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Review Article

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Role of Alarmone in Biofilm Development: A Weapon to Survive Under Stress

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Abstract

The bacterial association within the biofilm makes the organisms less susceptible to many antibiotics, immune responses, oxidative stress, and several harsh conditions. The biofilm formation provides pathogenicity to the organisms, leading to serious human diseases and device-related infections such as by growing on catheters, contact lenses, cardiac pacemakers, etc. The alarmone collectively called (p)ppGpp synthesis stimulates biofilm development in many bacterial cells. RelA SpoT homologs (long RSH family) and RelP RelQ (small alarmone synthetases) are responsible for the generation of (p)ppGpp. The phosphorylation of GTP or GDP using ATP as a phosphate group donor produces pppGpp or ppGpp respectively, under the stressed situation. When normal conditions appear, the (p)ppGpp is converted to GTP or GDP by hydrolases, preventing the excessive unregulated accumulation of (p)ppGpp. Mutant cells lacking the abovementioned synthetases show very little or no biofilm formation in *Vibrio cholerae*, *Staphylococcus aureus*, *Escherichia coli*, etc. Here, we focus on making an overall idea about the synthetase and hydrolase enzymes required to generate and degrade the alarmone in bacterial cells. It emphasizes the involvement of alarmone formation in the biofilm development of *Vibrio cholerae*, *Staphylococcus aureus*, and *Escherichia coli* and also the use of alarmone as novel therapeutic target.

Keywords: Biofilm, alarmone, RelA, SpoT, RelP, RelQ

Introduction

Initially, at a very ancient stage, the feature of a bacterium was contemplated as a single free-floating microorganism. During the 1970s Robert Koch's pure culture discovery opened a new field to biological sciences enabling the study of many deadly bacteria and the development of biocides to kill them. However, the rapid appearance of drug resistance properties of bacteria drew the attention of scientists to reexamine their life cycle. Now, this re-identification confronts the bacterial association in the self-produced Extracellular Polymeric Substances (EPS) matrix, providing a defence mechanism to the bacteria against several antibiotics. Anthony Van Leeuwenhoek first noticed the bacterial aggregation on tooth surfaces by using his own constructed simple microscope and marking them as 'scurf'(Roy *et al.*, 2018a). A biofilm is defined as an association of bacterial cells with the surface forming aggregates surrounded by EPS matrix, as observed in *Pseudomonas aeruginosa*, *Streptococcus mutans*, etc. The matrix usually comprises polysaccharides, proteins, nucleic acids, lipids, and extracellular DNA making the biofilm

more mechanically stable and allowing the adhesion to the surface. A variety of bacterial cell surface proteins, flagella, fimbriae mediate the attachment of bacterial cells on the surface. Such living protects the bacterial cells to become resistant against immune responses and many antibiotics that can only kill the planktonic bacteria. So, they can easily form biofilm on devices used for medical purposes such as cardiac pacemakers and prosthetic heart valves, intravenous catheters, indwelling urinary catheters, nephrostomy tubes, and contact lenses leading to many human infections. A study involving patients suffering from chronic lung infections with cystic fibrosis have revealed that *Pseudomonas aeruginosa* becomes resistant gradually against immune responses and many antibiotics by forming biofilm structure. The mucoid arrangement within the biofilm can easily destroy the beta-lactam group of antibiotics (Ciofu *et al.*, 2012).

When nutrients are depleted in the bacterial environment, they respond to such stressful conditions by producing signaling nucleotides, guanosine 5´- diphosphate 3´- diphosphate (ppGpp), and guanosine 5´- triphosphate 3´- diphosphate (pppGpp), togetherly known as alarmone. They are synthesized by phosphorylation of GTP and GDP utilizing ATP as a phosphate group donor. Under suitable conditions, these signaling nucleotides are made at a very low level. Accumulation of these second messenger molecules to a sufficient level reshapes the bacterial metabolism and physiology to cope-up with such harsh conditions (Potrykus and Cashel, 2008). The synthesis of (p)ppGpp enhances virulence, antibiotic tolerance, and also biofilm formation abilities of bacterial cells. Their synthesis usually targets transcription, translation, DNA replication, and different cell cycle stages. By altering the specificity of RNA polymerase to alternative sigma factor, they change the rate of transcription cell growth under the stressed condition and save the bacterial cells from environmental attack. Alarmone synthesis is mainly regulated by RelA-SpoT Homologs (RSH), usually conserved among bacterial cells. The synthetase domain of RSH produces the alarmone by phosphorylating GTP or GDP and the hydrolase domain causes the degradation of (p)ppGpp back to GTP or GDP. A study on *Vibrio cholerae* found that mutant strains lacking the ability to make (p)ppGpp are deficient in forming biofilms. RelA, SpoT, and RelV are three synthases that are responsible for switching on the stress response in *V. cholera* (He *et al.*, 2012). *Escherichia coli* cells having a mutation in *relA-spoT* genes have a reduced ability to form biofilm on minimal media (Balzer and McLean, 2002). The emergence of antibiotic resistance property of bacteria in the biofilm can be therefore challenged by designing novel compounds that imbalance the rate of alarmone synthesis. The inhibition in the pathway of alarmone synthesis is not able to turn on the stringent response under environmental attack. As a result, the biofilm development will be inhibited, preventing the rapid dispersal of biofilm-related infections. This review paper aims to draw an overall picture about the mechanism of alarmone in a stressed condition, their correlation with biofilm development along with an emphasis on the generation of biofilm in *Vibrio cholerae*, *Staphylococcus aureus* and *Escherichia coli* monitored by alarmone.

Development of biofilm:

One of the important causes of the emergence of antibiotic resistance properties of bacteria is their ability to form biofilm on the surfaces of both biotic and abiotic sources. The association of bacteria in the form of ‘group’ makes them resistant to many environmental harsh situations and also immune responses. The biofilm formation within the human body and on medical devices is the onset of many biofilm-associated infections that are difficult to treat (Renner and Weibel, 2011). Figure 1 depicts that biofilm formation is a cyclical process comprising of following sequential stages: 1) the attachment of bacteria to surfaces, 2) monolayer formation surrounded by self-produced extracellular polymeric substance matrix (EPS), 3) microcolony formation, 4) generation of structured mature biofilm, 5) finally dispersion and reversal to the planktonic stage again (Tolker-nielsen, 2015). The initial attachment of bacterial cells on surfaces is driven by electrostatic interaction and van der Waals forces. Gravitational forces and Brownian motion also facilitate the adhesion of bacteria to a surface. After attachment, bacterial cells are enclosed in an EPS matrix composed mainly of polysaccharides, glycoproteins, and extracellular DNA (Flemming, Neu and Wozniak, 2007). Extracellular DNA (eDNA) plays an important role in biofilm formation in several organisms such as *Pseudomonas aeruginosa*, *Streptococcus mutans*, *Enterococcus faecalis*. Autolysis is the general mechanism by which extracellular DNA is liberated into the environment (Liao *et al.*, 2014). The anionic nature of eDNA chelates the positive charge of cationic antimicrobial peptides, thereby increasing resistance in the biofilm of *Pseudomonas aeruginosa*

(Mulcahy, Charron-Mazenod and Lewenza, 2008). The degradation of eDNA by introducing DNA degrading enzymes could be a new strategy to disrupt the conventional antibiotic-resistant biofilms. The study depicted that *Enterococcus faecalis* showed a reduced biofilm formation upon treatment with DNase 1. This finding proved that eDNA is an important constituent in biofilm formation. Several organisms need an additional matrix protein to generate a mature biofilm. This requirement may vary from species to species. Mucoid arrangement of *Pseudomonas aeruginosa* biofilm requires an important matrix component, alginate, a polyanion polysaccharide. However, the nonmucoid arrangement of *P. aeruginosa* biofilm involves the expression of *psl* operons. If the *pslA* and *pslB* genes are mutated, *P. aeruginosa* shows a dramatically decreased level of biofilm formation. The overexpression of *psl* genes has a high probability of adhering to surfaces and forming biofilms (Ma *et al.*, 2006).

After forming EPS surrounded monolayer, other bacterial cells belonging to the same or different species attach to the surface. This assemblage of bacterial cells forms a structured complex network arrangement forming a ‘mushroom’ or ‘tower’ like structure. In such an organization bacterial cells find their places according to their metabolism and oxygen need. Anaerobic organisms prefer the deeper side to bypass the presence of oxygen whereas aerobic organisms usually lie outside of the biofilm. Within this biofilm bacteria interact with each other through a quorum sensing mechanism. This communication is mediated through releasing, sensing, and responding to diffusible signaling molecules by bacterial cells. When the bacterial population reaches a maximum density, the quorum sensing mechanism upregulates many genes involved in biofilm formation and maturation. c-di-GMP is a secondary signaling molecule having an important role in biofilm maturation and dispersal observed in *Pseudomonas aeruginosa*, *Vibrio cholerae*, *Escherichia coli* (Cotter and Stibitz, 2007). In *Vibrio cholerae* VieA controls the transcription of *Vibrio* exopolysaccharide synthesis genes (vps) that regulate biofilm formation. It has been found that VieA lowers the level of intracellular c-di-GMP concentration and thus reducing the activity of c-di-GMP synthetase proteins. In other words, the biofilm formation ability of *V. cholerae* is modulated by the c-di-GMP synthetase or phosphodiesterase altering the c-di-GMP level (Tischler, A. D., & Camilli, 2004). The magical role of alarmone is to stimulate biofilm formation in many organisms. This secondary signaling molecule is produced in response to various environmental stimuli such as changes in temperature, pH alteration, osmotic shock, oxygen availability, etc. The production of (p)ppGpp monitors the biofilm formation capability in *Bordetella pertussis*, whooping cough pathogen. Mutant B. pertussis cells having a deletion in *relA* and *spoT* genes show a defective biofilm along with increased susceptibility to oxidative stress (Geiger *et al.*, 2010). A variety of chemical signaling molecules participate in quorum sensing mechanisms such as acyl-homoserine lactones (AHL), furanosyl borate diester, *cis*- unsaturated fatty acids and peptides (Solano, C., Echeverz, M., & Lasa, 2014). *P. aeruginosa* possesses two quorum sensing components LasI/LasR and RhlI/ RhlR. Both these systems have LuxI synthase and LuxR receptor. At high population density accumulation of AHL binds with LuxR receptor, which regulates the transcription of the destined genes by binding to them. In some cases, AHL complexed with LuxR causes the activation of *luxI* gene, via an autoinduction signal amplification mechanism (Solano, C., Echeverz, M., & Lasa, 2014). Finally, the dispersion of biofilm takes place due to the exposure of specific environmental factors. Loss of nutrients, accumulation of toxic products, excessive competition, oxygen imbalance could cause biofilm dispersion. A small part or the overall structure of biofilm can be dispersed depending on the circumstances. This dispersion process again reverts the bacterial cells to the planktonic stage again and initiate another biofilm cycle (Roy *et al.*, 2018b).

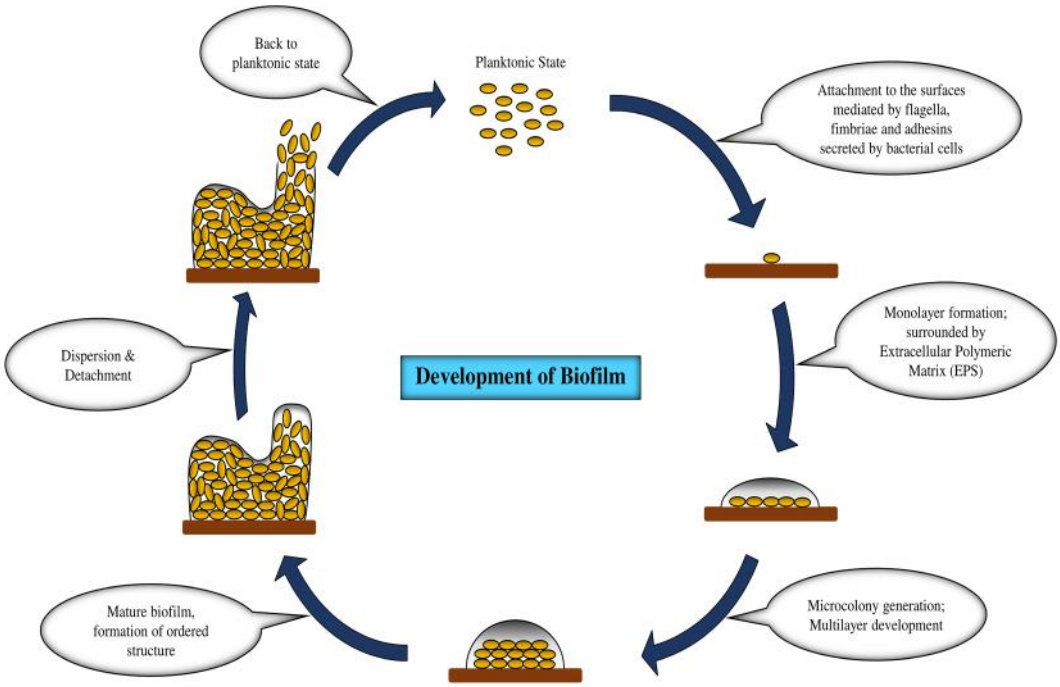

Figure 1: Development of biofilm in a sequential manner

Mechanism of alarmone synthesis and an overall structural view about alarmone synthesizing:

RelA-SpoT Homologs (RSH) globally modulate the alarmone synthesis and is highly conserved among bacteria, plants, and algae (Atkinson, Tenson and Hauryliuk, 2011). RSH comprises two domains. The synthetase domain is responsible for transferring the pyrophosphate group (PPi) from ATP to the 3´OH of GTP or GDP forming pppGpp i.e., guanosine 5´triphosphate 3´diphosphate or ppGpp i.e. guanosine 5´diphosphate 3´diphosphate respectively, known as alarmone. The hydrolase domain of RSH degrades pppGpp or ppGpp back to PPi and GTP or GDP respectively (Figure 2) (Atkinson, Tenson and Hauryliuk, 2011). One of the important roles of (p)ppGpp is the induction of biofilm formation in several organisms. The stringent response is turned on when bacteria survive from a stressed condition such as nutrient deprivation. The accumulation of intracellular (p)ppGpp i.e., alarmone upregulates many stress response genes that lower the growth rate of bacterial cells. It also makes bacteria suitable to use maximum resources available in their surroundings. *Enterococcus faecalis* is the predominant nosocomial pathogen and inhabits the human and animal intestine. Being a nosocomial pathogen the close association of this organism in hospital and medical system increases the resistance property towards many antibiotics and stressed conditions (Gao, Howden and Stinear, 2018). The biofilm formation ability of this organism contributes to its virulence and pathogenicity. RelA and RelQ are two synthetase enzymes producing (p)ppGpp in *E. faecalis*. Studies found that RelA is the predominant enzyme for (p)ppGpp synthesis under antibiotic stress whereas RelQ produces the basal level of (p)ppGpp under normal conditions. *relA* mutant cells were very much sensitive to stressed conditions and *relAQ* double mutants were able to restore many stress-susceptible phenotypes of *relA* mutant. The complete removal of (p)ppGpp showed a dramatically decreased biofilm formation compared to wild type cells. These studies reveal that (p)ppGpp has an important effect on biofilm development under specific stressed circumstances. The effect of alarmone also alters DNA replication during exposure to nutritional stress. In *Bacillus subtilis*, the limitation of amino acids stops the progression of replication forks thus controlling the DNA replication within minutes. The study found that (p)ppGpp blocked the recruitment of primase required in DNA replication (Wang, Sanders and Grossman, 2007). (p)ppGpp causes the transcription switching to stress response genes that can survive organisms from stress. In *Escherichia coli* the survival genes and virulence genes are turned on by altering the specificity of sigma factor binding to core polymerase. The nucleophilic transfer of phosphate group to GTP or GDP occurs in an S_N2 manner when both the substrates are positioned in the correct orientation favorable for the reaction

(Steinchen and Bange, 2016). There are three enzyme groups under the RSH superfamily: long RSH enzymes, small alarmone synthetases (SASs), and small alarmone hydrolases (SAHs) (Steinchen and Bange, 2016).

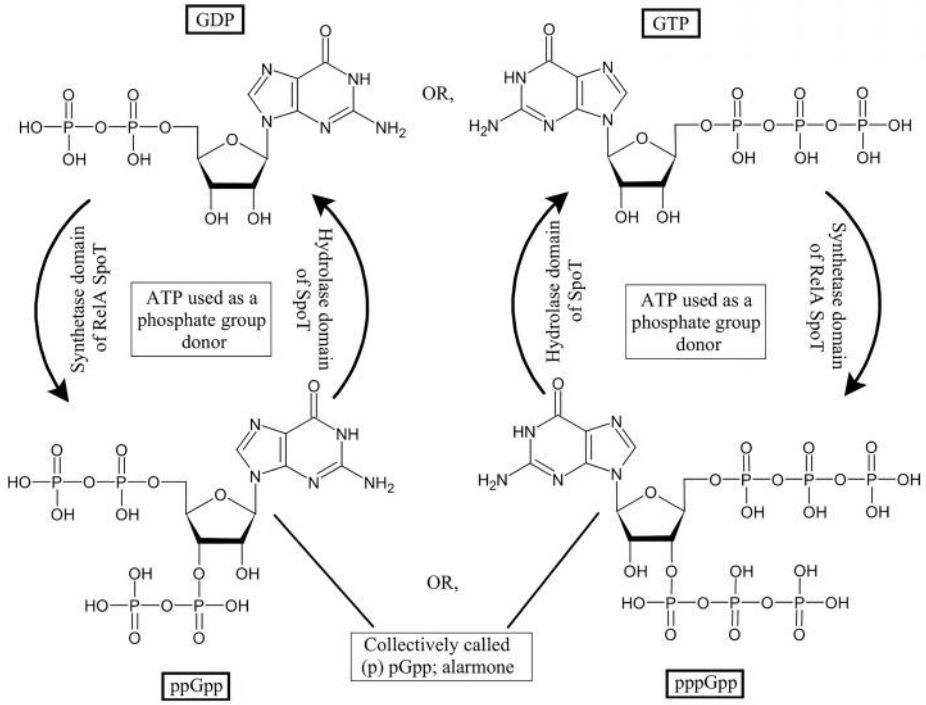

Figure 2: Mechanism of alarmone; (p)ppGpp synthesis and its degradation

Long RSH enzymes:

These enzymes have multi-domain characteristics containing the N-terminal catalytic domain (RSH-NTD) and C-terminal regulatory domain (RSH-CTD). Synthetase and hydrolase domains are confined to the N-terminal catalytic domain. The synthetase and hydrolase domain and their regulation maintain a balance of (p)ppGpp production in response to environmental factors. The active hydrolase domain causes a decrease in the accumulation of alarmone under normal conditions (Steinchen and Bange, 2016; Irving, Choudhury and Corrigan, 2021). RSH-CTD domain contains the following portions: 1) TGS domain (ThrRS, GTPase and SpoT), 2) α helical domain, 3) a putative zinc finger domain (CC) and 4) The ACT domain (aspartate kinase, chorismate mutase, and TyrA) (Steinchen and Bange, 2016). *Escherichia coli* and many others belonging to the class of Gammaproteobacteria and Betaproteobacteria harbor one monofunctional RSH protein, RelA. RelA possesses only the synthetase domain, incapable of degrading alarmone because they have degenerated hydrolase domain (Atkinson, Tenson and Hauryliuk, 2011). Another bifunctional RSH is also observed in this class of bacteria named SpoT, carrying both the synthetase and hydrolase domain (Steinchen and Bange, 2016). When GDP interacts with ATP to the synthetase domain, the structure of the enzyme is opened and the catalytic activity of the hydrolase domain is inhibited. Likewise, when (p)ppGpp binds to the hydrolase domain, a conformational change takes place that turns off the function of the synthetase domain. As a result, the degradation of alarmone back to GTP or GDP occurs (Irving, Choudhury and Corrigan, 2021). However, the function of these two domains is in-turn regulated by the interaction of the C-terminal domain with its cognate partner. In *Escherichia coli*, under amino acid starvation, the alarmone synthesizing enzyme RelA recognizes the stalled ribosome where the A site is occupied by deacetylated tRNA. RelA senses this condition and becomes activated to produce alarmone that can fight this amino acid depleted environment (Kudrin *et al.*, 2018). Cryo-electron microscopy study of E. coli RelA protein complexed with stalled ribosome occupied by deacetylated tRNA depicts an important regulatory mechanism of (p)ppGpp synthesis. The N- terminal

domain of RelA containing synthetase and hydrolase domain projects towards the solvent. It does not interact with the stalled ribosome. The interaction with ribosome takes place through C- terminal domain which acquires an 'open' conformation stabilizing A/T like structure of the deacetylated tRNA in the A site of the ribosome. This open conformation of RelA is necessary for (p)ppGpp synthesis (Arenz *et al.*, 2016).

Small alarmone synthetases (SASs):

Small alarmone synthetases are not widely expressed in all bacterial cells like long RSH enzymes. They are restricted in *Bacillus subtilis*, *Streptococcus mutans*, *Staphylococcus aureus*, and some other bacteria belonging to *Firmicutes* phyla (Lemos *et al.*, 2007; Nanamiya *et al.*, 2008; Manav *et al.*, 2018). Hydrolase domain and C-terminal regulatory domain are completely absent from this enzyme family as compared to the long RSH family. The activation of enzymes takes place under alkaline shock, cellular stress i.e., exposure to antibiotics which ultimately induces the synthesis of (p)ppGpp, providing fitness to the bacterial cells under shock. RelP and RelQ are two such SAS enzymes found in *B. subtilis* producing (p)ppGpp under alkaline shock (Nanamiya *et al.*, 2008). The importance of RelP and RelQ in the induction of alarmone synthesis in *S.aureus* has also been studied. After applying several cellular stresses like exposure to vancomycin and ampicillin, mutant cells lacking *relP* and *relQ* have a very reduced chance of survival (Geiger *et al.*, 2014). *Vibrio cholerae*, another pathogenic organism, contains an additional enzyme RelV which shares homology with SASs. The expression of this small RelV is stimulated by low carbon and membrane stress. It is also a synthetase enzyme for the production of (p)ppGpp.

Small alarmone hydrolase (SAHs):

These enzymes have been recently identified containing the hydrolase domain. SAHs as a bacterial representative were first determined from *Corynebacterium glutamicum* based on the amino acid sequence similarity with the hydrolase domain (Ruwe *et al.*, 2018). Both in-vivo and in-vitro analyses were examined to understand the pyro phosphohydrolase activity of RelH protein. In the in-vivo experiment, the enzyme expression was noticed in two separate *E. coli* strains, and in the in-vitro experiment, the purified protein was identified. If a deletion event occurs within the *relH* gene, a very minor change arises on growth in both wild type and mutants having a deletion in (p)ppGpp synthetase enzymes. This research reveals that the activation of this enzyme occurs under specific environmental cues.

Relationship between alarmone synthesis and biofilm:

In a natural environment, microorganisms assemble on the surface of living or nonliving objects, forming a complex ordered structural arrangement called biofilm. This structural organization of bacterial cells is encased in a self-producing extracellular polymeric substances (EPS) matrix (Flemming and Wingender, 2010), restricting antibiotics penetration towards the bacterial cells. The emergence of resistance property of bacterial cells in the biofilm can lead to several biofilm-associated infections like cystic fibrosis, urinary infection, periodontal diseases (Donlan, 2001). Within this ordered network bacterial cells form a suitable channel through which nutrients can easily travel to the deepest layer of the biofilm. Cells within a biofilm have a reduced growth rate compared to the planktonic stage that provides a defense mechanism to biofilm-associated cells under stress (Olsen, 2015). Microorganisms growing on medical devices enter into the human body during transplantation and lead to serious infections because cells in biofilm bear all the resistant characteristics against antimicrobial. The alarmone synthesis turns on the stringent response that makes bacterial cells more tolerant against external attack. It has been observed that the generation of (p)ppGpp imparts an effect on biofilm development in many bacterial cells. *Vibrio cholerae*, *Staphylococcus aureus*, *Bordetella pertussis*, *Escherichia coli*, and many more bacteria show the involvement of (p)ppGpp synthesizing synthetase enzymes on biofilm growth (He *et al.*, 2012).

The involvement of (p)ppGpp on biofilm growth in *Vibrio cholerae*:

The illness and death of most humans caused by cholera is a major trouble globally. The pathogen responsible for cholera disease is gram-negative gammaproteobacterium *Vibrio cholerae*. It causes profuse watery diarrhea and leads to rapid dehydration of the body and sometimes to death also. The causative agent of cholera is found in the marine environment such as surfaces of the river, lakes, estuaries. It enters into the human intestine and colonizes there (Faruque, Albert and Mekalanos, 1998). The biofilm formation by *V. cholerae* helps the organisms to fight the harsh environmental condition

and protect it from the gastric acidic barrier (Hammer and Bassler, 2003). Biofilm forming genes are localized in two operons: one operon containing *vpsU* and *vpsA-K* and another with *vpsL-Q*. These two operons are transcriptionally modulated by two activators VpsT and VpsR (Casper-Lindley and Yildiz, 2004). If *vpsT* is disrupted, cells are deficient to form biofilm along with a reduced expression of *vps* genes. The disrupted *vpsR* gene also does not yield a mature biofilm (Yildiz and Schoolnik, 1999). *V. cholerae* possess three stringent response regulatory proteins: RelA, SpoT, and an additional synthetase called RelV. RelV has only a synthetase domain lacking hydrolase activity (He *et al.*, 2012). One research study investigated whether the biofilm formation of *V. cholerae* depends upon alarmone synthesis by constructing *relA*, *relV*, *relA spoT*, *relA relV*, and *relA spoT relV* mutants. They found that wild type cells and *relA spoT* mutants produced the most detectable (p)ppGpp compared with others. Wild type cells have all the three synthetases, thus producing maximum (p)ppGpp level. Although *relA spoT* mutants lack spoT hydrolases, they still contain *relV*, thereby producing a sufficient level of (p)ppGpp. Less (p)ppGpp was found in *relA* mutant and a very low level was observed in *relA relV* mutant. (p)ppGpp production was almost negligible in *relA spoT relV* mutants (He *et al.*, 2012). They found that *relA spoT relV* mutants show a remarkable decreased amount of biofilm formation because of a lack of synthesis of (p)ppGpp. *relA spoT* mutants form a very little biofilm. They produce an excessive level of (p)ppGpp without regulation because of the absence of SpoT hydrolase, inhibiting cell growth. This research study predicts that the synthesis of (p)ppGpp i.e., alarmone has an important role in the biofilm formation of *V. cholerae*.

RelP and RelQ stimulate the biofilm formation in *Staphylococcus aureus*:

Staphylococcus aureus, being an opportunistic pathogen, causes serious infections in the health care system. They cause mild skin infection to fatal necrotizing pneumonia and they are also responsible for some deadly infectious diseases such as bacteremia, infective endocarditis, osteoarticular and pleuropulmonary infections. The biofilm-forming ability makes the pathogen suitable to form biofilm layers on medical devices and surgical wounds. A variety of adhesins play an important role in the attachment of *S. aureus* to the surface, gradually forming a mature biofilm. *S. aureus* possesses Rel, RelP, and RelQ synthetases that produce (p)ppGpp under a stressed condition. Rel has hydrolase activity that can degrade (p)ppGpp produced by RelP and RelQ, preventing the toxic accumulation of (p)ppGpp. RelP and RelQ only have the synthetase function (Geiger *et al.*, 2014). To determine the transcription activation of *relP* and *relQ*, the cells were undergone many stimuli such as treatment with cell wall synthesis inhibitors vancomycin and ampicillin. Mutant cells having a deletion in *relP* and *relQ* indicated a lower survival rate upon induction with vancomycin and ampicillin . The formation of biofilm of *S. aureus* began under cell wall stressed conditions by RelP and RelQ. When the sub-inhibitory vancomycin concentration was applied, *relPQ* double mutants and (p)ppGpp^0 mutants produced a very little amount of biofilm than wild type cells. Wild type cells could generate a thick layer of biofilm at vancomycin treatment because of having synthetase enzymes. It depicted that RelP and RelQ imparted an effect on biofilm development. To characterize which synthetase affected biofilm formation, the single mutants of *relP* and *relQ* were designed. They found that both the single mutant could induce biofilm formation at vancomycin treatment. But, *relPQ* double mutant could not stimulate biofilm formation. Thus, RelP and RelQ act synergistically to produce biofilm under cell wall stressed conditions (Geiger *et al.*, 2010).

Effect of alarmone in *Escherichia coli* biofilm development:

The gram-negative facultative anaerobic organism *Escherichia coli* is a natural inhabitant of the intestine of most warm-blooded animals. They prevent the colonization of other pathogenic organisms by growing there. However, under certain situations, the biofilm of *E. coli* causes severe urinary tract infections. They also form many device-related infections by developing biofilm on intravascular catheters, prosthetic joints, prosthetic grafts. The filaments of flagella, their motility, and also chemotaxis play an important role in the biofilm development of *E. coli*. The biofilm formation depends on the specific adhesins poly-β-1,6-N-acetyl-glucosamine whose synthesis, in turn, relies on secondary signaling molecules (p)ppGpp i.e., alarmone (Boehm *et al.*, 2009). E. coli harbors RelA SpoT enzymes for synthesizing either pppGpp or ppGpp. To determine the role of RelA and SpoT on biofilm development, the mutant cells possessing a

mutation in *relA spoT* were created (Balzer and McLean, 2002). Both the wild type and *relA spoT* mutants were allowed to grow on MOPS i.e., a defined media and M9 + CAA minimal media. The mutant cells showed a significantly decreased amount of biofilm on MOPS and M9 + CAA media than wild type cells. But in the LB medium, the mutant cells show enhanced biofilm formation because of the absence of the amino acid depleting condition. These findings suggest that the alarmone synthesis or in other words the (p)ppGpp generation is important to develop biofilm under low nutrient conditions. If *relA spoT* genes were mutated, the chance of biofilm formation on MOPS media was also lowered (Balzer and McLean, 2002).

Eradication of biofilm by inhibiting the alarmone synthesis: the journey ahed

The altered behaviors of bacterial cells in the biofilm restrict the use of several antimicrobials to disrupt the biofilm. This challenging problem shifts the attention of scientists towards the inhibition of different regulatory pathways to eradicate the biofilm. If the alarmone synthesis can be blocked or imbalanced, bacteria are unable to use the stringent response. As a result, they can't survive in a stressful situation. Anti-biofilm peptides show a broad range of activity against biofilm-associated infections (Pletzer and Hancock, 2016). They belong to a specified group of antimicrobial peptides which are usually cationic. They comprise about 12 to 50 amino acids having 2-9 positively charged lysine or arginine residues and about 50% hydrophobic amino acids. These cationic antimicrobial peptides also termed cationic host defense peptides, have a wide role in infection and inflammation by regulating immune responses. Several studies revealed that these anti-microbial peptides can degrade (p)ppGpp, inhibiting the bacterial association in biofilm. Anti-microbial peptides 1018, DJK-5, DJK-6 show an inhibitory effect on biofilm by degrading the synthesis of (p)ppGpp. Coprecipitation of anti-microbial peptide 1018 with ppGpp and nuclear magnetic resonance spectrometry showed the direct interaction of peptide 1018 with ppGpp (de la Fuente-Núñez, C., Reffuveille, F., Mansour, S. C., Reckseidler-Zenteno, S. L., Hernández, D., Brackman, G., Coenye, T., & Hancock, 2015). It was observed that the sensitivity towards this peptide was decreased by gradually increasing the level of (p)ppGpp due to the addition of serine hydroxamate and overexpression of *relA*. When *relA* and *spoT* genes were mutated, bacteria showed an increased susceptibility towards this peptide 1018. Nuclear magnetic resonance spectrometry and chromatography results indicated that this anti-microbial peptide 1018 interacted directly to cells and degraded the ppGpp within 30 minutes. Treatment with anti-microbial peptide 1018 and DJK-5 can suppress the *spoT* promoter activity in *Pseudomonas aeruginosa* biofilm (Pletzer and Hancock, 2016).

Conclusion

Biofilm contributes towards bacterial pathogenicity, tolerance against antibiotics, and immune responses, implying a major clinical challenge because of their difficulty in management. The alarmone i.e., (p)ppGpp synthesis alerts organisms to form biofilm under harsh conditions. Synthetase enzymes such as RelA SpoT or RelP RelQ produce (p)ppGpp by phosphorylating GTP or GDP utilizing ATP as a phosphate group donor. The hydrolase domain reverts such a reaction by degrading (p)ppGpp back to GTP or GDP again. Alarmone targets replication, transcription, translation, cell division of bacterial cells under stress. In *Escherichia coli*, the (p)ppGpp synthesis alters the binding specificity of sigma factor to core RNA polymerase. This modification changes the transcription of normal housekeeping genes to stress response genes, protecting the bacterial cells from external attack. Under amino acid depletion conditions, the alarmone reduces the growth rate increasing the survival rate of organisms. The presence of extracellular polymeric substance matrix restricts the penetration of several antibiotics to the bacterial cells, making them highly resistant. The degradation of (p)ppGpp could be a novel strategy to destroy biofilms. Several antimicrobial peptides 1018, DJK-5, DJK-6 modulate (p)ppGpp synthesis, targeting biofilm maturation. Peptide 1018 directly binds with intact cells, degrading (p)ppGpp within 30 minutes. Peptide 1018 and DJK-5 also inhibits the *Pseudomonas aeruginosa* biofilm by suppressing *spoT* promoter activity. The inhibitory role of several antimicrobial peptides on alarmone highly warrants to be explored more towards developing a novel strategy for biofilm-associated infections.

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