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.


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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 crotimoxazole 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]).

<table>
<thead>
<tr>
<th>Infection or disease</th>
<th>Common bacterial species involved</th>
</tr>
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<tbody>
<tr>
<td>Dental caries</td>
<td>Acidogenic Gram-positive cocci (<em>Streptococcus</em> spp.)</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>Gram-negative anaerobic oral bacteria</td>
</tr>
<tr>
<td>Otitis media</td>
<td>Nontypeable <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>Chronic tonsillitis</td>
<td>Various species</td>
</tr>
<tr>
<td>Cystic fibrosis pneumonia</td>
<td><em>Pseudomonas aeruginosa</em>, <em>Burkholderia cepacia</em></td>
</tr>
<tr>
<td>Endocarditis</td>
<td>Viridans group streptococci, staphylococci</td>
</tr>
<tr>
<td>Necrotizing fasciitis</td>
<td>Group A streptococci</td>
</tr>
<tr>
<td>Musculoskeletal infections</td>
<td>Gram-positive cocci</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>Various species</td>
</tr>
<tr>
<td>Biliary tract infection</td>
<td>Enteric bacteria</td>
</tr>
<tr>
<td>Infectious kidney stones</td>
<td>Gram-negative rods</td>
</tr>
<tr>
<td>Bacterial prostatitis</td>
<td><em>Escherichia coli</em> and other Gram-negative bacteria</td>
</tr>
<tr>
<td><strong>Infections associated with foreign body material</strong></td>
<td></td>
</tr>
<tr>
<td>Contact lens</td>
<td><em>P. aeruginosa</em>, Gram-positive cocci</td>
</tr>
<tr>
<td>Suture</td>
<td>Staphylococci</td>
</tr>
<tr>
<td>Ventilation-associated pneumonia</td>
<td>Gram-negative rods</td>
</tr>
<tr>
<td>Mechanical heart valves</td>
<td>Staphylococci</td>
</tr>
<tr>
<td>Vascular grafts</td>
<td>Gram-positive cocci</td>
</tr>
<tr>
<td>Arteriovenous shunts</td>
<td>Staphylococci</td>
</tr>
<tr>
<td>Endovascular catheter infections</td>
<td>Staphylococci</td>
</tr>
<tr>
<td>Peritoneal dialysis (CAPD) peritonitis</td>
<td>Various species</td>
</tr>
<tr>
<td>Urinary catheter infections</td>
<td><em>E. coli</em>, Gram-negative rods</td>
</tr>
<tr>
<td>IUDs</td>
<td><em>Actinomyces israelii</em> and others</td>
</tr>
<tr>
<td>Penile prostheses</td>
<td>Staphylococci</td>
</tr>
<tr>
<td>Orthopedic prosthesis</td>
<td>Staphylococci</td>
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</table>
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 adherence, 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
antibiotics [27] has been shown to delay bacterial coloniza-
tion. However, these catheters have a short duration of anti-
microbial efficacy and carry the risk of selecting for antibi-
otic-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 infec-
tions remains inconclusive with contradictory results published
in the literature [29,30].

Diagnosis
Physicians have been trained to think of live bacteria as cul-
turable 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 ster-
ilre effusions has demonstrated that traditional culturing meth-
ods 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]. Anal-
ogous 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 suc-
cessful when serum antibiotic levels were held at least tenfold
above the MBC [34]; but even with 8 weeks of parenteral anti-
biotic 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 concern-
ing 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 com-
promised 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

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.
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...
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 aminoglycosides 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
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 coordinate 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.](image-url)
phase genes. The exposure of *P. aeruginosa* to AHL increases the levels of QS [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 *Delicea 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 (SP0A, 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 iadABC gene cluster [109,110]. Ziebuhr and colleagues detected the iac 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]. 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.
[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...
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 liquefication 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.

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The discovery of cell–cell signaling


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


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


Papers of special note have been highlighted as:

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


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


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


- 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.


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


• 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.


• 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.


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


• 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.


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


- 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.


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