

# Mechanisms and Control Measures of Mature Biofilm Resistance to Antimicrobial Agents in the Clinical Context

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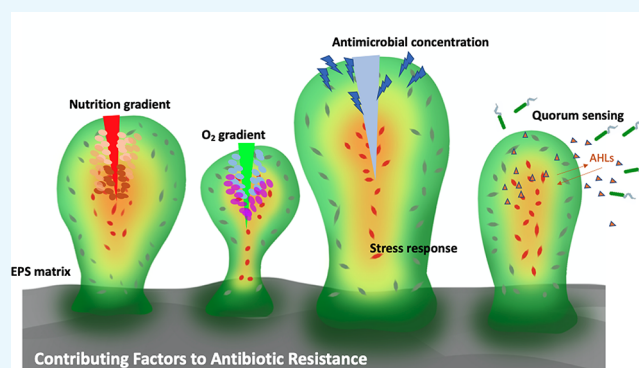
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**ABSTRACT:** Biofilms are the aggregation of micro-organisms, which are composed of extracellular polymeric substance (EPS) and many other biochemical components. Though they might be beneficial to some wastewater and soil treatment processes, they may expose chronic infection and risk to personal hygiene in the industrial as well as the clinical context. Despite having a well-established disinfection and hygiene monitoring program for the prevention of formation and growth, biofilm persistently remains in the medical settings because of its antibiotic resistance to antimicrobial agents and even the immune system. In this paper, the contributing factors of antibiotic resistance and the corresponding mechanisms, including heterogeneity inside biofilms, the roles of the EPS matrix, cell density, and quorum sensing, and cell mutability, are reviewed. Moreover, current clinical practice and strategic applications are also suggested to address the biofilm resistance issues.



## INTRODUCTION

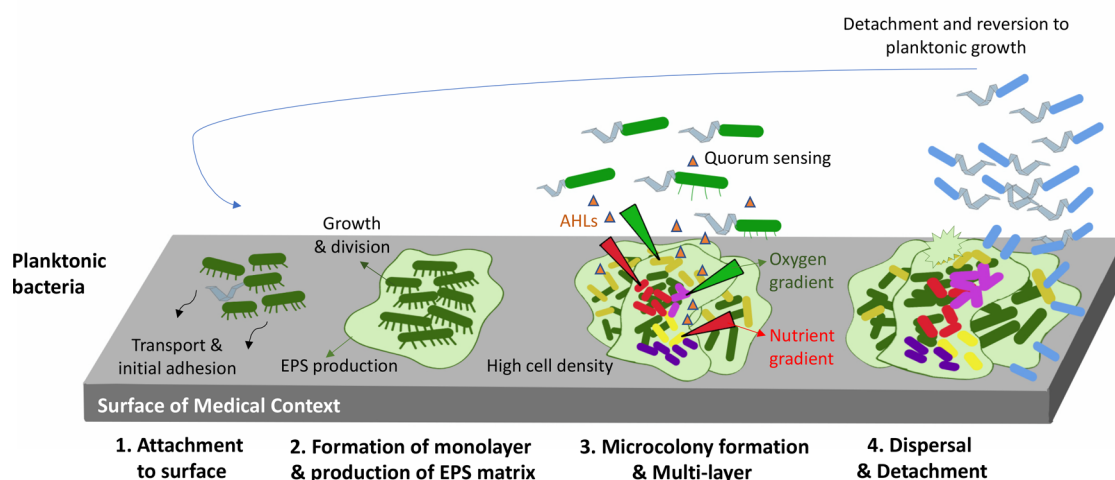
Biofilms are aggregations of micro-organisms living in an extracellular matrix, which are composed of extracellular polymeric substances (EPS), including polysaccharides, proteins, lipids, nucleic acids, and other minor components, at a liquid interface. Although biofilms could be beneficial to some processes such as wastewater treatment, they are more known for their problematic nature: in clinics, biofilms often account for chronic infections; in industries, biofilms may cause the clogging of filters and corrosion of pipes. The removal of biofilms is remarkably difficult using antimicrobial agents, which exhibit perfect eradication outcome on planktonic cells. Biofilm resistance to antimicrobials is also another ubiquitous problem across diverse fields, ranging from medical settings to surgical environments. The capability of biofilms to protect and preserve themselves, including dangerous or opportunistic pathogens, has received great attention in recent years as multi-drug-resistant “superbugs” can even overcome the last resort, colistin.<sup>1</sup> Sensational in nature, the surge of interest in medical biofilm emphasizes the focal term—antibiotic resistance. In fact, in biofilm-related infections, biofilm-growing cells are resistant not only to conventional antibiotic treatment but also to the host’s immune system, causing the recurrence and recalcitrance of the infection.<sup>2</sup> In another biofilm habitat, medical systems and even personal protective equipment are known to harbor pathogens. Despite having well-established disinfection and monitoring programs, biofilms also persistently remain in the

medical settings, serving as a protective barrier for microbials and containing a seed reservoir for pathogens.<sup>3</sup>

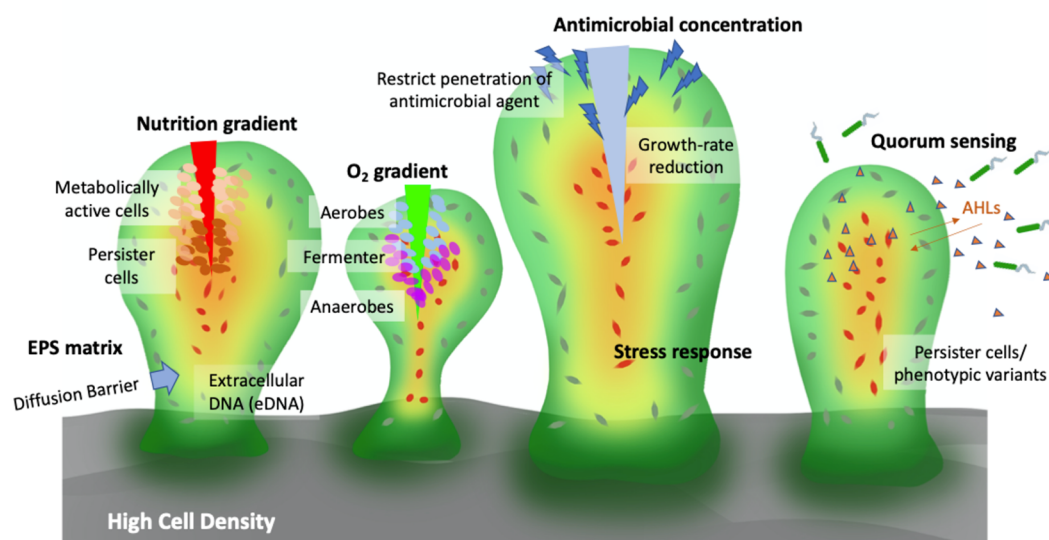
Typically, the biofilm attachment, formation, and detachment (Figure 1) begin with (i) the transport and initial adhesion of the planktonic bacteria through the adsorption of suspended particles and organic species from the bulk fluid, (ii) transport and attachment of the planktonic cells, (iii) microbial multiplication and EPS production, and (iv) dispersal and detachment of the mature clusters. It was estimated by the National Institutes of Health (NIH) back in 2002 that more than 80% of microbial infections in the human body are associated with biofilms, and about 65% of chronic infections are biofilm-related, revealing the medical importance of biofilms.<sup>4</sup> Biofilm infections are particularly significant in foreign bodies such as intrauterine catheters, pacemakers, and stents and in cystic fibrosis (CF) patients infected with biofilm-growing mucoid strains of *Pseudomonas aeruginosa*.<sup>5</sup> Persistent biofilm infections lead to tissue damage because of the combined action of pathogens and the indiscriminate release of oxidative species by the phagocytes.<sup>2,6</sup> However, it needs to be

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**Figure 1.** Schematic representation of biofilm formation on a medical context surface.



**Figure 2.** Schematic representation of antibiotic resistance of bacterial biofilms.

determined whether the EPS matrix becomes truly the main contributing factor to biofilm resistance or other factors could be at play; for example, are the biofilms heterogeneous in terms of their stratification and cell populations and are the metabolic activities controlled by concentrations of nutrients and oxygen availability within biofilms. Hence, the increasing tolerance of bacterial biofilms toward antimicrobial agents may also reinforce the idea of a metabolic growth rate heterogeneity inside the biofilm.

In this report, the causes of mature biofilm resistance to antimicrobials (e.g., antibiotics, biocides, heterogeneity inside biofilms, EPS matrix, and quorum sensing) are separately discussed in clinical habitats. Hypotheses, including diffusion limitation, physiological adaptation, protected biofilm phenotypes, persisters, and other species-specific or antibiotic-specific resistance mechanisms, will be examined with suggestions of plausible solutions.

## ■ ANTIBIOTIC RESISTANCE OF BACTERIAL BIOFILMS

Several mechanisms have been proposed and investigated to explain the remarkable resistance of biofilm-growing bacteria to antibiotic therapy and phagocytosis, as indicated in Figure 2. Bacteria have stratified metabolic activities inside a biofilm due to the concentration gradient of nutrients and oxygen, making the deeper cells in the biofilm become less accessible to nutrients and oxygen.<sup>7</sup> Many antibiotics are targeting the actively growing bacteria; therefore, less active bacteria will be inherently less susceptible to antibiotics. The nutrient limitation also triggers the stress responses in bacteria, leading to altered gene expressions and increased antibiotic tolerance.<sup>8</sup> The EPS matrix may act as a protective shelter, a diffusion barrier, and a reservoir of enzymes that can degrade antibiotics. Components like extracellular DNA (eDNA) may also play a role in the resistance by triggering the activation of certain cellular systems.<sup>7</sup> The high cell density and proximity nature of cells living in a biofilm trigger the quorum sensing (QS) circuits, which will detect as well as react to cell density via

gene regulation. QS influences the development of the biofilm and regulates the production of virulence factors such as enzymes or toxins, which are important for the phagocytosis resistance.<sup>8</sup> Increased mutation and horizontal gene transfer rates are also observed in biofilms as a combined result of high cell density and increased oxidative stress.<sup>9</sup> The existence of a subpopulation called “persister cells”, which can survive transiently under the lethal effect of antibiotic treatment, also contributes to the extraordinary resistance of biofilms.<sup>10</sup> Other species-specific and/or antibiotic-specific mechanisms have also been widely explored.<sup>11,12</sup>

Before the discussion about the causes of biofilm resistance, several definitions should be clarified to obtain a better understanding of this report. The first term is “antibiotic resistance”, which is defined as the inherited ability acquired through genetic mutations to survive and multiply under the lethal effects of antibiotics for bacteria cells. Antibiotic resistance, which occurs through permanent genetic modifications and several mechanisms for antibiotic resistance in planktonic cells, has been justified, including modifications of the antibiotic targets, enzymatic inactivation of antibiotics, and the increased activity of efflux pumps. The next term is “adaptive resistance” used by de la Fuente-Núñez et al.<sup>13</sup> to describe the temporary genetic alterations that lead to the resistance exhibited by bacteria in biofilms, but it will disappear after the bacteria return to the planktonic state. When we talk about the resistance in biofilms, we normally refer to the bacteria resistance regardless of whether it is permanent or transient.

**Heterogeneity Inside Biofilms. Concentration Gradient.** There is a clear stratification in the bacterial metabolic activity inside the biofilm due to the different concentrations of nutrients and oxygen accessible to cells at the surface and deep in the biofilm, as proven by the work done by Sternberg et al.<sup>8</sup> In their study, fluorescent tags were used as reporters for specific metabolites to demonstrate the growth activity and the position of single cells in biofilms in real time. The results showed that cells in the center had down-regulated growth activity compared with cells at the bulk liquid interface. The growth activity of the less active cells in the biofilm was successfully restored when adding proper nutrients, indicating that nutrient availability is a critical factor for biofilm-growing bacteria to perform the metabolic activities. In another correlated work, de Beer and colleagues constructed the oxygen concentration profiles with varying depths of biofilms using microelectrodes.<sup>14</sup> The results demonstrated that the oxygen distribution was strongly associated with the biofilm structure, and the oxygen was decreased by as much as 30-fold in the center of larger microcolonies. These studies indicate that nutrients, oxygen, and other essential compounds will be depleted toward the center of the microcolonies and lead to the stratified metabolic activity, growth rate, and gene expression. Antibiotics such as  $\beta$ -lactams will only work on dividing bacterial cells, and they are not efficient against those more or less dormant cells in the biofilm.

The nutrient limitation not only changes the growth activity of the bacteria but also triggers the activation of certain stress responses, which are known to confer increased antibiotic tolerance or resistance. Recent studies show that the antibiotic tolerance induced by nutrient limitation is not simply dependent on the result of reduced metabolic activity, it is more dependent on a firmly controlled response involving complex regulatory pathways. It has been reported that the

stringent response is involved in the increased antibiotic tolerance in starved *P. aeruginosa* and the fluoroquinolone tolerance in *Escherichia coli* biofilms.<sup>5</sup> The survival response and the heat shock response have also been associated with the fluoroquinolone resistance and aminoglycoside resistance in planktonic *Pseudomonas*,<sup>6</sup> but how these stress responses will participate in the resistance in the biofilm state is still awaiting further investigation.

**Persister Cells.** There exists a subpopulation of bacterial cells called “persister cells”, which are described as genetically identical to the active cells. However, due to their different physiological states, these cells are more dormant and tolerant to the antibiotics. The mechanism inside persister cells is complex. The presence of persister cells has been considered as a neglected mechanism for the recurrence of infections. It has been reported that persister cells are able to exist in the exponentially growing bacterial population before the antibiotic treatment is applied. Thus, it has been proposed that the formation of persister cells is an adaptive strategy used by bacteria to cope with the possible environmental change, so that they can resume their growth quickly once the environmental stress is withdrawn. Persister cells have been described in several bacterial species such as *Mycobacteria* and *Borrelia*, and they have been considered to be the most important resistance mechanism in *Staphylococcus epidermidis* biofilms.<sup>15</sup>

The presence of persister cells possesses a great challenge in the antibiotic treatment of biofilms as different phenotypes are proven to exist. The state-of-the-art antipersister strategy is to sensitize persister cells by introducing specific carbon sources and terminal electron acceptors.<sup>16</sup>

**Roles of the EPS Matrix. Diffusion Barrier.** It was originally believed that the reduced penetration of antibiotics confers the resistance in biofilms. To investigate how the EPS matrix would affect the antibiotic penetration, Suci and colleagues used a germanium crystal substratum in an infrared (IR) field to determine the penetration ability of ciprofloxacin through the biofilm of *P. aeruginosa*.<sup>17</sup> The germanium crystal was IR-transparent, and the IR radiation could travel unimpeded to the distance at a maximum of 0.2  $\mu\text{m}$  above the substratum. Once antibiotics penetrated the distance of 0.2  $\mu\text{m}$  above the substratum, IR signals of the compound would be generated and detected, and it could be also monitored in situ and in real time. The results showed that the biofilm could significantly reduce but not entirely block the antibiotic penetration. The subsequent study conducted in this group revealed that the penetration rate of antibiotics through biofilms was dependent on the chemical nature of antibiotics and did not directly account for the biofilm recalcitrance. These results also suggested that decreased antibiotic penetration might be crucial for the reduced efficacy of certain antibiotics, but this reduction was not supposed to account for the overall resistance. Another important role that this diffusion barrier nature played was to help with the accumulation and retention of enzymes that could degrade antibiotics in the extracellular matrix. For the enzyme,  $\beta$ -lactamase, which was overproduced by *P. aeruginosa* biofilms, was present in the biofilm matrix and might destroy the functionality of  $\beta$ -lactams before they could reach the bacteria cells or at least reduce the possibility that  $\beta$ -lactams can be in touch with cells. The  $\beta$ -lactamase released into the extracellular space from the lysed cells due to the antibiotic exposure or from the membrane vesicles.

**Additional Induced Resistance Mechanisms.** Extracellular DNA is present in the EPS matrix as structural support for the biofilm architecture. Recent studies have reported that eDNA contributes to the increased resistance in biofilms by inducing additional resistance mechanisms. Mulcahy et al.<sup>7</sup> have shown the extracellular DNA induced antibiotic resistance by contributing to cation gradients and the release of genomic DNA. eDNA can chelate cations that stabilize lipopolysaccharide and the outer membrane, leading to cell lysis with the release of cytoplasmic content and genomic DNA. The increased DNA concentration in the biofilm matrix created cation limitation, which led to the induction of the PhoPQ- and PmrAB-regulated cationic antimicrobial peptide resistance operon PA3552–PA3559 in *P. aeruginosa*. The DNA-induced expression of this operon greatly increased the resistance to cationic antimicrobial peptides and aminoglycosides with no impact on  $\beta$ -lactam and fluoroquinolone resistance.

**High Cell Density and Quorum Sensing.** Bacteria live in high cell density and proximity in biofilms. This high cell density has been suggested to account for part of the enhanced resistance of biofilms to antibiotic treatment. Larsen<sup>18</sup> tested the susceptibility of planktonic *Porphyromonas gingivalis* to amoxicillin, doxycycline, and metronidazole using cell densities equal to the ones identified in biofilm populations ( $10^7$  to  $10^8$  cells/mL). Increased minimum inhibitory concentrations (MICs) have been observed for planktonic cultures of equal cell numbers to the population in biofilms, suggesting that there is an inoculum effect on the increased resistance of biofilms. It is also noted that the minimum bactericidal concentrations (MBCs) of biofilms were at least 2-fold greater than the MBC values for planktonic cultures. The molecular mechanism behind this inoculum effect has been speculated to be quorum sensing. QS describes the ability of bacteria to sense and respond to changes in cell density via various regulations. QS influences the development of biofilms and regulates the production of virulence factors including extracellular enzymes and cellular lysins such as rhamnolipid, which are critical for the phagocytosis resistance in *P. aeruginosa* biofilms. The use of QS inhibitors has been proposed as one of the strategies to overcome the resistance of biofilms.

**Mutation.** Increased mutability and horizontal gene transfer have been reported in the biofilm compared with the planktonic state.<sup>9</sup> This increased mutability has been associated with increased oxidative stress in biofilms. The increased production of endogenous reactive oxygen species and oxidative burst from the immune system together with an insufficient antioxidant defense lead to the overall increase of oxidative stress. The oxidative stress has also been linked with the occurrence of hypermutable *P. aeruginosa* strains in CF patients. Boles and Singh<sup>19</sup> found that endogenous oxidative stress led to the break of double-stranded DNA, which could be repaired via a mutagenic mechanism involving recombinatorial DNA repair genes, thus generating genetic variants. They also demonstrate that the addition of antioxidants can reduce the occurrence of genetic variants in the biofilm.

## NOVEL STRATEGIES FOR BIOFILM RESISTANCE CONTROL

The current clinical practices to prevent chronic *P. aeruginosa* biofilm infections in CF patients include prevention of cross-infection from other chronically infected CF patients by patient isolation and hygienic measures, early aggressive

antibiotic treatment to eradicate intermittent colonization, and daily nebulized DNase to reduce the viscosity of the sputum. When chronic *P. aeruginosa* biofilm infections are developed, the recommended remedy becomes to apply chronic suppressive antibiotic therapy together with daily nebulized DNase.<sup>20</sup> This maintenance method has gained clinical success in terms of slowing down the degradation of the pulmonary function and extending the lifespan of patients. However, the side effects are very obvious; that is, the continuous antibiotic treatment leads to a high level of resistant/persistent strains and possible allergic adverse events. Høiby et al.<sup>21</sup> have suggested popularizing the practices used in chronic *P. aeruginosa* biofilm infections to foreign-body biofilm infections by applying prophylactic antibiotic treatment to prevent the biofilm formation, early aggressive eradication of planktonic cells or early stage biofilms and chronic suppressive antibiotic treatment to maintain the function of an inserted medical device. The trend of biofilm resistance research is still using different compounds to destroy the biofilm matrix and release the cells back to the planktonic state. It is assumed that the susceptibility to conventional antibiotics will be restored once the bacteria become planktonic. Quorum sensing inhibitors have also been extensively studied to interfere with biofilm development and the formation of persisters. Other nonbiological agents such as electrical current and antimicrobial coating have also been applied in the battle against biofilm resistance, as indicated in Figure 3.

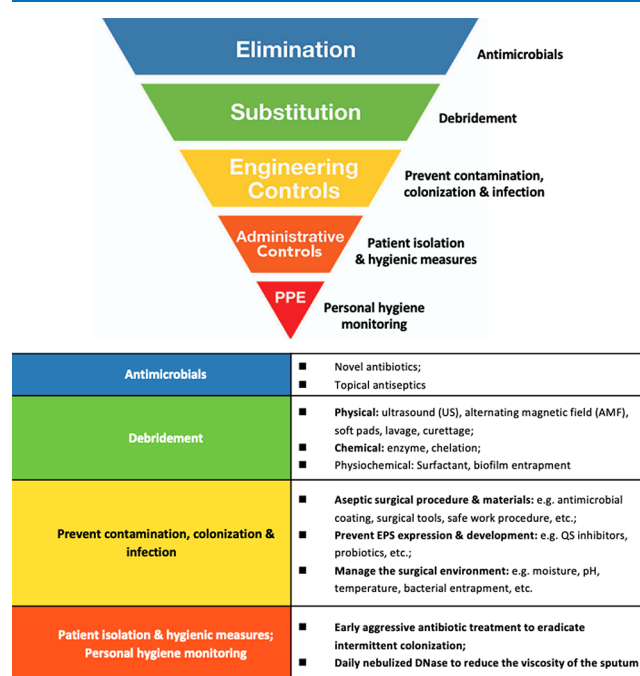


Figure 3. Strategies for biofilm resistance control in a clinical context.

**Compounds to Disrupt the Biofilm Matrix.** A number of studies have investigated the ability of different compounds to disrupt the biofilm matrix and hence increase the efficacy of conventional antibiotics. DNase and alginate lyase have been introduced to the *P. aeruginosa* biofilms to dissolve the biofilm matrix, and increased efficacy of tobramycin was shown in terms of the ability to reduce the sputum bacterial counts.<sup>22</sup> Some biofilm-dispersing molecules have also been used to disrupt the biofilm matrix.<sup>23</sup> Dispersin B is capable of

degrading the poly-*N*-acetylglucosamine component of the biofilm matrix. Dispersin B, together with antibiotics, indicates efficacy in the prevention of bacterial infections.<sup>24</sup> One thing that should be noted is the possibility of pathogen release after the biofilm matrix has already been disrupted, as there is increasing evidence showing the existence of intracellular pathogens in both phagocytic and nonphagocytic resistance.<sup>25</sup>

**Inhibition of Biofilm Formation: Synthetic Cationic Peptides and Quorum Sensing Inhibitors.** It has been observed that the natural human cationic peptide LL-37 and bovine neutrophil peptide indolicidin, which are part of the host defense system, could inhibit the biofilm formation in vitro at a very low concentration and have an impact on existing *P. aeruginosa* biofilms. Therefore, the use of synthetic cationic peptide variants has been proposed as antibiofilm agents, and the smallest peptide being reported with this property is only nine amino acids in length that can significantly prevent biofilm formation by *P. aeruginosa*, *B. cenocepacia*, and *L. monocytogenes*.<sup>26</sup> In addition, azithromycin (macrolide), ciprofloxacin (fluoroquinolone), and ceftazidime ( $\beta$ -lactam) are also found to exhibit strong quorum sensing inhibitory activity using transcriptomic and phenotyping analysis.<sup>27</sup> The use of quorum sensing inhibitors (QSIs) has been proposed as a solution with great potential to solve the current antibiotic resistance crisis. According to the current understanding, QSI resistance can only occur due to mutations, which render the QS-deficient bacteria unable to produce virulence factors; that is, the bacteria become nonvirulent, similar to the outcome of the QSI therapy. If this holds, then the antibiotic resistance crisis we face today will be greatly relieved.

Some macrolide antibiotics such as azithromycin have been reported to inhibit QS in *P. aeruginosa* at sub-MIC concentrations, which could inhibit the production of the virulence factors. Most CF patients with chronic *P. aeruginosa* lung infections are therefore now treated continuously with azithromycin. However, the use of antibiotics as QSIs is expected to develop resistance in other pathogenic bacteria such as *S. aureus* in CF patients. QSIs without conventional bacteriostatic or bactericidal properties are, therefore, desired.<sup>28</sup> One example of such a QSI has been found in garlic extract, which both in vitro and in vivo have been able to render otherwise resistant *P. aeruginosa* biofilms susceptible to antibiotic therapy and to immune response and eradication of the biofilm both with the effort of antibiotic therapy and polymorphonuclear neutrophils, which dominates the inflammatory response in CF patients. It is also noted that the usage of those probiotics will also prevent gene expression of various bacterial factors like bioluminescence, antibiotic biosynthesis, plasmid conjugation, and virulence, finally preventing EPS expression and development.

**Anti-adhesion Coatings.** There are mainly four chemical cleaning methods that are commonly applied to clinical surfaces for the removal of biofilms. These methods may include detergent, hydrogen peroxide cleaning, bactericidal/bacteriostatic, or anti-adhesion coatings. Typically, the anti-adhesion coating can prevent biofilm formation at its early stage, which should be more desirable in clinical settings. The anti-adhesion coating surface generally needs considerations for four characteristics, which includes chemical composition and reactivity, hydrophilic/hydrophobic wettability, surface textures, and surface charges.<sup>29</sup> For example, the recent research obtained by Li et al.<sup>30</sup> reveals that the modified

polyurea antibiofouling coating indicates a hydrophobic characteristic; the nanotitanium dioxide may generate reactive oxygen species to kill the bacteria, and the riblet surface textures formed by nanotitanium dioxide are able to enhance the drag reduction effect and the antibiofouling performance, as well. Moreover, such coatings may extend the maintenance interval and exhibit its commercial values for practical applications.

## ■ CONCLUSION AND POSITION

The increased resistance of biofilms has been shown to have a multilayer defense. The most promising direction to solve the resistance problem is likely to be a combination therapy, either a combination of different antibiotics or antibiotics with compounds that can disrupt the biofilm matrix. For example, colistin works against nondividing the central part of *P. aeruginosa* biofilms in vitro, whereas the active cells on the surface of the biofilm are susceptible to ciprofloxacin. The experimental results have shown that this combination therapy was able to kill all cells in the biofilm in vitro. Clinical efficacy of this combination therapy has also been demonstrated for the early eradication treatment of *P. aeruginosa* in CF patients. The future treatment strategies require a deeper understanding of the biofilm formation process, biofilm mechanics, and the cellular behavior of different cells in the biofilm. The improved understanding at both an individual and a collective level could lead to a breakthrough in the battle against harmful biofilms.

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### Notes

The authors declare no competing financial interest.

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