Understanding the Bacterial Biofilm Resistance to Antibiotics and Immune Evasion

Surekha Challa, G. Mohana Sheela, and Nageswara Rao Reddy Neelapu

Abstract Biofilm is a multicellular lifestyle for bacteria to survive in adverse environmental conditions. Biofilms withstand antibiotics, immune defenses, disinfectants, nutritional changes and high temperatures. The present chapter reviews information of biofilm and also provide insights on how biofilms are able to tolerate antibiotics and evade immune system.

Keywords Biofilm · Antibiotic resistance · Immune evasion

Introduction

Microorganisms thrive in nature by existing either as free living individuals (planktonic mode) or as community known as biofilm. It was assumed that the standard mode of growth for some bacterial species is formation of biofilms whereas the planktonic growth is an in vitro work of art [1]. The term biofilm was coined by William J. Costerton in 1978 to describe the 'surface-attached microbial agglomerations' [2]. The alternative description available according to Donlan and Costerton [3] is …." communities of microorganisms attached to a surface, producing extracellular polymeric substances (EPS) and exhibiting an alternate phenotype when compared with corresponding planktonic cells….". Biofilm is made up of water, bacterial cells, dead cells, and EPS [4]. EPS (referred as matrix) is 90% of the biofilm and EPS matrix consists of exopolysaccharides, DNA, proteins and other macromolecules [5]. The composition of the bacteria is different in the biofilm's. Bacteria form a biofilm either by recruiting the same bacterial species or by recruiting other bacterial species. If the bacterium recruits the same bacterial species then the biofilm formed is known as monospecies biofilm. Whereas, if the bacterium

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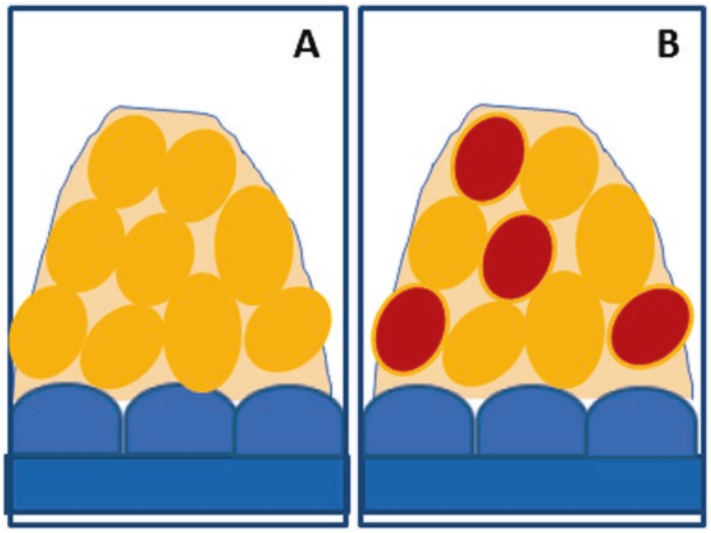

Fig. 1 Bacterial biofilm formed by the (**a**) same bacterial species (monospecies biofilm), (**b**) other bacterial species polymicrobial biofilm)

recruits the other bacterial species then the biofilm formed is known as polymicrobial biofilms (Fig. 1). Some available examples for polymicrobial biofilms are *Pseudomonas aeruginosa* mixed with *Staphylococcus aureus* [6]; *Prevotella* mixed with *S. aureus* [7]; and *Escherichia coli* mixed with *Bacteroides fragilis* [8]. Polymicrobial biofilms increase the rate of infection and survival of bacteria and thereby becomes recalcitrant [9]. *P. aeruginosa* and *S. aureus* biofilms [6]; and *Prevotella* and *S. aureus* biofilms [7] increased the infection rates of pathogens in a rat and mouse models respectively. *E. coli* with *B. fragilis* increased abscess formation in a mouse model [8].

Stoodley et al. [10] proposed a model to demonstrate how a bacterium like *P. aeruginosa* forms biofilm. The development of a biofilm (Fig. 2) includes the following five steps –

1. The first step includes initial or reversible adherence of bacterial cell to a surface in the host. This initial adherence of the bacterium to the surface is influenced by the factors like specific bacterial surface molecules (secreted adhesins and extracellular adhesive appendages), motility and chemotaxis. The forces acting or involved between bacterial cells and the surface of attachment are hydrophobic or electrostatic interactions.
2. The second step includes multiplication of the bacteria forming microcolonies. The microcolonies in the biofilm grow up both horizontally and vertically in size. The bacterial cells generate EPS on all sides of the microcolonies resulting in irreversible adhesion.
3. The third step includes development leading to formation of an early structure like matrix for biofilm.
4. The fourth step includes maturation of matrix leading to formation of biofilm. The mature biofilm is a either a “thick and mushroom-like or tower-like

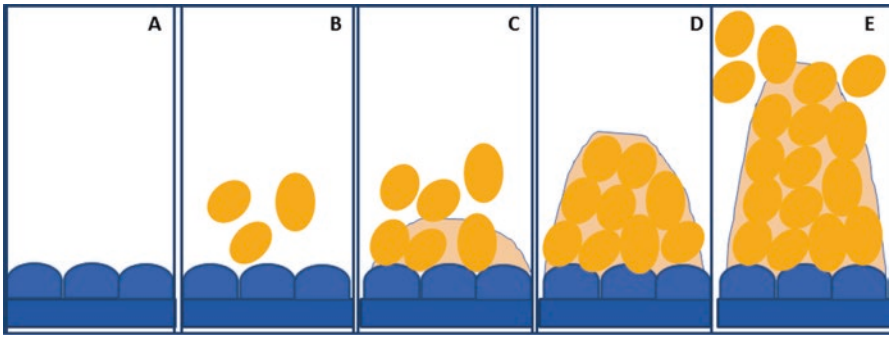

Fig. 2 The sequence of events involved in formation of a biofilm (**a**) surface/substrate for the formation of a biofilm, (**b**) bacterial cells adhering to the surface, (**c**) bacterial cells generating EPS resulting in irreversible adhesion, (**d**) development leading to formation of an early structure like matrix for biofilm, (**e**) maturation of matrix leading to formation of biofilm and dispersal of cells from the matrix of biofilm

Table 1 Biofilms related to devices

S. No	Devices	Reference
1	Orthopedic alloplastic devices	[11, 12]
2	Indwelling urinary catheters or urethral stents	[13, 14]
3	Intravenous catheters	[15]
4	Vascular prostheses	[16]
5	Cardiac pacemakers and prosthetic heart valves	[13, 17, 18]
6	Endotracheal tubes	[19]
7	Cerebrospinal fluid shunts	[20]
8	Peritoneal dialysis catheters	[21]
9	Biliary tract stents	[22]
10	Intrauterine devices	[23, 24]
11	Contact lenses	[25]
12	Tissue fillers	[26, 27]
13	Dentures	[28]

structures". The 3-dimensional structures filled with cells in groups as the number of bacteria increase. These structures form ducts between the groups allowing transport of water and nutrients; and removal of waste.

5. The fifth step includes dispersal of cells from the matrix of biofilm. Thereby biofilms display crucial disbanding mechanisms and release cells which are circulated to further sites. Fluctuation in oxygen, nutrient availability, other stress-generating situations, and toxic products are the factors persuading dispersal of biofilm.

Generally, biofilm is formed on medical devices; or in the tissue of the host; or on fresh fruits and vegetables; or on agricultural products used for food consumption (Tables 1, 2, and 3). Biofilm generally provides a strong platform for interaction

Table 2 Biofilms related to tissues

S. No	Disease	Pathogen	Tissue	Reference
1	Cystic fibrosis	*P. aeruginosa*	Lungs	[29]
2	Chronic obstructive pulmonary diseases	*P. aeruginosa*	Lungs	[30]
3	Tuberculosis	*Mycobacterium tuberculosis*	Lungs	[31]
4	Chronic wound infections	Invasive infectious agents like *Staphylococcus aureus*	Tissue with wounds	[32]
5	Chronic otitis media	*S. pneumoniae, Haemophilus influenzae, Moraxella catarrhalis,* and *S. aureus*	Ear	[33]
6	Chronic sinusitis	Viral or bacterial infection	Nasal passages (sinuses)	[34]

Table 3 Biofilms on fresh fruits, vegetables or agricultural products used for food consumption

S. No	Pathogen	Fruit/vegetable	Reference
1	*S. enterica serovar Saphra*	Cantaloupe melons	[35, 36]
2	*E. coli*	Apples	[37–39]
3	*E. coli* O157:H7	Lettuce and spinach	[40]
4	*Shigella sonnei*	Fresh parsley	[40]
5	*Shigella boydii*	Bean salad	[41]
6	*Shigella*	Parsley plants	[40]

and communication among the individuals present in the colony and also withstand antibiotics, immune defenses, disinfectants, nutritional changes, high temperatures etc., In this section, a detailed discussion on how biofilms tolerate antibiotics and evade immune system are given below.

Antibiotics Resistance

Though modification of the antibiotic molecule, reducing drug permeability, and modification of target binding sites are the known mechanisms for antibiotic resistance; formation of biofilm is another mechanism for antibiotic resistance. Biofilms when exposed to antibiotics show several phenotypic changes and alteration in signaling pathways. Changes in biofilm structure, cell morphology, growth rate, induction of extracellular DNA (e-DNA) and bacterial membrane vesicles (BMVs) are the phenotypic changes reported when exposed to antibiotic. The signaling mechanisms like Cyclic dimeric guanosine monophosphate (c-di-GMP) signaling, oxidative stress response, quorum sensing, SOS response and starvation stress response involved in the biofilm. These signaling mechanisms are altered when exposed to antibiotics (Fig. 3).

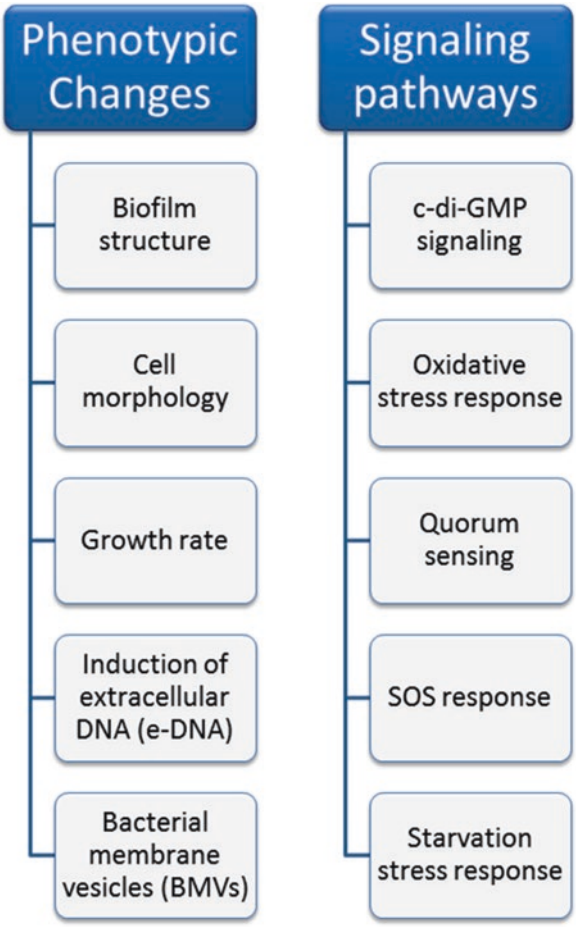

Fig. 3 Phenotypic changes and alteration in signaling pathways in a biofilm providing resistance to antibiotics

Alterations in bacterial cell morphology were reported in *Klebsiella pneumonia*, *E. coli*, and *Streptococcus mutans* when exposed to sub-lethal concentration of antibiotics and other compounds. *K. pneumonia* when exposed to carbapenem, imipenem, meropenem and doripenem; morphological alterations of *K. pneumonia* cell was observed. Round cells of *K. pneumonia* when exposed to carbapenem modified there size and shape through RpoS-dependent regulation [42]. When *K. pneumonia* was exposed to imipenem for 24 h significant cell shortening was observed, whereas significant cell lengthening was observed when *K. pneumonia* was exposed to meropenem and doripenem. *E. coli* when exposed to piperacillin or a combination of piperacillin and tazobactam, changed its morphology to filamentous form [43, 44]. *S. mutans* when exposed to xanthorrhizol (extract of *Curcuma* xanthorrhiza), changed its surface and contour of cell wall and membrane [45]. Thus, bacterial cells when exposed to antibiotics alter the shape with a possible connection to antibiotic response.

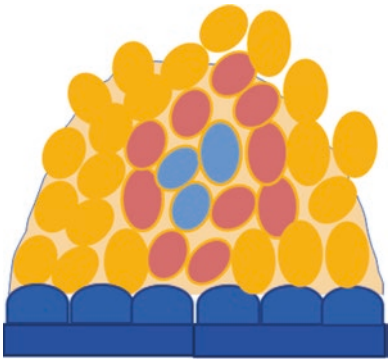

Fig. 4 Surface layer cells, middle layer cells and deepest layer cells of the biofilm

The change in the growth rate of cells in a biofilm when exposed to antibiotic is another notable feature. Cells in the biofilm can typically be classified as surface layer cells, middle layer cells and deepest layer cells (Fig. 4). Cells present at the surface, middle, and deepest of the biofilm are metabolically active, non-growing but alive, and dormant respectively. Cell surface cells of the biofilm are sensitive to antimicrobials, whereas middle layer cells acquire tolerance to some agents, and inner layer cells are tolerant to antimicrobial agents. The lowered metabolic activities of the middle layer cells; and zero metabolic activities in the inner cell layers of the biofilm are responsible for the resistance to antibiotics. Thus, biofilms when exposed to antibiotics exhibit reduced growth leading to antibiotics resistance.

eDNA is known for formation, sustaining and maintenance of biofilm [46–48]. The sources of eDNA can be external to the biofilm or can be one of the cells lysed in the polymicrobial species biofilm. This eDNA via horizontal gene transfer is absorbed by other competent cells of the biofilm leading to antibiotic resistance [49]. Further, eDNA binds to antibiotics [50, 51], or activates genes concerned with resistance leading to antibiotic resistance. Thus, role of eDNA in antibiotic resistance by various mechanisms is a fact.

BMVs have multiple roles like guarding the microbial cells from antibiotic stress, promoting biofilm formation; facilitating adherence; material delivery; retaining integrity of the cell membrane; and competing for growth factors. BMVs provide resistance to antibiotics such as polymyxin B, colistin, and melittin [52, 53]. In an experiment with *P. aeruginosa* biofilm, drug-binding proteins were identified in the BMVs; and this signifys a likely drug-sequestering consequence by content in BMVs [54, 55]. In another study, BMVs of *S. aureus* carrying protein lactamase showed resistance to ampicillin [56]. The other possible role of BMVs is acting as an interspecies communication system to transfer DNA, proteins, RNA, and toxins [57]. Another role of BMVs is to promote biofilm formation, where addition of BMV to *Helicobacter* planktonic culture initiated the formation of *Helicobacter* biofilm. Thus, vesicles allow microbial cells in the biofilms to thrive against antibiotics in addition to other roles.

Starvation of the middle and inner layer cells of the biofilm is known and biofilm induces response to this starvation. These starvation responses are known to protect

bacterial biofilm when exposed to antibiotics [58, 59]. Nguyen et al. [60] reported antibiotic resistance when nutrients are limited to biofilms and bacteria. The plausible explanation is that starvation response signal like RelA-SpoT mediates decrease in prooxidants and increase in antioxidants to protect biofilm from antibiotic. Thus, starvation responses have the ability to defend the biofilm from antibiotics.

SOS responses generated by bacterial cells in the biofilm were known to provide tolerance to antibiotics. DNA damaging agents or antibiotics increase the mutation rate leading to a "hypermutator phenotype". Hypermutators have an advantage in colonizing the host as well as in exhibiting virulence [61]. Hypermutator phenotypes also hinder recombination and generate SOS response. SOS response activates DNA repair and facilitates recombination, and as a result DNA repair mutants can acquire antibiotic resistance genes [62]. In *P. aeruginosa* MMR deficient mutators were to able adjust as a biofilm community, whereas planktonic cells were not able to adjust. Fluoroquinolones and ciprofloxacin induced SOS response in pathogens resulting in bacterial persistence [63, 64]. Though, the clear connection between SOS response and antibiotic resistance is not established; the above evidences are in favor of SOS response and antibiotic resistance.

Oxidative stress responses generated by bacterial cells in the biofilm were known to provide tolerance to antibiotics. Oxidative stress induces double-strand breaks in bacterial DNA and as consequence bacteria activates the DNA repair mechanism. The DNA repair mechanism facilitates recombination allowing the mutants to acquire antibiotic resistance genes [62]. Boles and Singh [65] revealed that oxidative stress induce mutations in the bacteria cells of biofilm leading to variants. And identified that activation of DNA repair have a tendency to increase antibiotic resistance in biofilms against gentamicin [65, 66]. Thus, oxidative stress responses generated by bacterial cells in the biofilm provide antibiotic resistance.

c-di-GMP signaling by bacterial cells in the biofilm bestows tolerance to antibiotics. c-di-GMP is the secondary messenger involved in regulating the formation of biofilm and persister cell [67]. Hoffman et al. [68] proved that signaling of c-di-GMP in *E. coli* and *P. aeruginosa* improved biofilm mass in the presence of antibiotic tobramycin. Thus, c-di-GMP signaling improves tolerance to antibiotics.

Quorum sensing (QS) facilitates antibiotics resistance to the bacterial cells in the biofilm [69]. QS signaling provided resistance in *P. aeruginosa* for antibiotics ceftazidime and colistin. LasR mutants of *P. aeruginosa* acquired beta-lactamase activity and showed resistance to ceftazidime. QS in *P. aeruginosa* is regulated and colistin-tolerant cells migrate to the upper layer of the biofilm using "type IV pili-dependent motility" [70]. This allows the biofilm to grow in size and helps the pathogen to persist even in the presence of antibiotics. Thus, QS signaling also have an important role in contributing resistance to antibiotics.

Immune Defenses

Biofilms use a number of strategies to withstand host defense mechanisms. Literature reports key strategies used by biofilms to evade host immune system. The strategies are (1) leukocytes penetration into the biofilm is limited, (2) QS increases resistance to leukocytes, (3) leukocytes adeptness to engulf biofilm decreases, (4) activity of leukocyte is suppressed, (5) genetic switches of biofilms, [71] (6) dysfunctioning or destroying macrophages, and (7) biofilm shields (Fig. 5). In this section we discuss in detail the immune evading mechanisms used by biofilms of *S. aureus* and *P. aeruginosa*.

Mechanisms used by biofilms of *S. aureus* to evade immune system are evading recognition of TLR2 and TLR9 [72]; skewing the immune response; dysfunctioning of macrophage; and impaired phagocytosis of leukocytes [73]. Though, leukocytes penetrate into biofilm they were not able to kill bacteria in biofilm due to impaired phagocytosis of leukocytes [73]. Although, macrophages were able to engulf immature or disrupted biofilm of *S. aureus* [72]; macrophages were not capable of engulf a mature biofilm. At the same time dysfunctioning of macrophages is due to release of products by biofilm. Therefore, the above mechanisms are used by *S. aureus* to evade the host immune system.

The alternative mechanisms used by biofilms to evade immune system are by developing protective layers around biofilms. *P. aeruginosa* biofilms to evade immune system have protective layers like exopolysaccharide alginate and rhamnolipids. The exopolysaccharide alginate in *P. aeruginosa* biofilms shields bacteria from leukocyte phagocytosis, whereas rhamnolipids form a "biofilm shield" and

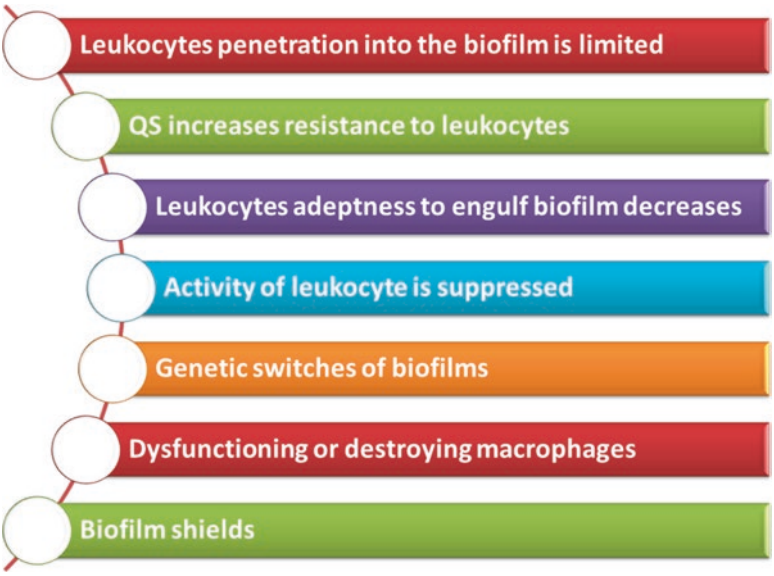

Fig. 5 Immunoevasion strategies used by biofilms to withstand host defense mechanisms

prevent the bactericidal activity of polymorphonuclear leukocytes (PMNs) [74]. Thus, biofilm shields prevent immune action against *P. aeruginosa* and protect it from host immunity.

Conclusion

Bacteria live in communities to provide a platform for interaction and communication among the individuals and also to withstand antibiotics, disinfectants, high temperatures, immune defenses, nutritional changes etc. Changes in biofilm structure, cell morphology, growth rate, induction of e-DNA, BMVs; and altered signalling mechanisms like c-di-GMP signaling, oxidative stress response, quorum sensing, SOS response and starvation stress response provide resistance to the biofilm. Limited leukocytes penetration into the biofilm, increased resistance to leukocytes, decreased leukocytes adeptness to engulf biofilm, suppression of leukocyte activity, genetic switches of biofilms, dysfunctioning or destroying macrophages, and biofilm shields form the important strategies of the biofilm to evade host immune system.

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Authors Contribution CS and NNR initiated the review, participated in writing and revised the manuscript.

Conflict of Interest Statement The authors declare that there is no potential conflict of interest.

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