Biofilm Detachment and Its Implication in Spreading Biofilm-Related Infections

Akshat Gupta, Anmol Srivastava, and Vishnu Agarwal(✉)

Department of Biotechnology, Motilal Nehru National Institute of Technology, Allahabad (Prayagraj) 211004, Uttar Pradesh, India
vishnua@mnnit.ac.in

Abstract. Biofilms are a community of microorganisms formed on both abiotic and biotic surfaces. These colonies play a vital role in the virulent life cycle of bacteria. Bacteria communicate intrinsically and extrinsically to grow and eventually disperse their virulent factors, ultimately leading to diseases. Biofilm dispersion is the last stage in this life cycle; at this stage, the biofilm has completed maturation. The microorganism then disperses as the biofilm ruptures and assumes a planktonic lifestyle until they find a new surface to attach to and repeat the cycle. This mechanism plays a vital role in the pathogenicity of the microorganism and can be triggered prematurely to disrupt the microorganism's virulent nature. In this mini-review, we have summarized biofilm dispersion, its mechanisms, and the factors influenced by, focusing on their effect on the pathogen's virulence. We have also discussed the significance of quorum sensing and the modern methods used to develop quorum sensing inhibitors through in-silico approaches.

Keywords: Biofilm · quorum sensing · pathogenicity · dispersion · quorum sensing inhibitor

1 Introduction

Biofilms are aggregates of microbial cells clumped together to form an irreversible attachment to the substratum [1]. These microbial communities produce EPS (extracellular polymeric substances), a matrix in which these cells are embedded. These microbial cells interact through quorum sensing, using chemotactic particles or auto-inducers (Acyl Homoserine Lactones and oligopeptides) [2]. In the case of *Pseudomonas aeruginosa*, two prominent quorum-sensing systems, las and rhl are present; the former is responsible for the synthesis of auto-inducer 3-oxo-C12-HSL (Homoserine lactone) and later synthesizes C4-HSL. A characteristic property is associated with the microbes in a biofilm. They are more resistant to antibiotics and U.V. exposure than free-living planktonic cells because of the semi-permeable nature of biofilm. In addition, biofilms also provide stable conditions for growth and enhance the metabolic activity of the prokaryote synthesizing it. The biofilm formation steps include attachment, irreversible attachment, proliferation, maturation, and detachment [3].

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It has been reported by the U.S. National Institute of Health (NIH) that almost 80% of infections involve biofilm formation. Patients with medical implants and individuals that are immunocompromised are more susceptible to infections related to biofilm. Biofilm dispersion is a process by which the sessile microbial cells from inside the biofilm are released to form motile planktonic cells due to internal or external stimuli, which then translocate to a new location to spread the disease [4]. The dispersed cells at times lose their characteristics of antibiotic resistance. Hence, it is essential to neutralize these dispersed cells, as otherwise, it could lead to a more severe infection [5]. Dispersal agents include enzymes produced by bacteria like proteases, nucleases, hydrolases, and rhamnolipids [6].

Infections caused by biofilms encompass both infections related to medical devices such as catheters, mechanical heart valves, and pacemakers [7] and non-device-associated infections, which include infections like periodontitis [8] (infection in gums) and osteomyelitis (disease of bones) [9]. These infections result from the property of a biofilm that is recalcitrance against antibiotics, resistance to U.V. light and heavy metals, phagocytosis, etc. One such organism, *Pseudomonas aeruginosa*, is responsible for nosocomial infections like catheter-associated urinary tract infections (CAUTI) and ventilator-associated patients (VAP) [10]. Here in this review paper, we will discuss biofilm detachment, its mechanism, and its role in controlling the spread of infections.

2 Mechanism of Biofilm Detachment

Biofilm detachment can be an active or passive process; when the bacteria itself initiates dispersion due to any environmental disturbances like pH, temperature, unavailability of nutrients, and changes in concentration of gases like oxygen and nitric oxide, it is termed active dispersion. On the other hand, the passive process is triggered by various external forces, including fluid shear, mechanical interventions, or the abrasion caused by the collision of particles from the environment with the biofilm, predator grazing by eukaryotic organisms [6], and human interference. The mechanism of biofilm dispersion (Fig. 1) includes three characteristic phases:

1. Detachment of the cells from the biofilm colony.
2. Translocation of these cells to a new area.
3. Attachment of these detached cells to this new place to initiate biofilm formation.

2.1 Significance of Quorum Sensing in Biofilm Dispersion

This mechanism was first reported in *Vibrio fischeri* for its bioluminescence property. It is a process by which bacterial cells or communities interact and regulate their complex physiological processes due to signaling molecules termed “auto-inducers” and their receptors. Specific properties like virulence and antibiotic resistance result from genes expressed after the interaction of auto-inducers with their receptor [11]. Gram-negative bacteria produce acylated homoserine lactones (AHLs) as auto-inducers. Gram-positive bacteria secrete peptides as signal molecules that interact with membrane-bound two-component signal transduction systems [12]. It has been reported that the quorum sensing

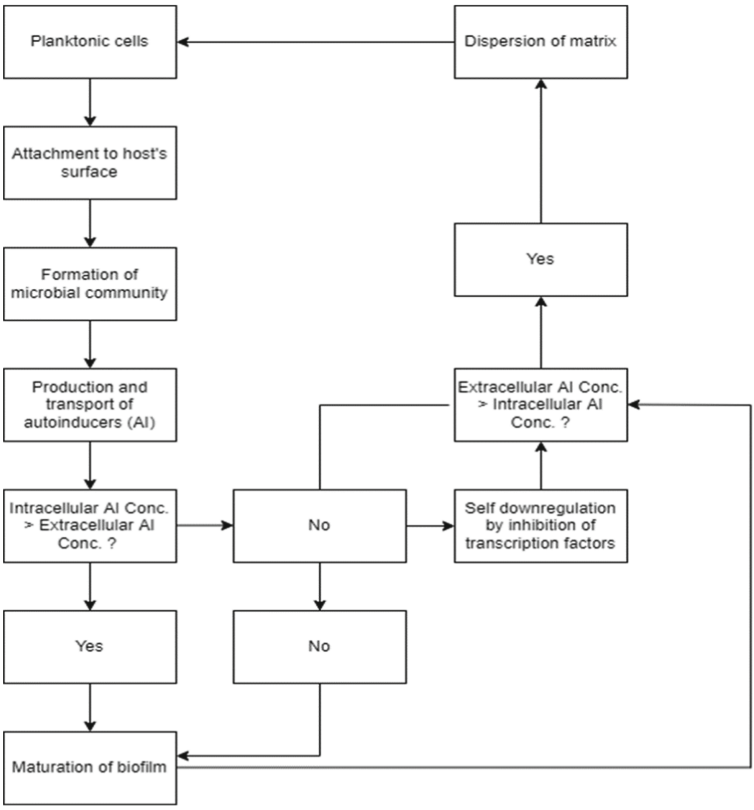

Fig. 1. The biofilm lifecycle and role of autoinducer concentration in the dispersal of the biofilm.

mechanism helps regulate social activities and physiological processes in many bacteria, including symbiosis, spore or fruiting bodies formation, bacteriocin production, genetic competence, programmed cell death, virulence, and biofilm formation [13]. Several antibiotics have been developed to counter the bacterial infections caused by biofilms.

However, other innovative and novel strategies are required to combat infections as the nature of biofilm is continuously changing. These strategies include interfering with the quorum sensing system, inhibiting cell adhesion to the substratum, and altering biofilm architecture by disrupting extracellular matrix production [14]. It has been found that the bacterial quorum sensing system and genes associated with it can be quenched by certain compounds that can interact with the signal molecules and their receptors. Such compounds are abundantly present in nature, like in plants or algae. In addition, secondary metabolites like essential oils, flavonoids, amino acids, terpenoids, sterols, and brominated furanones have antibiofilm and antibacterial activity. Table 1 provides a few examples of organisms that naturally produce biofilm and quorum sensing inhibitors to combat microbial biofilm development. Quorum sensing is one of the most suitable methods that can be used to trigger biofilm dispersion, as disrupting the bacterial communication structure can limit pathogenicity sustainably without the adverse effects due

Table 1. Examples of naturally occurring biofilm and quorum sensing inhibitors and their mechanism of inhibition of biofilm formation.

Organism/Source	Quorum Sensing inhibitor produced	Mechanism of inhibition	Reference
Delisea pulchra	Furanone compounds	Furanone interferes with quorum sensing (acyl-HSL) and makes *P. aeruginosa* biofilm susceptible to tobramycin.	[15]
Combretum albiflorum (Bark)	Flavonoid-Flavan-3-ol cathechin	inhibition in biofilm formation, and it also reduces the production of pathogenic compounds like elastase and pyocyanin	[16]
Zingiber officinale	Zingerone	Zingerone is detrimental to the motility of planktonic cells, and hence it inhibits the initial attachment of bacteria to the substratum. It also leads to thinner biofilms because of the reduction in EPS production	[17]

to selection pressure. However, like humans, bacteria operate on a spectrum of individualism and collectivism. This quality can breed conflict, but collaboration and interspecies quorum sensing can take both forms.

2.2 c-di-GMP's Role in Biofilm Dispersion

Cyclic dimeric guanosine monophosphate (c-di-GMP) is a secondary messenger first reported in *Acetobacter xylinum* as an activator of cellulose production [11]. Elevated levels of c-di-GMP promote sessile biofilm structure. However, when there is a decrease in the level of c-di-GMP, it favors planktonic cells that show characteristics like increased motility and low resistance to anti-microbial agents and growth. The concentration of c-di-GMP is maintained by the activity of two enzymes, diguanylate cyclases (DGCs) and phosphodiesterases (PDEs). DGCs are responsible for synthesizing c-di-GMP, whereas PDEs have antagonist activity towards DGCs. Hence, they hydrolyze c-di-GMP to produce 5' phosphoguanylyl-(3'–5')-guanosine (pGpG) or GMP [12]. When biofilm dispersion initiates, PDEs hydrolyze c-di-GMP, as discussed above. Due to the low concentration of this secondary messenger, there is increased expression of genes involved in polymeric matrix degradation. The scarcity of gases like oxygen, nitric oxide (NO), and nutrients [13] plays a significant role in this biofilm dispersion. Furthermore, it was found that cells towards the outer layer of biofilm were in a more metabolically active

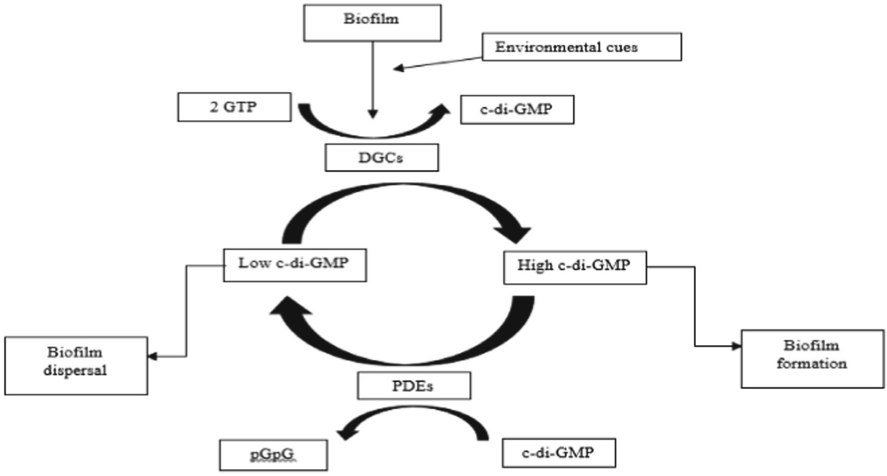

Fig. 2. Enzymes Diguanylate Cyclase (DGC) and Phosphodiesterase (PDE) regulate the concentration of c-di-GMP, leading to biofilm formation and dispersal

state. So, they harbor a high concentration of c-di-GMP compared to the cells embedded in the biofilm, which are less metabolically active [14, 15] (Fig. 2).

2.3 Role of Native Agents and Signals in Dispersal

The Nitric oxide (NO) molecule is among the first few compounds reported to initiate biofilm dispersal. At a concentration of 500 nM, NO could significantly reduce biofilm biomass in *Pseudomonas aeruginosa*; sodium nitroprusside is the NO donor. As discussed above, it induces the expression of phosphodiesterases (PDEs), which results in the formation of 5' phosphoguanylyl-(3'–5')-guanosine (pGpG). NO also leads to the release of LapG, a proteinase, from the gene LapD that disrupts matrix-bound proteins LapA and CdrA [16]. The proteins are crucial for the biofilm's stability; as they are disrupted, matrix degradation occurs, which in turn causes biofilm dispersion. Some examples of other native agents include Apolipoprotein B (ApoB)-derived peptide, Cathelicidin-derived shorter peptide FK13, *Enterococcus faecalis* bacteriocin EntV, Bovine Myeloid Antimicrobial peptide 27 (BMAP-27), BMAP-28, and Myeloid Antimicrobial Peptide 29 (SMAP-29). These are also found to play a key role in the disruption and dispersion of cystic fibrosis biofilms. Furthermore, lytic anti-microbial peptides (AMPs) are positively charged peptides derived from sheep, cows, and humans attached to the lipopolysaccharides because they are negatively charged and lead to the formation of pores on the outer surface of the biofilm and cause its disruption. The lytic Adenosine Monophosphate (AMP), PTP-7, is a synthetic analog of the Gaegurin 5 peptide isolated from an Asian frog which shows significant activity toward antibiotic-sensitive and resistant *S. aureus* biofilms [17].

In addition to dispersal agents, several signals have been found to initiate dispersals, like the fatty acid signaling molecule cis-2-decanoic acid (cis-DA) and Diffusible signal factor (DSF) and DSF of the important human pathogen *Burkholderia cenocepacia*

(BDSF) in *P. aeruginosa*, which can regulate several physiological processes in bacteria like motility, growth, virulence, and biofilm dispersal in both Gram-negative and Gram-positive bacteria as well as yeast [18, 19].

3 Regulation of Biofilm Dispersion

Various molecules and cell signaling pathways regulate biofilm dispersion. Here, we will discuss the regulation of dispersion in two different organisms through three different pathways. The first microorganism is *E. coli*. Under various culture conditions, the RNA binding global regulatory protein CsrA (carbon storage regulator) of *Escherichia coli K-12* serves as a repressor of biofilm formation and an activator of biofilm dispersal [1]. CsrA is a global regulatory protein that is encoded by the CsrA gene. This protein represses several metabolic pathways, including gluconeogenesis, glycogen biosynthesis, and catabolism. It has also been established that CsrA activates motility, glycolysis, and acetate metabolism [2, 3]. The experimental study performed by a group of researchers confirmed the regulatory role of the CsrA protein on both biofilm formation and inhibition/dispersal [1]. They utilized two strains of *E. coli,* the first one was the parental wild-type *E. coli* K-12 strain MG1655, and the other was its isogenic CsrA mutant (the CsrA gene has been knocked out). Upon culture, both strains developed the biofilm slowly until they reached the stationary phase of their cycle. Here, the biofilm accumulation was seen to be more rapid and extensive in the CsrA mutant. The biofilm also displayed all the characteristics of a mature biofilm, especially its increased adherence to the substratum. In the next phase, the CsrA gene was overexpressed using a multicopy plasmid vector, and its effects were then examined. This ectopic expression of CsrA was found to inhibit biofilm formation in both the wild-type *E. coli* K-12 and its CsrA mutant. These effects are primarily due to CsrA's glycogen synthesis and catabolism mediation [1–3].

The next microorganism is *P. aeruginosa*, which regulates biofilm formation via c-di-GMP signaling. The secondary messenger cyclic diguanosine-5′-monophosphate (c-di-GMP) is crucial for the bacterial biofilm life cycle. The high internal levels of c-di-GMP induce the production of adhesins and extracellular matrix components that enable bacteria to form biofilms. On the contrary, the low c-di-GMP levels downregulate the production of adhesins and extracellular matrix components and then lead the biofilm bacteria into dispersal and a planktonic growth mode. Although many proteins are involved in c-di-GMP metabolism in *P. aeruginosa*, only four proteins, Alg44, FimX, PelD, and FleQ, have been successfully identified as c-di-GMP effectors. The Alg44 protein is mainly involved in synthesizing the matrix exopolysaccharide alginate in *P. aeruginosa.* In the case of alginate overproduction, a mucoid phenotype frequently observed with bacterial strains isolated from the lungs of chronically infected cystic fibrosis patients is seen. Although alginate plays a crucial role in the final stages of infection, the exopolysaccharides Pel and Psl are more critical for biofilm formation by non-mucoid *P. aeruginosa* strains [4].

4 Impact of Quorum Sensing on Biofilm Dispersion and Its Virulence

Recently, antibiotic-resistant species have been on the rise and have undoubtedly contributed to an impending global crisis. It is difficult to find a substitute to combat such organisms to prevent the onset of a global pandemic in the future. Biofilm formation is a crucial characteristic that most bacterial strains share. By disrupting this mechanism and triggering dispersion, we can disrupt the virulence of the disease-causing pathogen. Quorum sensing is one of the most studied mechanisms of the past few decades. It is the most suitable target for biofilm disruption among the currently available methods discussed in this review. However, NO-based therapeutics that mediate biofilm dispersion has been found to cause systemic cytotoxicity [24]. Another critical factor is that c-di-GMP doesn't guarantee biofilm dispersion. For example, it has been seen that in *P.aeruginosa* biofilms, doxorubicin, an anti-cancer drug, reduces the intracellular c-di-GMP concentration and stimulates biofilm formation by triggering the synthesis of extracellular DNA (eDNA) [24].

Targeting quorum sensing is beneficial for two significant reasons. The first and apparent reason is that if it is disrupted, the internal communication of the microorganism tarnishes, leading to dispersion and elimination. The second factor is the disruption of external communication. Quorum sensing can and does occur between different bacterial species [25]. It has been shown that several species cannot produce their autoinducers but have receptors that are programmed to recognize the auto-inducers of other species. This allows them to sense and respond to environmental cues, including other bacterial species. This communication can be targeted and tapped, which can breed conflict amongst different species daily in our gut between the 'good' and 'bad' bacterial cultures. Another critical phenomenon based on this mechanism is that several bacterial strains work in cohesion to enhance each other's virulence. For example, a study has found that when *B. cepacia* is cultured in *P. aeruginosa* conditioned media, the concentrations of *B. cepacia's* protease and virulence factors also increase drastically [26]. This was accounted for by the presence of *P. aeroginosa's* autoinducers that changed *B. cepacia's* gene expression, leading to higher virulence. Another study found that *Streptococcus goronii* and *Porphyromonas gingivalis* employ the same autoinducer to operate their communication systems and use it to form a symbiotic relationship that leads to the production of mixed-species biofilm [25]. This biofilm's virulence has increased exponentially and causes a very potent periodontitis coinfection. Gram-negative bacteria operate through a LuxI/R-type quorum sensing system, where Acyl-Homoserine-Lactone (AHL) synthase (LuxI) synthesizes acyl-homoserine lactone autoinducers, which accumulate up to the desired concentration and then bind to LuxR type receptors; this complex then regulates the expression of target genes, including the genes responsible for producing virulence factors. This mechanism controls the production of antibiotics in *Erwinia carotovora*, plasmid conjugation in *A. tumefaciens*, and the expression of vital factors for the symbiosis of *Sinorhizobium meliloti* [27]. These examples justify and elucidate the importance of quorum sensing and the potential to control the virulence and pathogenicity of several species through disruption and interference in this intricate communication system [25, 27].

5 In Silico Approach for Developing Quorum Sensing Inhibitors (QSI)

The Quorum Sensing inhibitors (QSIs) target quorum sensing. Molecular signaling machinery and disable it within the bacterial species. It effectively renders the cells incapable of sensing the neighboring cells by modifying the regulation of genes by either repressing or inducing them. Furthermore, QSI also alters the expression of various genes involved in biofilm formation, production of secondary metabolites, and the expression of disease-causing virulence factors [28]–[30]. This inhibition can alter the microbial environment by redistributing the competitive advantage while forming a complex community. One of the most striking examples is *Pseudomonas aeruginosa*, a common human pathogen that can cause infections in cystic fibrosis (C.F.) patients. In *P. aeruginosa*, the QSI pressure can select quorum sensing -harmful strains [30].

Gram-negative bacteria synthesize acyl-homoserine lactone autoinducers, which can diffuse through their thin cell walls. In contrast, Gram-positive bacterial autoinducers are made of peptides. These quorum-sensing peptides (QSPs) initiate a signaling cascade of events via a two-component system or by directly binding to transcription factors; Because of the therapeutic potential of the Acyl Homoserine Lactones (AHLs) and Quantitative systems pharmacology (QSP) as drug targets, numerous in silico approaches have been used to find inhibitors and model them to aid in the fight against bacterial pathogenicity. Algorithms such as support vector machines (SVM) and hidden Markov models (HMM) help predict QSI agents. These are specifically used to identify novel and effective biofilm inhibitory peptides (BIPs). In addition, other algorithms like bidirectional recursive neural network (BRNN) and Random Forest (R.F.) algorithms were used in silico approaches for predicting and designing peptides having antibiofilm properties. Several Researchers have also used identification of the binding pocket(s), motif search, and other physicochemical properties to predict the three-dimensional structure of the target. Furthermore, ultra-high-throughput screening is another approach that reveals QSIs based on the characterization of natural products and screening for naturally occurring enzymes [30].

6 Limitations of Quorum Sensing Inhibitory Molecules

Despite the benefits and advantages the quorum sensing inhibitory molecules carry, some limitations need to be considered before they can be authorized for administration in humans. The quorum sensing inhibitory molecules target signaling molecules in the bacterial system, preventing them from further communicating and commencing growth. This specific signaling molecule which is generally an autoinducer may be common in more than one species of the bacteria of the same or, at times, a different genus. This lack of specificity of a particular bacterial autoinducer may prove harmful to the human microbiota when administered as a drug [31]. It may render the human microflora, often termed "good or beneficial microorganisms," unable to form biofilms in the gut, which may lead to gastrointestinal diseases. Another critical limitation studied over the past years is the instances where inhibition of quorum sensing leads to an increase in the virulence of the microorganism. Several studies have found that gram-negative bacteria

like *Vibrio cholera* and *Helicobacter pylori* showed increased biofilm aggregation upon inhibiting their quorum-sensing molecules [31]. Thus, it is crucial to understand the limitations of employing quorum sensing molecules before these can be administered in drugs to inhibit microbial biofilm formation.

7 Conclusion

Biofilm formation is a significant problem that needs to be addressed. It is a leading cause of severe bacterial infections and poses a significant challenge in treating such infections due to the antibiotic resistance property of biofilm. Removing the entire, sessile bacterial community using conventional or mechanical methods is impossible, so novel methods that include biofilm dispersal or biofilm dispersal in synergy with anti-microbials are being looked into. The available techniques used to trigger biofilm dispersion include quorum sensing, native agents, and the c-di-GMP pathway. Among these agents, quorum sensing or the microbial communication network presents the best possible solution to trigger biofilm dispersion. Quorum sensing inhibitors are thus being predicted in-silico through various computational algorithms. These inhibitors will disrupt the internal and, in some cases, the microbe's external communication system, leading to dispersal and, consequently, reduction and elimination of its virulence and pathogenicity. Lastly, we have discussed some limitations that quorum sensing inhibitors have and the need to carefully understand all the nuances of such molecules through sufficient study and experiments before use as a biofilm dispersal agent.

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