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Review

Effects of biotic and abiotic factors on biofilm growth dynamics and their heterogeneous response to antibiotic challenge

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Over the last couple of decades, with the crisis of new antimicrobial arsenal, multidrug-resistant clinical pathogens have been observed extensively. In clinical and medical settings, these persistent pathogens predominantly grow as complex heterogeneous structures enmeshed in a self-produced exopolysaccharide matrix, termed as biofilms. Since biofilms can rapidly form by adapting new environmental surroundings and have potential effect on human health, it is critical to study them promptly and consistently. Biofilm infections are challenging in the contamination of medical devices and implantations, food processing and pharmaceutical industrial settings, and in dental area caries, periodontitis and so on. The persistence of infections associated with biofilms has been mainly attributed to the increased antibiotic resistance offered by the cells growing in biofilms. In fact, it is well known that this recalcitrance of bacterial biofilms is multifactorial, and there are several resistance mechanisms that may act in parallel in order to provide an enhanced level of resistance to the biofilm. In combination, distinct resistance mechanisms significantly decrease our ability to control and eradicate biofilm-associated infections with current antimicrobial arsenal. In addition, various factors are known to influence the process of biofilm formation, growth dynamics, and their heterogeneous response towards antibiotic therapy. The current review discusses the contribution of cellular and physiochemical factors on the growth dynamics of biofilm, especially their role in antibiotic resistance mechanisms of bacterial population living in surface attached growth mode. A systematic investigation on the effects and treatment of biofilms may pave the way for novel therapeutic strategies to prevent and treat biofilms in healthcare and industrial settings.

Keywords. Biofilm; heterogeneity; quorum sensing; resistance; sub-MBEC

1. Introduction

Bacterial cells can exist in free-living and biofilm growth modes. There is increasing evidence that both phenotypic states play significant role in weakening the healing and resulting in acute and chronic infections, respectively (Furukawa *et al.* 2006; Armbruster and Parsek 2018; Beebout *et al.* 2019). When antibiotics were discovered for clinical use more than 85 years ago, many believed that it was the final solution for acute infectious diseases caused by bacteria. Unfortunately, within a few years, bacteria controlled by antibiotics had developed marked resistance by developing as biofilms. Presently, biofilm associated infections have become a serious health problem worldwide, killing ∼17 million people each year. Now the supply of new antibiotics is drying up (Furukawa *et al.* 2006; Bjarnsholt 2013; Clinton and Carter 2015; Ciofu and Tolker-Nielsen 2019). Furthermore, microbial load has been exposed to insufficient dose at the beginning and end of dosing treatment, which cannot eliminate the biofilm-embedded bacteria. Subsequently, survivors were able to grow and develop a new biofilm within 24 h after stopping the treatment (Song *et al.* 2005; Hoiby 2011; Dang and Lovell 2016; Kırmusaoğlu 2016). To challenge the antibiotic response of biofilm, it is imperative to investigate new antibiotics and practice highly responsible use of conventional antibiotics.

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Biofilms are self-organized multicellular microbial aggregates, generally composed of tightly packed bacteria, fungi, and algae. As the biofilms mature, they form a viscous, shiny matrix comprised of extra cellular polymeric substances (EPS), making them easily visible. This EPS matrix acts as a protective membrane for the cells growing within a multicellular aggregate and hinders the antibodies from attacking them (Furukawa *et al.* 2006; Machineni *et al.* 2018; Trastoy *et al.* 2018). Biofilm formation can lead to delayed wound healing or non-healing chronic wounds and potentially increases the risk of infection for the patient. A chronic wound is a wound that is arrested in the inflammatory stage of healing process and cannot progress further to proliferative phase. Previous studies have reported that more than ∼90% of microbial infections in humans were associated with biofilm strata and about ∼ 50% of them were mainly due to indwelling medical settings (Clinton and Carter 2015; Hurlow *et al.* 2016; Singh *et al.* 2017). Chronic biofilm infections are still a significant challenge to clinical researchers, owing to their higher morbidity and mortality (Saxena *et al.* 2019).

Bacteria can form single and multispecies communities with distinct features based on microbial composition and microenvironment. Biofilms can exhibit extremely high resistance to conventional antibiotic arsenal and host immune defense system (Mulcahy *et al.* 2014; Santos-Lopez *et al.* 2019). For instance, alginate-overproducing *Pseudomonas aeruginosa (P. aeruginosa)*, biofilms developed in cystic fibrosis lung resulted in increased tolerance and are believed to be the leading cause of morbidity and mortality (Ciofu *et al.* 2015; Maurice *et al.* 2018). Persistence and resistance mechanisms of microbial species growing in biofilms present unique phenotype and novel challenges to clinicians and patients.

Biofilms display recalcitrance to multiple drugs, and the minimum eradication concentration for biofilm-associated bacteria is ∼1000 fold higher compared to their non-biofilm, free-floating counterparts (Hall and Mah 2017). However, frequent high dosages of antibiotics could easily damage the surrounding healthy tissues. Thus, rational use of antimicrobial agents is the key approach to enhance their efficiency and fight against infectious diseases.

A number of mechanisms have been reported to explain biofilms reduced susceptibility, including efflux-mediated removal of antimicrobial agents from cytoplasm (Zhang and Mah 2008; Hall and Mah 2017), occurrence of persistent subpopulations of cells (Stewart and Franklin 2008), reduced penetration of antibiotics (Ciofu *et al.* 2017), expression of specific genes (Resch *et al.* 2005; Dötsch *et al.* 2012) and stress responses (Poole 2012; Park *et al.* 2014a, b). Several studies have reported that biofilm resistance mechanisms are influenced by multiple factors, such as concentration gradients in nutrient (Stoodley *et al.* 1998; Hunt *et al.* 2004; Rochex and Lebeault 2007; Machineni *et al.* 2017), antibiotics (Tezel *et al.* 2016; Machineni *et al.* 2018), and heterogeneous metabolic activity of bacterial population (Wilkins *et al.* 2014). Moreover, quorum sensing regulated EPS secretion was supposed to reduce susceptibility (Tseng *et al.* 2013; Park *et al.* 2014a, b) and enzymatic degradation of antibiotic compounds are likely involved depending on the conditions (Wright 2005). This review presents a comprehensive description of the effect of cellular and physiochemical factors on surface–programmed biofilm growth, and associated reduced susceptibility. A greater understanding of these factors in stage specific resistance might pave the way for novel treatments for the prevention and eradication of biofilm-related infections.

2. Three stages of biofilm development

All abrasions or open wounds lack skin protection that have high tendency to support the attachment of living cells from endogenous or exogenous flora. Consequently, microbes on wound surface can actively proliferate, and result in biofilm formation. Biofilms generally develops in three steps. Initially, a small community of bacteria reversibly arrests themselves to a biotic or abiotic substratum through cell surfaces appendages, such as flagella and pili. At this step, clinical practitioners can quickly terminate the maturation of biofilm via cleaning and debridement. Within a couple of hours, the microbial consortia will develop as highly cohesive symbiotic community with permanent attachment to substratum. Finally, this community will start secreting EPS that provide the foundation for biofilm, and start to spread infection. EPS matrix helps to establish an environment conducive for biofilms longevity and protection against physical, chemical and biological stressors. This complete process takes around two to four days (Kostakioti *et al.* 2013; Armbruster and Parsek 2018). As the biofilm matures via these three stages, it becomes progressively more challenging for the patient's immune defense to fight it efficiently (Armbruster and Parsek 2018; Santos-Lopez *et al.* 2019). Even after debriding matured biofilm, it

can recur in about 24 h, which is interestingly rapid than initial development (de la Fuente-Nunez *et al.* 2013; Dang and Lovell 2016). At this stage, the risk of impaired wound healing and development of chronic infection is increased (Demidova-Rice *et al.* 2012). Consequently, the prevention of biofilm in the initial step is potential for rapid and highly effective eradication or inhibiting of chronic wounds (Santos-Lopez *et al.* 2019).

Bacterial cells growing within biofilm matrix exhibits complex communication process known as quorum sensing (QS), via self-secreted diffusible chemical mediators. As soon as the concentration of chemical mediators or signaling molecules called autoinducers reaches a threshold limit, it stimulates specific gene expression that alter the community behavior, allowing them to act more as a solitary unit as opposed to an individual cell (Atkinson and Williams 2009; Park *et al.* 2014a, b). QS phenomenon enables biofilm maturation to form heterogeneous structures with EPS production (Flemming *et al.* 2007; Maurice *et al.* 2018). In addition, bacteria living in biofilm manifest themselves to adapt local changes and new environments via cell-cell signaling system (Tsuneda *et al.* 2003; Nguyen *et al.* 2011; Jiang *et al.* 2019).

Biofilm formation is known as one of the microbial survival strategies because it provides potential advantages to microbes that include (i) sequestration to nutrient-rich area (colonization); (ii) protection against human immune response and antibiotics; (iii) utilization of mutual benefits (community), and (iv) protection against environmental stresses. Within 48–96 h of development, biofilm exhibited resistance to host immune system and therapeutic maneuvers (Kumar *et al.* 2017; Saxena *et al.* 2019). Over the past few decades, biofilm formation by microbial pathogens has received the most attention, mainly in health-related fields, such as medicine and dentistry, due to its substantial risks, including extreme resistance to biocides, persistence against phagocytosis, and virulence factor secretion (de la Fuente-Nunez *et al.* 2013; Ciofu and Tolker-Nielsen 2019).

3. Antimicrobial resistance of biofilms

Most of the acute infections caused by free-floating microbes have been extensively studied for well over past few decades. These infections affected millions of humans in earlier centuries, but they have been efficiently alleviated with the use of modern vaccines, and infection control measures. However, these acute infections have now been supplemented by a new category of chronic infections caused by bacteria living in EPS matrix. When bacteria develop a biofilm within the human host, the infection quickly becomes untreatable with conventional antimicrobial agents and mature into persistent state by evading the host defense system. Biofilm associated infections, including *P. aeruginosa* associated with cystic fibrosis, and multidrug resistant *Staphylococcus aureus (S. aureus)* associated wound infections attack millions of patients every year and cause innumerable deaths (Costerton *et al.* 1987; Bridier *et al.* 2012; Bjarnsholt 2013; Clinton and Carter 2015; Maurice *et al.* 2018). Despite the significance of microbial biofilms, it is important to understand the mechanism of biofilm development and associated resistance mechanisms.

Microorganisms growing within biofilm community display features that are distinct from their free-floating counterparts (Olsen 2015). The highly evident features, which are common to all observed biofilms, include genetic features, and cellular and physiochemical features. Expression of specific genes may allow the biofilm bacteria to actively adapt and survive external stresses (Pu *et al.* 2016). These genetic features contribute to antibiotic tolerance of microbial consortia at single-cell level (Pamp *et al.* 2008; Poudyal and Sauer 2018a, b). In addition, biofilms are predominantly characterized by altered microenvironments and growth dynamics, concentration gradients of nutrients or growth factors, diffusional limitation of antimicrobial agents, higher cell densities, and EPS. Bacteria growing in biofilms are physiologically heterogeneous, due in part of their adaptation to local environmental surroundings. They form different zones of fast growing bacterial population, with more metabolically active forms at the surface layers and slow growing or metabolically inactive forms in the interior portion of biofilm. Subsequently, distinct micro colonies may develop, in which the cellular physiology is different from their neighbors in terms of growth rates, concentrations of nutrients, signaling molecules, antimicrobial agents, and EPS production. This intrinsic cellular and physiochemical heterogeneity of biofilms may have considerably contributed to the protection of bacteria growing in biofilms (Xu *et al.* 1998; Sauer *et al.* 2002; Stoodley *et al.* 2002; Rice *et al.* 2005; Williamson *et al.* 2012a, b; Wood *et al.* 2013; Dang and Lovell 2016; Beebout *et al.* 2019) (table 1). Recent experimental finding showed that these metabolically quiescent cells could withstand multiple antibiotic challenges, and recur as biofilm within 24–48 h after

Table 1. Different biotic and abiotic factors

Biotic factors	Abiotic factors
Persisters (Rafii 2015; Meylan *et al.* 2018)	Penetration limitation (Singh *et al.* 2010; Staudinger *et al.* 2014)
Quorum sensing (Hall and Mah 2017)	Insufficient antibiotic dose (Stoitsova *et al.* 2016)
Oxidative stress (Drenkard 2003; Trastoy *et al.* 2018)	Nutrient accessibility (Nguyen *et al.* 2011; Williamson *et al.* 2012a, b; Amato and Brynildsen 2014; Stewart *et al.* 2016)
Growth rate heterogeneity (Singh *et al.* 2017)	Oxygen, temperature, and pH (Hajdu *et al.* 2010; Sønderholm *et al.* 2018)
Efflux pumps (Williamson *et al.* 2012a, b; Poudyal and Sauer 2018a, b)	Environmental drivers (Gothwal and Thatikonda 2017; Meylan *et al.* 2018)
Inactivation of antimicrobials by exo-polymers (Yang *et al.* 2011; Goltermann and Tolker-Nielsen 2017)	

treatment (Fernández-Barat *et al.* 2017). An increased understanding of the dynamics of micro-environments of chronic biofilm infections may shed light on the molecular and biophysical resistance mechanisms of biofilms.

It is commonly known that the basis for biofilm-associated persistence towards cellular and physiochemical stressors is multifactorial (Hall and Mah 2017; Ciofu and Tolker-Nielsen 2019; Santos-Lopez *et al.* 2019). The mechanisms of antimicrobial tolerance vary based on specific biocide and its concentration, microbial species, age and developmental stage of biofilm, and environmental conditions. In particular, there is no single mechanism likely to account for the enhanced resistance of multicellular communities. The following sections attempt to discuss the influence of several cellular and physiochemical factors on biofilm growth dynamics and their heterogeneous response to antibiotics.

4. Factors influencing biofilm growth dynamics and their heterogeneous response to antibiotic challenge

There are several key factors that influence biofilm growth dynamics and their heterogeneous response towards antibiotic, including biotic factors, cellular and physiochemical heterogeneity, and quorum sensing, and abiotic factors, antimicrobial concentration, and nutrient concentration (figure 1).

Additional understanding with respect to the relation between antimicrobial regimens and bacterial growth dynamics could provide new insights in order to design highly effective clinical antibiotic therapy, and develop novel antimicrobial agents in parallel. The following sections will thus discuss about the recent findings on the effects of above mentioned factors on biofilm growth dynamics and their response to antibiotic challenge in detail.

4.1 *Biotic factors*

4.1.1 *Cellular and physiochemical heterogeneity:* The major challenge in biofilm research is the intrinsic heterogeneity of its structure, such as non-uniform distribution of biomass, nutrients flux, and intercellular signaling, which leads to temporal and spatial variation in microbial metabolism and gene expression (Gu *et al.* 2013; O'Donnell *et al.* 2017; Beebout *et al.* 2019). Using advanced imaging tools, such as scanning electron microscope and confocal laser scanning microscope, biofilm researchers recently confirmed the presence of spatially scattered antibiotic sensitive subpopulations within the biofilms. For instance, antibiotic tobramycin specifically killed fast growing cells at the outer portion of *P. aeruginosa* biofilms, while other biocides, including colistin, sodium dodecyl sulfate, and gallium were effective on the cells in interior portion (Kim *et al.* 2008; Kim *et al.* 2009). Antibiotics that are effective against metabolically active cells can actually interfere the fundamental physiological processes of bacterial cells, such as replication or translation. In contrast, biocides that are effective against metabolically inactive cells can interfere with bacterial membrane structure and function, while active cells survive the treatment at the outer layer of biofilm due to their ability to induce adaptive stress response associated with genetic modifications (Haagensen *et al.* 2007; Park *et al.* 2014a, b). It has been reported that by using selective GFP labelling and cell sorting techniques, cells at the bottom of thick *P. aeruginosa* biofilm were in a slow-growth state and had reduced sensitivity to the antibiotic ciprofloxacin (Kim *et al.* 2009; Meylan *et al.* 2018). Similarly, a study that involved dose-response killing of *P. aeruginosa* biofilms by quinolones, ofloxacin and ciprofloxacin showed that most of the cells were effectively killed by low concentrations of antibiotics, which is not much different from what was observed with planktonic cells. However, after a rapid and significant decrease in biofilm bacterial load, an additional increase in the biocide concentration could

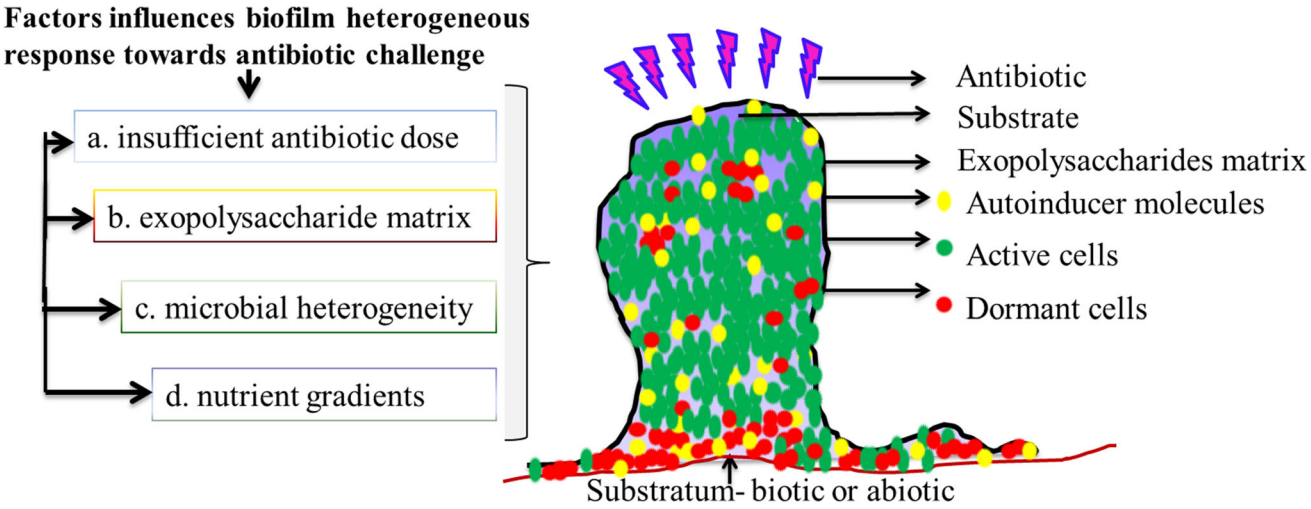

Figure 1. Biofilm growth and its predominant features.

not eliminate biofilm population (Broun *et al.* 2000; Meylan *et al.* 2018). Altogether, these experimental investigations demonstrated that only subpopulation of the cells have exhibited increased resistance and protected the biofilm from killing for later resuscitation when nutrients are available. While spatial heterogeneity in the biofilms is clearly important to select potential antibiotics for biofilm-related infections, only little information is available with respect to the process, in which local environmental conditions influence the spatially heterogeneous antibiotic response.

4.1.2 *Quorum sensing:* Bacterial cells growing in biofilms 'talk' with each other by sensing and responding to self-produced small diffusible chemicals called autoinducers (AIs) to regulate their cellular processes via QS phenomenon. The activities regulated by QS include biofilm formation in several opportunistic pathogens, EPS synthesis in *P. aeruginosa* (Quinones *et al.* 2005; Sakuragi and Kolter 2007; Tan *et al.* 2014; Saxena *et al.* 2019), induction of virulence factors in *S. aureus* (Novick and Geisinger 2008), sporulation in *Bacillus subtilis,* competence development in *Streptococcus* mutants, and bioluminescence in *Vibrio cholera* biofilms (de Kievit and Iglewski 2000; Koutsoudis *et al.* 2006; Ng and Bassler 2009; Saxena *et al.* 2019). Each cell in biofilm matrix is in one of the two states – up-regulated or down-regulated – between which it can instantaneously switch based on local AI concentration. In an up-regulated state, they typically produce the signalling molecule at increased rate compared with down-regulated state. Up-regulated sub-population can spontaneously down-regulate, resulting into low levels of gene expression and/or *vice versa* (Nadell *et al.* 2008; Wolska *et al.* 2016). For instance, the net result of quorum sensing regulatory cascade in mature biofilm of *S. aureus* was up-regulation of secreted virulence factors, such as α-toxin, and down-regulation of surface virulence factors, such as protein A (Queck *et al.* 2008; Kırmusaoğlu 2016).

The positive correlation between QS, EPS and EPS interactions with physio-chemical environment of clinically relevant pathogenic bacteria has brought together a large group of biologists interested in bacterial group dynamics. For example, in a *Pantoea stewartii* biofilm, approximately ten-fold increase in EPS production per up-regulated cells was observed than down-regulated cells upon QS induction (von Bodman *et al.* 1998; Jones and Wozniak 2017). Recent findings suggested that EPS harvested from *Acinetobacter baumannii* biofilms act as a 'universal protector' by inhibiting tobramycin activity against bacterial cells, irrespective of species. By using fluorescent labelling of antibiotics, alginate was shown to defend the microbial community from effective killing by hindering the diffusion of tobramycin at the periphery (Davenport *et al.* 2014). Studies of EPS-producing (mucoid) and non-EPS producing (non-mucoid) *S. aureus* biofilms found that the mucoid strain exhibited maximum resistance to aminoglycosides (Gristina *et al.* 1989; Owlia *et al.* 2014; Jones and Wozniak 2017). The mucoid phenotype, *P. aeruginosa* isolated from the lungs of CF patients was found to overproduce alginate. The contribution of QS regulated EPS production to biofilm architecture and resistance have been shown to vary depending on specific growth stages during biofilm formation (Vu *et al.* 2009; Jones and Wozniak 2017). However, the exact mechanisms behind EPS-

antibiotic interaction are still unclear; with some investigations supporting non-charge-based interactions (Xue *et al.* 2013) while others supporting ionic interactions (Tseng *et al.* 2013).

The inhibition of QS by using quorum sensing inhibitors (QSI) to potentiate the effect of existing antimicrobial agents is a promising alternative to the development of new antibiotic formulations. For example, QSI Hamamelitannin increased the antibiotic susceptibility of *S. aureus* biofilm towards vancomycin by affecting EPS synthesis (Brackman *et al.* 2016). The combination of tobramycin and QSI baicalin hydrate efficiently eliminated most of the cells of *Burkholderia cenocepacia* infection in the lungs of mice than clindamycin or vancomycin treatment alone (Brackman *et al.* 2016). The ability of QSI has been investigated in different animal models and are now used in the designing of anti-biofilm medical implants, including catheters, and wound dressings for developing therapeutic arsenal against microbes.

4.2 *Abiotic factors*

4.2.1 *Subminimal biofilm eradication concentration (sub-MBEC):* Minimal biofilm eradication concentration (MBEC) is the lowest concentration of an antimicrobial agent that results in the lowering of the number of cells by ≤99.99% after an overnight incubation (Ceri *et al.* 1999; Hoffman *et al.* 2005; Perumal and Mahmud 2013; Macia *et al.* 2014; Hall and Mah 2017). sub-MBEC, which is slightly lower than MBEC only inhibits biofilm growth, and cannot exterminate entire biofilm population (Verderosa *et al.* 2019). In addition, sub-MBEC of antibiotics namely, tobramycin, tetracycline, and norfloxacin treatment led to significant increase in the expression of specific genes – up to 7% of 555 genes in genomic array – relevant for colonization and further stress response of *P. aeruginosa* species (Linares *et al.* 2006; Hall and Mah 2017). The influence of sub-MBEC has clinical importance because biofilm population may be exposed to sub-lethal concentrations of antibiotics at the beginning and end of the dosing treatment or may be subjected to continuous low dose therapy (Machineni *et al.* 2018; Verderosa *et al.* 2019).

Biofilms became resilient when resistance was challenged with sub-MBEC (Tezel *et al.* 2016). For instance, the cell wall inhibitors cephalothin and cephalexin (Haddadin *et al.* 2010; Subrt *et al.* 2011), and the protein synthesis inhibitor linezolid (Frank *et al.* 2007) at sub-lethal concentrations (usually ≤ 1/2×MBEC), enhanced *S. aureus* biofilm formation as much as four-times on medical devices and tissues. It was shown that 1/4× MBEC of β-lactams induced *alg* gene that exhibited ten-fold increase in EPS synthesis, which in turn resulted in biofilm persistence, and it was extremely difficult to eradicate using aggressive antibiotic therapy (Bagge *et al.* 2004; Verderosa *et al.* 2019).

The outcome of above summarized studies suggested that sub-MBEC of antimicrobials use could lead to the adaptation and development of resistant bacterial population resulting in treatment failure, extended periods of hospital care, increased costs of medication, and increased morbidity. Therefore, it is necessary to stress the importance of maintaining effective bactericidal concentrations of antibiotics to fight biofilm-related infections.

4.2.2 *Nutrient accessibility:* Several experimental investigations have shown the direct influence of nutrient concentration on growth dynamics, physiological heterogeneity, and antibiotic resistance of biofilms (Amato and Brynildsen 2014). For instance, growth of microbes in a nutritionally limiting environment promoted the transition of cells from planktonic phenotype to sessile phenotype (Sauer *et al.* 2004; Rochex and Lebeault 2007; Machineni *et al.* 2017; Verderosa *et al.* 2019). Using *Acinetobacter baumnnii* as a model strain, they showed that the nutrient concentration modified the adherence ability and morphology of planktonic cells. Initially, low nutrient concentration stimulated the switch of small fee-floating cocci to biofilm phenotype. Subsequent increase in the nutrient levels led to modified morphology from cocci to rods (James *et al.* 1995; Ghanbari *et al.* 2016). Even in the very early stages of biofilm formation, heterogeneity in growth rates have been reported, which emerge from (and influence) spatial gradients in nutrient concentration (Melaugh *et al.* 2016; Machineni *et al.* 2017). Paul Stoodley's group visualized the growth of distinct cell clusters in mature *P. aeruginosa* biofilms by using digital time-lapse microscopy. They demonstrated that a biofilm could change its structure from flat ripples and streamers to densely packed mound-like structures when the nutrient concentration was increased by ten-fold. These densely packed biofilms exhibited greater tendency to slough off from the substratum than the ripples and streamers formed at lower concentration (Stoodley *et al.* 1998, 2001; Liu *et al.* 2017). Nutrient starvation induced cell death inside biofilm plays a key role in the metabolic and proliferation heterogeneity

and dispersal of biofilm population (Hunt *et al.* 2004; Fagerlind *et al.* 2012; Liu *et al.* 2017; Anutrakunchai *et al.* 2018).

A three-dimensional individual-based cellular automata model was developed and used to investigate the influence of nutrient availability and quorum sensing on microbial heterogeneity in growing biofilms. The model predicted the formation of metabolically dormant cellular microniches embedded within faster growing cell clusters. Biofilms utilizing quorum sensing were more heterogeneous compared to their non-quorum sensing counterparts, and resisted sloughing, featuring a cell-devoid layer of EPS atop the substratum upon which the remainder of the biofilm developed (Machineni *et al.* 2017) The same group has shown that only active subpopulation within biofilms died, whereas inactive cells shown highly increased resistance to treatment (Machineni *et al.* 2018). Dormant cells restricted to the interior of the community were unaffected by conventional antibiotics – β-lactams, fluoroquinolones (inhibit transcription), and aminoglycosides (inhibit translation) – that actively target dividing cells within biofilm regions that have greater access to exogenous nutrients (Kohanski *et al.* 2010; Singh *et al.* 2017). Conversely, polymyxins and detergents (that attack cellular membranes) eliminated inactive cells in the interior of *P. aeruginosa* biofilms (Berditsch *et al.* 2015). These investigations imply that the modification of nutrient levels is important to prevent and control the biofilm formation of clinically significant pathogens. In combination, however, these resistance mechanisms drastically decreased our ability to control and eradicate biofilm-associated infections with the current antimicrobial arsenal.

5. Conclusions and future prospects

Altogether, further investigation is required to determine the cellular and physiochemical factors that mostly affected the cells in biotic or abiotic surface attached form, and thus contributed in the identification of the treatments that may provide helpful results in the eradication of biofilm-associated infections in medical settings. In addition, recent clinical observations in support of experimental findings clearly proved that current antimicrobial therapies alone are not enough to inhibit biofilm-mediated infections. This situation was further convoluted by the paucity of new classes of antibiotics in development, and thus prompted renewed interest to tackle biofilms using the combination of antimicrobial therapies. However, these drug mixtures failed to kill the bacteria existing as a biofilm due to rapidly changing metabolic activity of cells within preformed biofilm and structural heterogeneity controlling drug penetrability. Thus, there is an urgent need to design effective approaches to prevent biofilm formation or treat established biofilms, which do not depend on conventional and modern antimicrobials.

Research on the effects and treatment of sessile communities is an extremely dynamic area, and medical practitioners should focus on the most recent clinical trials to ensure that they are providing the best possible health care solutions to patients. Understanding the factors influencing the underlying mechanisms of biofilm-specific antibiotic resistance will help in the development of therapies that could inhibit the underlying mechanism and render the cells more responsive to antimicrobial treatment. Considering the human health issues that were caused by chronic biofilm infections in recent decades, the characterization of stage specific structural heterogeneity throughout the development of biofilm matrix is highly important to facilitate the research that offer potential solutions to fight against biofilms.

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