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Biofilm formation mechanisms and targets for developing antibiofilm agents

Biofilms are communities of microorganisms that are attached to a surface and play a significant role in the persistence of bacterial infections. Bacteria within a biofilm are several orders of magnitude more resistant to antibiotics, compared with planktonic bacteria. Thus far, no drugs are in clinical use that specifically target bacterial biofilms. This is probably because until recently the molecular details of biofilm formation were poorly understood. Bacteria integrate information from the environment, such as quorum-sensing autoinducers and nutrients, into appropriate biofilm-related gene expression, and the identity of the key players, such as cyclic dinucleotide second messengers and regulatory RNAs are beginning to be uncovered. Herein, we highlight the current understanding of the processes that lead to biofilm formation in many bacteria.

Dating back to the seminal works of Robert Koch, the father of modern microbiology, to the 1970s, bacteria were largely considered as single free-floating microorganisms. Using the planktonic pure bacterial culture model, scientists have been able to study many deadly bacteria and developed biocides to kill such bacteria. The emergence of drug-resistant bacteria and the difficulty in killing some bacteria led to a re-evaluation of the bacterial lifestyle and it is now acknowledged that the aggregation of bacteria within self-produced matrices, called biofilms, endows bacteria with mechanisms to resist biocides. Biofilms were observed a few centuries before their relevance to the persistence of disease was realized. In 1684, a Dutch scientist, Antonie van Leeuwenhoek, saw dental plaque using his self-constructed microscope and he described it as scurf. During the early part of the 20th century many scientists reported that most bacteria were not free-floating but were attached to surfaces, such as the bottom of lakes [1,2]. Scientists began to realize that some sessile bacteria were directly related to disease when in 1977 *Pseudomonas aeruginosa* aggregation was found in sputum from the lungs of infected cystic fibrosis patients [3]. In 1978, Clark reported that an important component of *Streptococcus mutans* biofilm,

polysaccharide glycocalyx formed on teeth. The term 'biofilm' was formally introduced in 1978 by Costerton [4]. Biofilms have varied morphologies, depending on the constituent bacteria as well as the conditions under which that biofilm was formed. See Figure 1 for a few examples of bacterial biofilms.

In the last few years, interests in biofilm formation by a group of clinically relevant bacteria, which cause high mortality, called ESKAPE (*Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa* and *Enterobacter* spp.) have intensified. There is an interest in understanding biofilm formation by bacteria that cause dental caries, such as *Streptococcus sobrinus* and *S. mutans*, and also there are many efforts to delineate biofilm mechanisms of bacteria that foul implants, such as *Proteus mirabilis*. In the sections that follow, we highlight current understanding in how the aforementioned and other bacteria form biofilms and targets or processes, for which small molecules can be developed against to inhibit bacterial biofilm formation.

Biofilm structure

The structure of the extracellular polymeric substance (EPS) matrix of bio-

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Key terms

Extracellular polymeric substances: The biofilm is supported by a matrix of polymeric compounds. These compounds are secreted by bacteria into the environment. EPS are usually composed of exopolysaccharides, proteins and nucleic acids.

Exopolysaccharides: Exopolysaccharides are high-molecular-weight polymers that are composed of sugar residues and are secreted by bacteria into the surrounding environment.

films is composed of one or more of extracellular polysaccharides, DNA and proteins (Figure 2) [11]. Channels in the biofilm allow for water, air and nutrients to get to all parts of the structure [12].

Exopolysaccharides

Exopolysaccharides are either synthesized extracellularly or intracellularly and secreted into the outside

environment [13]. In electron microscopy, they look like linear or branched long strands that are attached to cell surfaces and stretched out to form large networks (Figure 3). Exopolysaccharides serve as scaffolds for other carbohydrates, proteins, nucleic acids and lipids to adhere. The components, structures and properties of the exopolysaccharides differ from one another.

Nelson and coworkers analyzed the compositions and linkages of EPS matrix from *E. faecalis*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. spp.* biofilms [15]. Mannose, galactose and glucose are the most abundant carbohydrates, followed by *N*-acetyl-glucosamine, galacturonic acid, arabinose, fucose, rhamnose and xylose. Most exopolysaccharides are not biofilm specific, but their production increases as a result of a stress response, such as colanic acid production in *Escherichia coli* [16] and alginate synthesis in *P. aeruginosa* [17]. Colanic acid (also known as M antigen) is an extracellular heteropolysaccharide found in

Figure 1. Bacterial biofilms. (A) SEM image of *Pseudomonas aeruginosa* isolates attaching to glass surfaces; (B) biofilm formation of *Escherichia coli* on titanium oxide surface; (C) SEM image of catheter-associated *Staphylococcus aureus* biofilm growth *in vivo*; (D) electron microscopy of *Staphylococcus epidermidis* biofilm formation on Teflon catheter; (E) SEM image showing attachment and biofilm formation by *Salmonella enterica* serovar Poona cells inside the netting of inoculated cantaloupe; (F) SEM of mature mixed biofilms formed on discs of hydroxyapatite, demonstrating the affinity of *Streptococcus mutans* to the hyphal elements of *Candida albicans*. SEM: Scanning electron microscopy.

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Figure 2. Biofilm structure.

Enterobacteriaceae [18]. The colanic acid repeat unit is made up of L-fucose, D-galactose, D-glucuronate and D-glucose and decorated with O-acetyl and pyruvate side chains [19]. The repeat units are assembled by glycosyl transferases on the cytoplasmic side of the inner cell membrane. Then the repeat units are flipped from the cytoplasm into the periplasm by Wzx. Wzy system is responsible for initial polymerization and high-level polymerization is conducted by Wzc and Wzb. The outer-membrane protein Wza transports colanic acid polymers into the extracellular space [20]. *E. coli* K-12 strain defective in colanic acid production has a slower initial attachment rate, but the attachment is not completely abolished [21].

There are three exopolysaccharides associated with *P. aeruginosa* biofilms: Pel [22], Psl [23,24] and alginate (Figure 4) [25]. Alginate is composed of D-mannuronic acid residues interspersed with L-guluronic acid residues (Figure 5B). Alginate is not involved in biofilm initiation, but it is a critical virulence factor in chronic infections. Alginate protects *P. aeruginosa* cells from antibiotics such as ciprofloxacin, gentamicin, ticarcillin and ceftazidime [26], and it suppresses host immune response [27]. AlgC enzyme was found to be involved in the synthesis of alginate and lipopolysaccharide (LPS) (Figure 5A) [28]. The enzyme, encoded by *algC* gene, has both phosphomannomutase activity and phosphoglucomutase activity. The conversions of Man-6-P into Man-1-P and glucose-6-P into glucose-1-P are catalyzed by this enzyme. Man-1-P is a crucial intermediate in the synthesis pathway of alginate and LPS [29]. Apart from alginate and LPS, Psl and Pel are also two major biofilm matrix exopolysaccharides. Pel and Psl are both important materials in mature *P. aeruginosa* biofilms. Psl polysaccharide is composed of a repeating pentamer consisting of D-mannose, L-rhamnose and D-glucose residues (Figure 6B) and it promotes the initial surface attachment process. *Pel*

genes are responsible for glucose-rich exopolysaccharides formation in the strain PA14[22] and *psl* genes are involved in mannose-rich exopolysaccharides formation in strains ZK2870[23] and PAO1[24]. A *psl*-deficient PAO1 strain could still form mature biofilm by upregulating *pel* genes [30]. It was shown that AlgC enzyme is also needed for the synthesis of Psl and Pel [28]. Man-1-P is a common intermediate in the synthesis of Psl, alginate and LPS, and the productions of these biofilm components were found to be influenced by each other (Figure 6A) [28]. That is, if one of them was over produced, then the production of the others would be reduced.

In total, there are 24 genes involved in the production and secretion of alginate in *P. aeruginosa*. Eight of them regulate the exportation of alginic acids, and 12 are responsible for the biosynthesis of the polysaccharide. The four remaining genes regulate the synthesis [32,33].

In *Staphylococcus*, the biofilm-related exopolysaccharide is polysaccharide intercellular adhesin (PIA),

Figure 3. Scanning electron microscopy visualization of biofilm. The bacteria and the extracellular polymeric substance matrix were visualized by SEM at $\times 10,000$ magnification. The white box indicates the enlarged section, and the white arrow indicates the connections between bacteria in 3-day old biofilm (scale bar = $1\ \mu\text{m}$).

SEM: Scanning electron microscopy.
Reproduced from [14].

Figure 4. Extracellular polysaccharides in *Escherichia coli* and *Pseudomonas aeruginosa*. (A) Atomic force microscopy image of alginate in *P. aeruginosa* FRD1. (B) Confocal laser scanning microscopy image of Psl in hydrated *P. aeruginosa* PAO1. (C) SEM image of Pel in *P. aeruginosa* PA14. SEM: Scanning electron microscopy. Reproduced from [31].

which was characterized by Mack and coworkers [34]. PIA, also known as poly *N*-acetyl glucosamine, is a linear polymer composed of β -1,6-linked glucosamine residues. More than 80% of the PIA residues in *Staphylococcus epidermidis* are *N*-acetylated and the rest are positively charged [34]. Biosynthesis of PIA is conducted by four proteins: IcaA, IcaB, IcaC and IcaD. The *N*-acetylglucosaminyltransferases IcaA and IcaD synthesize 20 residue oligomers from UDP-GlcNAc. IcaC is involved in long oligomer formation (>130 residues) and transportation of the oligomers to the extracellular space. Deacetylation by IcaB results in positive charges, which is essential for interactions with cell membranes [35].

In *Bacillus subtilis* the biofilm-related exopolysaccharide can be EPS or poly- δ -glutamate, depending on the strain and the conditions [36,37].

Extracellular proteins

Extracellular proteins are another major EPS matrix component [38]. Some proteins are attached to cell surfaces and polysaccharides to help with biofilm formation and stabilization. One example is the glucan-binding proteins (Gbps) in *S. mutans* biofilm [39]. Gbps play an important role in biofilm architecture maintenance by linking bacteria and exopolysaccharides. Biofilms produced by Gbps mutants have significantly reduced height.

Amyloids are also insoluble fibrous proteins that play a supportive role in biofilm architecture. One example is the Fap amyloids in *Pseudomonas* spp. Overexpression of Fap amyloids leads to cell aggregation and increased biofilm formation [40]. Amyloid protein TasA is one of the major components of *B. subtilis* biofilms [41]. TasA forms strong fibers that can hold biofilm cells together and tolerate harsh

destructive forces. Another example is the biofilm-associated protein (bap) family. The bap family includes Bap protein from *S. aureus* [42] and Esp protein from *E. faecalis* [43]. The bap family is involved in biofilm formation and infection processes [44]. Other examples include outer-membrane lectins of *Azospirillum brasiliense* [45] and the galactophilic lectin le-cA [46] and L-fucose binding lectin lecB [47] of *P. aeruginosa*.

Some enzymes are involved in degradation processes within biofilms. The enzymes' substrates include polysaccharides, proteins, nucleic acids, cellulose, lipids, other EPS matrix components and objects that are trapped in the EPS matrix. These enzymes can break down biopolymers and provide carbon and energy resources to biofilm cells, especially during starvation [48]. The detachment and dispersal of biofilm also require enzymatic functions. Degradation of EPS matrix internally by enzymes releases biofilm cells and initiate a new biofilm lifecycle. For example, DspB protein is responsible for the surface detachment of *Actinobacillus pleuropneumoniae* biofilms [49].

eDNA

Extracellular DNAs (eDNAs) were previously considered as leftovers from lysed cells until Mattick and coworkers found that DNase I could prevent *P. aeruginosa* biofilm formation [50]. The fact that eDNA not only comes from lysed cells but also is actively secreted [51] indicates that eDNA has an important role in biofilm formation. It is found to be critical for biofilm attachment (Figure 7). Its negative charge works as a repulse force in the initial attachment, but when the distance between cell and surface becomes a few nanometers, eDNA interacts with receptors on substratum surface to facilitate adhesion [52]. Also, eDNA was found to

Figure 5. Alginate (A) biosynthesis and (B) chemical structure.

coordinate cell movement in the twitching motility-mediated *P. aeruginosa* biofilm expansion [53]. Due to its negative charge, eDNA is able to chelate metal cations and some positively charged antibiotics. eDNA can chelate Mg^{2+} and activate PhoPQ/PmrAB two-component systems, leading to antimicrobial peptide resistance in *P. aeruginosa* [54,55], *Salmonella enterica* serovar Typhimurium [56] and other Gram-negative bacteria. In *S. epidermidis*, eDNA was also found to inhibit the transportation of vancomycin within bio-

films and hence protect the bacteria embedded within the biofilm [57,58].

The current knowledge about the roles that eDNA plays in biofilm maturation and persistence is limited and more research is warranted to shed light on its role in biofilm development.

Reason for biofilm formation

Many bacteria can switch between planktonic form and biofilm form. The planktonic bacteria have relatively

Figure 6. Psl (A) biosynthesis and (B) chemical structure.

high cell growth and reproduction rate. However, the biofilm state appears to be natural and predominant state of bacteria. Several reasons could account for the need for a bacterial biofilm state [60]. First, biofilm can enhance the tolerance of bacteria to harsh environmental conditions. Bacteria can avoid being washed away by water flow or blood stream by simply attaching to a surface or tissue. The oral biofilms can stand up to repeated, strong, shear forces. Cells in biofilms are about 1000 times more resistant than their planktonic [61]. Second, the EPS matrix protects bacteria cells, in deeper layers, against antimicrobial agents

probably by limiting the diffusion of these agents. Biofilm restricts bacterial mobility and increases cell density, which also provides an optimal environment for eDNA (plasmid) exchange (via conjugation), some of which encode for antibiotic resistance. As reported by Hausner and Wuertz, the horizontal gene transfer rate is significantly higher in biofilms than in the planktonic cells [62].

Process of biofilm formation

A prerequisite of biofilm formation is that bacteria should get close enough to a surface. As bacteria

approach a surface several forces, both attractive and repulsive, come into play. At about 10–20 nm distance from the surface, the negative charges on the bacterial surface are repelled by negative charges on most environmental surfaces [63]. This repulsion could, however, be overcome by attractive van der Waals forces between the bacterial cells and the surface as well as the use of fimbriae and flagella to provide mechanical attachment to the surface [63]. Biofilm formation can be described in three stages: attachment, maturation and **dispersion** (Figure 8).

The attachment step could be further categorized as a two-stage process: initial reversible attachment and irreversible attachment [64]. The irreversible attached biofilm can tolerate stronger physical or chemical shear forces. [65] In the initial attachment, flagella and type IV pili-mediated motilities are important. Flagella are critical for initial interactions between cells and surface. Type IV pili-mediated twitching motilities enable attached cells to aggregate and form microcolonies. O'Toole and Kolter demonstrated that *P. aeruginosa* flagella-deficient mutants could not land onto surfaces and type IV pili-deficient mutants could not form microcolonies [66].

For the human pathogens *S. epidermidis* and *S. aureus* the initial step of biofilm formation is the attachment to human matrix proteins, such as fibronectin (Fn), fibrinogen (Fg), vitronectin (Vn), etc. Microbial surface components recognizing adhesive matrix molecules dependent adhesions are covalently linked to the peptidoglycan on the cell wall. *S. aureus* has more than 20 microbial surface components recognizing adhesive matrix molecule genes while *S. epidermidis* RP62A has only 12 [67]. Noncovalent adhesions, such as those mediated by autolysins, also contribute to initial attachment of biofilms [68].

The production of EPS matrix signifies the irreversible phase of bacterial attachment to a surface. The EPS matrix of *P. aeruginosa* has been well studied, probably due to the role that *P. aeruginosa* biofilms play in the progression of cystic fibrosis. Alginate, a major polysaccharide component of *P. aeruginosa* EPS matrix, is produced by bacteria that are attached to a surface (recently attached bacteria) in quantities that are several folds more than by planktonic cells [69]. The σ factor AlgT, which is also required for alginate production, downregulates flagella genes [70].

Once the first layer of the biofilm is established, cells of the same species or other species are recruited to the biofilm from the bulk fluid. Biofilm grows from a thin layer to a 'mushroom' or 'tower' shape structure. In a thick biofilm (>100 layers), bacteria are arranged according to their metabolism and aero tolerance. For example, anaerobic bacteria prefer to live in deeper lay-

Key terms

Planktonic cells: Bacteria that float or swim as single cells.

Dispersion: The release of planktonic cells from the biofilm as a result of unfavorable conditions for the biofilm form. This is the final step in biofilm development, from a mature biofilm to the planktonic form. Usually few parts of the biofilm are dispersed and it is a continuous process. This step allows the biofilm to spread and colonize on a new surface.

ers to avoid exposure to oxygen. Bacteria within biofilm communities 'talk' to each other and take specialized functions. As the biofilm matures, more biofilm scaffolds, such as proteins, DNA, polysaccharides, etc. are secreted into the biofilm by the entrapped bacteria.

After biofilm maturation the dispersal step, which is also critical for the biofilm life cycle, follows. Biofilms disperse because of myriads of factors, such as lack of nutrients, intense competition, outgrown population, etc. Dispersal could occur in the whole biofilm or just a part of it. Release of planktonic bacteria promotes the initiation of new biofilms at other sites.

Biofilm-related diseases

Biofilms are linked to various human diseases [71]. Many bacterial infections are biofilm related, for example, chronic lung, wound and ear infections [72]. Biofilms are also able to colonize on medical devices such as catheters and implants. According to the NIH more than 80% of all microbial infections are biofilm related [73]. These kinds of infections are hard to diagnose and treat.

Cystic fibrosis

Cystic fibrosis (CF) in the lung causes the formation of thick and sticky mucus, which blocks airway and

Figure 7. Extracellular DNA in *Enterococcus faecalis* biofilm. Visualization of labeled strands by using an anti-dsDNA monoclonal antibody conjugated to an immunogold particle (asterisks; scale bar = 250 nm). Reproduced from [59].

Figure 8. Biofilm formation process.

makes it hard for patients breathe [74]. Eighty percent of CF patients are chronically infected by *P. aeruginosa*. Medical instruments, devices and tools can also be contaminated with *P. aeruginosa*. Several cases of hospital-acquired *P. aeruginosa* infections have been reported [75,76], but unfortunately, there are not many good antibiotics that are available for treating recalcitrant *P. aeruginosa* infection [77]. For patients who are infected with *P. aeruginosa*, antibiotic treatment often relieves the symptom of the infection but not necessarily cures the infection. The lack of complete cure is mainly due to *P. aeruginosa* biofilms that act as reservoirs for disease recurrence [78]. This calls for the development of drugs that can act on *P. aeruginosa* biofilms. These future drugs could either kill the bacteria in the biofilm or cause the dispersal of the biofilm into planktonic state and by that enabling drugs that work on planktonic bacteria to take effect [79]. Beyond acting as disease reservoirs, the components of biofilms, such as the major polysaccharide of *P. aeruginosa* matrix (alginate), are virulence factors. For example, alginate can cause lung damage [80].

Dental plaque

Biofilms play an important role in dentistry and there have been many studies on dental biofilms [81,82]. Over 700 species of bacteria and archaea have been reported to exist in dental plaque [83,84]. Dental plaque is actually composed of mixed biofilms [85]. However, the composition of the healthy plaque biofilm is significantly different from disease-associated plaque biofilms.

The mouth is a unique microenvironment that is characterized by water, temperature fluctuations, hard surface, and carbon and nitrogen input. Changes in the local environment influence the biofilm bacterial composition. Acid is a major cause of tooth decay and innocuous bacteria species in healthy biofilms, including *Streptococcus sanguis*, *Streptococcus gordonii*, *Streptococcus oralis*, *Actinomyces* species, etc. often show low acid tolerance [86]. On the contrary, bacteria involved in dental caries, such as *S. sobrinus* and *S. mutans*, have very high acid tolerance. These acid-tolerant bacteria that can also form robust biofilms are responsible

for many oral cavity diseases, such as dental caries, periodontitis, gingivitis, etc. [87].

Communication among bacteria is essential for initial colonization and successive biofilm formation on the enamel surfaces. If bacteria do not retain on the tooth surface, the bacteria are washed with the mouth fluid. If they do manage to hold to the surface, they are organized as a multispecies to form a dental plaque. Oral microorganisms adhere to surfaces such as teeth, epithelial tissues or to other bacteria that is immobilized on the surface (cohesion). Cell-cell interaction between genetically distinct cells can also occur in suspension (coaggregation). Oral bacteria have at least one coaggregation partner. A diagram illustrating competition and co-operation among early and late colonizers of the tooth is shown in Figure 9. The initial colonizers are streptococci and actinomyces [88,89]. Receptors are recognized by the primary colonizers of dental plaque. Among the receptors are found phosphate-rich proteins such as statherin, and enzymes such as α -amylase. Streptococci recognize receptors in the salivary pellicle (coats the enamel). Actinomyces bind to proline-rich proteins and statherin (Figure 9). Fusobacteria, anaerobic Gram-negative bacteria, also bind to statherin.

Bacteria communicate in the oral biofilm through the secretion and accumulation of small molecules. Autoinducer (AI)-2 is a universal intergeneric signaling molecule that plays an important role in biofilm development [91,92]. AI-2 stimulates the coaggregation of *S. oralis* and *Actinomyces oris* T14V. Small concentrations of AI-2 are sufficient for coaggregation of oral bacteria, less for oral pathogens. As the cell density of the commensal bacteria increases, the AI-2 concentration increases. At the highest concentration, the pathogens grow and develop the biofilm. At low concentration the conditions do not support their growth. Fusobacteria produce high levels of AI-2, then coaggregate with variety of colonizers and become dominant bacteria in biofilms associate with healthy gums and plaque.

Wounds

Biofilms are commonly found in chronic wounds [93]. Compared with an acute wound, which is usually not associated with biofilm, a biofilm-related chronic wound persists and only slowly heals. Biofilms usually form on the outer layer of wounds but some biofilms are also embedded in the deep layers of a wound, such as *P. aeruginosa* biofilms, and they are difficult to diagnose using a traditional wound swab culture [71].

Urinary infection

Biomaterials in the urinary tract, such as catheters, increase the chance of bacterial biofilm formation,

Figure 9. Oral bacterial colonization. Spatiotemporal model of oral bacterial colonization showing recognition of salivary pellicle receptors by initial colonizing bacteria and coaggregations between initial colonizers, fusobacteria and late colonizers of the tooth surface. Collectively, these interactions are proposed to represent development of dental plaque. Starting at the bottom, initial colonizers, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus sanguinis*, bind to complementary salivary receptors (sialylated mucins, proline-rich protein, α -amylase, salivary agglutinin and bacterial cell fragments) in the acquired pellicle coating the tooth surface. Late colonizers bind to previously bound bacteria. Sequential binding results in the appearance of nascent surfaces that bridge with the next coaggregating partner cell. Coaggregation is different from aggregation that occurs between genetically identical cells and from agglutination of cells through interaction of cells with soluble molecules, for example, antibodies. Most coaggregations are between cells of different genera; *Fusobacterium nucleatum* strains, for example, coaggregate intergenerically with representatives of all oral bacterial species. However, intrageneric coaggregation among fusobacterial strains is only rarely observed. In sharp contrast, streptococci exhibit broad intrageneric coaggregation partnerships (e.g., *S. gordonii* and *S. oralis*) as well as intraspecies partnerships (e.g., *S. gordonii* DL1 and *S. gordonii* 38). Each bacterial strain exhibits specificity in partners. For example, some streptococci are capable of coaggregating with certain *Veillonella* spp., whereas other streptococci cannot coaggregate with those veillonellae but do coaggregate with a separate group of veillonellae. Reproduced with permission from [90] © Macmillan Publishers Ltd (2010).

leading to urinary infection [94]. The synthetic foreign body provides surfaces for bacteria to attach. Almost all urinary catheters (both the inner and outer surfaces) are infected with bacterial biofilms (Figure 10). These biofilms, for example, *P. mirabilis* biofilms, which can be crystalline in nature, can block catheters and patients have to replace the blocked catheters [95].

Prosthetic joint infection

Bacteria causing prosthetic joint infection are usually Gram-positive, such as staphylococci [96]. Most often immediately after surgery, bacteria (which could come from blood or the lymph) attach to the surface of prosthetic joints to form biofilms. Unlike typical bacterial

infections that give symptoms like fever, it could take a while before symptoms of the biofilms on these implants, such as pain, emerge [97].

Cardiac valve infection

Bacterial biofilm on mechanical cardiac valve causes a disease called prosthetic valve endocarditis. The species involved in endocarditis are *S. epidermidis*, *S. aureus*, *Streptococcus* spp., *Corynebacterium* spp., *Enterococcus* spp. and *Candida* spp [98]. Accumulated biofilm can disrupt or block the artificial cardiac valve, resulting in diminished flow, turbulence or even leaking. Detached biofilm cells can migrate along with the blood stream and cause infection in other organs. Biofilm fragments in the blood circulation can block blood at the terminal

Figure 10. Biofilm on urethral stents. Scanning electron microscopic picture of a developing biofilm on urethral stents that is lying completely within the urinary tract. Reproduced with kind permission from [94] © Springer Science and Business Media (2006).

points, such as the brain and kidney.

Biofilm resistance to antibiotics & host immune system

Bacteria in a biofilm are more resistant to antibiotics or the reactive molecules that are produced by the host immune systems than planktonic bacteria. It has been estimated that biofilm cells can be up to 10,000 times more resistant to antibiotics than planktonic cells [99,100]. Costerton and coworkers observed that when they treated *P. aeruginosa* biofilm and planktonic cells with tobramycin, planktonic cells could not survive greater than 50 µg/ml tobramycin, but biofilm cells could tolerate 1 mg/ml tobramycin [100]. Abee and coworkers also reported that with two different disinfectants, benzalkonium chloride and the oxidizing agent sodium hypochlorite, the effective inhibitory concentrations on *S. aureus* biofilms are 50- and 600-times higher than planktonic cells, respectively [101]. The reason(s) for increased antibiotic resistance by bacterial biofilms is/are not yet fully understood. It is highly probable that multiple factors work together to protect biofilm cells from antibiotic treatment. Some of the possible mechanisms for antibiotic resistance exhibited by bacterial biofilms are as follows.

Limited antibiotic penetration

As previously described, EPS matrix plays an important role in antibiotic resistance by limiting antimicrobial agents' penetration into the biofilm. Charged polysaccharides and eDNA can trap several kinds of antibiotics. The penetration property of antibiotics has been measured by the concentration at the base of the biofilm by Suci and coworkers. Results showed that ciprofloxacin concentration in *P. aeruginosa* biofilm was dramatically reduced, but not completely blocked [102]. Steward and coworkers investigated the penetration limitation of ampicillin and ciprofloxacin on *K. pneumoniae*. Ciprofloxacin has a much better penetration capability than ampicillin. As a result,

biofilm cells could tolerate concentrated ampicillin but their resistance to ciprofloxacin is poor [103].

Horizontal gene transfer

Some bacteria can acquire antibiotic resistance via random mutations on genes. Others also harbor antibiotic-resistant genes on plasmids. Plasmids can be easily passed on to other cells by horizontal gene transfer. In biofilms, the frequencies of horizontal plasmid transfer are much higher than between planktonic cells. Studies on *S. aureus* biofilms showed that biofilms promote the spread of plasmid-borne antibiotic resistance genes by conjugation/mobilization [104].

Reduced growth rate

There is limited availability of oxygen and nutrients inside biofilms, so biofilm cells, especially those in the deep layers, have a slow metabolic rate, as well as growth rate and division rate. These features make biofilm bacteria insensitive to antibiotic drugs that target dividing cells. For example, the targets of β-lactams are dividing cells, so when they are used on *E. coli* biofilms, their bacteriolytic activity is diminished [105].

Persister cells

In biofilms, there is a small subpopulation of cells called persister cells [106,107]. Their growth rate is zero or extremely slow. Most of the antibiotics that are currently used in the clinic, which target processes that are relevant for cell growth or division, are not effective against persister cells. Therefore, these cells act as disease reservoirs that could reactivate into infectious particles once the antibiotic stress has been removed.

Efflux pumps

Efflux pumps allow bacteria cells to pump intracellular toxins out, including antibiotic drugs. Efflux pumps are also expressed in planktonic cells, but some efflux pump genes are upregulated in biofilm, indicating that they contribute to antibiotic resistance. Zhang and coworkers identified a novel *P. aeruginosa* efflux pump gene PA1874–1877 and the expression level of PA1874–1877 gene in biofilm is much higher than in planktonic cells [108]. Efflux pump encoded by this gene increases the resistance to tobramycin, gentamicin and ciprofloxacin.

EPS matrix protection

EPS matrix provides physical protection to the aggregated biofilm cells. Lei and coworkers showed that exopolysaccharide alginate in *P. aeruginosa* biofilms kept biofilm bacteria from human leukocyte killing [109].

Signaling in biofilm formation

Quorum sensing

In order to form a biofilm, bacteria have to synchronize their gene expression and some use **quorum-sensing** (QS) systems to achieve this. QS is a mechanism by which bacteria apparently monitor cell density and regulate collective behaviors [110]. It is worth pointing out that the issue of bacterial population monitoring via QS is still hotly debated and many alternative hypotheses have been proposed for the role of molecules that are involved in QS [111]. Bacterial populations coordinate their gene expression by producing and responding to a variety of intra and intercellular signals termed ‘autoinducers’ [112]. Bacteria ‘talk’ to each other using chemicals for ‘words’ including small molecules (Figure 11A). For example, acyl-homoserine lactones (AHLs) mediate QS in Gram-negative bacteria. There are several types of AHL, depending on the length and functionalization of the acyl side chain. Small peptides regulate QS-mediated gene expression in Gram-positive bacteria and AI-2 mediate QS in both Gram-positive and Gram-negative bacteria, although its role in cell signaling is still controversial [113]. At low cell densities, a small amount of autoinducer is present in the extracellular media; however, it is too dilute to be detected. As cell density increases, the autoinducer concentration reaches a threshold and at this point the autoinducer/regulatory protein (the receptor)

Key term

Quorum sensing: Cell – cell communication process that regulates gene expression at high cell density. At high cell density the concentration of an autoinducer, which is secreted by the bacteria to the environment, reaches to a certain threshold, which leads to gene expression and regulation of diverse physiological activities.

complex acts to induce or repress expression of target genes (Figure 11B) [114]. QS controls diverse physiological processes: for example, bioluminescence, secretion of virulence factors, biofilm formation, antibiotic resistance and more. QS has been shown to regulate biofilm formation in several bacterial species [115].

In PAO1 strain of *P. aeruginosa*, AHL QS systems are responsible for eDNA release and for biofilm structure [116]. In PA14 strain, the QS system regulates the production of PEL exopolysaccharide [117]. In *S. aureus*, the QS system regulates biofilm dispersal. The QS molecule in *S. aureus* is a peptide (AIP) derived from the product of the *agrD* gene, which is then detected by AgrC and activate the regulator AgrA. AgrA regulates the transcription of genes that code proteases that are involved in biofilm dispersal [118,119]. In *B. subtilis*, the QS molecule surfactin is important for biofilm formation [120]. Surfactin causes potassium leakage from the cytoplasm which triggers the expression of the genes involved in extracellular matrix production.

Figure 11. Quorum sensing. (A) Main quorum-sensing autoinducers. **(B)** Quorum-sensing circuit. AHL: Acylhomoserine lactone; AIP: Autoinducing peptide; AI: Autoinducer.

Figure 12. Cyclic dinucleotide signaling pathway.

c-di-GMP

C-di-GMP has emerged as an important second messenger involved in biofilm formation. It was originally found to allosterically activate cellulose synthase in *Acetobacter xylinus* [121,122]. This signaling molecule has been found to modulate a variety of bacterial growth phenotypes including but not limited to biofilm formation, motility and virulence factor production [123]. In the cell, c-di-GMP is synthesized from two GTP molecules by diguanylate cyclases characterized by a GGDEF domain, a set of highly conserved active site residues, whereas the second messenger is degraded into pGpG or GMP by phosphodiesterases (PDEs) containing EAL domain or HD-GYP domain, respectively (Figure 12) [122,124–127]. These enzymes function in concert to regulate the intracellular c-di-GMP levels.

The correlation between intracellular c-di-GMP concentration and biofilm formation has been observed in several clinically relevant bacteria (Table 1). For a detailed review of how fluctuating levels of cellular

c-di-GMP affect biofilm formation and some effector systems involved, the reader is referred to an excellent review by Boyd and O’Toole [128]. Briefly, in most bacteria the transition from motile to sessile mode of life is regulated by various effector proteins that bind c-di-GMP resulting in varying outputs via cell–surface protein localization, protein–protein interaction, transcriptional activation and DNA binding [128]. In *P. fluorescens*, biofilm formation is initiated when an inner membrane effector protein, LapD binds cellular c-di-GMP to facilitate the transport of the biofilm adhesion LapA to the cell surface [129,130]. Other studies have revealed that in the presence of RapA PDE (suggesting decreased cellular c-di-GMP levels), the secretion of LapA is inhibited and hence biofilm formation is abolished [131]. A protein–protein interaction was observed in *E. coli*, with YcgR protein integrating cellular c-di-GMP levels and interacting with components of the flagellar motor to modulate motile-to-sessile transition [132–134]. Other studies showed that a VieS/VieA

Table 1. Clinically relevant bacteria that use c-di-GMP second messenger.		
Bacteria	Diseases	Ref.
<i>Vibrio cholerae</i>	Cholera	[127]
<i>Pseudomonas aeruginosa</i>	Pulmonary and urinary tracts infections Burn injuries infections Blood infections	[137]
<i>Yersinia pestis</i>	Plague of Justinian Black Death Third Pandemic	[138]
<i>Klebsiella pneumoniae</i>	Pneumonia Urinary tract Lower biliary tract Wound infections	[139]
<i>Legionella pneumophila</i>	Legionnaires’ disease	[140]
<i>Vibrio vulnificus</i>	Cellulitis Septicemia	[141]

sensor kinase/response regulator pair function to reduce cellular c-di-GMP concentration and inhibit biofilm formation in *Vibrio cholerae* [135]. It was determined that VieA PDE degraded c-di-GMP when phosphorylated by VieS sensor kinase. The decreased cellular c-di-GMP concentration was concomitant with a repressed expression of *vps* (vibro exopolysaccharide synthesis) genes, responsible for EPS synthesis and biofilm formation in *V. cholerae* [135,136]. Previous studies also observed an increased *vps* gene expression and subsequent induction of biofilm formation either by overexpression of putative diguanylate cyclases or inactivation of VieA PDE; both of which correspond to a high cellular c-di-GMP concentration [127].

sRNAs

Small noncoding RNAs (sRNAs) also play an important role in the bacterial regulatory networks controlling the switch between planktonic mode and biofilm mode. The regulatory functions of sRNAs are achieved by base pairing with other RNAs or binding with proteins. After Romeo and coworkers found the RNA-binding proteins CsrA and sRNA CsrB and revealed their roles in *E. coli* biofilm formation and dispersal [142–144], more sRNAs have been identified to participate in the biofilm regulatory networks in many bacteria.

One well-studied example is *P. aeruginosa* GAC signal-transduction network (Figure 13). GacS is a transmembrane sensor kinase, which induces the phosphorylation of its cognate regulator, GacA. GacA phosphorylation is also promoted by sensor kinase LadS and blocked by RetS [145]. Phosphorylated GacA stimulates the production of two sRNAs: RsmY and RsmZ [146]. RsmY and RsmZ have multiple binding sites for the regulatory protein RsmA. As a translational repressor, RsmA binds to the target mRNAs to promote bacterial motility and repress biofilm formation. Sequestration of RsmA by sRNAs RsmY and RsmZ switches planktonic mode to biofilm formation.

The first identified global biofilm regulator is CsrA [142]. The structure of CsrA regulatory network in *E. coli* is similar to the previously described RsmA system. CsrA's target mRNA binding ability is inhibited by two sRNAs, CsrB and CsrC. In the absence of CsrB and CsrC sRNAs, CsrA represses mRNA translation and leads to increased cell motility. CsrB and CsrC sRNAs trap CsrA, enable mRNA translation and promote biofilm formation [148].

Another master biofilm regulator in *E. coli* and *S. Typhimurium* is CsgD. CsgD regulation is at transcriptional level, by selectively activating curli fimbriae and extracellular polysaccharides genes and repressing flagella genes [149]. The activity of CsgD is under the regulation of sRNAs (McaS, RprA,

Figure 13. *Pseudomonas aeruginosa* GAC regulatory network. The phosphorylation of regulatory protein GacA is activated by GacS and LadS, but inhibited by RetS. Phosphorylated GacA promotes the production of two sRNAs, RsmY and RsmZ. RsmY and RsmZ sequester RsmA and block its mRNA-binding function. In the absence of RsmY and RsmZ, RsmA binds to target mRNA, prevents translation and resulted in increased cell motility and decreased biofilm formation. Adapted from [147].

Figure 14. Biofilm aggregates of *Pseudomonas aeruginosa* in a chronic infected cystic fibrosis lung. Using a specific *P. aeruginosa* PNA fluorescence *in situ* hybridization probe, the bacteria are visualized in red, whereas the inflammatory cells surrounding the biofilm patches are counterstained with 4',6-diamidino-2-phenylindole, DAPI (blue). Reproduced with permission from [158] © Wiley-Liss, Inc. (2009).

Figure 15. Evidence of catheter-associated biofilm growth *in vivo*. (A) Original magnification $\times 200$ demonstrating the irregular undulating pattern of the biofilm surface. (B) Higher magnification of a tower from the image shown in (A) revealing a predominantly hollow interior with numerous cocci at the margins (original magnification $\times 800$). The image has been pseudocolored to highlight *Staphylococcus aureus* (gold) and presumably matrix material (green), which likely aggregated during the SEM dehydration process, from remaining biofilm structure (gray). The smooth surface at the upper right represents the catheter (salmon) with biofilm visible on the internal face. (C) Original magnification $\times 3000$ of an *Staphylococcus aureus* cluster within the tower depicted in (B). SEM: Scanning electron microscopy.

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OmrA/OmrB, GcvB, etc.). These sRNAs are produced in response to different environmental conditions. Some sRNAs act as CsgB repressors, such as OmrA/OmrB, but some are CsgB activators, such as ArcZ [150].

For more detailed information, the reader is referred to a more thorough review of the role of small regulatory RNAs and biofilm formation by Chambers and Sauer [151].

Biofilms *in vivo*

The dynamics of the microenvironment *in vivo* is different from that seen *in vitro*. This raises the question of whether observations from *in vitro* experiments are directly amenable to *in vivo* studies. It is therefore important to study biofilms *in vivo*. Animal models such as mice [152,153], rabbits [154] and rats [155] have been used to investigate the effects of biofilm-mediated infections. It is important to note that these models may not exactly depict conditions in a human host but can provide good predictive power.

One of such infection caused by *P. aeruginosa* is the catheter-associated urinary tract infection. Cole *et al.* demonstrated that the biofilms formed in a murine model of catheter-associated urinary tract infection were independent of extracellular polysaccharides [156]. This observation was achieved by using mutant PA14 and PAO1 strains, which lacked the ability to produce exopolysaccharides. The biofilm formed was found to be composed of eDNA, as DNase 1 treatment decreased the biofilm. Watters *et al.* used streptozotocin to induce diabetes in healthy mice. They infected a surgical excision wound with *P. aeruginosa* PAO1 and investigated the effect of the infection on the murine model wound healing [157]. It was observed that wound healing for diabetic mice was slow relative to healthy mice. Analysis of tissue extract of wound from diabetic mice revealed aggregates of *P. aeruginosa*. The biofilm did not respond to gentamicin treatment as

was expected. *P. aeruginosa* biofilms are also known to cause CF. Bjarnsholt *et al.* showed using various imaging techniques that *P. aeruginosa* biofilms were localized in the conductive zone of CF-infected lungs as opposed to the respiratory zone, which is enriched with polymorphonuclear leukocytes (Figure 14) [158]. In the conductive zone, the study suggested that the mucoid biofilms formed by the bacteria in the mucus are protected from antibiotics and host defense.

In an earlier study, Dalton *et al.* seeded multispecies biofilms from an *in vitro* culture into the wounds of mice to generate a polymicrobial wound infection [152]. This study also observed an impaired wound healing of wounded mice infected with the multispecies biofilm, and an increased tolerance to antimicrobial treatment. The *in vitro*-to-*in vivo* multispecies biofilm was made up of *S. aureus*, *P. aeruginosa*, *E. faecalis* and *Finnegoldia magna*. It was observed within 48 h postup that *P. aeruginosa* took over the infection and was typically present at the leading edge of the infection.

The response of the innate immunity system to biofilm infection has been investigated. *S. aureus* biofilms were established on surgically implanted catheters in a mouse model (Figure 15) [7]. The study observed that the biofilms evaded TLR2 and TLR9 which are bacterial recognition receptors used by macrophages. Host inflammatory responses such as TNF- α , IL-1 β and several others were found to be attenuated. The levels of iNOS, responsible for synthesizing reactive nitrogen intermediates which have microbicidal activities, were observed to be significantly reduced. A corresponding increase in arginase-1 expression levels was observed. In fact, the overall response of the innate immunity was observed to be altered; a plausible explanation for the persistence of biofilms *in vivo*.

Conclusion & future perspective

A major setback in the treatment of biofilm-related infections is the ineffectiveness of existing antibiotics due to the protective layers built by cells in the biofilm. There is therefore limited antibiotic penetration, so the community of sedentary cells persists even in the presence of antibiotics effective against their motile counterparts. The dispersion of matured biofilms may primarily require the disruption of the EPS matrix. Components of the EPS matrix such as alginate, Pel and Psl in *P. aeruginosa* biofilms could, therefore, be vital targets in the disruption of biofilm structure. With cells exposed, the drug compound may then exhibit bactericidal activity or act by dispersing the cells. On the other hand, the formation of new biofilms could be inhibited by preventing initial processes like attachment of cells to surfaces. This would be crucial in the development of medical and dental implants as they are easily colonized by biofilm-forming pathogenic bacteria. Nevertheless, the gap between observations made from an *in vitro* model and an *in vivo* model continues to be problematic. Choice of animal model, depending on how closely related the model is to humans, would be a critical parameter in the successful development of any antibiofilm drug.

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Executive summary

Background

- Biofilms are defined as aggregated microorganism communities attached to surfaces and embedded in a self-produced matrix which is composed of extracellular polysaccharide, DNA and proteins.
- There are three steps in biofilm formation: attachment, maturation and dispersion.
- The biofilm structure provides the bacteria with the ability to tolerate harsh environmental conditions, resistance to antibiotics and host immune systems, and provides an optimal environment for extracellular DNA (plasmid) exchange.
- Various human diseases are related to biofilm. Biofilms play an important role in dental diseases.

Signaling in biofilm formation

- Bacteria synchronize their gene expression by producing and responding to autoinducers. Quorum sensing regulates biofilm formation in several bacterial species.
- c-di-GMP is a second messenger that modulates a variety of bacterial growth phenotypes including biofilm formation.
- sRNAs are also biofilm regulators

Biofilms *in vivo*

- The microenvironment *in vivo* is different from that *in vitro*. It is important to investigate biofilms inhibition *in vivo* in animal models.

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