-dimensional biofilm architecture. Biofilms, in their turn, provide a sufficient density of bacterial recipients to assure high transfer rates of plasmids (77). The high expression level of prophages found in Gram-negative (39) and Gram-positive biofilms (62) is another indicator of a very active transfer of mobile genetic elements within biofilms.

From an epidemiological point of view, horizontal gene transfer is especially important within polymicrobial biofilms formed by the oral and intestinal flora (78, 79). In that environment, resistance genes can be transferred from apathogenic to highly virulent strains, both within and beyond species borders (78, 80). Considering that, for example, only 5% of the oral flora are detected by routine culture techniques, this gene pool available for horizontal transfer may still be profoundly underestimated.

All in all, biofilms play a triple role in the spread of antibiotic resistance: First, the treatment of biofilm-related infections requires long-term (and often recurrent) antibiotic therapy, exposing colonizing bacteria to prolonged antibiotic selection pressure. Second, biofilm physiology enables embedded bacteria to survive antibiotic exposure long enough to acquire specific resistance to the drug. Finally, the high cell density and the accumulation of mobile genetic elements within biofilms provide an ideal stage for efficient horizontal gene transfer.

8 Treating Biofilm Infections

Current therapeutic strategies are based on two pillars: (19) high-dose, long-term antibiotic therapy and (13) the removal of infected foreign-body material and any necrotic tissue. In bacterial endocarditis, for example, antibiotic treatment was shown to be more successful when serum antibiotic levels were held at least tenfold above the MBC (81). But even with 8 weeks of parenteral antibiotic treatment, few patients with prosthetic heart valve endocarditis have been cured by antimicrobial therapy alone (82).

The sterilization of a biofilm infection is highly demanding, both for the patient and the treating physician. The patient may face recurrent surgery, prolonged hospitalization for intravenous therapy, adverse drug reactions to the antibiotic agent(s), infectious complications related to intravascular devices, the disturbance of the colonizing flora, and tremendous costs. From an epidemiological point of view, any prolonged exposure to antibiotics selects for resistant organisms within the bacterial flora, and represents another step toward the postantibiotic era. Considering these risks and the considerable failure rate of current strategies, it cannot be stressed enough that any therapy should be based on a thorough diagnostic workup and treatment plan. Advances in molecular biology make culture-independent diagnostic strategies (such as the detection of bacterial 16S ribosomal DNA by polymerase chain reaction, or the detection of specific organisms with FISH-probes) available for clinical practice. Selan and coworkers have recently developed a non-invasive test for endovascular staphylococcal biofilms

that detects IgM antibodies directed against an epitope that is exclusively expressed on staphylococci growing in a biofilm (83). However, all these new techniques cannot provide the antibiogram of infecting organisms – a major shortcoming for the treatment of a smoldering chronic infection, where treatment success or failure may not be evident for weeks.

Therapeutic approaches for specific biofilm infections have been reviewed elsewhere (84–86). They have lately been complemented by new experimental approaches, such as the exposure of biofilms to ultrasound, or to an electrical field to facilitate matrix penetration or disturb the integrity of bacterial membranes (87, 88). We will attempt to crystallize the discussion of the clinical management of biofilm infections by focusing on two classical biofilm diseases: hip prosthesis infection and central venous catheter infection.

When dealing with infected prostheses, acute exacerbations respond well to antibiotic therapy, but sterilization is difficult. Debridement without removal of the implant, combined with 4–6 weeks of intravenous antibiotic treatment and subsequent long-term oral therapy, has a failure rate between 32 and 86% (86). Successful prosthesis sterilization relies upon intact surrounding host tissue, vigorous debridement surgery, and antibiotics with sufficient efficacy against surface-adhering, metabolically inactive microorganisms. Such antibiotics include rifampicin combined with quinolones, fusidinic acid or cotrimoxazole for staphylococci, and quinolones for Gram-negative rods (89–92). For microorganisms like enterococci, quinolone-resistant *P. aeruginosa*, or any type of multi-resistant bacteria, there are no potent oral antimicrobial agents. These cases require the removal of any foreign body material for a definitive cure (86).

The sterilization of infected central venous catheters with systemic antibiotic therapy failed in 33.5% of 514 published cases (85). One reason for treatment failure is insufficient local antibiotic concentrations to sterilize biofilms. This obstacle can be overcome for endoluminal catheter infections by periodically filling the catheter with pharmacological concentrations of antibiotics (i.e., 1–5 mg/mL). This "antibiotic lock" – with and without systemic antibiotic therapy – has been successful in 82.6% of 167 selected episodes (85).

9 Conclusion

In the industrialized world, acute bacterial infections caused by rapidly proliferating planktonic cells (e.g., *Salmonella typhi*) have been gradually replaced by chronic infections due to environmental organisms (e.g., *Staphylococcus epidermidis*) growing in biofilms. Biofilm eradication requires the elimination of all bacteria, otherwise infection recurs and its chronicity established. Current antimicrobial therapies are not aimed at growth-restricted bacteria protected by a biofilm mode of growth. To clear the residual fraction of dormant cells, we need antibiotics reaching far beyond the MBC definition of killing (≥3log) and the design of what we could call "antipathogenic" drugs. The latter may interfere with bacterial signaling or the expression of specific effector genes in order to convert resistant and virulent phenotypes into susceptible commensal organisms. Modulation of the host response is another strategy to promote biofilm clearance. Reviewing the redundancy of strategies providing tolerance within biofilm communities, the discovery of a single ON/OFF-switch for biofilm formation seems unlikely. Rather, biofilm eradication may depend on combined treatments.

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