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Journal of Advanced Scientific Research

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ISSN
0976-9595
Review Article

COMBATING STRATEGIES OF ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA* BIOFILM: A REVIEW

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ABSTRACT

Antibiotic resistant pathogen pose an ever increasing threat to mankind at any time of cost. The bacteria accumulate and encased in an extracellular matrix are called a biofilm and are challenging to eradicate with antibiotic treatment. *Pseudomonas aeruginosa* biofilm is particularly deadly in cystic fibrosis patients and is the root cause of chronic wounds, chronic -obstructive pulmonary disease (COPD), and medical device-related infections. Recent studies have shown that *P. aeruginosa* biofilm reported a higher prevalence in causing drug resistance and infection in intensive care units (ICUs). Bacteria within the biofilm are more tolerant of antibiotics; hence it is critically important to develop effective strategies to eradicate biofilm-related infections. Dispersion of biofilm is one of the major factors to tackle the antibiotic tolerance of biofilms. The purpose of this review is to summarize and provide a critical written account of the efficient approaches for biofilm dispersal and thus to make the dispersed bacterial cells susceptible to antibiotics. This points the way forward for further research in combating antibiotic resistance and the successful treating of biofilm-associated infections.

Keywords: *Pseudomonas aeruginosa* biofilm, antibiotic resistance, biofilm dispersal, biofilm eradication, combating strategies.

1. INTRODUCTION

Biofilm associated antibiotic drug resistance is a major contributing factor to chronic infections such as cystic fibrosis, wounds, otitis media, endocarditis, and periodontitis. The bacteria accumulate as microcolonies enclosed in a self-produced extracellular matrix called a biofilm. Bacteria within biofilms are more tolerant of antibiotics and this tolerance suppresses antimicrobial treatment [1]. *Pseudomonas aeruginosa* is known to form biofilms on the tissues of the cystic fibrosis lung and abiotic surfaces such as contact lenses, catheter lines, and ventilator tubes [2, 3].

The exopolysaccharide matrix of a biofilm is comprised of polysaccharides, lipids, proteins, and extracellular DNA [4]. The three exopolysaccharides namely Pel, Psl, and alginate are the main constituents of the EPS matrix [5, 6]. EPS matrix is a physical barrier against environmental factors and contains antibiotic degrading enzymes. The distinctive characteristics of the EPS matrix lead to the high antimicrobial tolerance and resistance of biofilms. Hence it is critically important to develop effective strategies to eradicate biofilms.

Dispersion of biofilm is one of the major factors to tackle the antibiotic tolerance of biofilms [7]. Biofilm dispersion can be induced by regulation of c-di-GMP, *Pseudomonas* Quinolone Signal (PQS), nutrient availability, and exposure to nanoparticles, phytocompounds, nitric oxide, polysaccharide degrading enzymes [8]. The biofilm dispersed cells with lower c di GMP have more virulence compared to planktonic cells. A recent study showed that the dispersal of biofilm by glycoside hydrolases results in hypervirulent dispersed cells leads to fatal septicemia in mice wound model [9]. However, the combinatorial effect of dispersion trigger and antibiotic prevents septicemia and results in biofilm eradication. The successful prevention of septicemia depends on the dispersion trigger and antibiotic used. In the present review, we summarize the general mechanisms that are involved in antibiotic tolerance and resistance in biofilm. Subsequently, we focus on biofilm dispersal strategies that give a future scope for further research in combating antibiotic resistance.

2. BIOFILM ASSOCIATED ANTIBIOTIC RESISTANCE IN *PSEUDOMONAS AERUGINOSA*

The antibiotic tolerance mechanisms and mutations contribute to antibiotic resistance in biofilms. Antibiotic tolerance in biofilms caused by restricted antibiotic

penetration, accumulation of antibiotic degrading enzymes in the EPS matrix, inactivity of antibiotic targets due to low metabolic activity, persister formation, pumping out of antibiotics by efflux pump, and expression of specific genes [10].

2.1. Restricted antibiotic penetration

Antibiotics should cross the extracellular biofilm matrix to reach enclosed bacterial cells during the treatment of biofilm-related infections. Various investigations proved that the antibiotics bind to components of the biofilm matrix or the bacterial membranes restricting the penetration of antibiotics. The biofilm exopolysaccharide matrix is a physical barrier to antibiotics [11], and the adsorption sites on the matrix limit antibiotic penetration [12].

2.2. Slow metabolic growth

Slow metabolic growth in biofilms is because of oxidative stress [13], and nutrient limitation or starvation [14]. Microcolonies at the periphery of the biofilm consume more nutrients and oxygen than the bacterial cells located in the inner part of the biofilm results in low metabolic growth responsible for the inactivation of antibiotic targets [15]. Persister cells are formed due to oxidative stress and exposure to antibiotics and are highly resistant to antibiotics. Persisters exhibit low metabolism and escape the activity of antibiotics [16].

2.3. Quorum sensing

Quorum sensing (QS) is cell to cell communication in biofilms and affects biofilm formation. The QS system in *P.aeruginosa* consists of Las and Rhl, and PQS systems and regulates each other in a complex fashion. Rhl and Pqs systems regulate biofilm formation and induce tolerance to immune cells [17].

2.4. Expression of specific genes

A secondary messenger c-di-GMP and transcription regulator BrlR plays a major role in antibiotic tolerance of *P.aeruginosa* [18, 19]. High c-di-GMP induces the production of extracellular matrix components to form a biofilm, whereas low c-di-GMP levels downregulate the production of extracellular matrix components and cause biofilm dispersal [20].

2.5. Efflux pumps

Efflux pumps are the membrane proteins and contribute to biofilm formation. Different modes of stress can induce efflux pumps in *P.aeruginosa* biofilms results in the contribution of antibiotic tolerance. Efflux pumps are capable of pumping antibiotics out of the bacterial cell [21].

2.6. Tolerance to antibiotics

Beta-lactam antibiotics have a poor antibiofilm effect. Beta lactamases present in the outer biofilm layer hydrolyses the beta-lactam antibiotics. Quinolones have a good antibiofilm effect but low oxygen concentration in biofilms induces tolerance to quinolones. Psl, Pel and alginate are the biofilm matrix components and protect the bacterial cells from aminoglycosides and anti-microbial peptides [22-24].

2.7. Horizontal gene transfer

Biofilms are the banks of genetic diversity. Antibiotic resistance genes can be transferred by horizontal gene transfer (HGT). Extracellular DNA plays a physical role in horizontal gene transfer of antibiotic resistance genes among microorganisms in biofilm [25].

3. COMBATING STRATEGIES FOR ANTIBIOTIC RESISTANCE

Biofilm dispersion can be induced by regulation of c-di-GMP, PQS, nutrient availability, and exposure to nano-particles, phytocompounds, nitric oxide, polysaccharide degrading enzymes. Biofilm dispersal by the following mechanisms shows high dispersal rates and the dispersed biofilm bacteria were susceptible to antibiotics.

3.1. Pyruvate dehydrogenase

Microcolony formation is an essential feature of biofilm structure in *P.aeruginosa* biofilms [26]. Metabolically active *P.aeruginosa* biofilm cells at the periphery secrete pyruvate, which diffuses into the central anoxic zone. Microcolonies at the central anoxic zone fermentatively utilize pyruvate to cope with stressful, oxygen limiting, and electron-rich conditions referred to as reductive stress (too much NADH/electrons, not enough O_2) [27]. The pyruvate fermentation process involves the conversion of pyruvate to lactate and acetate by lactate dehydrogenase and acetate kinase respectively. Inactivation of lactate dehydrogenase severely impairs pyruvate fermentation. Hence biofilm-dependent utilization of pyruvate requires lactate dehydrogenase.

The enzyme pyruvate dehydrogenase (PDH) is used to induce pyruvate depleting conditions, which catalyzes the conversion of pyruvate to acetyl-CoA in the presence of cofactors CoA and NAD^+ creates reductive stress that results in the dispersion of biofilm. This dispersion mechanism makes dispersal cells more susceptible to

antimicrobial agents. Co-treatment of PDH with tobramycin enhances the efficacy of tobramycin in the killing of biofilm cells. The continuous pyruvate-depleting conditions in the growth medium resulted in the prevention of biofilms [28].

3.2. Cinnamaldehyde

Cinnamaldehyde (CAD) is a phytocompound of cinnamon and biofilm inhibitor of *Pseudomonas aeruginosa*. *P.aeruginosa* consists of Las, Rhl, and Pqs quorum sensing (QS) systems to control the expression of the virulence factors and biofilm genes that contribute to its pathogenicity [29]. CAD interferes with Las, Rhl, and Pqs systems of *P. aeruginosa* and subinhibitory levels of CAD down-regulates Las and Rhl which results in quorum sensing inhibition [30]. Quorum sensing inhibitors reduce virulence and weaken the bacterial biofilm cells thus making them susceptible to antibiotics. A combination of CAD with colistin and tobramycin effectively inhibits biofilm formation and preformed biofilm dispersion, when compared to the individual treatments. Therefore, a combination of quorum sensing inhibitor (CAD) with antibiotics synergistically removes established biofilms thus making *P.aeruginosa* more susceptible to antibiotics [31].

3.3. Naringin

Naringin is a flavonoid glycoside that is commonly found in citrus fruits [32]. Naringin possesses antioxidant, anti-inflammatory, anti-apoptotic, and anti-carcinogenic properties [33, 34]. Naringin has both antimicrobial and antibiofilm activities against *P. aeruginosa*. Naringin depletes EPS of *P. aeruginosa* biofilm that allows the penetration of antibiotics into biofilm [35]. *P. aeruginosa* forms pellicle biofilm at an air-liquid interface that contributes to resistance [36]. *P. aeruginosa* exhibits swarming motility that assists in surface colonization and biofilm formation [37]. The combinatorial effect of Naringin with ciprofloxacin or tetracycline eradicates biofilm formation through suppression of pellicle formation and swarming motility in *P. aeruginosa* [38].

3.4. Nitric oxide

Nitric oxide (NO) is a biological signal molecule that initiates biofilm dispersal which prompted its thought for a therapeutic approach to treat biofilms and biofilm-related diseases. NO mediates biofilm dispersal by increasing phosphodiesterase activity with collateral reduction of the biofilm regulator cyclic-di-guanosine monophosphate (c-di-GMP) [39]. The generation of oxidative or nitrosative stress inside microcolonies induces the dispersal of biofilms and cell lysis in *Pseudomonas aeruginosa* biofilm. Nitrosative stress involves the production of reactive nitrogen intermediates (RNI), ensuing damage to DNA, lipids, and proteins [40]. RNI are the by-products of anaerobic respiratory metabolism. NO donor sodium nitroprusside (SNP) induces biofilm dispersal and exposure of established biofilm to SNP enhances the antibiotic efficiency against dispersed bacterial cells.

3.5. Boronic Acid Derivative SM23

The boronic acid derivative SM23 inhibits β-lactamase activity and makes the bacterial cells sensitive to β-lactam antibiotics [41]. *P.aeruginosa* elastase, which is capable of inactivating immunological agents, is significantly dampened by SM23 treatment. SM 23 inhibits β-lactamase and also acts as a powerful inhibitor of *P. aeruginosa* biofilm recommending that it may have a potential application in the prevention and treatment of biofilm-associated *P. aeruginosa* infections. Among several mechanisms of antibiotic resistance, the production of β-lactamases is the most concerning one. In *Pseudomonas aeruginosa*, class C β-lactamases express a high level of resistance to β-lactam antibiotics. The combination of the β-lactam antibiotics together with a β-lactamase inhibitor is the relevant strategy to overcome resistance to these drugs [42]. Boronic acid transition state analog inhibitors (BATSIs) are the most promising class of new β-lactamase inhibitors and restore the β-lactam activity both in vitro and in vivo. Meropenem is a new combination of boronic acid and the β-lactam that has entered the market for the treatment of infections caused by multidrug-resistant *Pseudomonas aeruginosa*.

3.6. Zinc oxide-Cadmium sulfide Nanoparticles

Recently, metal nanoparticles were reported for anti-microbial and antibiofilm activities. The photo-catalytical activity of ZnO nanoparticles is the reason for its wide applications in antimicrobial and antibiofilm research. In the presence of ZnO-CdS nanocomposite, *P.aeruginosa* shows a decline in biofilm development and is one of the excellent factors for inhibiting biofilm formation. ZnO nanoparticles produce reactive oxygen intermediates results in oxidative stress which causes damage to DNA, lipids, and proteins [43].

The generation of oxidative stress results in cell lysis and dispersion. ZnO particles are conjugated with CdS to suppress the recombination of photogenerated electron-

hole pairs in ZnO nanoparticles. The ZnO-CdS nanocomposites inhibited the microbial population inside the biofilm and eliminate the biofilm-forming ability of the microorganisms [44]. The disadvantage of using nanoparticles is their potential toxicity in humans; Nanoparticles are very reactive due to their high surface area to mass ratio, which may cause unwanted reactions in the human body and can induce systemic toxicity [45].

3.7. Silver nanoparticles

Biologically synthesized AgNPs were shown anti-biofilm activity against *P.aeruginosa* biofilms resulted in excellent biofilm reduction [46]. The antibiofilm activity of AgNPs involves biosorption, the major factor responsible for the inactivation of biofilm formation in *P.aeruginosa* [47]. The biologically synthesized AgNPs reduced 95% to 98% biofilm formation in *P.aeruginosa* [48, 49]. AgNPs arrest exopolysaccharide synthesis thus the organism cannot form biofilms [50]. This hypothesis was the basis of the anti-biofilm activity of AgNPs. A combination of AgNPs with antibiotics enhances the antibacterial and anti-biofilm effect. The synergistic effect of AgNPs and ampicillin inhibited biofilm activity by 70% and killed more than 80% of bacteria in *P.aeruginosa* [51]. Thus the combined treatments with AgNPs and antibiotics enhanced both the inhibition of biofilm activity and the levels of cell death.

3.8. Alginate lyase

Alginate is the biofilm matrix component and one of the main virulence factors associated with *P. aeruginosa* biofilms. Alginate protects the bacteria from harsh environmental conditions and helps in adhesion to solid surfaces [52].

Alginate lyase is an enzyme that degrades alginate through hydrolysis [53]. Alginate lyases degrade biofilm acts as good dispersal agents. Alginate lyases A1-II' and Alg2A have high enzymatic capability against different biofilms because they degrade a greater variety of alginate matrixes. A1-II' and Alg2A have polymannose lyase and poly-Glucuronide lyase (polyG/M) activity and dissolve *P. aeruginosa* biofilms. Polymannose lyase and polyglucuronide lyase enzymes were combined with ciprofloxacin antibiotic for the synergistic activity against biofilms [54].

3.9. Chitosan oligosaccharide - streptomycin conjugate

Chitosan oligosaccharide (COS) is a hydrolyzed product of chitin and have low molecular weight and high water solubility. COS also has significant biological activities, such as antimicrobial, antioxidant, anticancer, and immune-stimulant effects [55]. COS is conjugated with Streptomycin to eradicate established *P. aeruginosa* biofilms.

COS-Streptomycin conjugate impairs the structural integrity of biofilm and makes the *P. aeruginosa* biofilms susceptible to antibiotics. COS-Streptomycin suppresses the activation of MexX-MexY drug efflux pump through up regulating the expression of MexY suppressor that is *mexZ*. COS - Streptomycin down - regulates the expression of *pelA* gene which plays an important role in controlling biofilm cell density and inhibits the biosynthesis of alginate exopolysaccharide results in the eradication of biofilms [56]. Chitosan oligosaccharides are non-toxic and biocompatible hence this novel strategy might open up another road to conquer the intrinsic resistance of biofilms to antibiotics.

4. CONCLUSION AND FUTURE PERSPECTIVES

The treatment of *P.aeruginosa* biofilm-associated infections is a major challenge because of antibiotic tolerance and resistance of biofilms. Even the development of new antibiotics is ongoing; *P.aeruginosa* biofilms acquiring resistance to those new antibiotics due to the overuse of antibiotics and mutations. Biofilm dispersal is the key factor to tackle antibiotic resistance in biofilm. Biofilm dispersion can be induced by anti-biofilm molecules, enzymes that degrade the EPS matrix, exposure to nanoparticles, and several biofilm dispersal technologies. However, the dispersed cells from biofilm can be susceptible to antibiotics or can increase the risk of reinfection. Thus the combination of anti-biofilm molecules with antibiotics shows the synergistic effect and eradicates *P.aeruginosa* biofilms.

In the present review, biofilm dispersal by Pyruvate dehydrogenase, Cinnamaldehyde, Naringin, Nitric oxide, Boric acid transition state analog inhibitors, ZnO/CdS nanoparticles, silver nanoparticles, Alginate lyases, and Chitosan oligosaccharide are discussed because biofilm dispersal by these mechanisms makes the dispersed biofilm cells more susceptible to antibiotics. The combination of these dispersal mechanisms with antibiotics would inhibit and eradicate *P.aeruginosa* biofilms. Hence this innovative therapy can be used to treat biofilm-associated infections. We suggest that the origin of biofilm-related infections is the combination of both the release of bacteria from the biofilm and the enhanced virulence potencies of the dispersed bacteria, a prominent aspect to consider in future preventive and therapeutic strategies.

5. ACKNOWLEDGMENT

The corresponding author acknowledges Acharya Nagarjuna University, Guntur, Andhra Pradesh, India.

Conflict of interest

The author declares no conflict of interest.

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