Bacterial biofilms: a diagnostic and therapeutic challenge

Christoph A Fux†, Paul Stoodley, Luanne Hall-Stoodley and J William Costerton

Bacteria have traditionally been regarded as individual organisms growing in homogeneous planktonic populations. However, bacteria in natural environments usually form communities of surface-adherent organisms embedded in an extracellular matrix, called biofilms. Current antimicrobial strategies often fail to control bacteria in the biofilm mode of growth. Treatment failure is particularly frequent in association with intracorporeal or transcutaneous medical devices and compromised host immunity. The rising prevalence of these risk factors over the last decades has paralleled the increase in biofilm infections. This review discusses the shortcomings of current therapies against biofilms both in theory and with clinical examples. Biofilm characteristics are described with a focus on new diagnostic and therapeutic targets.

Expert Rev. Anti-infect. Ther. 1(4), 667–683 (2003)

CONTENTS

†Author for correspondence
Center for Biofilm Engineering
366 EPS Building, PO Box 173980, Montana State University-Bozeman
Bozeman, MT 59717, USA
Tel.: +1 406 994 4770
Fax: +1 406 994 6098

KEYWORDS:
antibiotic resistance, biofilm, human disease, gene expression, metabolic activity, protein expression, quorum sensing, shear forces

The power of biofilms

The biofilm concept

Traditionally, bacteria have been regarded as individual organisms growing in homogeneous planktonic populations. Current antimicrobial strategies have largely been developed in order to control acute infections caused by these planktonic bacteria. However, bacteria in natural environments usually form biofilm communities of sessile organisms embedded in a hydrated matrix of extracellular polymeric slime with polysaccharides, proteins and nucleic acids [1]. The formation of a 3D biofilm structure with functionally heterogeneous bacterial communities is a dynamic process. It involves a co-ordinated series of molecular events, which are partially controlled by quorum sensing, an interbacterial communication mechanism dependent on population density [2]. Embedded bacteria encounter a different microenvironment with higher-osmolarity conditions, nutrient limitation and higher cell density and behave differently with respect to growth rates and gene transcription than bacteria in the liquid phase [1,3].

Biofilms are inherently resistant to both antimicrobial agents and host defenses and therefore, are the root of many persistent bacterial infections. Antibiotic resistance has been attributed to the restricted penetration of antimicrobials and host defense cells into biofilms and the metabolic inactivity of starved bacteria in deep biofilm layers because of the limited diffusion of nutrients [1,4]. In addition, the expression of a resistant biofilm phenotype has been proposed [1,4]. Treatment failure of biofilm infections is particularly frequent in association with intracorporeal or transcutaneous medical devices and compromised host immunity. It has been estimated that as many as 60% of bacterial infections treated by physicians in the developed world are related to biofilm formation [1]. A partial list of biofilm diseases is presented in TABLE 1. Biofilms may grow over months or even years before causing symptoms. Diagnosis is often complicated by the reproductive inactivity of bacteria within biofilms delaying or inhibiting growth in diagnostic cultures [5,6]. As the eradication of biofilms depends on prolonged, high-dose antibiotic therapy and almost invariably requires the replacement of infected foreign body material, biofilm infections contribute significantly to hospitalization days and healthcare costs.

This review illustrates the diagnostic and therapeutic challenge of managing biofilm infections based upon two classical biofilm diseases, hip prosthesis infection and central venous catheter (CVC) infection. The clinical strategies against biofilms are described and the mechanisms of antimicrobial resistance within these bacterial communities are reviewed. The currently known biofilm regulation mechanism are then discussed with a focus on new diagnostic and therapeutic targets.

Clinical experiences

Hip prosthesis infections

Infections of orthopedic implants are rare but difficult to eradicate [7]. In cases of intraoperative contamination, the biofilm-mode of bacterial growth may delay overt symptoms for months or years. Diagnostic aspirations of the articulation are often falsely negative, possibly because the microorganisms persist only within a biofilm on the synovia but not in planktonic form. Consistent with this, the sonication of removed implants and PCR amplification techniques have shown increased sensitivity to detect bacteria sequestered in biofilms. Tunney and colleagues reported detection rates of 4% in cultured tissue, 22% in cultured tissue and fluid from sonicated implants and 72% in sonicated samples analyzed by PCR amplification [5]. Considering the limited sensitivity of conventional culture, many cases of so-called aseptic prosthesis loosening may actually be undetected biofilm infections.

Acute exacerbations respond well to antibiotic therapy but prosthesis 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% [7]. Therefore, this strategy should be reserved for patients with a stable implant and symptoms not lasting more than a few weeks. Successful prosthesis sterilization relies upon vigorous debridement surgery and antibiotics with sufficient efficacy against surface-adhering microorganisms. Such antibiotics include rifampicin combined with quinolones, fusidinic acid or cotrimoxazole for staphylococci and quinolones for Gram-negative rods [8–11]. For microorganisms such as enterococci, quinolone-resistant *P. aeruginosa*, or any type of multiresistant bacteria, there are no potent oral antimicrobial agents. These cases require the removal of any foreign body material for a definitive cure [7].

Central venous catheter infections

More than 200,000 nosocomial bloodstream infections occur each year in the USA; most of these infections are related to different types of intravascular devices, in particular CVCs [12]. Microbial colonization of CVCs over time is inescapable. The bacterial spread from the skin insertion site along the external surface of the device is progressively followed, after 1 week, by hub contaminants colonizing the inner surface [13]. Electron microscopy has documented early biofilm formation on CVCs [13]. Biofilms as shown in FIGURE 1 eventually cause systemic infections by detached cells and clumps of cells. The transition from catheter colonization to infection however is incompletely understood and difficult to document. The low positive predictive value of catheter-blood cultures for infection (63% in [14]) is partially overcome by (semi-)quantitative catheter culture techniques, which, however, require catheter removal.

Table 1. Partial list of human infections involving biofilms (adapted from [1]).

Infection or disease	Common bacterial species involved
Dental caries	Acidogenic Gram-positive cocci (*Streptococcus* spp.)
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
Suture	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

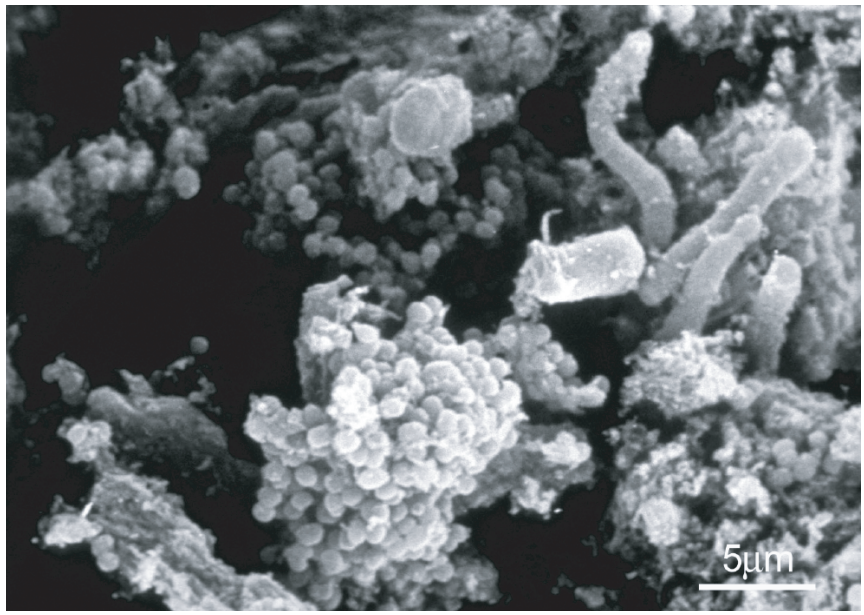

Figure 1. Biofilm on a hemodialysis catheter of a patient with symptomatic bloodstream infection. *S. epidermidis* and *C. albicans* were cultured from the catheter. The extracellular polymeric substance is reduced due to the dehydration process necessary for electron microscopy. Courtesy of Costerton WJ.

Emboli from *Staphylococcus aureus* biofilms frequently cause metastatic biofilm infections including endocarditis or osteomyelitis, which again require prolonged antibiotic therapy [12]. In contrast, catheter-related bloodstream infections caused by coagulase-negative staphylococci may resolve with the removal of the catheter and no antibiotic therapy. As a general rule, infected CVCs should be removed for a definite cure. The sterilization of an infected catheter with systemic antibiotic therapy failed in 33.5% of 514 published cases [12]. One reason for treatment failure is the inability of most antibiotics to sterilize biofilms with therapeutically achievable concentrations. This obstacle can be overcome in catheter infections originating from the hub by periodically filling the catheter-lumen with pharmacological concentrations of antibiotics (i.e., 1–5 mg/ml in 5–100 U of heparin). This 'antibiotic lock' technique – with and without systemic antibiotic therapy – has been successful in 82.6% of 167 selected episodes [12]. Awaiting controlled clinical studies, the use of 'antibiotic locks' is currently confined to uncomplicated infections of surgically implanted catheters involving coagulase-negative staphylococci [12].

Stages in biofilm formation & maturation

Based on microscopy, sequential steps of biofilm development have been characterized [15]: microbial attachment, the formation of microcolonies, biofilm maturation and detachment. Mature biofilms commonly demonstrate a complex architecture consisting of towers interspersed with water channels which facilitate nutrient supply [16]. The 3D biofilm architecture is the result of continuous growth and detachment events resulting in a structural heterogeneity as schematized in FIGURE 2.

Attachment results in a phenotypic change in the bacteria. In *P. aeruginosa*, the downregulation of flagella and the upregulation of pili mirror the surface-induced switch from flagella-based attachment to pili-associated motion known as swarming or twitching motility [17]. The morphological changes correlate with remarkable differences in protein expression. Bacteria grown in a biofilm differed from their planktonic counterparts by more than 50% of the expressed proteins [15]. Protein expression patterns between individual maturation stages changed by approximately 35%, or 500 proteins. When assessed by DNA microarrays, gene expression in biofilms differed from planktonic cultures by only 6% in *Bacillus subtilis* (as assessed after 24 h) and 1% in *P. aeruginosa* (assessed after 5 days of culture) [18,19]. The greater differences found in proteomics compared with genomics may be explained by differing test sensitivities and biofilm culturing techniques as well as the much longer half-life of proteins compared with mRNA. Where mRNA provides a 'snap shot' of expression the proteome provides a historical record over a longer time period.

Current strategies against biofilm infections

Prevention

Strategies to prevent biofilm formation range from systemic approaches controlling any bacterial invasion of sterile sites to local biofilm inhibition on medical devices. The latter focuses on the elimination of planktonic cells before they adhere to the surface and initiate biofilm formation. Both material properties and host factors determine bacterial adhesion to medical devices. Bacterial adherence to silicone, for example, has been found to be significantly higher than to polyurethane or Teflon® (DuPont, DE, USA) [20]. Host factors, such as fibronectin, fibrinogen or platelets may be deposited on the foreign body material and provide specific ligands for bacterial adhesins [21,22].

A variety of strategies have proven to be effective in reducing biofilm-related infections by preventing bacterial adhesion, at least in high-risk populations. They range from antiseptic irrigations of the operative site or the use of antibiotic-impregnated cement in orthopedic surgery [23] to the prophylactic use of 'antibiotic catheter locks' containing vancomycin and heparin [24] or minocycline and EDTA [25]. The impregnation of catheter surfaces with antiseptics [26] or

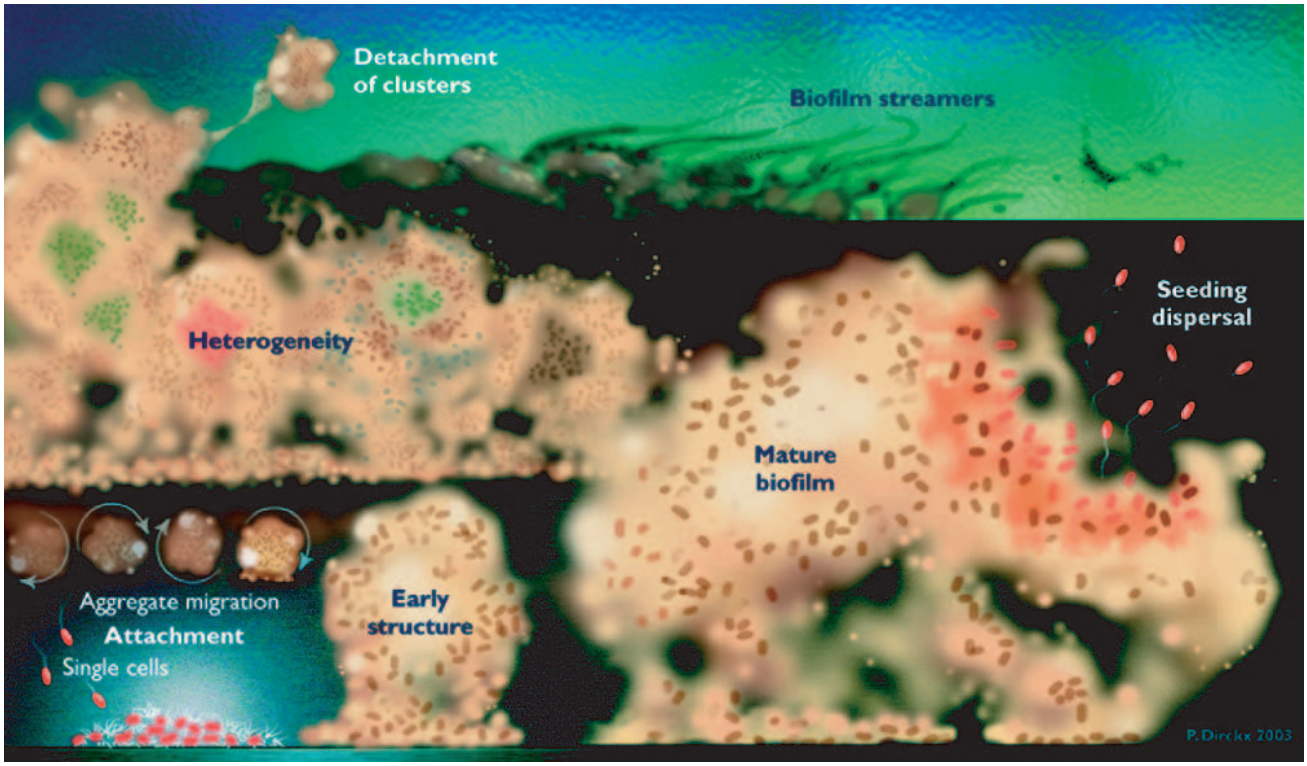

Figure 2. The structural heterogeneity of biofilms is the product of continuous growth and detachment. This cartoon illustrates the various mechanisms involved in this process. P. Dirckx, Center for Biofilm Engineering.

antibiotics [27] has been shown to delay bacterial colonization. However, these catheters have a short duration of antimicrobial efficacy and carry the risk of selecting for antibiotic-resistant bacterial strains. Degradation of the active antibiotic agent results in bacterial exposure to subinhibitory antimicrobial concentrations after a few weeks [28]. The value of urokinase-flushes for the prevention of catheter-related infections remains inconclusive with contradictory results published in the literature [29,30].

Diagnosis

Physicians have been trained to think of live bacteria as culturable and thus assume that the inability to grow cultures from a specimen is proof that viable pathogens are absent. Bacteria growing in biofilms may be in a dormant but viable state and therefore may initially fail to grow in culture. This dilemma is best illustrated by the controversy concerning the etiology of chronic otitis media. The 40 to 60% of culture-negative chronic otitis media with effusion have long been considered a sterile inflammatory process [6]. However, the detection of *H. influenzae* DNA and mRNA in 29 of 82 sterile effusions has demonstrated that traditional culturing methods may be inadequate to detect viable bacteria [6]. Electron and confocal scanning laser microscopy have provided visual evidence that biofilms form in this disease (FIGURE 3) [31]. Analogous findings suggest that chronic prostatitis also represents a biofilm infection [32].

Treatment

Bacterial biofilms are inherently resistant to antimicrobial agents and the host's immune system [1]. *In vitro*, the minimal bactericidal concentration (MBC) against adherent organisms can be three to four orders of magnitude higher than for planktonic bacteria [33]. Prolonged and high-dose antibiotic therapy as well as the elimination of infected foreign-body material are the cornerstones of a successful therapy. Antibiotic treatment of bacterial endocarditis was shown to be more successful when serum antibiotic levels were held at least tenfold above the MBC [34]; but even with 8 weeks of parenteral antibiotic treatment, few patients have been cured from prosthetic heart valve endocarditis by antimicrobial therapy alone [35]. The combination of rifampicin and a fluoroquinolone has proven especially successful in the treatment of various *S. aureus* biofilm infections, ranging from orthopedic prosthesis infections [8] to right-heart endocarditis [36]. Reports concerning the exposure of biofilms to an electrical field have shown promising results [37]. The reported 'bioelectric effect' may facilitate matrix penetration, disturb membrane integrity by cation depletion or generate oxidizing ions, such as peroxide.

Role of the host immune system

Host immunity plays a key role in biofilm clearance and a compromised immune system is a risk factor for many biofilm infections. However, the biofilm mode of growth includes a broad variety of bacterial defense strategies. Phagocytes have

reduced efficacy in ingesting sessile bacteria and biofilm clumps [38]. Biofilm fragments of eight to ten cells survived pulmonary host defenses even when deposited into the lungs of healthy animals [39]. Interestingly, leukocytes were able to penetrate *S. aureus* biofilms when grown under shear but not under static conditions [38]. The much larger amount of extracellular matrix in the latter may have hindered leukocyte penetration. Conflicting results concerning the penetration of immunoglobulin (Ig)G may be explained by variations in extracellular slime composition between different types of biofilm [40,41].

Biofilms stimulate the production of antibodies and cytokines [38]. Ensuing immune-complex depositions and the oxidative burst of macrophages, however, cause greater damage to the local host environment than to the biofilm itself [42]. In the latter, reactive oxygen intermediates are deactivated in the outer layers of the biofilm faster than they can diffuse into the biofilm [43].

Why antimicrobials fail

Resistance & tolerance

The minimal inhibitory concentration (MIC) and the MBC are standard values in antibiotic susceptibility testing and serve as important references in the treatment of acute infections. Specifically, they assess the effect of antibiotics against planktonic organisms in exponential growth. In biofilms, the MBC may be three to four logs higher compared with exponential planktonic cells [33,44]. Bacterial growth inhibition within a biofilm is poorly evaluated. Most studies have relied upon conventional MIC testing based on optical density, which is more reflective of the prevention of growth of planktonic bacteria shed from the biofilm, than of cell growth within the biofilm. Thus, it is not necessarily surprising that similar MIC values have been reported for biofilms and planktonic cultures [33]. Taken together, biofilms are highly tolerant to antibiotics in terms of killing but have not been shown to be much more resistant to growth inhibition than exponential planktonic cells. The standardization of a minimum biofilm-eradicating concentration (mBEC) has been postulated in the attempt to correlate *in vitro* measurements with therapeutic outcomes in biofilm treatment [45].

Interestingly, bacteria rapidly regain their antibiotic susceptibility after they have been mechanically dispersed from the biofilm architecture and transferred in fresh medium [46,47]. This underscores that antibiotic tolerance within biofilms is not acquired via mutations or mobile genetic elements but represents a functional characteristic of biofilm formation. Disruption of the biofilm may allow cells previously starving in deep layers new access to nutrients, which rapidly (i.e., within a few hours) brings them into exponential growth phase and subsequently renders them susceptible to antibiotics. Alternatively, disruption of the biofilm may result in the dilution of cell signals, which mediate metabolic inactivity and antibiotic resistance within a biofilm.

Antimicrobial susceptibility & growth phase

Virtually all antimicrobials are more effective at killing rapidly growing than stationary cells [48]. Some antibiotics, such as penicillin and ampicillin, have an absolute requirement for cell growth in order to kill [49]. Eng and colleagues were able to demonstrate, by controlling the growth rate of bacteria through nutrient limitation, that only fluoroquinolones produced bactericidal effects against stationary-phase Gram-negative organisms [48]. No class of antimicrobial agents was bactericidal in growth-limited *S. aureus*.

Antibiotic tolerance was found to be similar in biofilms and stationary-phase planktonic cultures of *P. aeruginosa* [50]. The comparable concentrations of catalase and a stationary phase sigma factor in both cultures suggest that at least some bacteria in biofilms exhibit stationary phase characteristics [47,51]. The antimicrobial tolerance of biofilms may thus be due to analogous metabolic and reproductive inactivity. The bacteria's 'dormant state' could be triggered by substrate depletion and the

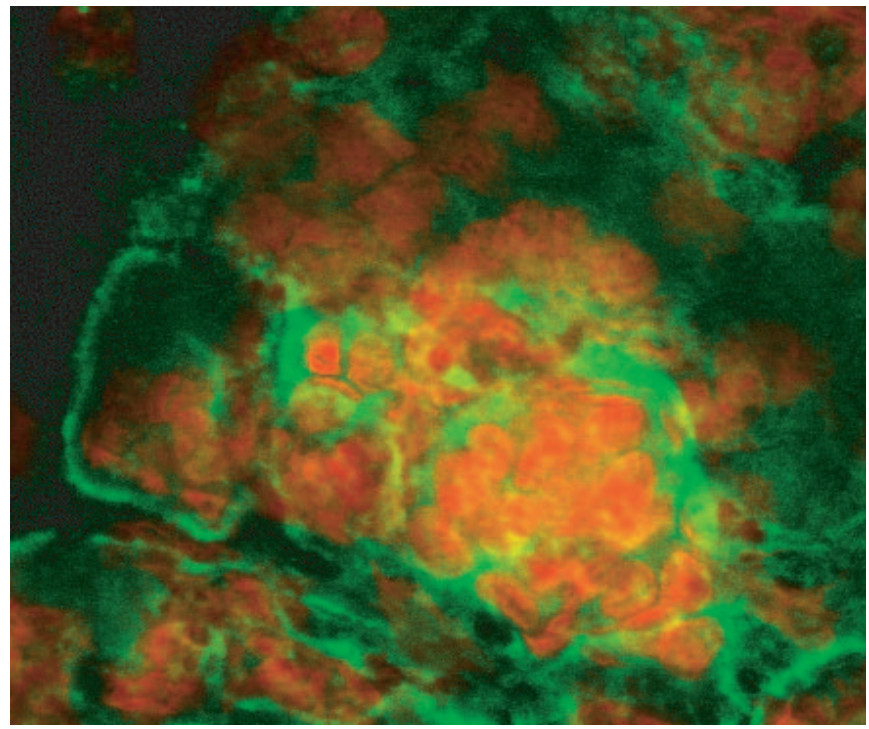

Figure 3. Biofilm in the middle ear of a chinchilla infected with *H. influenzae*. Confocal Laser Scanning Microscopy stained with LIVE/DEAD®BacLight™ nucleic acid stain (Molecular Probes, Eugene, Ore). SYTO 9 (green) shows live bacteria, propidium iodide (red) stains bacteria with compromised cell membranes and host cell nuclei. The latter measure approximately 5 mm in diameter. Courtesy of Rick Veeh, Center for Biofilm Engineering.

accumulation of inhibitory waste products. Alternatively, it may be the effect of an active, biofilm-specific regulation process. Another hypothesis suggested that biofilm tolerance is the result of restricted penetration of antimicrobials.

Hypothesis 1: antimicrobials fail to penetrate biofilms

The diffusion of antibiotics through biofilms has been assessed by concentration measurements and visualization of bactericidal effects in the depths of *in vitro* biofilms [44,47]. Most studies have documented unimpaired antimicrobial penetration [44,52]. Although antibiotic transport into biofilms is not the limiting step in general, three exceptions have been noted. In a β-lactamase-positive *Klebsiella pneumoniae* biofilm, β-lactam antibiotics were deactivated in the surface layers more rapidly than they diffused [47]. Second, the biofilm penetration of the positively charged aminogylcosides is retarded by binding to the negatively charged matrix polymers [53]. This retardation may allow more time for bacteria to implement adaptive stress responses. Aminoglycoside penetration into a *P. aeruginosa* biofilm was significantly hindered by binding to the extracellular alginate but markedly improved after the addition of alginate-lyase [54]. Third, extracellular slime derived from coagulase-negative staphylococci reduced the activity of glycopeptides even in planktonic bacterial cultures [55,56].

Hypothesis 2: starvation-induced stationary phase mediates tolerance

Nutritional starvation was documented in the depth of endocarditis vegetations using radiolabeled amino acids [57]. *In vitro,* biofilm-imaging with microsensors, fluorescent probes and reporter gene technologies allowed the comparison of the spatial distribution of nutrient supply with metabolic activity (FIGURE 4) [4,58]. Both oxygen and glucose are completely consumed in the surface layers of the biofilms, leading to anaerobic niches in the depths [47]. Areas of active protein synthesis, as for example demonstrated by the expression of inducible green fluorescent protein, were restricted to surface layers with sufficient oxygen and nutrient availability [53]. This biologically active zone could be expanded from 2 µm in biofilms grown in nitrogen to 46 µm when they were grown in oxygen [58].

While bacterial starvation through restricted diffusion of nutrients explains antimicrobial tolerance in the depth of a biofilm, surface layers should remain fully susceptible. In this case, biofilms would be cleared – layer by layer – with conventional antibiotics. However, antibiotic therapy may only damage but not kill these bacteria [4]. Their continuous consumption of nutrients would thereby shield underlying cells from nutrient exposure keeping them in a nongrowing, 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 [59,60].

Slowing growth rate and a hostile environment trigger the transformation of planktonic bacteria into a less susceptible phenotype [61,62]. New defense strategies are mediated by stress-response genes and phase-variation [63,64,119]. As both mechanisms are encountered in biofilms, they offer potential therapeutic targets. However, their relative contributions to biofilm tolerance as well as the role of persisters (see below) remain to be further characterized.

Stress response genes

Upon entry into stationary phase and under stimulation by environmental stresses (such as alterations in nutritional quality, temperature, pH or osmolarity), bacteria express stress-response genes including certain σ factors [63,65]. These genes protect bacteria from killing by antibiotics, the host immune system and environmental toxins [63]. Improved survival may be explained by an altered reaction to cell damage. Salmonella lacking alternative σ factors were found to have an increased susceptibility to oxidative stress during stationary phase [66]. The alternative transcription factor σ(B) was found to promote bacterial attachment and biofilm formation in *S. aureus* [67]. *RpoS*, another σ factor expressed in Gram-negative bacteria during stationary phase, has been detected in *P. aeruginosa* biofilms *in vitro* [51] as well as in sputa of cystic fibrosis (CF) patients [68]. Whereas *RpoS* mutant *Escherichia coli* were dramatically impaired in biofilm growth [69] *RpoS* mutant *P. aeruginosa* grew thicker biofilms providing higher antimicrobial tolerance [19,70]. Therefore, the role of *RpoS* for biofilm formation remains unclear, yet may depend on strain-specific cofactors and specific growth conditions.

Phase variation

While the transcription control of most bacterial genes permits a gradual response, phase variation exhibits an 'all-or-none' mechanism. The high-frequency ON–OFF switching of phenotype expression is apparently random but modulated by environmental conditions [64]. Phase variation has been discovered in a variety of bacterial species, including *P. aeruginosa* and *S. aureus* [64,71]. Phenotypic variation to small colony variants occurred in the former under the influence of antibiotics both *in vitro* and in the lungs of patients with CF. Interestingly, small colony variants exhibit increased antimicrobial resistance and biofilm formation. The specific gene product that modulates the phenotypic 'switch' from small colony variants to the susceptible phenotype in *P. aeruginosa* presents a promising therapeutic target. However, the impact of the biofilm mode of growth on phase variation has not been studied so far.

Persisters

Lewis postulated that antibiotic-tolerant biofilm cells are identical to the highly resistant cells in planktonic cultures that he had called persisters [72,73]. The 'persister concept' is based on the assumption that antibiotics do not kill cells but cause damage that triggers cell suicide. The development of antibiotic tolerance would result from the inhibition of programmed cell death in a subpopulation of the bacteria, while keeping them in a nongrowing state. Notably, the persister-state is fully reversible under growth stimulating conditions and does therefore not depend on genetic alterations. The regulatory mechanisms leading to persisters are as yet uncharacterized but might

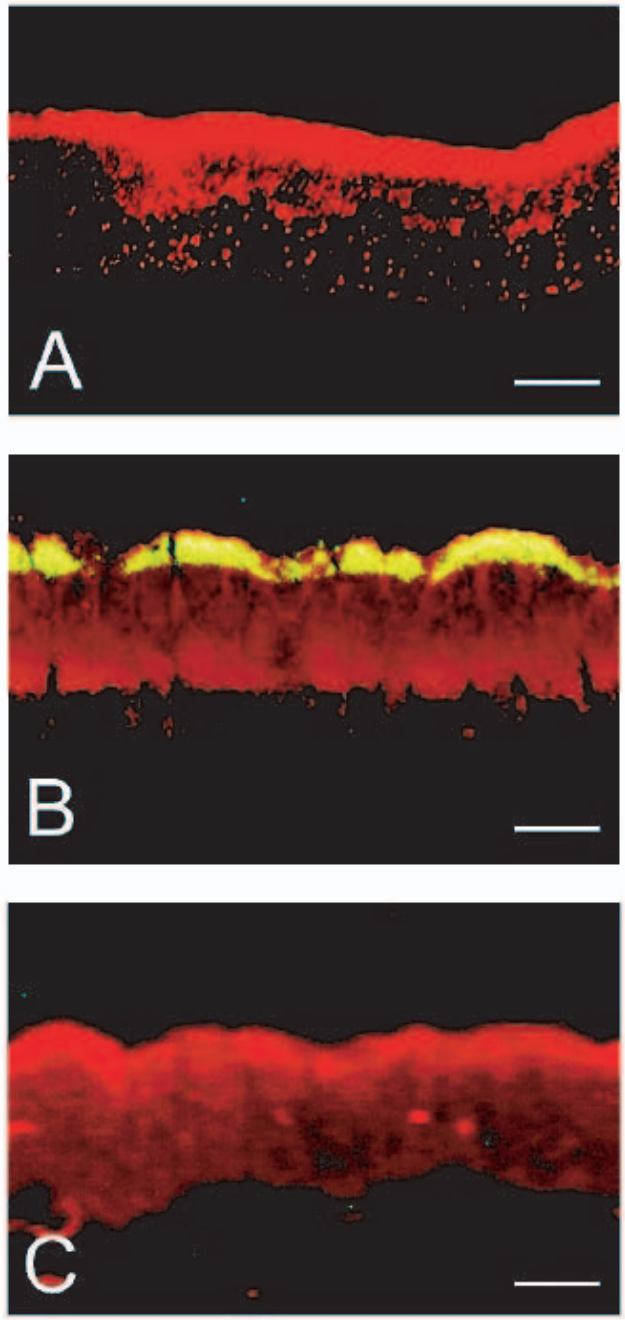

Figure 4. Visualization of the spatial heterogeneity in respiratory activity, protein synthesis and bacterial growth by epifluorescent microscopy. A *P. aeruginosa* biofilm was grown on a surface (bottom) covered by bulk fluid containing nutrients. **(A)** CTC-staining showing respiratory activity in red. **(B)** Fluorescent staining of alkaline phosphatase (green-yellow) indicating *de novo* protein synthesis under phosphate starvation; counterstaining of alkaline phosphatase-negative cells with propidium iodide (red). **(C)** Biofilm section hybridized with a eubacterial oligonucleotide probe. The slightly 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 [4] with permission of the publisher.

include stationary-phase triggered stress-response genes or phase-variation. Additional genes that promote survival under antimicrobial exposure may also be involved [73]. However, none of these genes has been evaluated in the context of persisters or biofilms to date.

Hypothesis 3: tolerance is an active adaptive process

This hypothesis postulates a genetically controlled, biofilm-specific phenotype. Altered gene expression in growing biofilms would thereby lead to the co-operative development of a specific architecture and the expression of increased antimicrobial tolerance. The hypothesis is supported by studies documenting antimicrobial tolerance in biofilms too thin to pose a barrier to the diffusion of metabolic substrates [74,75]. The concept of a biofilm-specific phenotype is of particular interest, since the expression control of its key genes or the inactivation of their products would offer excellent therapeutic options. Cell–cell signaling has been shown to mediate a biofilm-specific phenotype. The biofilm phenotype itself has been addressed by several studies analyzing protein and gene expression within biofilms.

Quorum sensing & biofilms

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 is widely used to co-ordinate gene expression within a species [76]. The bioluminescent marine bacterium *Vibrio harveyi* regulates its light production in response to cell density. Its transmitter, the autoinducer AI-2, has been found to allow interspecies communication between Gram-positive and -negative bacteria [103].

Given the tremendous metabolic and structural 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. In 1998, quorum sensing was found to modulate the transformation of *P. aeruginosa* from planktonic to a biofilm mode of growth [2]. However, quorum sensing is not indispensable for biofilm formation but one of several biofilm inducers. Mutants for the *las* quorum-sensing genes were unable to form mature biofilm under static conditions [2]. When grown under flow conditions, however, the mutants showed identical biofilm architecture and antimicrobial tolerance as the wild type [77]. Furthermore, quorum-sensing signaling may be overcome by environmental influences such as the nutritional status [78]. The stimulatory effect of quorum-sensing signals on early *P. aeruginosa* biofilm formation, for example, was abolished in a glucose-free medium [79].

Besides inducing biofilm formation, quorum-sensing signals converge with starvation-sensing pathways to regulate cell entry into stationary phase [62].

The prophylactic or therapeutic manipulation of quorum-sensing signals is promising, yet still far away from clinical practice. The only exceptions may be the RNA-III inhibiting protein, RIP, and synthetic derivates of natural furanone compounds, which are discussed in more detail below.

Gram-positive bacteria

Gram-positive bacteria regulate a variety of cellular processes via peptide-mediated quorum-sensing [76]. These systems have been well-characterized for several organisms but their involvement in biofilm formation is poorly defined. For example, protein expression in *S. aureus* is regulated in response to population density and growth state [65]. Proteins that promote adherence and colonization are expressed in early exponential phase. When cell growth reaches high densities, proteins involved in host damage, metabolism and dissemination predominate. Most of these staphylococcal products are under control of the accessory gene regulator *agr* and the staphylococcal accessory regulator *sar* [65,80,81]. *Agr* is activated during the transition from the exponential to the stationary growth phase. Its expression is negatively correlated with the ability to adhere to polystyrene and to form biofilms [82]. Consequently, *agr* is not essential for biofilm development [81].

In *S. mutans*, a quorum-sensing signaling system essential for genetic competence was found to function optimally in biofilms [83]. Transformation frequencies of biofilm-grown bacteria were ten- to 600-fold higher than those of planktonic cells. The inactivation of different genes within the system resulted in the formation of abnormal biofilms. The observed variability in architecture suggests that multiple signal transduction pathways are involved in biofilm control.

Therapeutic targets

FIGURE 5 schematizes some aspects of the complex regulatory networks within the quorum-sensing system of *S. aureus*, including the *agr*-operon, the *sar*- and the TRAP-system. The latter is of particular interest as it is the putative target of promising antibiofilm agent RIP, currently the only prophylactic agent in Gram-positive quorum-sensing signaling [84–86]. The RIP is a competitive inhibitor of the RNA-III-activating peptide RAP. RIP significantly reduced staphylococcal adherence and biofilm formation on epithelial cells as well as dialysis catheters *in vitro* and in a rat model [84,85]. In addition, RIP increased the efficacy of antibiotics in preventing biofilms [86]. Since RIP significantly reduced the attachment of highly adherent *agr*-mutants, the antiadhesive and antibiofilm properties of RIP seem to relate more to the direct effects of its phosphorylated and unphosphorylated target protein TRAP ('target of RAP') [84].

Gram-negative bacteria

Many Gram-negative bacteria utilize *N*-acyl homoserine lactone (AHL)-dependent quorum-sensing systems, which are involved in virulence gene expression and biofilm formation [2]. *In vivo*, AHLs have been detected in the urine of patients with a catheter infection [87] and in the lungs of patients with CF, thereby coinciding with the development of respiratory biofilms [88]. The so-called autoinducer (AI)-1 signaling system in Gram-negative bacteria consists of an AHL- synthetase belonging to the LuxI protein family and a transcriptional regulator, a LuxR protein [76]. *P. aeruginosa* possesses two AHL-dependent quorum-sensing systems, *las* and *rhl*. While *las* mediates biofilm architecture and the production of the extracellular polymeric slime, *rhl* is, together with the outer membrane protein OprF, required for optimal anaerobic biofilm viability [2,89,90]. Both systems, however, show a significant overlap in their functions. The lack of *rhl* leads to an accumulation of toxic nitric oxide; OprF, which was found to be upregulated 40-fold under anaerobic conditions *in vitro* and which can be detected in CF mucus, supports nitrite reductase [89]. In addition, *P. aeruginosa* produce the pseudomonas quinolone signal (PQS) molecule, which structurally resembles the quinolone antibiotics and modulates AHL-mediated quorum sensing [91].

Besides their impact on biofilm architecture, quorum-sensing signals induce protective stationary phase genes and stimulate inflammation in the host. The correlation between quorum sensing and stationary phase has recently been reviewed by Lazazzera [62]. In many Gram-negative bacteria, σS is the key transcription factor required for the expression of stationary

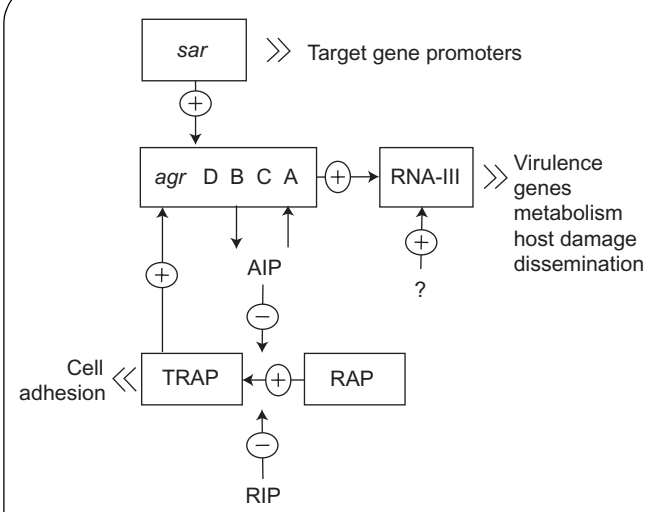

Figure 5. Quorum sensing in *S. aureus*: the complex interaction of the agr, sar and TRAP system. The auto-inducing peptide AIP is generated from its precursor AgrD and secreted through the action of the AgrB membrane protein [65]. The peptide interacts with the agrAC two-component system. Signal transduction ultimately results in the production of the effector molecule RNA-III, which modulates virulence factors both at their transcriptional and posttranscriptional level [65]. In an experimental endocarditis model, RNA-III activation was time and cell- density dependent and occurred in vegetations with agr mutants, thus arguing for additional agr-independent induction [151]. RNA-III downregulated *S. aureus* adherence to fibrinogen under static conditions and upregulated adherence to fibronectin and epithelial cells under both static and flow conditions [22]. The sar-encoded protein binds to the agr promoter to stimulate RNA-III transcription but also interacts directly with target gene promoters to control gene expression [81]. Both the activation and inactivation of sarA have been found to inhibit bacterial adherence. Mutation in sarA resulted in a reduced capacity to form biofilm [152].
The RNA-III-activating peptide RAP belongs to a third regulatory system. The phosphorylation of its target protein TRAP ('target of RAP') stimulates agr-transcription but also leads to increased cell adhesion and interacts with biofilm formation. AIP inhibits the phosphorylation of TRAP [85].

phase genes. The exposure of *P. aeruginosa* to AHL increases the levels of σS [92]. In a rhizobium subspecies, a greater percentage of cells survived in stationary phase if cells were starved at high density and the decreased survival of cells at low density was rescued by the addition of an AHL [93]. Purified AHL stimulated the production of interleukin (IL)-8 in human lung bronchial epithelial cells as well as the migration of monocytes, neutrophils and T-cells [94]. It further upregulated the expression of cyclooxygenase-2 inducing endothelial permeability [94]. As quorum sensing induces catalase and superoxide dismutase genes [43], *P. aeruginosa* neutralize the deleterious effects of attracted host defense cells within the biofilm, while the surrounding host tissues are severely compromised.

Two recent studies used microarray analysis to identify quorum-sensing controlled genes in *P. aeruginosa* [95,96]. The quorum-sensing regulated genes represented 6% [95] and over 10% [96] of the genome. The two studies showed a more than 50% agreement regarding AHL-induced genes but less than 5% concordance regarding the genes listed as being AHL-repressible [97]. This disagreement may mirror different experimental conditions, as suggested by the one study demonstrating the impact of medium composition and oxygen availability on gene expression [96]. Interestingly, Schuster and colleagues found the timing of gene expression to be similar in the wild type and in the quorum-sensing signal mutant grown in the presence of saturating levels of added signals [95]. This observation suggests that the trigger for quorum-controlled gene activation is independent of signal accumulation. The authors hypothesized that the receptor levels of *lasR* and *rhlR* may govern the onset of induction.

Therapeutic targets

Strategies to therapeutically influence quorum sensing were extensively reviewed by Camara and coworkers [76]. They include the depletion of signal molecules (by inhibition of synthesis or destruction) or the inhibition of their signal transmission. AHL synthesis may be blocked by targeting the fatty acid metabolism which supplies the acyl-chains, by blocking the binding sites of the involved catalytic protein LuxI, or by interfering with the enzymatic process itself. The inactivation of AHL by opening of the lactone ring is a pH-dependent, reversible reaction. AHL degrading enzymes have recently been isolated from *Bacillus* species [98]. Such enzymes may be used topically, yet are unlikely to be useful for systemic administration.

AHL-signaling can also be antagonized by blocking their binding with LuxR transcriptional regulator proteins, thereby switching off virulence gene expression. Several studies have shown the ability of various analogs to inhibit the action of the corresponding AHL [76]. The seaweed *Delisea pulchra* utilizes halogenated furanones to discourage bacterial colonization by blocking bacterial cell–cell communication [99]. Halogenated furanones, which are structurally related to AHLs, block specific Gram-negative signaling but also inhibit autoinducer-2, a signaling system found in Gram-negative and -positive bacteria [76]. Furanones affected the architecture and enhanced detachment of a *P. aeruginosa* biofilm [100], but also inhibited growth, motility and biofilm formation of *B. subtilis* [101]. In *P. aeruginosa*, 1.7% of the genes were significantly affected by furanones as quantified by microarray technology [102]. Furanone-repressed genes were not restricted to the *las* and *rhl* systems, which both seem to be inhibited by furanones at the post-transcriptional level.

A universal bacterial language?

With the autoinducer, AI-2, an interspecies quorum-sensing system was discovered [103]. The underlying gene, LuxS, is widely conserved among both Gram-negative and -positive bacteria [76]. AI-2 was found to control mixed-species biofilm formation in dental plaques, a complex biofilm community comprising more than 500 different bacterial species [104]. Plaque formation follows a relatively well-defined bacterial succession of commensals, such as *Streptococcus gordonii* and pathogens, such as *Porphyromonas gingivalis*. In the absence of AI-2, *S. gordonii* were unaffected in their biofilm formation but unable to construct a mixed-species biofilm with *P. gingivalis*. The role of luxS for *S. mutans* biofilms is controversial, conflicting results most likely being due to differing culture techniques [105,106].

Remarkably, the true role of AI-2 in quorum-sensing signaling has recently been questioned, suggesting that in most bacteria AI-2 is simply a metabolic side product [107].

Biofilm-specific gene expression

Gene expression patterns in biofilms have been analyzed in the search for key proteins that offer new diagnostic and therapeutic approaches. The early detection of biofilm-specific antigens or antibodies might result in greater treatment success, since younger biofilms are more susceptible to antimicrobial agents. For example, Anwar and coworkers demonstrated increased tolerance to antimicrobial therapy of a 13-day old compared with a 4-day old biofilm [108]. Biofilm-specific epitopes could further be used for vaccinations. Finally, targeting biofilm-specific signaling proteins, transcription factors or key enzymes could block bacterial adherence, biofilm formation or promote detachment.

A recent DNA microarray study of *B. subtilis* identified several transcription factors involved in the transition from a planktonic state to a biofilm [18]. Most of these transcription factors were maximally active after 8 hours of culture, when only 7% of the bacteria grew as a biofilm. Their increased activity under anaerobiosis (ResE), starvation and high cell density (SPo0A, σH) 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, a phenomenon known as catabolite repression [18].

Staphylococcal biofilm formation is mediated by the polysaccharide intercellular adhesin PIA, a product of the *ica*ADBC gene cluster [109,110]. Ziebuhr and colleagues 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 [111]. This power to discriminate between invasive and noninvasive coagulase-negative staphylococci, however, could not be confirmed [112]. Further observations suggested that many strains carry the *ica*-locus but do not form biofilms, thus stressing the importance of gene expression control. Virtually all *S. aureus* strains contain the *ica* gene cluster but do not necessarily express the operon and produce biofilms [113]. In as much as 44% of the strains, biofilm formation was only seen in certain media or after the addition of specific sugars. As UDP-N-acetylglucosamine is the limiting substrate for both PIA production and the formation of cell wall components, the shortage of this nucleotide sugar may have inhibited PIA production despite excessive *ica*-mRNA [114]. This again illustrates the strong link between biofilm formation and nutritional conditions. In addition, PIA synthesis is altered by subinhibitory antibiotic concentrations [115], phase variation [116], quorum-sensing systems [81] or icaR [117], a transcriptional repressor of ica expression under environmental control [114].

Despite the apparent relevance of the *ica* gene cluster and PIA for biofilm formation no diagnostic or therapeutic targets have been found to date, the search being complicated by the vast number of covariables. A recent study even reported equal efficiency of *ica*-negative and *ica*-positive staphylococci in causing foreign body infections in an animal model [118]. Thus, *ica* appears to be relevant but not indispensable for biofilm formation.

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 [15,19,119–121]. Finelli and colleagues described five 'indispensable' genes for *P. aeruginosa* biofilm formation [120]. 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. The most highly activated genes in *P. aeruginosa* biofilms as detected by DNA microarray were bacteriophages, which may be of importance for horizontal gene transfer in biofilms [19] as well as biofilm dispersal. Genes involved in attachment and motility were downregulated [19]. In *S. aureus* biofilms, five genes were identified as being upregulated compared with planktonic cultures, encoding enzymes needed for glycolysis, fermentation, amino acid metabolism as well as a general stress protein [119]. 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.

The biofilm matrix

Although a biofilm consists of up to 80% extracellular polymeric slime, this compartment is still poorly characterized. Biofilm matrix contains complex bacterial polysaccharides and minor fractions of bacterial debris, secreted proteins and nucleic acids, as well as various host products [122]. In endocarditis vegetations, bacteria become buried within a platelet-fibrin matrix, which makes up to 80% of the vegetation's volume [123]. The amount of produced slime may depend on quorum-sensing signals. In a 3-day old *P. aeruginosa* biofilm, polysaccharide production of the *las*-mutant was only 36% of the *rhl*-mutant and the wild type [89]. With reduced matrix protection, bacteria appeared to be more prone to detachment from the top of the biofilm.

The emergence of mucoid *P. aeruginosa* by mutation from nonmucoid isolates heralds chronic pulmonary infection in patients with CF. Mucoid strains produce the exopolysaccharide alginate, whereas nonmucoid strains use another, as yet unidentified exopolysaccharide for biofilm synthesis [2,124]. Alginate production is upregulated in response to environmental factors, such as high osmolarity, low oxygen tension, ethanol exposure or nitrogen limitation [125]. It has been shown to provide increased resistance to opsonization and phagocytic engulfment, as well as protection from toxic oxygen radicals

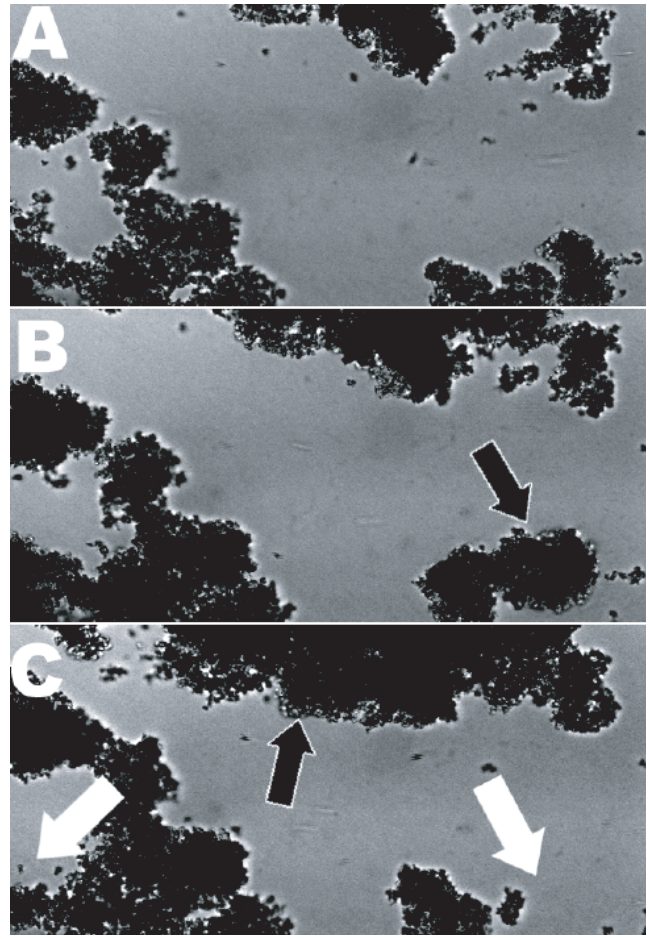

Figure 6. Growth (black arrows) and detachment (white arrows) dynamics of a 7 day old *S. aureus* biofilm in a glass flow-cell (BioSurface Technologies) visualized by time-lapse microscopy. Individual frames were taken at 1 h intervals. Courtesy of Wilson S, Center for Biofilm Engineering.

[126]. On the other hand, the mutation to a mucoid strain is associated with the loss of motility. These alterations are so fundamental, that other biofilm characteristics attributed to the nonmucoid laboratory strain PA01 may not be applicable to mucoid FRD1-strains.

Therapeutic targets

Macrolide antibiotics have demonstrated favorable therapeutic effects on chronic CF lung disease caused by *P. aeruginosa*, even though they do not exhibit intrinsic antipseudomonal activity. The inhibition of bacterial protein synthesis by macrolides has been attributed to decreased sputum viscosity, impaired cell–cell signaling or reduced expression of pro-inflammatory cytokines [127,128]. Similarly, clarithromycin (Biaxin®, Abbott Lab. Inc., IL, USA) causes reduced slime production in *S. aureus* biofilms, enabling imipenem and polymorphonuclear leucocytes to clear the biofilm [129,130].

In vitro, several drugs have been found to restore the antibiotic susceptibility of biofilms by interacting with extracellular polymeric slime. A treatment with bismuth dimercaprol initially failed to kill bacteria but reduced slime production in a *P. aeruginosa* biofilm [131]. With the reduction of biofilm matrix over time, bacteria became susceptible to the agent. The degradation of extracellular DNA inhibited the formation of a *P. aeruginosa* biofilm in another study [132].

In a rabbit endocarditis model, anticoagulant treatment increased antibiotic efficacy, however, at the price of higher fever, more constant bacteremia and increased mortality [133]. Platelet aggregation inhibitors significantly altered the course of endocarditis in another study [134]. The clinical role of anticoagulants in the treatment of endovascular biofilm infections remains to be elucidated.

Biofilms under flow conditions

Effect of flow on biofilm growth & detachment

There is increasing evidence that flow conditions, as observed in almost any physiological environment, crucially influence biofilm formation. Their importance is highlighted by the potential to re-establish the classical biofilm architecture in quorum-sensing knockout strains, which do not form biofilms in static culture [77]. Flagella and pili were indispensable for biofilm formation of *P. aeruginosa* under static conditions, whereas their deletion did not significantly affect biofilm formation in a flow system [79]. Certain bacterial adhesion factors have been shown to promote efficient adhesion to host cells only when subjected to shear stress, as provided by blood flow along an endothelium, mucus flow along intestinal mucosa or urine flow along urinary tract epithelium [135]. Biofilms grown under higher shear showed stronger attachment and cohesiveness than those grown under lower shear [136]. Shear stress was also able to reverse certain stress-resistance defects of an *E.coli RpoS* mutant [137].

The dispersal of biofilm cell-clusters subject to shear forces is of fundamental importance for both colonization and infection in clinical and public health settings. Examples range from catheter-related bloodstream infections to drinking water systems. The dissemination of biofilms formed inside endotracheal tubes may account for the high rate of ventilatory-associated pneumonias [138].

Digital time-lapse microscopy allows the continuous observation of biofilm growth and detachment in a glass flow cell (FIGURE 6) [139]. Various dispersal mechanisms can thereby be visualized: Spreading through continuous growth, the shear-mediated movement of biofilm ripple structures along a surface (as found in endotracheal tubes [140]), detachment and reattachment of single cells and cell clusters or the rolling

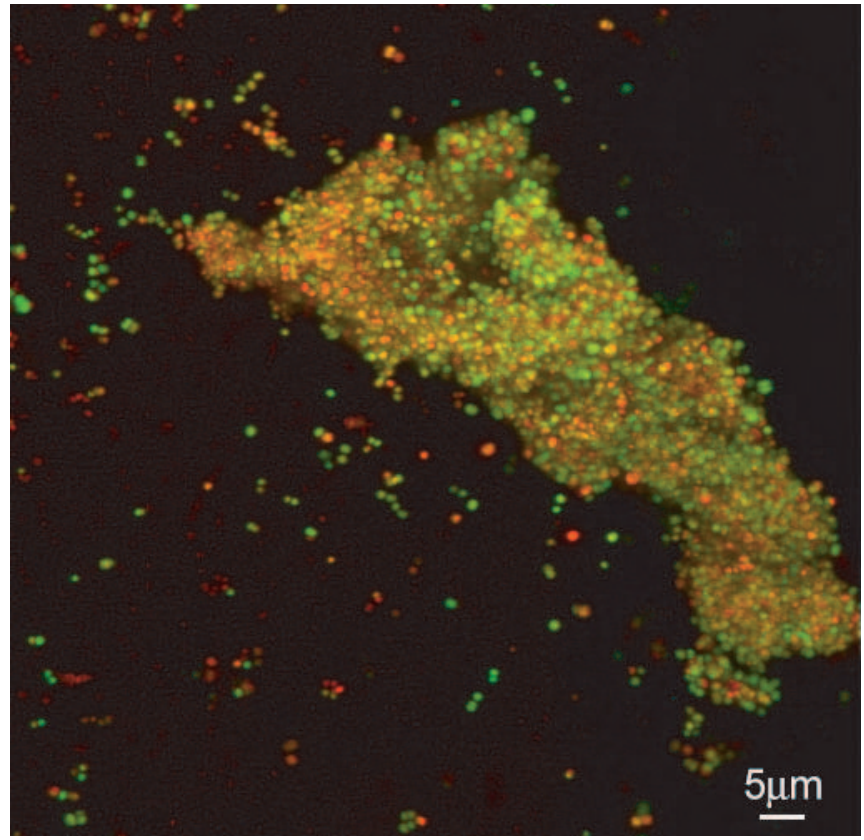

Figure 7. *S. aureus* **biofilm embolus by Confocal Laser Scanning Microscopy.** The LIVE/DEAD®BacLight™ nucleic acid stain discriminates viable (green) from dead cells (red). The embolus was captured in the effluent of a biofilm flow-cell. Courtesy of Wilson S, Center for Biofilm Engineering.

motion of microcolonies along a surface [201]. The size distribution of detached particles, ranging from single cells to emboli of more than 1000 cells, has shown species-specific patterns [STOODLEY P, UNPUBLISHED DATA]. For example, *S. aureus* mainly detach clumps, whereas nonmucoid *P. aeruginosa* primarily shed single cells. Large clumps from *S. aureus* biofilms (FIGURE 7) have demonstrated reduced susceptibility to antibiotics [STOODLEY P, UNPUBLISHED DATA]. Such inter-species differences may explain varying rates of symptomatic embolization from infected CVCs or endocarditis vegetations.

Control mechanisms

The mechanisms by which shear stress improves mechanical biofilm stability remain unclear. Possible explanations included the induction of a specific bacterial phenotype [141], increased nutritional transport [142], a more compact arrangement of biofilm matrix polysaccharide strands [143] or the passive selection of shear-tolerant subpopulations.

Detachment occurs both passively due to hydrodynamic forces [144] and as an active process in response to population density [15], changes in substrate concentration [145] or exposure to antimicrobials [146]. Active detachment strategies include the dissolution of extracellular polymeric slime through secreted enzymes or the downregulation of surface-associated binding sites [147,148]. Oxygen depletion was found to stimulate a specific exopolysaccharide lyase, which digested the matrix of a *Pseudomonas fluorescens* biofilm and liberated cells [149]. Swarming dispersal, the release of individual bacteria from a liquefied biofilm microcolony, is best described for nonmucoid, motile *P. aeruginosa* [15]. The liquification has recently been attributed to prophage-mediated cell lysis [150]. Prophage-mediated bacterial death could be an important mechanism of differentiation inside microcolonies that facilitates dispersal of a surviving subpopulation. In this respect it is of particular interest that high expression levels of prophage genes have been documented both in Gram-negative [19] and -positive biofilm cells [18].

Therapeutic targets

Comparative studies of protein expression patterns between biofilms and their detached particles are under way in search of key proteins regulating detachment. As RIP interacts with microbial attachment factors in staphylococcal biofilms, it may be effective in promoting detachment [85]. Strategies involving the dissolution of extracellular polymeric slime have been discussed in the previous chapter.

Key issues: the redundancy of biofilm regulation systems

Molecular techniques have identified a multitude of genes that appeared to be essential for biofilm formation [2,18,19,110,119–121]. Mostly, however, the knockout of one pathway at best alters biofilm expression and mature biofilm formation can be restored by varying the growth conditions. In fact, research has made clear that biofilms make use of an entire repertoire of regulatory and protective systems that allow them to adapt to a variety of environmental conditions. Reviewing this redundancy of antimicrobial strategies and their complex interactions the discovery of a single ON–OFF-switch for biofilm formation seems unlikely.

Expert opinion

The key role of environmental factors, in particular the accessibility of nutrients and the exposure to flow, is only now beginning to emerge. Many contradictory studies in the field of biofilm regulation can be explained by differing growth conditions. Specific factors for biofilm formation may be necessary in one but without any effect in another environment [77,114]. In this respect, the need for a standardized methodology is critical.

Antimicrobial tolerance in biofilms is increasingly related to the stationary growth phase and has been attributed to metabolic inactivity and active death-preventing strategies [4,47,62]. However, much more needs to be learned about the antimicrobial impact on stationary-phase bacteria and their response to damage. In particular, pathophysiological steps between damage and cell death need clarification. Strategies to overcome the stationary phase in biofilm bacteria by interfering with cell–cell signaling, reducing diffusion barriers and attacking biofilm cells with cyclic therapies and pulsatile nutrient stimulation all show promise at this time.

Five-year view

We expect the biofilm concept to spread within the medical world similar to the development observed in oral healthcare. The biofilm concept marks a milestone in the development of dentistry. Not only have the new insights in the pathogenesis of oral plaque and periodontitis found broad acceptance in the academic world but there is growing awareness about their relevance among healthcare companies, clinical dentists and patients. New strategies in disease prevention and therapy have evolved. This knowledge transfer, however, could only be achieved by co-ordinated educational efforts.

The implementation of the biofilm concept into the medical arena stands at its early stages. Within the next few years, much research will have to be repeated for bacteria in the biofilm mode of growth. The growing awareness of the chronicity of biofilm infections by both patients and clinicians will influence the healthcare market.

Due to the redundancy of biofilm regulation systems, more and more combined therapeutic strategies will evolve, however remaining based on conventional antibiotic regimes. The noninvasive detection of biofilm-specific gene expression may improve the outcome of biofilm infections by earlier diagnosis.

Acknowledgements

This work was supported by the Swiss National Science Foundation grant 81BE-69256 (C.F.) and the National Institutes of Health RO1 grant GM60052-02 (P.S and W.C.). We thank Dirckx P and Meyer J for their graphic assistance.

References

Papers of special note have been highlighted as:

- • of interest
- •• of considerable interest

1 Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 284, 1318–1322 (1999).

•• **This concise review is considered the benchmark description of the biofilm concept.**

2 Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280, 295–298 (1998).

•• **The discovery of cell–cell signaling mediating biofilm architecture and resistance constitutes a landmark in biofilm research.**

3 Prigent-Combaret C, Vidal O, Dorel C, Lejeune P. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. *J. Bacteriol.* 181, 5993–6002 (1999).

4 Xu KD, McFeters GA, Stewart PS. Biofilm resistance to antimicrobial agents. *Microbiology* 146, 547–549 (2000).

•• **Describes how the spatial heterogeneity of physiological activity within a biofilm can explain antimicrobial resistance.**

5 Tunney MM, Patrick S, Curran MD *et al.* Detection of prosthetic hip infection at revision arthroplasty by immunofluorescence microscopy and PCR amplification of the bacterial 16S rRNA gene. *J. Clin. Microbiol.* 37, 3281–3290 (1999).

• **The authors illustrate the difficulties to detect *in vivo* biofilm infections by conventional sampling and culture techniques.**

6 Rayner MG, Zhang Y, Gorry MC, Chen Y, Post JC, Ehrlich GD. Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. *JAMA* 279, 296–299 (1998).

7 Zimmerli W, Ochsner PE. Management of infection associated with prosthetic joints. *Infection* 31, 99–108 (2003).

• **This clinically highly relevant review covers both diagnostic and therapeutic problems in the management of prosthetic joint infections.**

8 Zimmerli W, Widmer AF, Blatter M, Frei R, Ochsner PE. Role of rifampin for treatment of orthopedic implant-related staphylococcal infections: a randomized controlled trial. Foreign-Body Infection (FBI) Study Group. *JAMA* 279, 1537–1541 (1998).

9 Drancourt M, Stein A, Argenson JN, Roiron R, Groulier P, Raoult D. Oral treatment of *Staphylococcus* spp. infected orthopaedic implants with fusidic acid or ofloxacin in combination with rifampicin. *J. Antimicrob. Chemother.* 39, 235–240 (1997).

• **Much more needs to be learned about the potential of individual antibiotic classes to fight surface-adherent bacteria in a biofilm-mode of growth.**

10 Stein A, Bataille JF, Drancourt M *et al.* Ambulatory treatment of multi-drug resistant Staphylococcus-infected orthopedic implants with high-dose oral co-trimoxazole (trimethoprim-sulfamethoxazole). *Antimicrob. Agents Chemother.* 42, 3086–3091 (1998).

11 Widmer AF, Wiestner A, Frei R, Zimmerli W. Killing of nongrowing and adherent Escherichia coli determines drug efficacy in device-related infections. *Antimicrob. Agents Chemother.* 35, 741–746 (1991).

12 Mermel LA, Farr BM, Sherertz RJ *et al.* Guidelines for the management of intravascular catheter-related infections. *Clin. Infect. Dis.* 32, 1249–1272 (2001).

• **These guidelines describe the state of the art therapy for one of the most important biofilm infections.**

13 Raad I, Costerton W, Sabharwal U, Sacilowski M, Anaissie E, Bodey GP. Ultrastructural analysis of indwelling vascular catheters: a quantitative relationship between luminal colonization and duration of placement. *J. Infect. Dis.* 168, 400–407 (1993).

14 DesJardin JA, Falagas ME, Ruthazer R *et al.* Clinical utility of blood cultures drawn from indwelling central venous catheters in hospitalized patients with cancer. *Ann. Intern. Med.* 131, 641–647 (1999).

15 Sauer K, Camper AK, Ehrlich GD, Costerton JW, Davies DG. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J. Bacteriol.* 184, 1140–1154 (2002).

• **The authors correlate different microscopic stages of biofilm development with changes in protein expression demonstrating multiple bacterial phenotypes during biofilm formation.**

16 deBeer D, Stoodley P, Lewandowski Z. Liquid flow in heterogeneous biofilms. *Biotechnol. Bioeng.* 44, 636–641 (1994).

17 Sauer K, Camper AK. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J. Bacteriol.* 183, 6579–6589 (2001).

18 Stanley NR, Britton RA, Grossman AD, Lazazzera BA. Identification of catabolite repression as a physiological regulator of biofilm formation by Bacillus subtilis by use of DNA microarrays. *J. Bacteriol.* 185, 1951–1957 (2003).

19 Whiteley M, Bangera MG, Bumgarner RE *et al.* Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413, 860–864 (2001).

•• **The first characterization of gene expression in biofilms is a milestone in biofilm research.**

20 Lopez-Lopez G, Pascual A, Perea EJ. Effect of plastic catheter material on bacterial adherence and viability. *J. Med. Microbiol.* 34, 349–353 (1991).

21 Vaudaux PE LD, Waldvogel FA. Host factors predisposing to and influencing therapy of foreign body infections. In: *Infections Associated with Indwelling Medical Devices. 2nd Edition.* Bisno AL, Waldvogel FA (Ed.), American Society for Microbiology Press, Washington DC, USA 1–29 (1994).

22 Shenkman B, Varon D, Tamarin I *et al.* Role of agr (RNAIII) in *Staphylococcus aureus* adherence to fibrinogen, fibronectin, platelets and endothelial cells under static and flow conditions. *J. Med. Microbiol.* 51, 747–754 (2002).

23 Hanssen AD, Rand JA, Osmon DR. Treatment of the infected total knee arthroplasty with insertion of another prosthesis. The effect of antibiotic-impregnated bone cement. *Clin. Orthop.* 44–55 (1994).

24 Carratala J, Niubo J, Fernandez-Sevilla A *et al.* Randomized, double-blind trial of an antibiotic-lock technique for prevention of Gram-positive central venous catheter-related infection in neutropenic patients with cancer. *Antimicrob. Agents Chemother.* 43, 2200–2204 (1999).

25 Raad I, Hachem R, Tcholakian RK, Sherertz R. Efficacy of minocycline and EDTA lock solution in preventing catheter-related bacteremia, septic phlebitis and endocarditis in rabbits. *Antimicrob. Agents Chemother.* 46, 327–332 (2002).

26 Veenstra DL, Saint S, Saha S, Lumley T, Sullivan SD. Efficacy of antiseptic-impregnated central venous catheters in preventing catheter-related bloodstream infection: a meta-analysis. *JAMA* 281, 261–267 (1999).

27 Raad I, Darouiche R, Dupuis J *et al.* Central venous catheters coated with minocycline and rifampin for the prevention of catheter-related colonization and bloodstream infections. A randomized, double-blind trial. The Texas Medical Center Catheter Study Group. *Ann. Intern. Med.* 127, 267–274 (1997).

28 Raad I, Darouiche R, Hachem R, Mansouri M, Bodey GP. The broad spectrum activity and efficacy of catheters coated with minocycline and rifampin. *J. Infect. Dis.* 173, 418–424 (1996).

29 Kalmanti M, Germanakis J, Stiakaki E *et al.* Prophylaxis with urokinase in pediatric oncology patients with central venous catheters. *Pediatr. Hematol. Oncol.* 19, 173–179 (2002).

30 Aquino VM, Sandler ES, Mustafa MM, Steele JW, Buchanan GR. A prospective double-blind randomized trial of urokinase flushes to prevent bacteremia resulting from luminal colonization of subcutaneous central venous catheters. *J. Pediatr. Hematol. Oncol.* 24, 710–713 (2002).

31 Ehrlich GD, Veeh R, Wang X *et al.* Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA* 287, 1710–1725 (2002).

32 Nickel JC, Costerton JW. Bacterial localization in antibiotic-refractory chronic bacterial prostatitis. *Prostate* 23, 107–114 (1993).

33 Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* 37, 1771–1776 (1999).

34 Joly V, Pangon B, Vallois JM *et al.* Value of antibiotic levels in serum and cardiac vegetations for predicting antibacterial effect of ceftriaxone in experimental *Escherichia coli* endocarditis. *Antimicrob. Agents Chemother.* 31, 1632–1639 (1987).

35 Hancock E. Artificial valve disease. In: *The Heart Arteries and Veins. (8th Edition).* Schlant RC, O'Rourke RA, Roberts R, Sonnenblick EH (Ed.), McGraw-Hill, Inc., NY, USA 1539–1545 (1994).

36 Heldman AW, Hartert TV, Ray SC *et al.* Oral antibiotic treatment of right-sided staphylococcal endocarditis in injection drug users: prospective randomized comparison with parenteral therapy. *Am. J. Med.* 101, 68–76 (1996).

37 Wellman N, Fortun SM, McLeod BR. Bacterial biofilms and the bioelectric effect. *Antimicrob. Agents Chemother.* 40, 2012–2014 (1996).

38 Leid JG, Shirtliff ME, Costerton JW, Stoodley AP. Human leukocytes adhere to, penetrate and respond to *Staphylococcus aureus* biofilms. *Infect. Immun.* 70, 6339–6345 (2002).

39 Lam J, Chan R, Lam K, Costerton JW. Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. *Infect. Immun.* 28, 546–556 (1980).

40 de Beer D SP, Lewandowski Z. Measurement of local diffusion coefficients in biofilms by microinjection and confocal microscopy. *Biotechnol. Bioeng.* 53(2), 151–158 (1997).

41 Zhu M, Takenaka S, Sato M, Hoshino E. Extracellular polysaccharides do not inhibit the reaction between *Streptococcus mutans* and its specific immunoglobulin G (IgG) or penetration of the IgG through *S. mutans* biofilm. *Oral Microbiol. Immunol.*16, 54–56 (2001).

42 Hoiby N, Fomsgaard A, Jensen ET *et al.* The immune response to bacterial biofilms. In: *Microbial Biofilms.* Lappin-Scott HM, Costerton JW (Ed.), Cambridge University Press, Cambridge, UK 233–250 (1995).

• **Describes the deleterious effects of 'frustrated phagocytosis' on host tissue surrounding biofilms.**

43 Hassett DJ, Ma JF, Elkins JG *et al.* Quorum sensing in *Pseudomonas aeruginosa* controls expression of catalase and superoxide dismutase genes and mediates biofilm susceptibility to hydrogen peroxide. *Mol. Microbiol.* 34, 1082–1093 (1999).

44 Anderl JN, Franklin MJ, Stewart PS. Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* 44, 1818–1824 (2000).

• **Provide an elegant technique to evaluate the antibiotic penetration trough biofilms.**

45 Anwar H, Strap JL, Costerton JW. Eradication of biofilm cells of *Staphylococcus aureus* with tobramycin and cephalexin. *Can. J. Microbiol.* 38, 618–625 (1992).

46 Williams I, Venables WA, Lloyd D, Paul F, Critchley I. The effects of adherence to silicone surfaces on antibiotic susceptibility in *Staphylococcus aureus. Microbiology*143, 2407–2413 (1997).

47 Anderl JN, Zahller J, Roe F, Stewart PS. Role of nutrient limitation and stationary-phase existence in *klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin. *Antimicrob. Agents Chemother.* 47, 1251–1256 (2003).

48 Eng RH, Padberg FT, Smith SM, Tan EN, Cherubin CE. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrob. Agents Chemother.* 35, 1824–1828 (1991).

•• **Landmark paper demonstrating that antibiotic killing greatly depends on the growth rate, which itself is regulated by nutritional resources.**

49 Tuomanen E, Cozens R, Tosch W, Zak O, Tomasz A. The rate of killing of *Escherichia coli* by β-lactam antibiotics is strictly proportional to the rate of bacterial growth. *J. Genet. Microbiol.* 132, 1297–1304 (1986).

50 Spoering AL, Lewis K. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J. Bacteriol.* 183, 6746–6751 (2001).

51 Xu KD, Franklin MJ, Park CH, McFeters GA, Stewart PS. Gene expression and protein levels of the stationary phase sigma factor, RpoS, in continuously-fed *Pseudomonas aeruginosa* biofilms. *FEMS Microbiol Lett.* 199, 67–71 (2001).

52 Zheng Z, Stewart PS. Penetration of rifampin through *Staphylococcus epidermidis* biofilms. *Antimicrob. Agents Chemother.* 46, 900–903 (2002).

53 Walters MC 3rd, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of antibiotic penetration, oxygen limitation and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob. Agents Chemother.* 47, 317–323 (2003).

54 Gordon CA, Hodges NA, Marriott C. Antibiotic interaction and diffusion through alginate and exopolysaccharide of cystic fibrosis-derived *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* 22, 667–674 (1988).

55 Konig C, Schwank S, Blaser J. Factors compromising antibiotic activity against biofilms of *Staphylococcus epidermidis*. *Eur. J. Clin. Microbiol. Infect. Dis.* 20, 20–26 (2001).

56 Souli M, Giamarellou H. Effects of slime produced by clinical isolates of coagulase-negative staphylococci on activities of various antimicrobial agents. *Antimicrob. Agents Chemother.* 42, 939–941 (1998).

57 Durack DT, Beeson PB. Experimental bacterial endocarditis. II. Survival of a bacteria in endocardial vegetations. *Br. J. Exp. Pathol.* 53, 50–53 (1972).

58 Xu KD, Stewart PS, Xia F, Huang CT, McFeters GA. Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl. Environ. Microbiol.* 64, 4035–4039 (1998).

59 Stewart PS, Griebe T, Srinivasan R *et al.* Comparison of respiratory activity and culturability during monochloramine disinfection of binary population biofilms. *Appl. Environ. Microbiol.* 60, 1690–1692 (1994).

60 Mason DJ, Power EG, Talsania H, Phillips I, Gant VA. Antibacterial action of ciprofloxacin. *Antimicrob. Agents Chemother.* 39, 2752–2758 (1995).

61 Zambrano MM, Kolter R. GASPing for life in stationary phase. *Cell* 86, 181–184 (1996).

62 Lazazzera BA. Quorum sensing and starvation: signals for entry into stationary phase. *Curr. Opin. Microbiol.* 3, 177–182 (2000).

• **It is suggested that quorum-sensing and starvation-sensing pathways closely interact to regulate cell entry into the less susceptible stationary phase.**

63 Nystrom T. Aging in bacteria. *Curr. Opin. Microbiol.* 5, 596–601 (2002).

64 Drenkard E, Ausubel FM. Pseudomonas biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 416, 740–743 (2002).

65 Novick RP. Autoinduction and signal transduction in the regulation of staphylococcal virulence. *Mol. Microbiol.* 48, 1429–1449 (2003).

66 Testerman TL, Vazquez-Torres A, Xu Y, Jones-Carson J, Libby SJ, Fang FC. The alternative sigma factor sigmaE controls anti-oxidant defences required for Salmonella virulence and stationary-phase survival. *Mol. Microbiol.* 43, 771–782 (2002).

67 Bateman BT, Donegan NP, Jarry TM, Palma M, Cheung AL. Evaluation of a tetracycline-inducible promoter in *Staphylococcus aureus in vitro* and *in vivo* and its application in demonstrating the role of sigB in microcolony formation. *Infect. Immun.* 69, 7851–7857 (2001).

68 Foley I, Marsh P, Wellington EM, Smith AW, Brown MR. General stress response master regulator rpoS is expressed in human infection: a possible role in chronicity. *J. Antimicrob. Chemother.* 43, 164–165 (1999).

69 Schembri MA, Kjaergaard K, Klemm P. Global gene expression in *Escherichia coli* biofilms. *Mol. Microbiol.* 48, 253–67 (2003).

70 Heydorn A, Ersboll B, Kato J *et al.* Statistical analysis of Pseudomonas aeruginosa biofilm development: impact of mutations in genes involved in twitching motility, cell-to-cell signaling and stationary-phase sigma factor expression. *Appl. Environ. Microbiol.* 68, 2008–2017 (2002).

71 Massey RC, Buckling A, Peacock SJ. Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus. Curr. Biol.* 11, 1810–1814 (2001).

72 Lewis K. Programmed death in bacteria. *Microbiol. Mol. Biol. Rev.* 64, 503–514 (2000).

73 Lewis K. Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* 45, 999–1007 (2001).

• **Provides the interesting hypothesis that antimicrobial resistance in both biofilms and stationary growth phase depends on a subpopulation of 'persister cells' and discusses possible mechanisms.**

74 Cochran WL, McFeters GA, Stewart PS. Reduced susceptibility of thin *Pseudomonas aeruginosa* biofilms to hydrogen peroxide and monochloramine. *J. Appl. Microbiol.* 88, 22–30 (2000).

75 Das JR, Bhakoo M, Jones MV, Gilbert P. Changes in the biocide susceptibility of *Staphylococcus epidermidis* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. *J. Appl. Microbiol.* 84, 852–858 (1998).

76 Camara M, Williams P, Hardman A. Controlling infection by tuning in and turning down the volume of bacterial small-talk. *Lancet Infect. Dis.* 2, 667–676 (2002).

•• **Excellent review of quorum-sensing mechanisms in Gram-positive, Gram-negative and mixed-species environments with a focus on possible new therapeutic targets.**

77 Purevdorj B, Costerton JW, Stoodley P. Influence of hydrodynamics and cell signaling on the structure and behavior of *Pseudomonas aeruginosa* biofilms. *Appl. Environ. Microbiol.* 68, 4457–4464 (2002).

•• **The ability of cell signaling mutants to form biofilms in high shear flow demonstrates that biofilm formation is a multifactorial process regulated by both genetics and environmental growth conditions.**

78 Bollinger N, Hassett DJ, Iglewski BH, Costerton JW, McDermott TR. Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. *J. Bacteriol.* 183, 1990–1996 (2001).

79 De Kievit TR, Gillis R, Marx S, Brown C, Iglewski BH. Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. *Appl. Environ. Microbiol.* 67, 1865–1873 (2001).

80 Blevins JS, Beenken KE, Elasri MO, Hurlburt BK, Smeltzer MS. Strain-dependent differences in the regulatory roles of sarA and agr in *Staphylococcus aureus*. *Infect. Immun.* 70, 470–480 (2002).

81 Valle J, Toledo-Arana A, Berasain C, Ghigo JM, Amorena B, Penades JR, Lasa I. SarA and not sigmaB is essential for biofilm development by *Staphylococcus aureus. Mol. Microbiol.* 48, 1075–1087 (2003).

82 Vuong C, Saenz HL, Gotz F, Otto M. Impact of the agr quorum-sensing system on adherence to polystyrene in *Staphylococcus aureus*. *J. Infect. Dis.* 182, 1688–1693 (2000).

83 Li YH, Tang N, Aspiras MB, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *J. Bacteriol.* 184, 2699–2708 (2002).

84 Balaban N, Gov Y, Bitler A, Boelaert JR. Prevention of *Staphylococcus aureus* biofilm on dialysis catheters and adherence to human cells. *Kidney Int.* 63, 340–345 (2003).

85 Giacometti A, Cirioni O, Gov Y *et al.* G. RNA III inhibiting peptide inhibits *in vivo* biofilm formation by drug-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 47, 1979–1983 (2003).

86 Balaban N, Giacometti A, Cirioni O *et al.* Use of the quorum-sensing inhibitor RNAIII-inhibiting peptide to prevent biofilm formation *in vivo* by drug-resistant *Staphylococcus epidermidis*. *J. Infect. Dis.* 187, 625–630 (2003).

• **One of several papers describing the effect of the currently most promising signaling-inhibitory molecule, the RNA-III inhibitory peptide RIP.**

87 Stickler DJ, Morris NS, McLean RJ, Fuqua C. Biofilms on indwelling urethral catheters produce quorum-sensing signal molecules *in situ* and *in vitro*. *Appl. Environ. Microbiol.* 64, 3486–3490 (1998).

88 Erickson DL, Endersby R, Kirkham A *et al.* *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. *Infect. Immun.* 70, 1783–1790 (2002).

• **One of very few papers that demonstrate the role of cell–cell signaling in human biofilm infections *in vivo*.**

89 Shih PC, Huang CT. Effects of quorum-sensing deficiency on *Pseudomonas aeruginosa* biofilm formation and antibiotic resistance. *J. Antimicrob. Chemother.* 49, 309–314 (2002).

90 Yoon SS, Hennigan RF, Hilliard GM *et al.* *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev. Cell.* 3, 593–603 (2002).

91 Pesci EC, Milbank JB, Pearson JP *et al.* Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc. Natl Acad. Sci. USA* 96, 11229–11234 (1999).

92 You Z, Fukushima J, Tanaka K, Kawamoto S, Okuda K. Induction of entry into the stationary growth phase in *Pseudomonas aeruginosa* by N-acylhomoserine lactone. *FEMS Microbiol. Lett.* 164, 99–106 (1998).

93 Thorne SH, Williams HD. Cell density-dependent starvation survival of Rhizobium leguminosarum bv. phaseoli: identification of the role of an N-acyl homoserine lactone in adaptation to stationary-phase survival. *J. Bacteriol.* 181, 981–990 (1999).

94 Smith RS, Iglewski BH. *P. aeruginosa* quorum-sensing systems and virulence. *Curr. Opin. Microbiol.* 6, 56–60 (2003).

95 Schuster M, Lostroh CP, Ogi T, Greenberg EP. Identification, timing and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J. Bacteriol.* 185, 2066–2079 (2003).

- **Demonstrate that signal accumulation at a critical population density is not sufficient to activate quorum-controlled genes. The complex regulatory system includes growth phase and environmental factors.**

96 Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J. Bacteriol.* 185, 2080–2095 (2003).

- **Demonstrate that signal accumulation at a critical population density is not sufficient to activate quorum-controlled genes. The complex regulatory system includes growth phase and environmental factors.This study further demonstrates the impact of environmental factors as medium composition and oxygen availability on quorum sensing**

97 Vasil ML. DNA microarrays in analysis of quorum sensing: strengths and limitations. *J. Bacteriol.* 185, 2061–2065 (2003).

98 Reimmann C, Ginet N, Michel L *et al.* Genetically programmed autoinducer destruction reduces virulence gene expression and swarming motility in *Pseudomonas aeruginosa* PAO1. *Microbiology* 148, 923–932 (2002).

99 Givskov M, de Nys R, Manefield M *et al.* Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *J. Bacteriol.* 178, 6618–6622 (1996).

100 Hentzer M, Riedel K, Rasmussen TB *et al.* Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. *Microbiology* 148, 87–102 (2002).

101 Ren D, Sims JJ, Wood TK. Inhibition of biofilm formation and swarming of Bacillus subtilis by (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. *Lett. Appl. Microbiol.* 34, 293–299 (2002).

102 Hentzer M, Wu H, Andersen JB *et al.* Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J.* 22, 3803–3815 (2003).

103 Bassler BL, Wright M, Showalter RE, Silverman MR. Intercellular signalling in Vibrio harveyi: sequence and function of genes regulating expression of luminescence. *Mol. Microbiol.*.9, 773–786 (1993).

104 McNab R, Ford SK, El-Sabaeny A, Barbieri B, Cook GS, Lamont RJ. LuxS-based signaling in *Streptococcus gordonii:* autoinducer 2 controls carbohydrate metabolism and biofilm formation with *Porphyromonas gingivalis*. *J. Bacteriol.* 185, 274–284 (2003).

105 Merritt J, Qi F, Goodman SD, Anderson MH, Shi W. Mutation of luxS affects biofilm formation in *Streptococcus mutans*. *Infect Immun*. 71, 1972–1979 (2003).

106 Wen ZT, Burne RA. Functional genomics approach to identifying genes required for biofilm development by Streptococcus mutans. *Appl. Environ. Microbiol.* 68, 1196–1203 (2002).

107 Winzer K, Hardie KR, Williams P. Bacterial cell-to-cell communication: sorry, can't talk now – gone to lunch! *Curr. Opin. Microbiol.*. 5, 216–222 (2002).

108 Anwar H, van Biesen T, Dasgupta M, Lam K, Costerton JW. Interaction of biofilm bacteria with antibiotics in a novel *in vitro* chemostat system. *Antimicrob. Agents Chemother.* 33, 1824–1826 (1989).

109 Cramton SE, Gerke C, Schnell NF, Nichols WW, Gotz F. The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation. *Infect. Immun*. 67, 5427–5433 (1999).

110 Rupp ME, Ulphani JS, Fey PD, Bartscht K, Mack D. Characterization of the importance of polysaccharide intercellular adhesin/hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. *Infect. Immun*. 67, 2627–2632 (1999).

•• **Documents the importance of the ica-gene cluster encoding PIA for biofilm production in the pathogenesis of S. epidermidis foreign body infections.**

111 Ziebuhr W, Heilmann C, Gotz F *et al.* Detection of the intercellular adhesion gene cluster (ica) and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. *Infect. Immun.* 65, 890–896 (1997).

112 de Silva GD, Kantzanou M, Justice A *et al.* The ica operon and biofilm production in coagulase-negative Staphylococci associated with carriage and disease in a neonatal intensive care unit. *J. Clin. Microbiol.* 40, 382–388 (2002).

113 Knobloch JK, Horstkotte MA, Rohde H, Mack D. Evaluation of different detection methods of biofilm formation in Staphylococcus aureus. *Med. Microbiol Immunol* (Berl). 191, 101–106 (2002).

114 Dobinsky S, Kiel K, Rohde H *et al.* Glucose-related dissociation between icaADBC transcription and biofilm expression by *Staphylococcus epidermidis*: evidence for an additional factor required for polysaccharide intercellular adhesin synthesis. *J. Bacteriol.* 185, 2879–2886 (2003).

- **Underscores that the presence of a 'biofilm gene' does not necessarily lead to a biofilm-positive phenotype but that gene expression crucially depends on growth conditions.**

115 Rachid S, Ohlsen K, Witte W, Hacker J, Ziebuhr W. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming *Staphylococcus epidermidis*. *Antimicrob. Agents Chemother.* 44, 3357–3363 (2000).

116 Ziebuhr W, Krimmer V, Rachid S, Lossner I, Gotz F, Hacker J. A novel mechanism of phase variation of virulence in *Staphylococcus epidermidis*: evidence for control of the polysaccharide intercellular adhesin synthesis by alternating insertion and excision of the insertion sequence element IS256. *Mol. Microbiol.* 32, 345–356 (1999).

- **With this study, phase variation is introduced as a regulatory factor in biofilm formation.**

117 Conlon KM, Humphreys H, O'Gara JP. icaR encodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in *Staphylococcus epidermidis*. *J. Bacteriol.* 184, 4400–4408 (2002).

118 Francois P, Tu Quoc PH, Bisognano C *et al.* Lack of biofilm contribution to bacterial colonisation in an experimental model of foreign body infection by *Staphylococcus aureus* and *Staphylococcus epidermidis*. *FEMS Immunol. Med. Microbiol.* 35, 135–140 (2003).

119 Becker P, Hufnagle W, Peters G, Herrmann M. Detection of differential gene expression in biofilm-forming versus planktonic populations of *Staphylococcus aureus* using micro-representational-difference analysis. *Appl. Environ. Microbiol.* 67, 2958–2965 (2001).

120 Finelli A, Gallant CV, Jarvi K, Burrows LL. Use of in-biofilm expression technology to identify genes involved in *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* 185, 2700–2710 (2003).

121 Prigent-Combaret C, Vidal O, Dorel C, Lejeune P. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli. J. Bacteriol.* 181, 5993–6002 (1999).

122 Sutherland I. Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* 147, 3–9 (2001).

123 Durack DT. Experimental bacterial endocarditis. IV. Structure and evolution of very early lesions. *J. Pathol.* 115, 81–89 (1975).

124 Wozniak DJ, Wyckoff TJ, Starkey M *et al.* Alginate is not a significant component of the extracellular polysaccharide matrix of PA14 and PAO1 *Pseudomonas aeruginosa* biofilms. *Proc. Natl Acad. Sci. USA* 100, 7907–7912 (2003).

125 Zielinski NA, Maharaj R, Roychoudhury S, Danganan CE, Hendrickson W, Chakrabarty AM. Alginate synthesis in *Pseudomonas aeruginosa*: environmental regulation of the algC promoter. *J. Bacteriol.* 174, 7680–7688 (1992).

126 Nivens DE, Ohman DE, Williams J, Franklin MJ. Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J. Bacteriol.* 183, 1047–1057 (2001).

127 Nguyen T, Louie SG, Beringer PM, Gill MA. Potential role of macrolide antibiotics in the management of cystic fibrosis lung disease. *Curr. Opin. Pulm. Med.* 8, 521–528 (2002).

- **The therapeutic efficacy of clarithromycin against *P. aeruginosa* biofilms demonstrates that not only direct killing of bacteria but the destruction of biofilm-specific targets as the extracellular polymeric slime promotes biofilm clearance.**

128 Mitsuya Y, Kawai S, Kobayashi H. Influence of macrolides on guanosine diphospho-D-mannose dehydrogenase activity in Pseudomonas biofilm. *J. Infect. Chemother.* 6, 45–50 (2000).

129 Sano M, Hirose T, Nishimura M, Takahashi S, Matsukawa M, Tsukamoto T. Inhibitory action of clarithromycin on glycocalyx produced by MRSA. *J. Infect. Chemother.* 5, 10–15 (1999).

130 Yamasaki O, Akiyama H, Toi Y, Arata J. A combination of roxithromycin and imipenem as an antimicrobial strategy against biofilms formed by *Staphylococcus aureus*. *J. Antimicrob. Chemother.* 48, 573–577 (2001).

131 Huang CT, Stewart PS. Reduction of polysaccharide production in *Pseudomonas aeruginosa* biofilms by bismuth dimercaprol (BisBAL) treatment. *J. Antimicrob. Chemother.* 44, 601–5 (1999).

132 Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. *Science* 295, 1487 (2002).

133 Hook EW 3rd, Sande MA. Role of the vegetation in experimental Streptococcus viridans endocarditis. *Infect. Immun.* 10, 1433–1438 (1974).

134 Kupferwasser LI, Yeaman MR, Shapiro SM *et al.* Acetylsalicylic acid reduces vegetation bacterial density, hematogenous bacterial dissemination and frequency of embolic events in experimental *Staphylococcus aureus* endocarditis through antiplatelet and antibacterial effects. *Circulation* 99, 2791–2797 (1999).

135 Isberg RR, Barnes P. Dancing with the host; flow-dependent bacterial adhesion. *Cell* 110, 1–4 (2002).

136 Stoodley P, Cargo R, Rupp CJ, Wilson S, Klapper I. Biofilm material properties as related to shear-induced deformation and detachment phenomena. *J. Ind. Microbiol. Biotechnol.* 29, 361–367 (2002).

137 Wilson JW, Ott CM, Ramamurthy R *et al.* Low-Shear modeled microgravity alters the *Salmonella enterica* serovar typhimurium stress response in an RpoS-independent manner. *Appl. Environ Microbiol.* 68, 5408–5416 (2002).

138 Koerner RJ. Contribution of endotracheal tubes to the pathogenesis of ventilator-associated pneumonia. *J. Hosp. Infect.* 35, 83–89 (1997).

139 Stoodley P, Hall-Stoodley L, Lappin-Scott HM. Detachment, surface migration and other dynamic behavior in bacterial biofilms revealed by digital time-lapse imaging. *Meth. Enzymol.* 337, 306–319 (2001).

140 Inglis TJ. Evidence for dynamic phenomena in residual tracheal tube biofilm. *Br J Anaesth.* 70, 22–24 (1993).

141 Liu Y, Tay JH. Metabolic response of biofilm to shear stress in fixed-film culture. *J. Appl. Microbiol.* 90, 337–342 (2001).

142 Peyton B. Effects of shear-stress and substrate loading rate on *Pseudomonas aeruginosa* biofilm thickness and density. *Water Res.* 30, 29–36 (1996).

143 Flemming HC, Mayer C, Koerstgens V and Borchard W. Cohesiveness in biofilm matrix polymers. In: *Community Structure and Co-operation in Biofilms. SGM Symposium Series 59.* Allison D, Lappin-Scott HM and Wilson M (Ed.), Cambridge Uni. Press, Cambridge, UK 87–105 (2000).

144 Stoodley P, Wilson S, Hall-Stoodley L, Boyle JD, Lappin-Scott HM, Costerton JW. Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl. Environ. Microbiol.* 67, 5608–5613 (2001).

145 Characklis W. Biofilm processes. In: *Biofilms.* Characklis WG, Marshall DC (Ed.), John Wiley & Sons, NY, USA 195–232 (1990).

146 Daly B, Betts WB, Brown AP, O'Neill JG. Bacterial loss from biofilms exposed to free chlorine. *Microbios* 96, 7–21 (1998).

147 Boyd A, Chakrabarty AM. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* 60, 2355–2359 (1994).

148 Lee SF, Li YH, Bowden GH. Detachment of Streptococcus mutans biofilm cells by an endogenous enzymatic activity. *Infect. Immun.* 64, 1035–1038 (1996).

149 Allison DG, Ruiz B, SanJose C, Jaspe A, Gilbert P. Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. *FEMS Microbiol Lett.* 167, 179–184 (1998).

150 Webb JS, Thompson LS, James S *et al.* Cell death in *Pseudomonas aeruginosa* biofilm development. *J. Bacteriol.* 185, 4585–4592 (2003).

151 Xiong YQ, Van Wamel W, Nast CC, Yeaman MR, Cheung AL, Bayer AS. Activation and transcriptional interaction between agr RNAII and RNAIII in *Staphylococcus aureus in vitro* and in an experimental endocarditis model. *J. Infect. Dis.* 186, 668–677 (2002).

152 Beenken KE, Blevins JS, Smeltzer MS. Mutation of sarA in *Staphylococcus aureus* limits biofilm formation. *Infect. Immun.* 71, 4206–4211 (2003).

Website

201 www.erc.montana.edu/Res-Lib99-SW/Movies/2003/03-M003_4.htm Accessed November 2003

Affiliations

- *Christoph A Fux, MD*
  Visiting Scientist Center for Biofilm Engineering,
  Montana State University,
  christophf@erc.montana.edu
- *Paul Stoodley, PhD,*
  Assistant Research Professor
  Center for Biofilm Engineering and Departments of Civil Engineering and Microbiology, Montana State University
  paul_s@erc.montana.edu
- *Luanne Hall-Stoodley, PhD*
  Assistant Research Professor
  Veterinary Molecular Biology, Montana State University
  lhstoodley@montana.edu
- *J William Costerton, PhD*
  Director Center for Biofilm Engineering,
  Montana State University
  bill_c@erc.montana.edu