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Molecular mechanisms of biofilm resistance against antibiotics

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Abstract

Biofilms are immobile communities of microbes attached to biotic an abiotic surfaces and are embedded inside a self-produced cement-like extracellular polymeric substances. The resistance of biofilms against commonly used drugs has been implicated in the pathogenesis of various bacterial infections under medical and veterinary settings which normally cannot be eradicated by antibiotics. Biofilms are characterized by the ability to evade not only the antibiotic effects but also the host immune system clearance. Currently the most worrisome aspect of global human health is the rise and spread of antimicrobial resistance in bacterial pathogens and this crisis got deepened by the emergence of antimicrobial resistance of bacterial biofilms. Different antibiotic resistance mechanisms, processes by which a target pathogen curtails the interaction between an antimicrobial agent and its intended target molecules, adopted by biofilms have been discussed in this review. Different antibiotic resistance mechanisms are employed by the biofilms depending on the species of the bacteria, growth conditions, and the antibiotic involved. Commonly, the role of biofilm matrix polysaccharides, antibiotic-modifying or degrading enzymes, extracellular DNA, hypoxic conditions, presence of efflux pumps, quorum sensing, horizontal gene transfer, mutation frequency, etc. have been implicated in antibiotic resistance of biofilms. This review also discusses different approaches of overcoming biofilm infections or biofilm resistance. However, it is pertinent to mention that since no new class of antibiotics have been approved in last four decades there is the need of greater understanding of biofilm-associated antibiotic resistance to effectively utilise the therapeutic value of the existing antibiotics. Although a number of anti-biofilm strategies have been put forward as discussed in this review, they are still in nascent stage and need to undergo clinical trials to reach the commercial market.

1. Introduction

Biofilms are immobile communities of microbes attached to biotic an abiotic surfaces and are embedded inside a self-produced cement-like extracellular polymeric substances (EPS). Biofilm is the most prevalent and successful microbial lifestyle in natural as well as manmade environments (Flemming et al. 2016; Plusa 2019). Though term biofilm came into existence recently, it represents the oldest form of life on Earth (Bowler 2018) and predominates all the habitats on the surface of earth, forming approximately 80% of the bacterial population on earth (Flemming and Wuertz 2019). The EPS provides a hydrated conducive environment for microbial growth which helps them attach together as a colony on any available surface. Biofilms protect the bacteria from the hostile environmental conditions like antibiotic exposure, osmotic stress, metal toxicity, extreme temperature and pH, poor nutrients, etc. Globally, bacterial biofilms pose serious challenges to human and animal health because of their resistance against antibiotics, host immune system, and other environmental stresses. Therefore, biofilms cause chronic infections (de la Fuente-Nunez et al. 2013) which increase the treatment cost and induce mental-illness in patients (Hoiby et al. 2011). Bacterial biofilms potentially grow on all surfaces including the living tissues, surgical equipments, as well as the implants and the internal devices such as contraceptive devices, catheters, sutures, pacemakers, dental implants, contact lenses, etc (Reg Bott 2011; Dincer et al. 2020; Jamal et al. 2018). Owing to the difficulty in eradication of biofilms because of their significant protection against desiccation, antibiotics, and host immune system, their widespread growth has produced severe clinical complications in medical and veterinary settings (Abebe 2020).

The resistance of biofilms against commonly used drugs has been implicated in the pathogenesis of various bacterial infections under medical and veterinary settings which

normally cannot be eradicated by antibiotics. This has resulted in recalcitrance of subacute and chronic bacterial infections such as chronic lung infections in cystic fibrosis patients by *Pseudomonas aeruginosa* (Singh et al. 2000; Hall and Mah 2017) and infections associated with medical devices such as pacemakers (Dincer et al. 2020). It has been shown that bacteria in biofilms are 10-1000 times more resistant to antibiotics compared to their planktonic forms and about 80% of recurrent and chronic infections in humans were associated with bacterial biofilms (Mah 2012). Although the primary causes of antibiotic resistance were identified as changes in drug targets, antibiotic impermeability in bacteria, genetic changes, etc. which resulted in treatment failures, the development of bacterial films has been implicated in antibiotic resistance recently and is now considered as a primary cause of chronic infections and antibiotic resistance (Bowler 2018). In biofilms the growth of bacterial cells is very slow and results in production of persistent cells which have the ability to withstand unfavourable environmental conditions such as exposure to antimicrobials (Flemming et al. 2016; Hall and Mah 2017). Several studies have reported that exposure of bacteria to lower levels of antibiotics can potentially induce formation of biofilm which indicates the regulation of biofilm formation by presence of antibiotics (Cepas et al. 2019). The increasing trend of antibiotic resistance in past few decades has been attributed to the incongruous, excessive, and over-the-counter use of antibiotics in medical and veterinary settings paralleled by poor sanitation and hygiene, and the influx of drug residues into human body through consumption of animal products (Aslam et al. 2018; Begum et al. 2018) along with the increased global travel (Hawkey 2015).

2. Biofilm development

Biofilm is a sessile community of microbes which irreversibly attach themselves to a surface or any interface. The cells produce extracellular polymeric substances (EPSs), remain embedded in it, and display altered gene expression, protein synthesis, growth rate, and phenotypic characters (Flemming et al. 2016; Oxaran et al. 2018). The microbes undergo a phenotypic shift from a planktonic free-swimming lifestyle to a sessile mode as a biofilm which is a highly regulated process influenced by environmental as well as genetic factors (Southey-Pillig et al. 2005; Otto 2008; Monds and O'Toole 2009; Lopez et al. 2010). The biofilm formation is a multi-step process involving physiological and structural changes in the microbial population. The general steps are: a) initial attachment of a planktonic cell to a favourable surface, b) colony formation by cellular multiplication and differentiation, c) development of biofilm and EPS secretion, d) maturation and formation of mushroom like shape, and e) disassembly of the matrix and dispersion of microbes (Dufour et al. 2012; Mangwani et al. 2016; Maunders and Welch 2017; Jamal et al. 2018). The EPS is a hydrated matrix of proteins, cellulose, alginates, teichoic acid, poly-N-acetyl, and other organic compounds (Jolivet-Gougeon and Bonnaure-Mallet 2014; Flemming et al. 2016) in which the microbial cells remain embedded. It plays a crucial physiological role for cells in the synthesis of compounds like glucosamine, lipids, nucleic acids, phospholipids, extracellular DNA (eDNA), and fosters physical interaction among the cells (Flemming et al. 2016). After maturation of the biofilm colony the cells can disassociate and adopt the free planktonic lifestyle again and may again start the biofilm formation on a new surface (Petrova and Sauer 2016). The cells in the biofilm, lying in close proximity of each other, communicate via chemical messengers to respond as a unit to ecological, environmental, and host related signals (Matz 2011). The communication between the cells, known as quorum sensing, is mediated by several signalling molecules such as acyl homoserine lactone (AHL) and autoinducing peptide (AIP) in Gram-negative and Gram-positive bacteria, respectively; and the autoinducer-2 (AI-2) in both types of bacteria in a cooperative manner for a common goal (Brackman and Coenve 2014; Petrova and Sauer 2012). The biofilm development in microbes is induced by different environmental signals, such as exposure of *P. aeruginosa* and *Escherichia coli* to aminoglycoside antibiotics at sub-inhibitory levels induce biofilm formation (Hoffman et al. 2005). Its course of formation is determined by the interplay of many factors such as surface conditions, osmolarity of medium, availability of growth factors, environmental stressors, etc. (Kostakioti et al. 2013).

3. Biofilm and antibiotic resistance

Currently the most worrisome aspect of global human health is the rise and spread of antimicrobial resistance in bacterial pathogens and this crisis got deepened by the emergence of antimicrobial resistance in bacterial biofilms (Balcazr et al. 2015; Zhang et al. 2018; Cepas et al. 2019). The striking phenotypic difference between biofilms and their corresponding planktonic forms is that cells in biofilms are relatively highly resistant to antimicrobial agents (Hall and Mah 2017). The microbes in the biofilm have been reported tolerate 10-1000 times the antimicrobial concentration compared to the corresponding planktonic form (Myszka and Czaczy 2011; Pinto et al. 2020). The biofilms provide protection to pathogens not only against unfavourable pH, osmolarity, nutrient availability, and physical forces (Fux et al. 2005; McCarty et al. 2012) but also against the antibiotics and host immune system (Sharma et al. 2019). It is the biofilm formation and consequent entrenching of microbes in the complex matrix that confers resistance against the antimicrobials and other sterilising agents making the eradication and control of microbes difficult (Satpathy et al. 2016; Khatoon et al. 2018; Lajhar et al. 2018). Thus, biofilms are important instruments leading to chronic infections and fostering the spread of antibiotic resistance resulting in the emergence of multi-drug resistant bad bugs.

The antibiotic resistance displayed by microbes in

biofilms is distinct from the natural antibiotic resistance exhibited by planktonic forms (Mauders and Welch 2017). The biofilms tend to develop different molecular strategies to avert the hostile conditions imposed by the antibiotics in the medium. The determinants of antibiotic resistance pertaining to biofilms are: a) type of antimicrobial agent, b) bacterial species/strain, c) developmental stage and age of biofilm, d) growth conditions of biofilm, e) nature of biofilm structure, etc (Ito et al. 2009; Alhede et al. 2011; Bowler et al. 2012; Haaber et al. 2012; Stewart 2015). A number of mechanisms have been put forward to substantiate the antibiotic resistance of biofilms, however, none of the mechanisms could individually account for this feature of biofilms. The commonly proposed mechanisms include: a) restriction of antibiotic diffusion in polymeric matrix, b) lowering of antibiotic activity by interaction with polymeric matrix, c) enzyme-mediated inactivation of antibiotics such as β-lactamase (Hoiby et al. 2010), d) altered metabolic activity inside biofilm, e) alterations in target genes or hiding of target genes, f) efflux pump mediated extrusion of antibiotics (Hoiby et al. 2010), g) production of persistent cells, h) easy transfer of resistance genes within the biofilm (Costerton et al. 2005; Balcazar et al. 2015; Lecuyer et al. 2018), etc. The genetic diversification of microbes in the biofilms has been largely held responsible for antibiotic resistance (Plusa 2019) because the resistant gene determinants undergo rapid horizontal transfer in the densely packed microbial biofilm (Costerton et al. 2005; Balcazar et al. 2015; Lecuyer et al. 2018).

4. Resistance mechanisms

The resistance mechanism is a process by which a target pathogen curtails the interaction between an antimicrobial agent and its intended target molecules (Lewis 2008). It can be either because of mutations or by exchange of resistant genetic elements (Cox and Wright 2013; Blair et al. 2015) or it may be an intrinsic property of the microbes to resist the effect of antimicrobial agents; such as the relatively greater impermeability of Gram-negative bacteria to antibacterial agents compared to Gram-positive bacteria. Such resistance mechanisms strongly circumvent the efficacy of antimicrobials to treat the infections, particularly the biofilm linked infections. Therefore, the development of an appropriate treatment strategy against the biofilm based infections warrants the better understanding of mechanisms underlying the biofilm based antibiotic resistance. The details of the commonly proposed mechanisms of biofilm based antibiotic resistance are as follows:

4.1 Biofilm matrix polysaccharides and antibiotic resistance

The component cells of the biofilm are entrenched in EPS which prevents the spread of antimicrobial agent in to the inner layers of the film. Since EPS is composed of charged molecules such as proteins, glycoproteins, and glycolipids, it forms a physical barrier to antimicrobial agents by binding the oppositely charged antimicrobials and render them ineffective (Nadell et al. 2015). On the other hand, it has also been proposed that EPS matrix of biofilm hampers the dispersal of antibiotic agent which provides enough time for the biofilm cells to adapt the environment with gradually increasing antibiotic concentration (Tseng et al. 2013). Biofilm matrix induced antibiotic resistance has been observed in *P. aeruginosa* biofilms where *Pel* exopolysaccharide hindered the action of aminoglycosides by spreading the cationic antibiotics. Lacking of *Pel* exopolysaccharide gene locus, vital for structural integrity of biofilms, in wild-type biofilms has made them more susceptible to aminoglycosides (Colvin et al. 2011). Another exopolysaccharide, *Psl*, has been declared indispensable for biofilm formation in most of the *P. aeruginosa* strains and confers resistance at early stages of biofilm development against colistin, polymyxin B, tobramycin, and ciprofloxacin (Billings et al. 2013). Similarly, the reduced penetration of oxacillin, cefotaxime, and vancomycin was also reported in *Staphylococcus aureus* and *S. epidermidis* biofilms which resulted in low susceptibility of these biofilms to the above three antibiotics (Jefferson et al. 2005; Singh et al. 2010, 2016).

However, this limitation to the diffusion of antibiotics seems to be dependent on the experimental conditions, bacterial strain involved, and the growth conditions of the biofilm. It is pertinent to mention that decreased antibiotic penetration as a results of biofilm EPS is occasionally linked to biofilm antibiotic resistance. Even the antibiotics which swiftly diffuse within the biofilm do not result in a significant death of cells. For example, no effect on cell viability was observed even after complete dispersal of tetracycline in the biofilm of uropathogenic *E. coli* within 10 minutes (Stone et al. 2002). The role of *Pel* in antibiotic resistance of biofilms was marred by controversy when it was demonstrated that *PelA P. aeruginosa* biofilms deficient in *Pel* have shown four time more resistance compared to wild type biofilms against the aminoglycosides (Khan et al. 2010). Similarly, the overexpression of *Psl* in certain *P. aeruginosa* strains did not increase their resistance against tobramycin (Colvin et al. 2011). Furthermore, even at concentrations much higher than MIC values for planktonic forms, ampicillin and ciprofloxacin could not affect the cells within the *Klebsiella pneumoniae* biofilm (Hall and Mah 2017). Similar observations have been reported with staphylococcal biofilms against the antimicrobial agents such as rifampin, daptomycin, amikacin, and ciprofloxacin (Stewart et al. 2009; Singh et al. 2010, 2016; Boudjemaa et al. 2016).

4.2 Antibiotic-modifying enzymes and biofilm resistance

Another aspect of biofilm matrix associated antibiotic resistance is the production of antibiotic modifying/degrading enzymes, such as β-lactamases which degrade the antibiotics and render them ineffective or unreachable to their intended targets. Under the influence of imipenem and ceftazidime the

matrix of *P. aeruginosa* biofilm secrete and accumulate high amount of β-lactamases which hydrolyse these antibiotics in defence of the biofilm (Bagge et al. 2004). Furthermore, as a result of greater accumulation of *β*-lactamases in mature *P. aeruginosa* biofilms they were reported to be more resistant to ceftazidime and meropenem compared to younger biofilms (Bowler et al. 2012). Similarly, the biofilms of *K. pneumoniae* were reported to secrete *β*-lactamase which hydrolysed ampicillin and prevented it from reaching the target cells deeper within the biofilm (Hall and Mah 2017; Dincer et al. 2020). However, even after the deletion of *β*-lactamase, *K. pneumoniae* biofilms were still resistant to ampicillin compared to their planktonic forms, which indicates the presence of additional mechanisms of resistance (Hall and Mah 2017; Dincer et al. 2020).

4.3 Extracellular DNA and role in antibiotic resistance

Again, extracellular DNA (eDNA) is another aspect of bacterial biofilm matrix induced antibiotic resistance. The role of eDNA in antimicrobial resistance is an extensively studied molecular mechanism in *P. aeruginosa*. The release of eDNA in the biofilm may be endogenously mediated by quorum-sensing and fratricidal lysis of cells within the biofilm; and exogenously by polymorphonuclear WBCs at the site of infection (Allesen-Holm et al. 2006; Jakubovics et al. 2013; Hall and Mah 2017). Irrespective of the source, the eDNA has been indicated in biofilm resistance to certain antimicrobials (Chiang et al. 2013). The exogenous eDNA gets incorporated into the biofilm matrix of *P. aeruginosa* and confers resistance to tobramycin and gentamicin (Chiang et al. 2013). The release of eDNA is fostered by exposure of the cells within the biofilm to the sub-therapeutic levels of the antibiotics. For example, the release of eDNA occurs in the biofilm of the *S. aureus* in response to low levels of methicillin, however, the mechanism behind this eDNA release is yet to be understood clearly (Kaplan 2011). On the similar lines, in *S. epidermidis* biofilm the exposure to sub-therapeutic levels of vancomycin resulted in two times increase of eDNA which strongly binds vancomycin and prevents its access to the cells of the biofilm (Doroshenko et al. 2014).

One of the mechanisms of antimicrobial resistance by eDNA is alteration of the mineral concentration in the extracellular environment within the biofilm. The anionic nature of eDNA causes the chelation of magnesium ions which reduces their effective concentration in the biofilm environment of *P. aeruginosa* and *Salmonella enterica* and in turn this low magnesium ion concentration signals the PhoPQ and PmrAB two-component system activation to elicit the antimicrobial resistance (McPhee et al. 2006; Mulcahy et al. 2008; Johnson et al. 2013; Wilton et al. 2016). Also, *P. aeruginosa* was shown to have acidic microdomains because of the accumulation of eDNA and this acidic environment also acts an environmental signal for the PhoPQ and PmrAB two-component system activation (Wilton et al. 2016). Furthermore, spermidine, a polyamine gene product of PmrA-regulated PA4773-4775 locus, is induced by eDNA which gets localised in the outer membrane of *P. aeruginosa* and reduces the membrane permeability to cationic antimicrobials, such as aminoglycosides (Johnson et al. 2012). Because of higher eDNA content in biofilm of *Clostridium jejuni* the resistant colonies against chloramphenicol and kanamycin were recovered 6.5 times more than the colonies of the planktonic forms (Bae et al. 2014). Similarly, natural transformation of antibiotic resistance genes was fostered in biofilms of *Streptococcus pneumoniae* with eDNA produced by fratricide compared to their planktonic counterparts (Wei and Havarstein 2012; Marks et al. 2012). In addition to the above discussed physical defense against antibiotics, the eDNA also plays a significant role in the horizontal transfer of resistance genes between the member cells of a biofilm (Hall and Mah 2017).

4.4 Hypoxia and antibiotic resistance

The biofilms are characterized by component cells with different metabolic activities as a result of oxygen and nutrient gradient across the biofilm depth. The cells near the surface consume oxygen and nutrients to the maximum level before reaching the deeper layers of the biofilm (Stewart and Franklin 2008). This nutrient and oxygen gradient results in bacterial cells within the biofilm with different growth rates (Blanco et al. 2016). Various researchers have reported the presence of steep oxygen gradient in biofilms of different bacterial species having oxygen deprived deeper layers and this hypoxic condition in deeper layers results in reduced growth rate with stationary phase-like condition in such cells (Borriello et al. 2004; Werner et al. 2004; Stewart et al. 2016). As a result, these cells undergo reversible transformation into persistent or dormant cells, which are typically retrieved from chronic urinary tract infections and cystic fibrosis-affected lungs (Hall and Mah 2017). Growth rate of the microbes is one of the major determinants of antibiotic efficacy because the target macromolecules of the antibiotics are synthesised in metabolically active cells and hence the slow growing cells in deeper layers of the biofilm exhibit antibiotic resistance. Furthermore, there is a report of hypoxic conditions in *P. aeruginosa* biofilm which confer resistance against aminoglycosides by reducing the outer membrane potential which reduces their transport into the cell (Stewart 2015). The hypoxic conditions in *P. aeruginosa* biofilm upregulated the expression *mexEF-oprN* efflux pump which shows resistance to multiple antibiotics (Schaible et al. 2012). However, contrary to the above discussion the determinants of antibiotic resistance are transferred efficiently at oxygen rich air-liquid interface in *E. coli* (Krol et al. 2011) which indicates that cell in different environmental conditions exhibit different mechanisms of antibiotic resistance.

4.5 Efflux pumps and antibiotic resistance

Antimicrobial efflux pumps, the membrane proteins coded by either bacterial chromosome or mobile genetic elements, are present in all bacterial species which export the antimicrobial agents out of the cell and confer resistance. These efflux pumps, having evolutionary significance, make the pathogens exhibit antibiotic resistance naturally and their overexpression also confers acquired resistance. Certain multidrug efflux pumps do contribute to formation of biofilms as well. There is a report on mutant *E. coli* that lack of genes associated with various efflux pumps results in severely reduced biofilm formation (Matsumura et al. 2011). Efflux pumps facilitate the formation of biofilm by regulation of genes associated with formation of biofilms indirectly and influence the aggregation of cells in biofilms as well (Alav et al. 2018). The biofilm resistance locus regulator (BrlR) in *P. aeruginosa* was declared vital for antibiotic resistance as it resulted in upregulation of *mexAB-oprM* and *mexEF-oprN* efflux pumps in the biofilm (Liao et al. 2013) and role of *MexAB-OprM* was also indicated in biofilm resistance to low concentration of ofloxacin (Brooun et al. 2000). On similar lines, the presence of azithromycin caused upregulation of efflux pumps - *MexAB-OprM* and *MexCD-OprJ* in resistant *P. aeruginosa* biofilms (Gillis et al. 2012). In *P. aeruginosa* the biofilm-specific multidrug efflux pump – PA1874-1877 is a four-gene operon which is expressed 10 times more in biofilms with respect to the planktonic cells (Zhang and Mah 2008). However, it is pertinent to mention that deletion of PA1874-1877 operon has no effect on the formation of biofilm, even though PA-1874 has sequence homology with *Bap* protein which is necessary for biofilm formation in *S. aureus* (Zhang and Mah 2008). However, the authors reported that there was 2-4 fold increase in susceptibility of the biofilm to tobramycin, gentamicin, and ciprofloxacin whereas, the susceptibility of planktonic forms remained unaltered (Zhang and Mah 2008).

In other species such as *Burkholderia cepacia* the resistance nodulation-division family efflux pumps (RND-8 and RND-9) confer resistance to biofilms against tobramycin, whereas, RND-3 pump conferred resistance to both ciprofloxacin and tobramycin (Buroni et al. 2014). Similarly, in *Helicobacter pylori* biofilm the expression of RND efflux pumps was higher compared to its planktonic form which could be the reason for resistance to clarithromycin (Yonezawa et al. 2013). The treatment of *B. pseudomallei* biofilm with an efflux pump inhibitor resulted in decreased resistance to ceftazidime and doxycycline (Sirijant et al. 2016). However, it is noteworthy to mention that the multidrug efflux pumps, such as *MexAB-OprM*, primarily confer antibiotic resistance to planktonic cells (Poole 2011). A number of researchers have reported that multidrug efflux pumps have no role in antibiotic resistance in biofilms (de Kievit et al. 2001; Stewart 2015). And, such contrasting results can be ascribed to the presence of different experimental setups.

4.6 Quorum Sensing and antibiotic resistance

Though biofilms are considered self-sufficient, the microbial cells within interact with each other through certain chemicals to achieve the collective goals. The biofilm acts as a collective enterprise which responds to external stimuli in a highly coordinated way to achieve the common goals of the unit (Matz 2011; Oliveira et al. 2015). This cell to cell interaction or communication at a cellular level within the biofilm community is called quorum sensing. It is a process in which the constituent microbial cells produce and perceive the chemical signal molecules to coordinate their activity towards a common goal (Brackman and Coenye 2014). This quorum sensing is mediated by different chemical signalling molecules such as AIP in Gram-positive bacteria, AHL in Gram-negative bacteria, and AI-2 in both types of bacteria (Petrova and Sauer 2012; Bhardwaj et al. 2013; Brackman and Coenye 2014). A fascinating hypothesis involving an interplay of quorum sensing molecule and eDNA has been put forward in *P. aeruginosa* biofilms which helps explain the contribution of quorum sensing to antibiotic resistance (Hazan et al. 2016). The authors state that 2-n-heptyl-4-hydroxyquinolone-N- oxide (HQNO), a quorum sensing-regulated molecule, inhibits cytochrome *bc*1 complex of electron transport chain which causes accumulation of ROS and in turn the fratricidal release of eDNA. This eDNA, as discussed in previous section, contributes to or promotes antibiotic resistance.

A number of studies have reported on the quorum sensing and biofilm associated antibiotic resistance. The biofilms formed by certain strains of *P. aeruginosa*, deficient in quorum sensing phenomenon, were highly susceptible to tobramycin compared to their wild counterparts (Bjarnsholt et al. 2005). The *P. aeruginosa* biofilm becomes more susceptible to tobramycin when the population of quorum-sensing mutants increase compared to wild-type (Popat et al. 2012) and colistin resistance in *P. aeruginosa* was reported to be driven by quorum sensing (Chua et al. 2016). However, an intriguing converse of the above observation made by Popat et al. (2012) was reported earlier that quorum-sensing mutants in the biofilm of *P. aeruginosa* increased the resistance of biofilm to tobramycin (Amini et al. 2011). The authors furnished an explanation to validate their observation in which they called such quorum-sensing mutants as 'social cheaters' who take the advantage of quorum sensing staged by other members of the biofilm without actually participating in this energy consuming process. On the similar lines, quorum sensing deficient *S. aureus* biofilms were 2-3 times more susceptible to rifampin compared to their wild-type counterparts (Yarwood et al. 2004). The quorum sensing system (*fsr*) and quorum-regulated protease (*geIE*) were reported to be fundamental in biofilm antibiotic resistance against gentamicin, daptomycin, and linezolid, but neither of them were required in planktonic forms (Dale et al. 2015).

4.7 Horizontal gene transfer and antibiotic resistance

The biofilms are characterized by close proximity of cells, high

density of cells, and accumulation of genetic elements in the biofilm matrix. This close proximity of cells provides an ideal environment for horizontal transfer of antibiotic resistance encoding plasmids. The horizontal transfer of resistance genes between the cells is called as conjugation and can potentially increase the resistance against antibiotics by 700 fold compared to planktonic cells (Flemming et al. 2016). Furthermore, in addition to the exchange of resistance elements between the cells via conjugation, bacteria may also internalise the eDNA present in the biofilm matrix. The eDNA is also linked to horizontal transfer of resistance elements to the cells and confers them antibiotic resistance (Hall and Mah 2017). However, conjugation is considered as most efficient means of horizontal transfer of resistance genes in biofilms compared to planktonic forms because of close proximity and sessile nature of cells within the biofilm (Madsen et al. 2012; Krol et al. 2013; Savage et al. 2013; van Meervenne et al. 2014). The *S. aureus* biofilm is considered as an unprecedented site for conjugal transfer of multidrug resistance conferring plasmids with an efficiency of 10,000 times as that of its planktonic forms (Savage et al. 2013). The biofilms of *Enterococcus faecalis* revealed a 2 fold increase in plasmid copy number associated with antibiotic resistance genes which suggests the furtherance of resistance genes in the biofilms (Cook and Dunny 2013). Similarly, the conjugal transfer of plasmids carrying antibiotic resistance determinants in *E. coli* occurred efficiently at air-liquid interface (Krol et al. 2011) which is an interesting contrast to the importance of hypoxia in promoting biofilm resistance as discussed above.

4.8 Mutation frequency and antibiotic resistance

Not enough conclusive literature is available which substantiates the mutation as a mechanism of antibiotic resistance in bacterial biofilms. However, inherently higher mutation rates have been anticipated in biofilms because of higher endogenous ROS production which damages the DNA more frequently compared to planktonic cells (Boles and Singh 2008). In line with this hypothesis, the presence of antioxidants in the culture of *S. aureus* biofilms revealed lower mutation frequency which was comparable to the mutation frequency in their planktonic counterparts (Ryder et al. 2012). Compared to planktonic life style, biofilms were reported to promote mutation at higher frequency which results in emergence of stable hypermutable strains (Driffield et al. 2008). In cystic fibrosis patients, the *P. aeruginosa* isolates with defective DNA oxidative repair mechanism were found to be more resistant to antibiotics compared to normal isolates (Oliver et al. 2000; Mandsberg et al. 2009). The mutation frequency in ciprofloxacin resistant mutants in *P. aeruginosa* biofilm was 100 times (Driffield et al. 2008) and significantly higher in *Campylobacter jejuni* biofilm (Bae and Jeon 2014) compared to their planktonic forms. Similar observations were reported in *S. aureus* biofilms where muciprocin and rifampin-resistant mutants were recovered at higher frequency compared to planktonic cells (Ryder et al. 2012).

5. Approaches to overcome biofilm resistance

The biofilm-associated infections paralleled by antibiotic resistance represents the most gruesome clinical picture in terms of both therapeutic cost and outcome. Keeping in view the ubiquity and consequences of biofilm formation in clinical settings, new methods are warranted urgently to treat such biofilm-associated infections. The correct choice and dosage of an antibiotic significantly affects the outcome of a treatment because some antibiotics may act as agonists or antagonists of biofilm formation (Dincer et al. 2020). Under hospital settings the application of altered designs of drains and water outlets, heat, electromechanical vibrations, anti-biofilm agents such as acetic acid and oxidising agents have been successfully tested in the removal of biofilms in the environment to keep check on biofilm-associated infections (de Jonge et al. 2019; Garvey et al. 2019; Smolders et al. 2019). Furthermore, under *in vivo* conditions the mechanical disruption of biofilms of wound provides a therapeutic window of 24-48 hours during which the antibiotic therapy is most effective (Wolcott et al. 2010) and it indicates that biofilm formation takes place within 24-48 hours of initiation. Therefore, there is a need of new combinations of antibiotics and biofilm-disrupting agents to effectively overcome biofilm-associated infections.

Keeping in view the increasing frequency of resistant pathogens, the quorum sensing inhibitors in combination with the effective antibiotics can exert complementary effects against the target pathogens. Such combinations are useful in the treatment of chronic biofilm-associated infections – such as urinary tract infections, cystic fibrosis, infection of prosthetic tools (Dincer et al. 2020). When patulin, a biofilm-disrupting agent targeting the AHL – a quorum sensing molecule, is used in combination with tobramycin it brings an unprecedented killing of *P. aeruginosa* cells (Rasmussen et al. 2005). The combination of quorum sensing inhibitors and tigecycline antibiotic caused a four-fold increase in the death rate of *S. aureus* and the treatment efficiency of ciprofloxacin in combination with cis-2-decenoic acid increased from 11% to 87% in *S. aureus* infection (Simonetti et al. 2013). The commonly tested quorum sensing inhibitors are halogenated furanone (Lonn-Stensurd et al. 2009), acyclic diamine (Kaur et al. 2017), ginseng and garlic extract (Bjarnsholt et al. 2005; Song et al. 2010), and nitric oxide (Beloin and Ghigo 2005). There are certain naturally produced molecules – D-amino acids and nor-spermidine, which disperse the mature biofilms in *S. aureus* and *E. coli* and in combination with the antibiotics they can help prevent biofilm-associated infection (Kolodkin-Gal et al. 2010, 2012; Hochbaum et al. 2011). Similarly, the combination of antibiotics with N-acetyl-cysteine (NAC) and Tween 80 effectively destroyed the biofilms of rapidly growing mycobacteria and Tween 80 was more effective than NAC because of higher mycolic acid content in mycobacteria (Munoz-Egea et al. 2016). Use of biofilm matrix degrading

enzymes such as DNase I, Dispersin B, and α-amylase degrade eDNA, biofilm matrix, and exopolysaccharides, respectively (Tetz et al. 2009; Sun et al. 2013), which have prominent role in biofilm resistance against antimicrobial agents as discussed above. They disperse the biofilms, prevent the formation of new biofilms, and increase the penetrance of antibiotics in the biofilms of many bacteria such as *S. aureus*, *Vibrio cholerae*, *P. aeruginosa* etc. (Kalpana et al. 2012). Furthermore, nano-formulations have offered a promising alternative to regular antibiotics to overcome drug resistance and biofilm-associated infections because of their high penetration power through the biological membranes.

6. Conclusions

Biofilm-associated infections represent a serious medical challenge because their eradication is difficult with the antibiotic levels normally use against their planktonic forms. Biofilms are characterized by their ability to evade not only the antibiotic effects but also the host immune system clearance. Different antibiotic resistance mechanisms as discussed in this review are employed by biofilms depending on the species of the bacteria, growth conditions, and the antibiotics involved, however, no mechanism is fully established yet. Therefore, a generalised mechanism applicable to all pathogens seems somewhat unrealistic which warrants the study of biofilm resistance of all pathogens individually to visualise the multifactorial nature of biofilm antibiotic resistance and to arrive at suitable therapeutic options. It is pertinent to mention that since no new class of antibiotics have been approved in last four decades there is the need of greater understanding of biofilm-associated antibiotic resistance to effectively utilise the therapeutic value of the existing antibiotics. Although a number of anti-biofilm strategies have been put forward as discussed above in this review, they are still in nascent stage and need to undergo clinical trials to reach the commercial market.

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