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Survival strategies of infectious biofilms

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Modern medicine is facing the spread of biofilm-related infections. Bacterial biofilms are difficult to detect in routine diagnostics and are inherently tolerant to host defenses and antibiotic therapies. In addition, biofilms facilitate the spread of antibiotic resistance by promoting horizontal gene transfer. We review current concepts of biofilm tolerance with special emphasis on the role of the biofilm matrix and the physiology of biofilm-embedded cells. The heterogeneity in metabolic and reproductive activity within a biofilm correlates with a non-uniform susceptibility of enclosed bacteria. Recent studies have documented similar heterogeneity in planktonic cultures. Nutritional starvation and high cell density, two key characteristics of biofilm physiology, also mediate antimicrobial tolerance in stationary-phase planktonic cultures. Advances in characterizing the role of stress response genes, quorum sensing and phase variation in stationary-phase planktonic cultures have shed new light on tolerance mechanisms within biofilm communities.

Introduction

Since the discovery of penicillin in 1938, antibiotics have proven tremendously successful in controlling acute bacterial infections. Microbiologists have learned to predict antibiotic effects *in vivo* by evaluating the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) *in vitro*. MIC and MBC assess the effect of antibiotics against planktonic organisms in the exponential phase of growth and therefore correctly predict antibiotic efficacy against rapidly dividing bacteria in acute infections, such as septicemia. In this review we will concern ourselves with the growing number of chronic and device-related infections in which antibiograms of the causative organism show sensitivity to standard antibiotics in readily attainable concentrations, but the infection fails to be cleared. Microscopic evaluations of these refractory infections have revealed bacteria growing as surface-adherent biofilms. The key characteristic of these slime-embedded bacterial communities – and the topic of this review - is their tolerance to antimicrobials and host defenses. Biofilm tolerance is of major clinical importance because more than 60% of the bacterial infections currently treated by physicians in the developed world are considered to involve biofilm formation (Table 1) [1]. Successful treatment in these cases depends on long-term, high-dose antibiotic therapies and the removal of any foreign-body material.

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Bacterial sanctuaries

Biofilms are inherently protected from host defenses and antibiotics. Secreted antibodies fail to penetrate biofilms because of matrix binding [2], and secreted catalase protects aggregated bacteria by preventing full penetration of hydrogen peroxide into the biofilm [3]. Host-specific

Table 1. Partial list of human infections involving biofilms[a]

Infection or disease	Common bacterial species involved
Dental caries	Acidogenic Gram-positive cocci (*Streptococcus* sp.)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Non-typeable *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
Cerebral spinal fluid-shunts	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

[a]Adapted, with permission, from Ref. [1].

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Box 1. Th1 and Th2-weighted immune responses

The Th1/Th2 concept describes the differentiation of $CD4^+$ T-helper cells into either a Th1 or Th2 subset, each directing specific immune response pathways [59]. Th1 and Th2 cells express distinct cytokine profiles. Typically, Th1 cells produce interferon-γ, tumor necrosis factor α, and interleukin (IL)-2 and IL-12. Th2 cells typically produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Th1 cells mediate 'cellular immunity' to fight viruses and other intracellular pathogens, to eliminate cancer cells, and also to mediate delayed hypersensitivity reactions. Patients suffering from *Mycobacterium tuberculosis* infection expressed a lower Th1/Th2 activity ratio than negative controls [60]. Th2 cells encourage 'humoral immunity' directed against extracellular organisms and promote antibody production (especially IgE) and allergic responses. Interestingly, Th1 and Th2 cells can antagonize the actions of each other. The differentiation of $CD4^+$ T-helper cells depends on the involved antigen-presenting cell, the balance of cytokines evoked by the antigen, the host genetic background and cofactors, such as intracellular antioxidants (e.g. glutathione) or hormones (e.g. cortisol, dehydroepiandrosterone and progesterone) [59,61]. The selective induction of Th1 and Th2-weighted immune responses represents a promising strategy to influence disease outcome.

differences in the balance between T-helper 1 (Th1) and Th2-weighted immune responses (Box 1) might determine both the healing rates of biofilm infections and the extent of collateral damage to host tissues due to immune-complex depositions and the oxidative burst of macrophages. Preliminary data from patients with cystic fibrosis suggest that chronification of pneumonia is associated with a Th2-type immune response and that a shift towards Th1 might improve prognosis in these patients [4]. Even if the immune response leads to the influx of phagocytes, they are ineffective in ingesting sessile bacteria and biofilm fragments. For example, aspirated fragments containing as few as ten cells survived pulmonary host defenses in healthy animals [5].

The biofilm matrix plays a key role in the protection of biofilm bacteria from host defenses. Vuong *et al*. [6] reported increased phagocytosis and killing of bacteria containing knockout mutations for polysaccharide intercellular adhesin (PIA, the major matrix component in staphylococcal biofilms) by polymorphonuclear leukocytes. In addition, these mutants were more susceptible to killing by major antibacterial peptides of human skin [6]. The recent discovery of intracellular biofilms in *Escherichia coli* urinary tract infections [7] has added another dimension to the pathogenesis of chronic and recurrent infections.

The diffusion of antibiotics across biofilms has been assessed by elegant concentration measurements and the visualization of bactericidal effects on the opposite side of *in vitro* biofilms [8]. Although most studies have documented unimpaired antimicrobial penetration [8], three exceptions must be noted. First, betalactamase-producing organisms increase enzyme production in response to antibiotic exposure [9]. Betalactamase is believed to accumulate in the biofilm matrix as a result of secretion or cell lysis, deactivating betalactam antibiotics in the surface layers more rapidly than they diffuse into the biofilm [8]. Second, biofilm penetration of positively charged aminogylcosides is retarded by binding to negatively charged matrices, such as alginate in *Pseudomonas aeruginosa* biofilms [10]. This retardation might provide 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 [11,12]. In addition to restricted diffusion into biofilms, tolerance could be related to restricted penetration into individual bacteria. A recent study identified a mutant of *P. aeruginosa* that expressed classical biofilm architecture, but remained fully susceptible to the three antibiotic classes tested [13]. Because the mutant lacked periplasmic glucans, which were shown to bind tobramycin, tolerance in wild-type biofilms was attributed to the sequestration of antimicrobial agents in the periplasm. In broth culture, antibiotic accumulation within the bacterium is antagonized by efflux pumps, which provide resistance to several antibiotic classes including tetracyclines, macrolides, betalactams and fluoroquinolones [14]. Therefore, the upregulation of efflux pumps appeared to be an attractive hypothesis to explain the class-overlapping 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 action of the four multidrug efflux pumps decreased over time with maximal expression occurring at the biofilm–substratum interface [15]. Bacterial exposure to subinhibitory antibiotic concentrations induces mucoid phenotypes, which generate thicker biofilms with additional matrix components, such as colanic acid in the case of *E. coli* [16] or alginate in the case of *P. aeruginosa* [17]. Colanic acid supports biofilm maturation [16], whereas alginate protects bacteria from phagocytosis by host leukocytes and absorbs antibiotics, in particular aminoglycosides [17].

Physiology determines antibiotic susceptibility

Biofilm formation occurs as a result of a sequence of events: microbial surface attachment, cell proliferation, matrix production and detachment [18]. This process is partially controlled by quorum sensing, an interbacterial communication mechanism that is dependent on population density and is associated with radical (more than 50%) changes in protein expression patterns [18]. Mature biofilms demonstrate a complex 3-dimensional structure with numerous microenvironments differing with respect to osmolarity, nutritional supply and cell density. This heterogeneity produces a variety of phenotypes within individual biofilms – a single 'biofilm phenotype' does not exist (Figure 1).

Heterogeneity also characterizes the antibiotic susceptibility of biofilm-embedded bacteria. Two patterns of damage have been observed by direct microscopy. First, a top-to-bottom gradient of decreasing antibiotic susceptibility. This gradient originates in the surface layers of biofilms where there is almost complete consumption of oxygen and glucose, leading to anaerobic nutrition-depleted niches with restricted metabolic activity in their depths [8,10]. Metabolic inactivity might reach an extent where biofilm bacteria are still viable, but have lost culturability. Second, there is a patchy distribution of bacterial survival within presumably identical

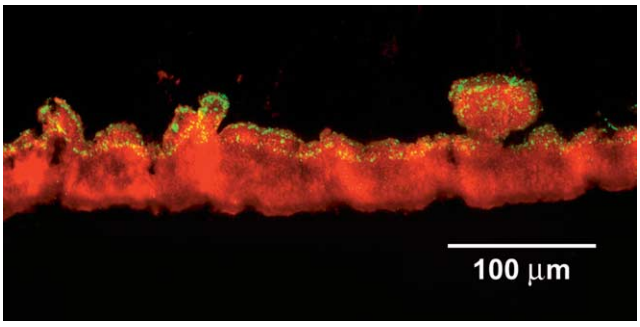

Figure 1. Biofilm-embedded bacteria show non-uniform distributions of physiological activity. In this *Pseudomonas aeruginosa* biofilm, green indicates cells capable of synthesizing a protein (alkaline phosphatase) in response to an environmental stimulus (phosphate starvation). Red indicates all cells independent of their activity. Image courtesy of Ruifang Xu.

microenvironments [19]. Both these phenomena have recently been described and analyzed in planktonic culture [20,21]. This knowledge might help to understand mechanisms of antibiotic tolerance within biofilms.

Learning from planktonic cultures

The physiology of bacteria in the depths of a biofilm shows striking similarities to stationary-phase planktonic cells. Both are affected by nutrient limitation and high cell densities and express similar degrees of antibiotic tolerance [22,23]. The impact of individual factors on antibiotic susceptibility can be illustrated with a set of experiments performed in planktonic culture (Box 2). The experiments showed that antibiotic tolerance is mediated by starvation, but also stressed the importance of high cell density that results in an accumulation of metabolic waste products and/or extracellular signaling molecules.

As originally described for luminescent organisms, population density is an important starvation-independent factor in bacterial physiology. For example, in rhizobium, more cells survived stationary phase if starved at high density [24]. In *E. coli*, high-density (6×10^8 bacteria/ml) cultures expressed stationary-phase characteristics with induced *RpoS*-dependent general stress-response genes and reduced outer membrane permeability while going through the exponential phase of growth [25]. Therefore, actively growing, high-density cultures are at least as differentiated from low-density cultures as exponential-phase bacteria are from those in stationary phase.

Similar to their biofilm counterparts, planktonic cells in stationary phase rapidly regain antibiotic susceptibility upon dilution in fresh medium [8], which not only provides nutrients but might also dilute protective cell-cell signaling. This reversibility proves that biofilm tolerance represents a phenotype rather than the product of genetic alterations.

Although bacterial starvation explains antimicrobial tolerance in the depths of a biofilm, surface layers should remain fully susceptible. Biofilms would then be cleared, layer by layer, with conventional antibiotics. By contrast, antibiotic therapy might only damage but not kill these bacteria. In this case, their continuous consumption of nutrients would shield underlying cells from nutrient exposure keeping them in a non-growing resistant state. This hypothesis is supported by the detection of persistent glucose and oxygen consumption and protein synthesis in biofilms suffering a 3-log bacterial reduction under treatment [26,27].

The survival of small pockets of bacteria on the surface of antibiotic-treated biofilms might be due to the patchy distribution of growth rates within any culture. Sufya *et al.* [21] demonstrated that growth rates within identical microenvironments are strikingly heterogeneous using elegant batch culture assays. For example, the mean doubling times of individual clones derived from late logarithmic culture varied between 500 and 45 minutes. For any time point between lag phase and stationary phase, a specific proportion of clones, the so-called persisters, exhibits growth rates below the threshold for antibiotic damage (Figure 2). This susceptibility threshold varies depending on the mode of action of the antibiotic used. The exponential increase over time of persister cells in planktonic cultures, which parallels the increase in starvation and cell density, might mirror the increase in the number of dormant cells as we progress from the biofilm surface into its depths. Stationary-phase physiology has resulted in 100% survivors for a betalactam and 0.1% to 1% for quinolones [20,22]. Remarkably, antibiotics with a good performance against stationary-phase bacteria *in vitro* have proven more successful in clearing biofilm infections *in vivo* [28].

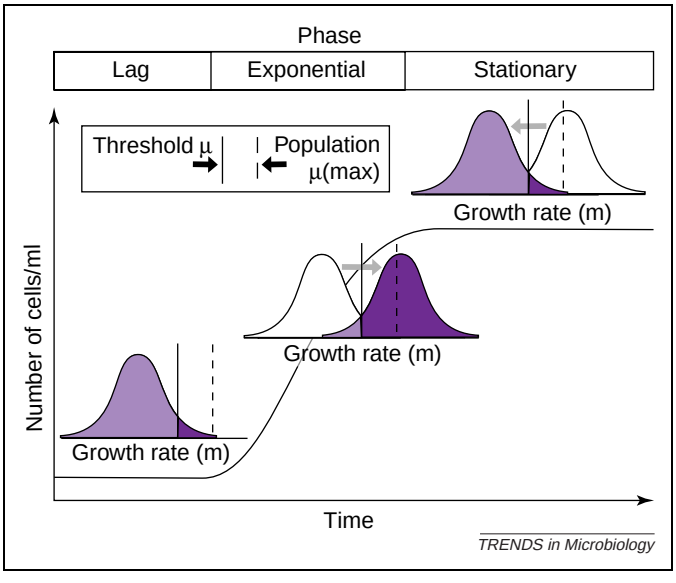

Figure 2. The antibiotic susceptibility of a bacterial population depends on the growth rate distribution of its individual clones. The traditional growth curve of a planktonic culture (solid line) illustrates the change in biomass over time. The slope (Δ Ln biomass / Δ time) represents the specific growth rate (μ). The maximum specific growth rate (μ_{max}) indicates the population average at the steepest part of the slope in exponential phase. If we assume that for every antibiotic a threshold μ (μ_{th}) is required for antibiotic susceptibility and that there is a normal distribution of μ within the population at any time point, some individuals will be growing faster than μ_{th} (dark purple areas under curve), and consequently be susceptible, and some slower (light purple areas under curve). Although in reality the distribution might not be bell-shaped, particularly in lag and stationary phase, the general hypothesis is still valid. As the whole curve shifts from a lower to higher μ when the population moves from lag to exponential phase, and then back to a lower μ in stationary phase, the proportion of the population lying on either side of the constant μ_{th} will vary. Importantly, neither proportion ever reaches zero.

Box 2. Illustration of the impact of growth conditions on antibiotic susceptibility

The impact of growth conditions on antibiotic susceptibility is illustrated in Figure I. In all cases, *Staphylococcus aureus* (ATCC 25923) from overnight cultures were grown to logarithmic phase in brain–heart infusion at 37 °C. Cells were pelleted and resuspended in the media indicated for the individual experiments. After exposing the samples to increasing oxacillin concentrations for 18 hours at 37 °C the bacteria were pelleted, washed, serially diluted and plated for quantification. Data points represent mean values and standard deviations of at least three repeats in a minimum of two individual experiments.

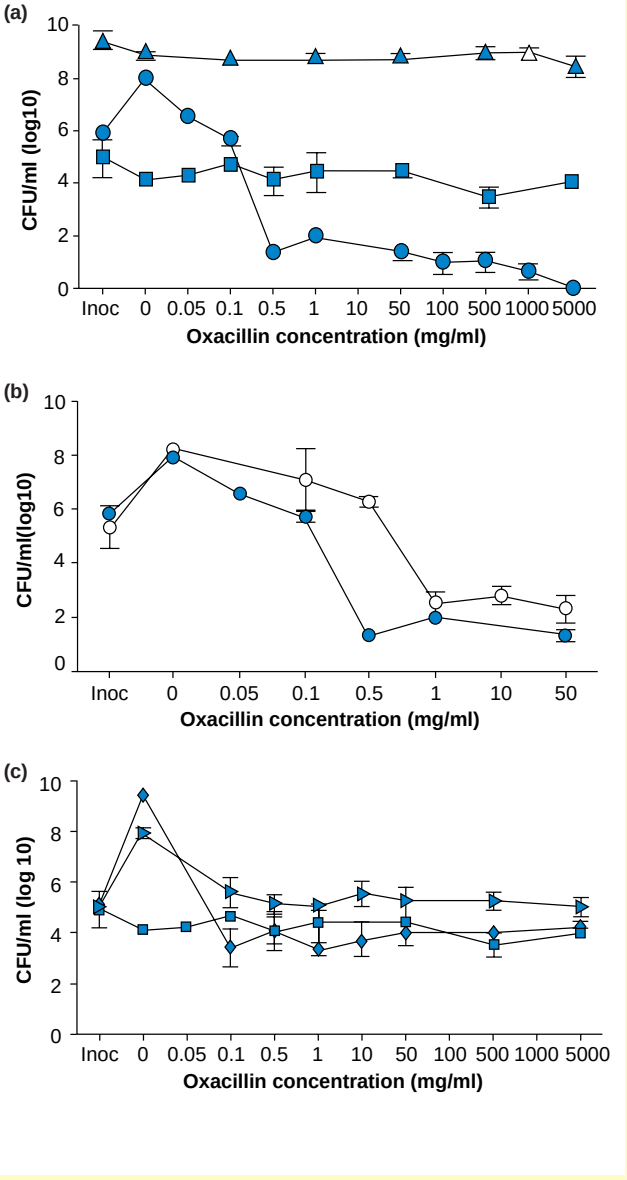

Figure I. The impact of growth conditions on antibiotic susceptibility. **(a)** Minimal bactericidal concentration (MBC) assays with an inoculum concentration of 5×10^5 *Staphylococcus aureus* per ml showed full oxacillin susceptibility (i.e. ≥3-log reduction) in fresh medium (circle), whereas no relevant killing was found when bacteria were starved in Ringer solution (square) or inoculated in fresh medium at a concentration of 10^9 bacteria per ml (triangle). The protective effect of high bacterial densities has been described previously [22] and is possibly due to intercellular signaling, the accumulation of metabolic waste products or a relative lack of antibiotic per bacterium. To evaluate the latter, the amount of free oxacillin was assessed in the supernatant of an antibiotic-treated 24-hour culture. **(b)** The supernatant of a 24-hour high-density culture with an initial oxacillin concentration of 1000 μg/ml [open triangle in graph (a)] was used as antibiotic source for an MBC assay. The oxacillin concentration in the spent medium was assumed to be unchanged from the nominal concentration added initially. The MBC (i.e. ≥3-log reduction) value increased from 0.5 μg/ml using a fresh stock of oxacillin (closed circle) to 1 μg/ml using serial dilutions from the supernatant (open circle). This suggests that the oxacillin concentration is not a limiting factor even in high bacterial densities. **(c)** Killing curves were performed using the supernatant of untreated 24-hour, high-density (inoculum concentration 10^9 bacteria/ml) cultures as medium. Pellets of 10^5 bacteria in exponential growth were resuspended in pure supernatant (arrow head) and supernatant that had been mixed 1:1 with double-strength fresh medium (diamond) to reconstitute the nutrients. Although the nutrients remaining in a 24-hour spent medium enabled a 3-log growth to occur in the untreated control (arrow head), the bacteria proved more tolerant to antibiotic stress than a culture starved in Ringer solution (square). This can be explained by the accumulation of metabolic waste products and/or intercellular signaling molecules. Nutritional reconstitution stimulated growth in the untreated control (diamond) to a higher extent, but failed to restore antibiotic susceptibility. Taken together, starvation is but one factor to explain tolerance.

Bacterial adaptations to stress and damage

In addition to the passive protection against antibiotics and host defenses provided by metabolic inactivity, bacteria actively adapt to stress. For example, biofilm organisms increase their capacity to withstand and neutralize monochloramine [29], stimulate catalase production [3], or induce the expression of chromosomal betalactamases [9] following prolonged treatment with the respective substances. Bacteria in biofilms and planktonic cultures can turn on stress-response genes and switch to more tolerant phenotypes upon environmental stresses, such as alterations in nutritional quality, cell density, temperature, pH or osmolarity [30]. Prolonged starvation induces loss of culturability under standard conditions, whereas the cells remain metabolically active and structurally intact [31]. This reversible 'viable but non-culturable state' is considered the main reason for the low detection rate of biofilm infections by routine culture. Adaptations for long-term survival presumably represent an active process because they are lost in knockout mutants for *rpoS* and ppGpp, two key components for the adaptation to stationary-phase conditions [32].

Stress-response genes can antagonize the deleterious effects of antibiotics, the host immune system and environmental toxins on bacteria [33]. Improved survival might be explained by an altered reaction to cell damage. For example, the SOS DNA-repair system, although not specifically reported in biofilms, is induced in ageing colonies on agar plates [34]. 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*, mutations of the *hipA* gene (homologues of which are present in a wide variety of Gram-negative organisms [35]) increased tolerance, probably by inducing (p)ppGpp synthesis, which potentiates the transition to a dormant state upon application of stress [35]. The *hipA7*

mutant contained 10 to 10 000 times more persisters during exponential growth than the wild-type [20]. Importantly, these mutants are tolerant but not resistant to antibiotics because their MIC remains unchanged [20]. A *P. aeruginosa* PPK mutant showed inhibited quorum sensing and failed to form thick differentiated biofilms [36]. 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* [37].

Sigma factors are key elements in general stress response. Bacteria lacking the sigma factor E had an increased susceptibility to oxidative stress during stationary phase [38]. *RpoS*, a sigma factor expressed in Gram-negative bacteria during stationary phase, has been detected in *P. aeruginosa* biofilms *in vitro* [39] and in the sputa of cystic fibrosis (CF) patients [40]. *RpoS* mutant *E. coli* were dramatically impaired in biofilm growth [41], whereas *rpoS* mutant *P. aeruginosa* grew thicker biofilms and showed higher antimicrobial tolerance [42,43]. Therefore, the role of *rpoS* in biofilm formation remains unclear, but could depend on strain-specific cofactors and specific growth conditions.

Phase variation, the random on-off switching of phenotype expression, has been discovered in a variety of bacterial species [44,45]. 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 CF [44]. 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 [44].

Genomics and proteomics

The observation of antimicrobial tolerance in biofilms that are too thin to represent a relevant diffusion barrier for metabolic substrates [46,47] proposes that starvation-induced dormancy is not the only reason for antimicrobial tolerance. This observation led to the hypothesis of a genetically controlled biofilm-specific phenotype. This concept is of particular interest because the control of key biofilm genes would offer excellent options to overcome tolerance.

A multitude of strategies have been applied to compare gene and protein expression patterns in biofilms with those in planktonic cultures. When assessed by DNA microarrays, gene expression in biofilms differed from that in planktonic cultures by 6% in *B. subtilis* (as assessed after 24 hours) and 1% in *P. aeruginosa* (assessed after five days of culture) [42,48]. Staphylococcal biofilm formation has been attributed to the *staphylococcal accessory regulator* (sarA) [49] and the *icaADBC* gene cluster [50]. However, Knoblach and coworkers [51] reported that virtually all *Staphylococcus aureus* strains contain the *ica* gene cluster, but do not necessarily produce biofilms. This observation underlines the importance of additional control mechanisms, such as subinhibitory antibiotic concentrations [52], phase variation [53], quorum sensing [54] or *icaR* [55], a transcriptional repressor of *ica* expression under environmental control.

The remainder of the differentially expressed genes and proteins in biofilms that have been identified to date are involved in (mainly anaerobic) metabolism, the regulation of membrane permeability, the production of extracellular polymeric slime, cell-cell signaling and motility [18,42,49,56–58]. However, none of these differentially expressed genes and proteins was irreplaceable in its function or was reproducibly found among various species and therefore do not promise diagnostic or therapeutic potential.

Box 3. Antibiotic tolerance favors resistance

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. Subinhibitory antibiotic concentrations induce the production of biofilm matrix and further promote biofilm survival [52]. Finally, the high cell density, increased genetic competence and accumulated mobile genetic elements within biofilms provide an ideal stage for efficient horizontal gene transfer [62].

From an epidemiological point of view, horizontal gene transfer is especially important within polymicrobial biofilms formed by the oral and intestinal flora [63,64]. In that environment, resistance genes can be transferred from apathogenic to highly virulent strains both within and beyond species borders [63,65]. Considering that, for example, only 5% of the oral flora is detected by routine culture techniques this gene pool available for horizontal transfer might still be profoundly underestimated.

Concluding remarks

In the industrialized world, acute bacterial infections caused by rapidly proliferating planktonic cells (e.g. *Salmonella typhi*) have been gradually replaced by chronic infections owing to environmental organisms (e.g. *Staphylococcus epidermidis*) growing as biofilms. The failure of conventional culture techniques to predict antibiotic susceptibilities of biofilm communities explains part of our failure to eradicate biofilm-related infections. Furthermore, the biofilm mode-of-growth represents a major risk factor for the spread of antibiotic resistance (Box 3).

To clear growth-restricted matrix-embedded bacteria antibiotics are needed that reach far beyond the MBC definition of killing ($\geq$3 log). Much more needs to be learned about the antimicrobial impact on bacteria and their response to damage. In particular, pathophysiological steps between damage and cell death need clarification. Modulation of the host response and the design of what we could call 'antipathogenic' drugs are other strategies to promote biofilm clearance. Antipathogenic drugs might interfere with bacterial signaling or the expression of specific effector genes and thereby convert resistant and virulent phenotypes into susceptible commensal organisms. Reviewing the redundancy of strategies providing tolerance within biofilm communities, the discovery of a single on-off switch for biofilm formation appears unlikely.

Biofilm eradication might ultimately depend on combined treatments.

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