Bacterial Biofilm and Antibiotic Resistance

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Biofilm is a structural and functional unit of microbial consortia encased in self-produced polymeric substances including protein, polysaccharide and e-DNA. Biofilm formation by bacteria represents one of the group behaviors in bacterial world which help them to acquire emergent properties in order to survive under unfavorable conditions. Multidrug resistance in bacterial biofilm is thus an increasing threat to global health. For example, Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* are causal agents of biofilm borne infections. The mechanisms of antibiotic resistance are often conferred by horizontal gene transfer promoting evolution and genetic diversity. The inactivation of antibiotics is also advocated by extracellular polymers and modifying enzymes and survival of bacteria at high antibiotic pressure involves enzymes such as chromosomal beta-lactamase, up-regulated efflux pumps and mutation in antibiotic target molecules. Ironically, detailed studies on effect of antibiotics on bacterial biofilm architectures and unraveling the alterations in molecular networks involved in bacterial biofilm formation in presence of antibiotics would be an upcoming avenue for a deeper understanding of biofilm physiology to develop better therapeutic strategies against biofilm mediated bacterial infections.

Keywords: Bacteria, Biofilm, Infection, Antibiotic, Resistance

Introduction

Despite of antibiotic therapy and innate and/or adaptive immune response of the host, chronic bacterial infections persist due to colonisation of cells within the matrix produced by microbial association. Antimicrobial agents such as antibiotics and several biocidal drugs shows feeble activity on bacteria associated within the abiotic and biotic surface also known as biofilm [1,2, 3]. Bacteria persisting in such multi-cellular aggregation of biofilm are causal agent of chronic infection [4] and characterised by persistent inflammation and tissue damage [5]. The challenging resistance towards antibiotic is the key for persistence of bacterial cells viable under harsh and infectious conditions [6]. The infections include periodontitis, pneumonia, lungs of cystic fibrosis patients as well as various indwelling devices such as heart valve, prosthetics, catheters etc. For example, biofilm growth can be observed on the outer and/or inner surface of the foreign body. Natural surface including heart valves, teeth, lungs of cystic fibrosis (CF), patients with chronic rhinosinusitis, chronic osteomyelitis, bronchopneumonia, prosthetic joint infections, intravenous catheters and stents and chronic wounds [5,7-18]. The microbes within the biofilm matrix containing proteins, polysaccharides and DNA originate from microbes and bacterial consortia with one or more species [4,5,18,19]. The matrix provides protection to the biofilm from hash environment and offers mechanical and structural stability for survival. The modern microscopic techniques including confocal laser scanning microscopy enables study of biofilm development with green fluorescent protein (GFP)-tagged bacteria. This technique has recently been coupled with advanced in silico image analysis to produce three dimensional representation of biofilm maturation and have also been extended for research on drug discovery against biofilm borne infection [20-22]. The development of mature biofilm *in vitro* is initiated by planktonic bacteria by attaching to the surface reversibly with the support of matric made up of proteins or pellicle within a span of 5-7 days [7, 23]. At this stage of biofilm initiation, the microorganisms are susceptible to antibiotics till they bind to the surface irreversibly. The microorganisms start secreting self-produced polymeric substances and engulf cells within the matrix around the micro colonies [23]. The bacteria associated within the colony are placed with stacks of cells under specific growth condition and temperature to form a profuse mushroom like architecture. At this stage the biofilm offers maximum tolerance to antibiotics. Interestingly, in case of *Pseudomonas aeruginosa*, it was observed that specific bacteriophage contributes in making channels or canopies (by killing bacterial sub-population) within the biofilm structures to help in maintaining the required fluid dynamics [24]. The motile cells with the help of type IV pili mount within the biofilm and colonise with water filled channels resembling much similar to multi-cellular organisms [21].

Biofilm are studied by light microscopy, however, the specific details of species identification are done by DNA hybridization technique in clinical specimen [5]. Interestingly, the physiological properties of biofilm are different from planktonic counterpart where resistance towards antibiotics can be up to 100–1000-fold higher in the former [25-27]. New tests are being developed to understand the susceptibility of biofilm borne cells towards antibiotic [28, 29].

Mechanism of Antibiotic Resistance

The physiological properties of antibiotic resistance bacteria show interesting features to withstand harsh conditions and host defence. Two well-studied strains show active survival under vast antibiotic concentrations are *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Another example of microorganism which shows less susceptibility to antimicrobial drugs are the yeast *Candida albicans* [30] and the obligate anaerobe *Porphyromonas gingivalis* [31]. Notably, in most of the case studies the biofilm mode of growth showed greater resistance to antibiotics compared to free floating cells in aqueous suspension.

The resistance mechanisms among different strains of microorganisms show plethora of diversity. The adaptive resistance of the bacteria operated in biofilm contribute towards wide spectrum of defence. The mechanism may include alteration in efflux pump, low cell permeability, targeted mutation leading to modification in enzymes. For example, in biofilm mode of growth bacteria conferring less susceptibility towards drugs could be due to protective mutation in of plasmid or mobile genetic element or horizontal gene transfer among bacteria [32]. But, for a considerable number of cases, the antibiotic sensitivity was restored when bacteria engulfed within biofilm are dispersed to its free floating state. Therefore, it was concluded that the reversal of resistance is largely due to adaptive resistance mechanism rather being genetic. However, there are some exceptional cases where bacterial cells within biofilm accelerate the rate of plasmid transfer [33]. Therefore research on antibiotic resistance under biofilm environment has opened up a completely unexplored area for study. The cells engulfed in biofilm matrix enhance the chance of spontaneous mutation.

Genetic and physiological factors for antibiotic resistance of bacterial biofilm

(A) Mutation and horizontal gene transfer

The horizontal gene transfer results in enhanced rate of mutation in biofilm growing bacteria compared to the planktonic counterpart [34, 35]. The traditional resistance mechanism of biofilm borne bacteria against β-lactam antibiotics, aminoglycosides and fluoroquinolones depends on physiological conditions. Enzymes produced by bacterial cells in biofilms inactivate antibiotic action by degrading or breaking chemical bonds and increasing expression of efflux pump that have broad range of substrate. The hypermutable phenotype of *P. aeruginosa* was observed in cystic fibrosis lungs with infection where a bacterial population size is approximately 108-1010/ml of sputum [17, 36, 37].

This is due the fact of selection of isolate expressing multidrug efflux pump [38, 39]. This hypermutable phenotype possess mutation in either of two repair systems i.e. mismatch repair involving *mutS*, *mutL* and *uvrD* or DNA oxidative lesion repair system involving gene coded by *mutT*, mutY and *mutM* [38, 40]. Another crucial factor that leads to enhanced mutability in biofilm and bringing genetic adaptation with evolutionary stratification is oxidative stress due to increased production of reactive oxygen species [41-43]. The change in microcolony structure was observed because of antioxidant deficiency leading to recurring increase of oxidative stress [43]. The reactive oxygen species from activated polymorphonuclear leukocytes (PMNs) often cause oxidative stress in biofilm of hypermutable *P. aeruginosa* strain in CF patients [37]. An interesting study shows that due to consumption of oxygen by polymorphonuclear leukocytes creates a hypoxic environment in CF sputum resulting in stress within biofilm embedded bacterial cells [44]. This intern results in mutation conferring antibiotic resistance among patients receiving repeated antibiotic course. The resistance of antibiotic was found with large number of compounds which cause serious threat for present treatment option [45].

Recent finding suggests mutation occurring in regulatory genes of β-lactamase results in down regulation of AmpC β-lactamase enzyme [46]. On the other hand, mutation in *gyrA* and alteration of efflux system namely MexCD-OprJ and MexEF-OprN offer resistance to ciprofloxacin. Similarly, overexpression of MexXY-OprM multidrug efflux pump confers resistance towards tobramycin [47, 48]. Notably, another study demonstrated mutation in pmr system of lipopolysaccharide structure results in resistance to antibiotic colistin [49].

(B) Role of enzymes in entry of antibiotics

Entry of antibiotics within biofilm matrix was studied in details. The effective diffusion coefficient of solutes in biofilm is approximately 40% of the respective diffusion in pure water for solutes with molecular weight of 100-1000 [50]. For molecules such as glycoprotein or polysaccharide the value is about 36%-76% [51, 52]. However, the entry of antibiotics through the biofilm is always not ensured. The entry of antibiotic is greatly altered in case the drug losses its activity or even may get sequestered by binding and thereby slowing down the entry. For example, in case of betalactamase-positive bacterium, loss of entry of antibiotic penicillin was observed due to reaction diffusion interaction [32]. The interesting report showed entry of antibiotic ampicillin when administered on beta-lactamase negative mutant. Sometime binding of negatively charged biofilm matrix polymer with positively charged aminoglycosides retards the smooth entry of drugs [52-54]). However, complete neutralization or sorption of antibiotic is not recorded or observed [55]. The mechanism of antibiotic resistance of *P. aeruginosa* in infected cystic fibrosis lung is by overproduction of AmpC cephalosporinase [56]. In presence of β-lactam antibiotics, the partially depressed β-lactamase production is

activated within the CF isolates [46]. The phenotype of the microorganism is crucial in determining the resistance towards β-lactam antibiotics. Antibiotics carbapenems such as imipenem acts as strong inducer, however, not all antibiotics work in similar mechanism. Poor inducer such as piperacillin may induce resistance by inducing overexpression of the MexAB-OprM efflux together with β-lactamases. Interestingly, 2.5% cases of clinical CF isolate report total derepressed β-lactamases [46]. However, not all β-lactams are strong inducers and overexpression of the MexAB-OprM efflux pump may, together with β-lactamases, play an important role in resistance to poor inducers (e.g. piperacillin). Totally derepressed β-lactamase production is encountered in 2.5% of clinical CF isolates [46] and found to be independent of the efflux pump overexpression [57]. Another study reports identification of an insertion sequence (IS1669) that inactivate the *ampD* gene among different clinical isolates of *P. aeruginosa* with the ability for high expression of β-lactamase[57]. The β-lactamases present in the biofilm matrix often plays role in hydrolysis of β-lactam antibiotics before reaching the bacterial cells [46, 58] Antibiotic such as imipenem and piperacillin are able to induce β-lactamase production in biofilms of *P. aeruginosa* [59]. Another interesting study suggested as long as chromosomal β-lactamase is low, cells embedded in biofilm would be unable to protect against diffusion of β-lactam. The mechanism suggests cells expressing high level of β-lactamase within biofilm gets accumulated within polysaccharide matrix and protect the cells lying inside the matrix by inactivation of antibiotic penetrating inside it.

Treatment of biofilm cells with various antibiotics were tested in *P. aeruginosa* strains isolated from patients with cystic fibrosis. The insertion of *ampD* sequence resulting derepression of β-lactamase killed few cells of bacteria within biofilm compared to control on being treated with ceftazidime. A combinatorial treatment with aztreonam improved the efficiency of ceftazidime where the former acts as β-lactamase inhibitor [56]. Another study reported an efficient eradication of biofilm of *P. aeruginosa* by antibiotic meropenem, a modified antibiotic with β-lactamase-stable β-lactam ring [26, 60]. The role of biofilm matrix was investigated by several groups in connection with the resistance mechanism. Enhanced synthesis of alginate was observed in presence of β-lactam antibiotics within the biofilm of *P. aeruginosa* and few slime producing *Staphylococci* [61-63]. Repeated dose response of antibiotic also lead to saturation of binding site on the other hand tolerance of aminoglycoside is also due to transport limitation for positively charged antibiotic with negatively charged biofilm matrix [64-66]. The tolerance toward aminoglycosides and ciprofloxacin is also mediated by oxygen limitation and metabolic rate [67]. Within the respiratory zone, delayed entry of aminoglycoside through thick biofilm leads to poor access of antibiotic [5]. Recently combinatorial treatment with DNase and Alginate lyase resulted in dissolution of biofilm matrix and thereby increasing the activity of antibiotic tobramycin in biofilm [68].

Enzymes plays critical role in inactivation or modifying antibiotics. If the matrix is completely impervious to antibiotics the entry of the agent is inhibited naturally. But in most of the cases this does not hold true. Antibiotic inactivating enzymes include chloramphenicol acetyltransferases, beta-lactamases, aminoglycoside-modifying enzymes etc. The presence of such modifying enzymes diminish the antibiotic concentration to significantly lower level but do not completely omit the chance of killing the otherwise sensitive bacteria. Therefore antibiotic resistance only confer the protective mechanism of surviving bacteria within biofilm environment often referred as adaptive resistance mechanism [32,69,70].

(C) Bacterial growth

Bacterial growth phase, generation time and growth environment also play important roles in regulating antibiotic sensitivity and its killing action [71]. With the advent of modern microscopy and use of fluorescent probes and reporter genes, microbial growth in biofilm environment is studied in great details [20, 72, 73]. The recent study suggested the presence of stiff micro-gradient of key metabolic substrate and products within the biofilm [74]. Due the presence of chemical gradient, biofilm associated cells have prolonged stationary phase and growth rate is much lesser than the free living neighbours [75]. The conditions are applicable for a mixed population to even in single species where the rapidly growing cells become metabolically inactive. Biofilm cells growing under uniform intermediate rate are generally rare [76] and in several studies survival of non growing bacteria under antibiotic pressure is reported [70, 77]. Moreover, the attack of antibiotic will also not work in cases where the agent targets the macromolecular biosynthesis process in growing cells. For example the penicillin the most well studied antibiotic acts only on readily diving cells [78].

(D) Oxygen tension

The other factors which immensely contribute towards antibiotic resistance include oxygen tension within biofilm microenvironment. For example, oxygen availability modulates the action of aminoglycosides [79]. The same study demonstrated that the cells in biofilm inhabiting within the anaerobic environment are differentially protected from antimicrobial agents as reported in few studies. Similarly the pH gradient also negatively regulates the efficacy of antibiotics [80, 81].

(E) Metabolic state

Metabolic state of bacteria also plays important role in physiological state of biofilm associated cells living under antibiotic pressure. The biofilm defence therefore equally relies upon the gene expression of various key metabolic enzymes and its pathway for their survival. In context of studying the role of metabolic state of cells within biofilm environment in correlation to antibiotic resistance, three different case studies were described earlier. A mutant of *P. aeruginosa* with defective sigma factor rpoS was found to produce thicker biofilm than its wild type counterpart and interestingly they were found to be less sensitive to tobramycin [82, 83]. In another study mutant overproducing extracellular polymeric substance was generated and the thicker biofilm of the mutant was significantly less susceptible to antibiotic tobramycin [84]. Similar study was reported by Parkins et al., 2001[85] where mutant of *gacA*, the two component regulatory system required for normal biofilm development was generated. Interestingly the mutant failing to form mature structure was found more susceptible to antibiotics.

(F) Environment

In addition to the factors described, another important parameter that determines the success or failure of antibiotic action on biofilm associated cells is the adaptability with environmental fluctuation. Oxidative stress, low water activity, temperature changes, starvation and DNA damage alters biofilm architecture and defence mechanism [86, 87]. Sigma factor rpoS plays crucial role in antimicrobial susceptibility of biofilm detected in continuously fed *P. aeruginosa* [88]. The transcript of the same gene was found in sputa of cystic fibrosis patients [89]. Studies shown earlier that mutant of *rpoS* fail to support antimicrobial susceptibility of biofilm [82, 90]. Additionally, the multidrug efflux pump of the biofilm also contributes towards resistance. However, no elevated expression of efflux pump is documented prior to antibiotic challenge [91, 92]. Advanced DNA microarrays study was conducted to test the role of efflux pump in antibiotic pressure. Interestingly, the study described transcription of efflux pump gene within the biofilms of *P. aeruginosa* administered with antibiotic tobramycin [82]. Therefore, the environmental challenge also drives the stress responses within biofilm population as similar to the planktonic counterpart. The biofilm associated cells are able to express the traits more often than the planktonic cells due to decrease in antibiotic penetration and slow growth rate. Study conducted on activation of *katB* gene coding for catalase was found in response to treatment of 50 mM hydrogen peroxide [93]. However, the study confirmed no activation of the same gene in case of free living planktonic cells since the activity of 50 mM hydrogen peroxide took over before the stress response genes were activated.

(G) Persister formation

Finally, the resistance offered by biofilm associated cells are often due to persister formation. Persisters are a small group of cells within any biofilm mass or bacterial colony which neither grow nor die in presence of antibiotics [94]. Such persister cells were found to exist in presence of chemical disinfectants such as chlorine bleach and glutaratdehyde [1, 3]. Due to the presence of persisters, a fraction of cells could survive under prolonged antibiotic treatment [95, 96].

(H) High cell density and quorum sensing (QS)

Signalling molecule plays important role in bacterial communication and sensing the population density of microorganisms present in a niche [97-100]. The cells present in a limited space respond to its environment by means of activation of certain genes; for example production of virulence factor such as enzymes or toxins solely by quorum sensing mechanism. Small molecules secreted by microorganisms including N-acyl-l-homoserine lactones in Gram-negative bacteria and small peptides in Gram-positive bacteria are important in terms of cellular communications [100].

For example, in case of well-studied *P. aeruginosa* the secretion of virulence factors including a range of extracellular enzymes and lysins are regulated by quorum sensing [68, 101, 102]. Such virulence factors play important role in pathogenesis of infection and acts as protective shield against phagocytes. Thus, quorum sensing not only plays key roles in biofilm development and maturation, it also regulates the antibiotic resistance and therapeutic attribution of innate inflammatory response dominated by polymorphoneucleosite [103, 104].

The vast information on cellular crosstalk and quorum sensing originated with the advent of experiments on QS knockout mutants [105, 106]. There are numerous quorum sensing inhibitors (QSI) isolated and identified from nature [75]. Various groups have synthesized QSI and its biosimilar in laboratory and tested on experimental animal model in vivo [107]. QSI has shown its activity on experimental biofilm model with infection of bacteria *in vivo* for example cystic fibrosis patients with chronic infection *P. aeruginosa* [105,108, 109]. However, antibiotics such as azithromycin, ceftazidime and ciprofloxacin also showed quorum sensing inhibition leading to inhibition of virulence at sub-MIC concentrations without growth arrest [109,110]. Antibiotic azithromycin thereafter shown to have significant success in improving patients with chronic *P. aeruginosa* infection in the lungs of cystic fibrosis and now being regularly used in children and adults [111-114]. However, the development of resistance to other pathogenic bacteria has remained challenging [115]. One interesting natural product to address this issue is garlic extract which has shown remarkable efficiency *in vitro* and *in vivo* biofilm model of *P. aeruginosa* [103].

Foreign body infection is another uprising problem that steadily enhance medical problem related to intrauterine catheters, nasolaryngeal tubes, stents, artificial heart, alloplastic materials etc. [6]. When the biofilm forming bacteria colonise on foreign devises it often lead to chronic inflammation around it. A strategy was developed where antibiotic coated foreign bodies were introduced to prevent formation of biofilm and was found to be efficient [116, 117]. Presently, new strategies are being tested to develop compounds against bacterial resistance towards antibiotics and quorum sensing inhibitors.

Alternative treatment opportunities

With the increasing cases of multidrug resistance, demands for discovery of new antibiotics are ever rising. For example, Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* are causal agents of biofilm borne infections. An interesting study by Barksdale et al. 2017 [118] describes the discovery of cationic antimicrobial peptides (CAMPs) with an attractive possible therapeutic value against multi-drug resistant bacteria. The peptide was found to be effective as antimicrobial as well as anti-biofilm in nature [119-123]. The peptide acts on cytoplasmic membrane or trough any complex machinery is currently unknown. The antimicrobial peptides are active against Gram-negative and Gram-positive bacteria, few fungal strains as well as on membrane borne viruses. The strategy is very important in case of biofilm-infected war wounds as the CAMPs seldom cause genetic resistance in bacteria and are effective against antibiotic resistant bacteria. Strikingly the CAMPs were isolated from alligator mississippiensis (American alligator) which inhabits in bacteria-laden environments without being succumb to bacterial infections. The newly identified CAMP cathelicidin has shown to have strong activity against multiple Gram-negative bacteria, including clinical isolates of multidrug resistant (MDR) *Acinetobacterbaumannii* and carbapenem-resistant *Klebsiellapneumoniae*. The results also suggested that the peptides could successfully permeabilize the bacterial membrane and very less sensitive to salt inhibition than many other known CAMPs. The non-haemolytic activity and significantly less cytotoxicity against A549 human lung epithelial cells could bring an attractive platform of these peptides for further development as a group of potential therapeutic agents.

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