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Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies

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Bacteria have evolved the ability to form multicellular, surface-adherent communities called biofilms that allow survival in hostile environments. In clinical settings, bacteria are exposed to various sources of stress, including antibiotics, nutrient limitation, anaerobiosis, heat shock, etc., which in turn trigger adaptive responses in bacterial cells. The combination of this and other defense mechanisms results in the formation of highly (adaptively) resistant multicellular structures that are recalcitrant to host immune clearance mechanisms and very difficult to eradicate with the currently available antimicrobial agents, which are generally developed for the eradication of free-swimming (planktonic) bacteria. However, novel strategies that specifically target the biofilm mode of growth have been recently described, thus providing the basis for future anti-biofilm therapy.

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Introduction

Biofilms are structured aggregations of microorganisms associated with surfaces that have been widely studied over the past few decades in part because they cause 65% or more of all infections, being particularly prevalent in device-related infections, infections on body surfaces (skin and soft tissue, lung, bladder, endocarditis, etc.) and chronic infections [1–3]. They are particularly problematic due to their resistance to host defence mechanisms and to conventional antimicrobial therapy, which substantially hinders their treatment in the clinic [1–4]. From a broader perspective, biofilms are ancient phenotypic adaptations to the environment and are ubiquitous in Nature [1].

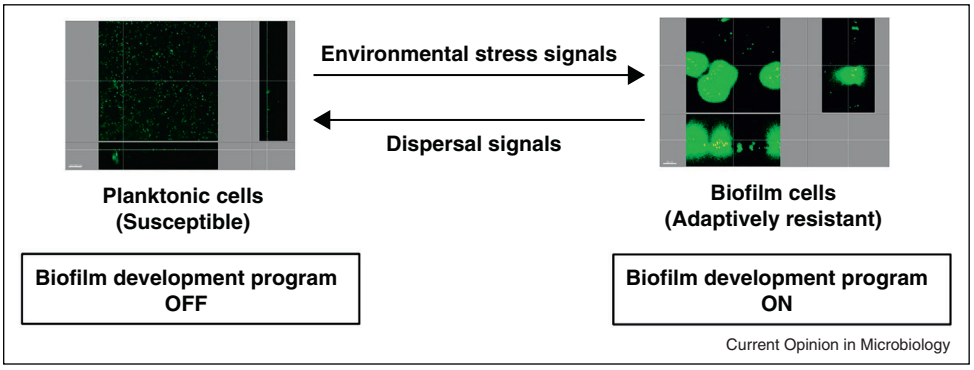
In this review, we will summarize the most recent advances in the field of biofilm research and analyze these findings from the perspective that biofilms have developed as a survival strategy of adaptation to environmental stress. In this context, we explain the most recent findings about the adaptive antibiotic resistance mechanisms displayed by biofilms, as well as the new therapeutic strategies aimed at effectively inhibiting and/or eradicating biofilms.

Biofilm formation: an adaptive response to hostile environments

Biofilms appear early in the fossil record (~3.25 billion years ago), and are common in a broad range of organisms, including not only bacteria but also archaea and eukaryotic microbes such as fungi [1]. The emergence of these primitive biofilms appears to have coincided with the first evidence of an evolutionary transition from unicellular to multicellular organization, which was provided by fossils of prokaryotic filamentous and mat-forming cyanobacteria-like organisms [1]. This suggests that bacteria that transitioned into a biofilm lifestyle might have been the first multicellular life forms. The deep evolutionary roots of this adaptation suggest that it was advantageous for survival in the harsh environment of early Earth. Indeed, a growing number of studies indicate that biofilm formation is closely linked to stress responses. With this in mind, we may speculate that around 3.25 billion years ago, a combination of stress signals triggered the activation of certain molecular pathways in bacteria. This eventually resulted in the emergence of the advantageous biofilm phenotype that increased the chances of survival under those particular conditions (Figure 1).

Thus while bacteria are usually thought of as free-living, unicellular organisms, we now know that they predominantly exist as adherent multicellular biofilms in diverse environmental niches including the majority of infections [1–3]. The transition from the planktonic state to biofilm growth occurs as a consequence of environmental changes that trigger the dysregulation of multiple regulatory networks [1–3]. Thus, upon sensing a stress signal, free-living (planktonic) cells will initiate attachment to a surface, which will lead to the formation of a biofilm that has a greater ability to withstand environmental challenges. Biofilm formation is therefore an environmentally driven developmental process that increases resistance to exogenous stresses, enabling bacterial survival under unfavorable conditions. Once the mature biofilm has developed, some cells within the population start to

Figure 1



Biofilm development initiates when flagella-propelled planktonic cells receive a stress signal from the environment. This stress signal, possibly combined with surface adherence, initiates the biofilm developmental program leading to increased (adaptive) resistance, enabling cell survival. Images shown correspond to *P. aeruginosa* biofilms grown in flow cell chambers, stained with SYTO-9 (green; live cells) and propidium iodide (red; dead cells) and visualized using confocal microscopy from above square panel and from two sides (bottom and right panels), demonstrating biofilm colonies as mounds arising from the surface.

dissociate (disperse) from the sessile structure [3]. This stage is designated dispersal and it is essential to complete the biofilm cycle by enabling the cells to spread and colonize new surfaces.

To avoid death of the entire population, the response to stress has to be faster than the adverse action of the stress signal. Therefore, the development of biofilms in response to stress signals must be an extremely rapid and efficient process. Even before a structured biofilm is evident, a 'biofilm program' is activated, as evidenced by the differential expression of an important number of genes [5,6]. Initially this might be a response to association with a surface, which can occur through adhesion organelles including pili, flagella and external microbial layers, or due to immobilization of the bacteria [2,3,7]. As bacteria start to grow they attach more firmly and the increased numbers of microbes can trigger so-called quorum sensing circuits that involve endogenous secreted signal molecules which, when accumulated to high enough local (threshold) concentrations, are taken up and trigger profound regulatory changes [3]. One such signal molecule is the quorum-sensing signal 3-oxo-C12-homoserine lactone of *Pseudomonas aeruginosa*, which is required for the efficient differentiation of planktonic into biofilm cells [8]. Mature biofilms eventually form and include prominent microcolonies that protrude from the surface, are held together by molecules termed collectively the extracellular matrix, including specific polysaccharides, proteins, and extracellular DNA (eDNA) [9], and contain water-filled channels enabling enhanced access of nutrients into the biofilm [1,2,10]. The biofilm program includes the production of these matrix components, that may be protective, as well as extracellular enzymes, enhanced induction of mechanisms for excreting toxic compounds (e.g. efflux pumps), as well as a change in metabolic processes, especially a decline in metabolism in bacteria deeper in the biofilm structure [1,2,3,10]. This suggests that bacteria possess a biofilm genetic program that is triggered by stressful conditions and propagated by association with surfaces and quorum sensing, impacting on both biofilm development and the characteristics of the resultant biofilm. Consequently, this program is an adaptation involving the triggering of regulatory circuits that cause temporary rather than permanent genetic alterations. We infer that biofilm formation is a deeply-wired genetic developmental process triggered by stress signals that has been selected through evolution to enable bacterial survival in harsh conditions.

Biofilm formation in Nature and in the clinic

Biofilms are formed in diverse environmental niches, including hydrothermal hot springs and deep-sea vents, freshwater rivers, rocks, etc. [1,2]. Additionally, these multicellular structures have been observed in various industrial and clinical settings [1,2,11,12,13•]. This suggests that the presence of stress signals in most natural and human ecosystems drives bacteria to exist predominantly within the protective milieu of a biofilm structure. Cells within biofilms have been shown to be resistant to many different environmental insults, including a range of chemically diverse biocides and antibiotics used in industrial and clinical settings, as well as UV damage, metal toxicity, anaerobic conditions, acid exposure, salinity, pH gradients, desiccation, bacteriophages, amoebae, etc. [1,2,14]. Importantly, biofilms play a fundamental role in infectious diseases as they can form on any body surface and persist after treatment with diverse antimicrobial agents [2]. Biofilm cells can also withstand host immune responses (both innate and adaptive), being particularly resistant to phagocytosis, and are between 10 and 1000-fold more resistant to treatment with most conventional

Table 1

Examples of different bacterial species involved in infections associated with biofilm development in immunocompromised patients and medical devices

Biofilm bacterial species	Surface	Disease/infections
Aerobic/anaerobic bacteria	Surface/deep skin	Chronic wound
Burkholderia cepacia	Lungs	Cystic fibrosis
Enterococcus faecalis	Heart valves Central venous catheters Urinary catheters	Endocarditis
Escherichia coli	Urinary tract Middle ear Prostheses	Urinary tract infections Otitis media
Haemophilius influenzae	Middle ear	Otitis media
Klebsiella pneumoniae	Central venous catheters	
Mycobacterium tuberculosis	Lungs	Tuberculosis
Pseudomonas aeruginosa	Lungs Middle ear Contact lenses Central venous catheters Prostheses	Cystic fibrosis Otitis media Nosocomial infections
Staphylococcus aureus	Middle ear Bones Sutures Central venous catheters Prosthetic heart valves Prostheses	Otitis media Musculoskeletal infections Nosocomial infections
Staphylococcus epidermidis	Surface/deep skin Heart valves Central venous catheters Prostheses	Chronic wound Endocarditis
Streptococcus sp	Tooth surfaces	Dental caries

antibiotics than their planktonic counterparts [2,14]. Indeed, antibiotic development pipelines rarely test the susceptibility of recalcitrant biofilm cells or utilize animal models in which bacteria form biofilm infections.

It is estimated that the majority of all medical infections are caused by bacterial biofilms that colonize either non-biological or biological surfaces [2,13•,14] (Table 1). Abiotic surfaces such as medical devices are commonly infected by biofilms. Examples include intravenous, endotracheal, Hickman and dialysis catheters, prosthetic heart valves, orthopedic devices, tissue fillers, cardiac pacemakers and cerebrospinal fluid shunts [1,2,13•,14]. Indeed, 60–70% of all nosocomial infections are due to the presence of biofilms on implants [15]. The microorganisms most frequently associated with medical devices are the staphylococci (particularly *Staphylococcus epidermidis* and *S. aureus*), followed by the bacterium *P. aeruginosa* and a plethora of other environmental bacteria that opportunistically infect hosts compromised by invasive medical intervention, chemotherapy or a pre-existing disease state [1,2,13•,14]. In addition, biofilms can associate with living biological surfaces, including those provided by the human body (Table 1). Indeed, biofilms play a significant role in human infections as diverse as dental caries, periodontitis, otitis media, chronic wounds, musculoskeletal infections, necrotizing fasciitis, biliary tract infection, osteomyelitis, bacterial prostatitis, native valve endocarditis, intra-amniotic infections, meloidosis, a wide range of nosocomial infections and cystic fibrosis (CF) pneumonia [1,2,12,13•,14–16].

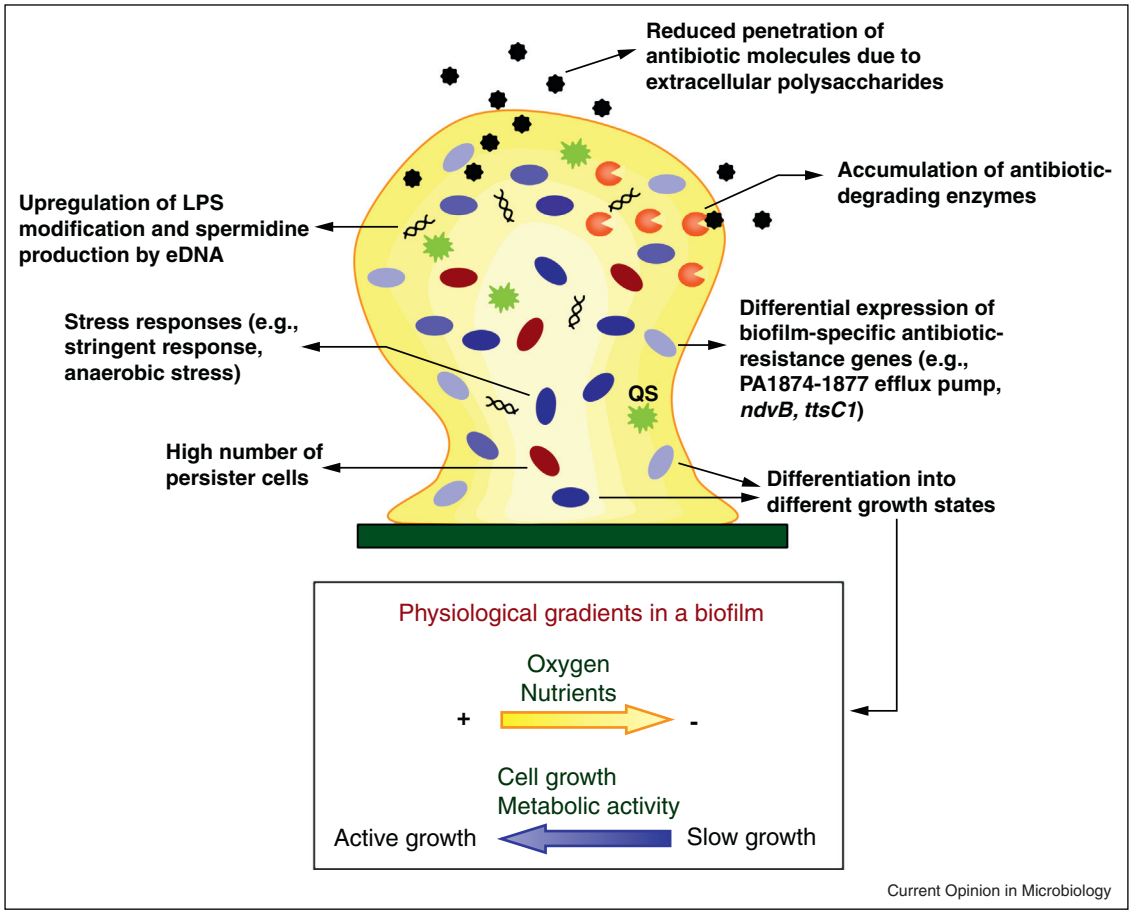
Bacterial colonization of the CF lung provides a good example of a biofilm-related infection. These lung infections are typically characterized by inflammation and tissue damage, as well as antibiotic and phagocytosis resistance [11,12]. In particular, the lower respiratory tract of adolescents and early adult CF patients becomes colonized and chronically infected, predominantly by the bacterium *P. aeruginosa*, a model organism for the study of biofilms due to its tendency to develop well-structured biofilms [12]. These infections lead to lung tissue damage as a result of the combined action of bacteria and increased inflammation, frequently resulting in the death of the patient (the median life expectancy of CF patients is about 30–48 years) [11,12,13•]. It has been

demonstrated that *Pseudomonas* can form biofilms in the lungs of CF patients, which clearly contributes to the difficulty of eradicating these infections. In this specific case, *P. aeruginosa* presumably develops biofilms in response to stress signals present in the CF lung environment, such as microaerobiosis or antibiotic pressure. Once the biofilm has fully developed, subsequent strategies of host defense (immune cells especially phagocytes, reactive oxygen species, etc.) and/or antimicrobial therapy are often ineffective and fail to completely clear the organism. As mentioned in the previous section, this ability to resist under conditions of extreme stress is inherent to the nature of biofilms. Consequently, considerable efforts have been made to attain a greater understanding of the mechanisms that explain this endurance, information that is critical to the development of novel therapeutic strategies.

Mechanisms of antibiotic resistance in biofilms

While many explanations have been advanced to explain the high antibiotic resistance displayed by bacterial biofilms, it constitutes a clear example of adaptive resistance, a phenomenon that is increasingly attracting the attention of clinical microbiologists [17]. The adaptive nature of biofilm resistance is evidenced by the fact that cells taken from a biofilm and brought back to the planktonic state generally recover their original susceptibility [18]. It is worth noting, however, that growth in a biofilm can favor the occurrence of processes that lead to the acquisition of inheritable resistance, such as horizontal gene transfer [19,20] or adaptive mutations [21]. The underlying mechanisms of adaptive resistance in the biofilm state are numerous and diverse (Figure 2). Some of these mechanisms are more general, as they relate to the drastically altered transcriptional program of biofilms or the inherent properties of biofilms, including their structure or composition, and as such they can be found in a wide range of species and affect the action of several antibiotic classes. In contrast, other mechanisms are very species-specific and/or antibiotic-specific. Here, we will give an overview of the different known factors contributing to biofilm resistance, most of which have been identified relatively recently.

Figure 2



Schematic representation of a *P. aeruginosa* biofilm indicating various examples of adaptive resistance mechanisms exhibited during this multicellular growth state. The box below shows the gradients of oxygen and nutrients formed within the biofilm structure and how they relate to cell differentiation into different growth states. Abbreviations: QS, quorum-sensing signal; eDNA, extracellular DNA.

Stress responses. As discussed above, biofilm formation is closely linked to the adaptations triggered by exposure to environmental stresses, with adherence and quorum sensing also playing a role. Stress responses (as well as adherence and quorum sensing) cause hundreds of genes to change expression. Some of these changes are induced to better enable the bacterium to resist harmful conditions, including chemicals such as antibiotics. We are increasingly appreciating that the resistomes for many bacteria, representing the total number of genes that, when altered in expression, lead to decreased susceptibility to a given antibiotic, can be quite extensive; for example, the tobramycin and ciprofloxacin resistomes of *Pseudomonas* involve more than 100 separate gene products [17].

Certain stress responses, such as those exhibited under starvation conditions, are known to confer increased antibiotic tolerance. It was originally thought that the slower growth rate of biofilms per se conferred a greater ability to survive an antimicrobial insult. However, recent evidence shows that the antibiotic tolerance associated with nutrient limitation is a tightly controlled response involving complex regulatory pathways. Indeed, Nguyen *et al.* [22••] recently demonstrated that the activation of the stringent response was essential for increased antibiotic tolerance during starvation in *P. aeruginosa*. Similarly, in *E. coli* biofilms, the stringent response was also found to participate in fluoroquinolone tolerance, although the possession of a fully functional DNA-stress SOS response is more important [23••]. In the case of *Pseudomonas*, the SOS response has been shown to be decisive for fluoroquinolone resistance in planktonic cells [24]; as a result, it would be interesting to determine whether it also plays a role in biofilms. Another stress response that has been related to antibiotic resistance in *Pseudomonas* is the heat shock response, which impacts on susceptibility to aminoglycosides through the intracellular protease AsrA [25]. Again, this link has only been demonstrated for planktonic cells, but it would be interesting to study its participation in the biofilm state. In other pathogens, such as *E. coli* and *Listeria monocytogenes*, genes encoding heat shock proteins are induced in biofilm cells; moreover, mutations affecting these genes exert a negative effect on biofilm formation [26,27]. Also worth noting are the adaptations related to growth under anaerobic conditions, as they too have been associated with antibiotic tolerance [28], and anaerobiosis evidently impacts on biofilm formation. It has been demonstrated that low-oxygen conditions can lead to the upregulation of certain efflux pumps in *P. aeruginosa* [29]. It would appear logical that such mechanisms might play a role in the resistance exhibited by the cells from the oxygen-deprived deeper biofilm layers.

Antibiotics themselves propagate adaptive resistance as it is well established that sub-inhibitory antibiotics lead to increased resistance [17]. In many cases, this has been shown to relate to altered regulation/expression of several resistance genes. Thus, any resistance that might occur due to other stress conditions, such as those encountered by cells in biofilms, would be amplified by antibiotic-mediated stress. For instance, exposure of *P. aeruginosa* biofilms to azithromycin induced the expression of the MexCD-OprJ efflux pump, although no such response was observed when planktonic cells were grown in the presence of this macrolide [30].

Heterogeneous population. Biofilm communities are very complex and typically consist of a heterogeneous population of cells in different growth states. This cell differentiation is proposed to be a consequence of the decreasing oxygen and nutrient gradients that exist between the surface and the deeper layers of the biofilm (Figure 2). Consequently, more metabolically-active cells at the surface and slow-growing cells in nutrient-deprived and oxygen-deprived layers exhibit markedly distinct responses when exposed to different antimicrobials. For instance, fluoroquinolones and tetracycline were only effective in killing the metabolically active cells in the upper layers of a *P. aeruginosa* biofilm [31]. In contrast, the lipopeptide colistin proved useful for the eradication of the slow-growing cells from deeper layers, but not the actively growing cells, which acquired adaptive resistance by upregulation of the LPS-modification (*arn*) operon [31].

Another phenomenon that contributes significantly to antibiotic resistance in biofilms is persistence, a property of the so-called persister cells, which are more numerous in biofilms than in planktonic populations [32,33]. Persister cells, which can withstand the presence of otherwise-inhibitory concentrations of antibiotics, are more able to overcome stressful conditions, including antibiotic challenges, likely due to transcriptional programming [32]. Indeed, possessing a large persister subpopulation has been described as the most significant resistance mechanism in *S. epidermidis* biofilms [33].

Extracellular matrix. Typically, the cells in a biofilm are embedded in a matrix of polysaccharides, extracellular DNA (eDNA) and proteins [10]. In addition to providing structural stability, this matrix has been reported to protect the cells from different agents, including antibiotics. Originally, it was thought that this was due to a filtration effect such that the antibiotics failed to effectively penetrate the matrix [34,35]. However, with increased understanding that the extracellular matrix has large water-filled spaces as well as channels, this seems increasingly less likely. On the other hand, it is possible that the matrix can facilitate the accumulation of antibiotic-degrading enzymes such as β-lactamases [36]. Conversely, increasing evidence indicates that the extracellular matrix might contribute by inducing additional adaptive resistance mechanisms. A clear example of this is

the effect of eDNA exposure on *P. aeruginosa* cells. Thus, chelation of divalent cations by subinhibitory levels of the negatively charged eDNA mimics growth in a Mg^{2+}-limiting environment and triggers the activation of the PhoPQ and PmrAB two-component systems. This, in turn, leads to aminoarabinosylation of the lipid A and makes the cells adaptively resistant to cationic peptides and aminoglycosides [37]. Exposure to eDNA also results in the accumulation of spermidine on the cell surface, which exerts a protective effect against polymyxins and oxidative stress [38•].

Specific mechanisms. While there are likely to be conserved regulatory mechanisms underlying biofilm adaptive resistance in multiple species, the effector mechanisms of resistance may vary considerably, although only modest characterizations have been undertaken to date. The identification of genetic determinants of species-specific or antibiotic-specific biofilm resistance has been facilitated by screening strategies targeted at finding mutants that form supersusceptible biofilms. Within this group, it is worth highlighting the participation of efflux pumps that are known to influence the rate at which antibiotics accumulate in cells and are synergistic with other mechanisms, such as low outer membrane permeability and β-lactamase mediated degradation. For instance, a novel pump from *P. aeruginosa* strongly influenced resistance to aminoglycosides and fluoroquinolones in biofilms but not in the planktonic state [39]. Another efflux pump, YhcQ, partly explained the increased resistance of *E. coli* biofilms [40]. Expression of this gene was regulated by the helicase-like protein RapA that also controlled the expression of another gene, *yeeZ*, necessary for biofilm-specific resistance. The *yeeZ* gene product has been thought to participate in polysaccharide production, thereby contributing by reducing antibiotic penetration into the biofilm, although as mentioned above this interpretation may not be correct. Nevertheless, in these cases, a biofilm-specific upregulation of efflux pumps could be observed. This is interesting in the context of recent data suggesting the participation of certain efflux pumps in the process of biofilm formation [41,42].

Another specific mechanism is the production of periplasmic glucans in *P. aeruginosa* biofilm cells, which requires the activity of the *ndvB* gene product. These glucans have been proposed to act by binding antibiotics before they reach their intracellular targets [43], although it was recently shown that they also increase the expression of ethanol oxidation genes, which confer a protective effect against tobramycin [44•]. Additionally, accumulation of these periplasmic glucans may have an impact on the Donnan potential, thereby affecting uptake of the antibiotics across the cell envelope. In other studies, a type VI secretion gene, *tssC1*, was found to be involved in biofilm antibiotic resistance, but not in biofilm formation, in *P. aeruginosa* [45].

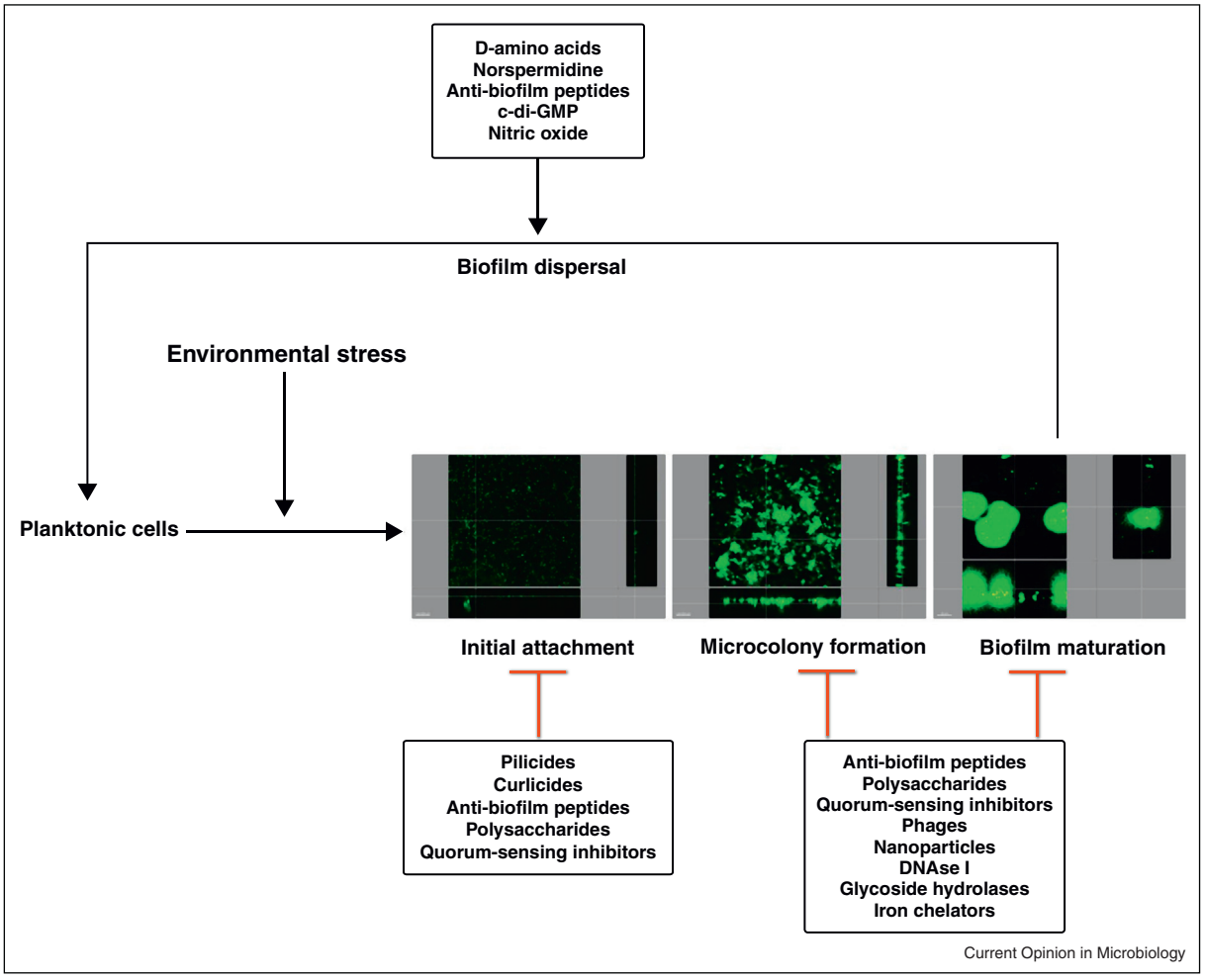
The above mechanisms highlight the diversity of the factors that participate in the decreased susceptibility of biofilm cells and there are many similar studies that we did not have the space to describe. Over the last few years, significant progress has been made towards understanding the nature of these mechanisms, despite the difficulties associated with the study of adaptive resistance due to its transient and often multi-genic nature. Nonetheless, considerably more work is required before we can fully decipher the molecular pathways that control this phenomenon and understand how it relates to biofilm formation and development.

New concepts in biofilm prevention and eradication

The traditional focus on discovering compounds that target the planktonic mode of growth, both in vitro and in vivo, and the insufficient level of understanding of the biofilm phenotype have resulted in a lack of available drugs that specifically target bacterial biofilms. In the clinic, biofilm infections are usually treated with combinations of antibiotics [11,12,13•,14]. Conversely, in the case of device-related biofilm infections, the device often has to be removed and replaced, a procedure that requires surgery, with all the costs, risks and complications involved [13•]. These treatments are clearly very aggressive but they represent the only truly effective solutions currently available to clinicians. However, recent efforts have provided novel strategies to both prevent and eradicate bacterial biofilms (Figure 3).

Cells within mature biofilms naturally produce compounds that induce their dispersal from the biofilm structure, to enable the colonization of new substrates; the resultant dispersed planktonic cells are then susceptible to conventional antibiotics. For example, Kolodkin-Gal *et al.* [46••] found that D-amino acids were produced by cells dispersing from *Bacillus subtilis* biofilms. Exogenous addition of low levels of these D-amino acids were then found to disrupt mature *B. subtilis* biofilms, and to additionally inhibit biofilm formation by the Gram-positive bacterium *S. aureus* and the Gram-negative pathogen *P. aeruginosa* [47]. Mechanistically, in *B. subtilis* D-amino acids were found to trigger the release of amyloid fibers (encoded by the *yqxM-sipW-tasA* operon), which form part of the matrix linking cells within the biofilm. The same group identified a second self-produced biofilm-dispersing molecule, the polyamine norspermidine [48•]. This molecule led to the disassembly of *B. subtilis* biofilms by targeting the exopolysaccharide present in the biofilm matrix. Norspermidine also prevented biofilm development by *S. aureus* and *E. coli*. Interestingly, combinations of D-amino acids and norspermidine were more effective at preventing biofilm development and disrupting mature biofilms than either of the compounds alone [48•]. The signaling molecule nitric oxide (NO) has also been shown to disperse biofilms. For instance, treatment

Figure 3



Examples of novel anti-biofilm therapeutics known to affect different stages of the biofilm developmental process. The black arrow in the upper box indicates molecules that work through induction of dispersal while the red lines represent inhibitory actions of the different molecules. The photographs represent confocal microscopy images of *P. aeruginosa* cells stained with SYTO-9.

of *P. aeruginosa* biofilms with non-toxic levels of exogenously added NO stimulates c-di-GMP-degrading phosphodiesterases, which induce a switch to planktonic growth [49].

Polysaccharides produced by bacteria are structural components of the biofilm extracellular matrix. Indeed, it is well known that these polysaccharides are responsible for mediating cell-to-cell and cell-to-surface interactions, which are key in the overall architecture of biofilms. Interestingly, recent studies have identified polysaccharides released exclusively by mature biofilms. These polysaccharides, added exogenously, limited biofilm formation by Gram-positive bacteria, and represent an interesting strategy for the prevention of biofilm development [50•].

Another strategy to target biofilms is the use of synthetic cationic peptide variants derived from natural peptides, based on the observation that the natural human peptide LL-37 and the bovine peptide indolicidin were able to inhibit biofilm formation and cause dissolution of bacteria in mature biofilms [51] at concentrations well below the MIC for planktonic bacteria. Intriguingly, although these peptides seem superficially similar to the cationic antimicrobial peptides (active against planktonic bacteria), containing cationic residues and a proportion of hydrophobic residues, these activities can be clearly distinguished. Indeed, peptides with good anti-biofilm but little anti-planktonic activity have been isolated and vice versa. Moreover, anti-biofilm peptides are active against biofilms of *Burkholderia cenocepacia*, which is completely resistant to killing by all antimicrobial peptides in the planktonic state [52•]. Peptides active on biofilms are increasingly being reported [53]. The smallest appears to be a peptide of only 9 amino acids in length that can effectively prevent biofilm

formation by *P. aeruginosa*, *B. cenocepacia* and the Gram-positive microorganism *L. monocytogenes* [52•], despite very high MICs for planktonic bacteria [52•]. Similar peptides have also been shown to exhibit immunomodulatory and anti-inflammatory activities [54], although it has not been established if this is an additional property of these peptides (except LL-37).

Other strategies have been recently explored to target bacterial biofilms, although many of these are anticipated to be relatively species specific in their action on biofilms. For instance, altering the biofilm developmental process might also be achieved by interfering with the signaling pathways involved. For example, targeting the quorum-sensing circuitry is a potentially attractive anti-biofilm approach that is being pursued [55–57]. In addition, interfering with the second messenger signaling pathway (c-di-GMP, cAMP) has proven to be effective at inhibiting *Pseudomonas* biofilms [58,59]. Enzymes that interact with essential biofilm components are also of interest as anti-biofilm therapeutics. Some examples include DNAse I, an enzyme that degrades eDNA, an essential structural component in developing biofilms [9]. Moreover, a glycoside hydrolase produced by *Actinobacillus* biofilms was capable of breaking down the β 1–6-N-acetylglucosamine polymers present in the peptidoglycan layer of bacterial cells. This enzymatic reaction led to the inhibition of biofilm formation without affecting growth and also dispersed preformed *Actinobacillus* biofilms [60,61]. Surface appendages necessary for cell adhesion to other cells and surfaces and subsequently biofilm development have also been exploited as anti-biofilm targets. Thus, treatment with compounds that blocked biogenesis and/or assembly of pili and curli inhibited *E. coli* biofilm formation [62]. Other innovative strategies for the treatment of bacterial biofilms include the use of enzymatic bacteriophages [63], iron chelators (iron being essential for biofilm formation) [64], and nanoparticles [65,66].

All the examples provided here show how our greater insight into the molecular mechanisms that participate in biofilm formation and dispersal has been essential for the development of new therapeutic strategies that specifically target these structures.

Conclusions and future directions

Throughout their evolution, bacteria have gradually adapted to endure situations of environmental stress. One such adaptation entails the formation of biofilms, multicellular specialized structures that have become very efficient at tolerating external insults. On the basis of our current knowledge about the biology of biofilms, we can speculate that the evolutionary adaptation to the biofilm mode of growth may have been driven by stress signals present in the natural environment. One consequence of this adaptation is the adaptive resistance to antimicrobial compounds that is displayed by biofilm-forming cells. This characteristic has made biofilms a particular challenge for the treatment of infectious diseases linked to biofilm formation. However, during the last decade we have made considerable progress in the understanding of the signalling pathways and molecular mechanisms that govern the cycle of biofilm formation and dispersal. This knowledge has opened the door to the development of new therapeutic strategies directed at inhibiting biofilm formation and inducing biofilm dispersal. With this in mind, we anticipate that in the near future some of these therapeutics will be introduced into clinical trials and eventually help in the treatment of biofilm-related infections. At the same time, further research on the molecular biology of biofilms and the exogenous stress signals responsible for their formation will be decisive in discerning the role that these structured colonies play in the bacterial world. Furthermore, it is tempting to speculate that this knowledge might perhaps hint at the processes that led to the origin of multicellular organisms.

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- • of special interest
- •• of outstanding interest

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