CHARACTERISTICS AND REGULATION OF BIOFILM FORMATION IN SALMONELLA

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Received in February, accepted in May 2021

Abstract: The ability to form biofilms, which is a common feature in Salmonella serovars, is the main cause of persistent infections and permanent contamination in both clinical and industrial systems. Because the biofilm structures are significantly more resistant to environmental stress conditions than the planktonic forms of bacteria, it is often impossible to remove them through conventional disinfection or sterilization practices. Therefore, it has become necessary to develop effective strategies in combating biofilms, which are defined as the dominant form of microbial life. To achieve this goal, it is necessary to understand the genetic regulatory mechanisms that control the transition from planktonic form to the biofilm form and the related changes in gene expression. In this review, the current state of knowledge regarding gene regulation systems that affect the biofilm formation in Salmonella, has been summarized and discussed.

1. Introduction. 2. Regulation of biofilm formation in Salmonella. 2.1. csgD. 2.2. BarA/SirA and Csr system. 2.3. PhoPQ and RstA. 2.4. The interaction of cells in the biofilm structures through signal molecules. 2.5. sRNAs. 2.6. dam and seqA. 2.7. MarT. 3. Conclusion

K e y w o r d s: Salmonella Typhimurium, biofilm, genetic regulation

1. Introduction

Biofilm can be defined as a structured consortium attached to a living or inert surface, which is formed as a result of encircling microorganisms by the extracellular polymeric substance (EPS) produced by them [1]. Biofilms usually contain 10–25% cells and 75–90% EPS depending on the species that make up them [2]. As will be discussed later, EPS has much more function than a sticky substance that holds cells together. Enormous advances in omic technologies, molecular biology and computer technology have revolutionized biofilm research. The fact that no habitats are occupied by only one bacterial species emphasizes the importance of working with biofilms. The types of microorganisms found in different habitats are capable of establishing various forms of interaction and communication between them to create stable communities. All these determinations brought together the research of the structure, formation and regulation mechanisms of biofilms. Researchers have found that biofilms consisting of more than one species are more stable and exhibit a lower level of nutritional requirements than biofilms formed by single cell species [3, 4]. Also multi-species biofilms exhibited higher tolerance to disinfectants, antimicrobial agents and predation. With the light of these findings recent researches focus on autotrophic-heterotrophic interactions between various microbial species using chemical signals, other interactions, competition and cooperation [5].

EPS generally consists of polysaccharides, proteins, nucleic acids and lipids. These components form the three-dimensional polymer network structure that provides the mechanical stability of the biofilm, forming the adhesive form, adhering to a surface and communication between the cells forming the biofilm [2]. The purpose of the biofilm is to protect microorganisms from external factors or to gather nutrients within it. Biofilms are of great importance in the food industry due to their negative effects on both industrial production processes and health. The damage caused by microbial biofilms on medical and industrial tools and production surfaces, energy and product losses they cause in production processes and persistent recurrent infections are among the most important microbial problems in the world [6].

The use or control of any process or activity of biofilms for scientific or technological purposes is possible by knowing the formation and regulation of biofilms significantly. Biofilm formation is generally a multi-stage process. The first stage is the process of bacterial attachment to biotic or abiotic surfaces. This process is examined in two phases as reversible adhesion and irreversible adhesion. Although the bacterial cell is very close to the biotic and abiotic surface in reversible adhesion, it is the stage where no physical contact is provided. In this process, besides electrostatic forces, surface pH, nutrient concentration, temperature and hydrophobicity are also active. In addition, especially extracellular proteins attached to the surface may play
a role in achieving the first physical contact with bacteria. In the irreversible adsorption – the second phase of the adsorption- dipole-dipole interactions, ionic and covalent bonds, hydrophobic interactions and hydrogen bonds play a critical role. After irreversible attachment, bacteria attached to the surface divide and form microcolonies. Many microcolonies can be produced in a biofilm structure depending on the number of bacteria attached to the surface from different regions. The bound cells are then matured and taken into the extracellular polymeric substance (EPS) produced by the cells that form the microcolonies. This EPS is responsible for the formation of the three-dimensional architectural structure and the stabilization of biofilms. Among the microcolonies within the EPS, a three-dimensional form of water channels and a primitive discharge system network, which serves in the transmission of food and waste, are formed. It has a linear or branched molecular structure formed by a repeating sugar (homopolysaccharides) or a mixture of different sugars (heteropolysaccharides). Although the EPS matrix varies according to the cell type that forms the biofilm; in general it contains 94–97% water, 1–2% extracellular nucleic acid and different lipids and 1–2% proteins. Biofilms that have completed their maturation are in the process of disintegration at the last stage. At this stage, the disruption of the enzymatic processes and matrix integrity due to other physical and chemical factors and the separation of planktonic cells from the biofilm matrix are involved [7] (Figure 1). In the light of the information obtained to date, it is believed that bacteria generally use environmental signals, flagella, outer membrane proteins, pili or lipopolysaccharides (LPS) for the formation of microcolonies and quorum sensing (QS) molecules to form biofilm structure [8]. However; many questions regarding genetic and biochemical mechanisms involved in the perception of the surface by bacteria and the production of different stages during biofilm formation have not yet been lightened yet [9].

Members of the food-borne Salmonella enterica species, belonging to the Enterobacteriaceae family, have the ability to form biofilms both on biotic and abiotic surfaces in their natural life cycle [10, 11]. It is imperative to target the biofilms created by these bacteria in minimizing the industrial problems they cause, in addition to the control and treatment of infections caused by Salmonella species in humans and animals, which have been identified with more than 2500 serotypes to date. Main components of extracellular polymeric matrix (EPS) in Salmonella biofilms are curli fimbria and cellulose. These components, together or individually; plays a key role in the attachment of the bacteria to a surface, cell clustering and the formation of the biofilm structure [12–16]. The expression of curli fimbria in Salmonella in most cases has common regulation systems with cellulose production in which cell to cell and cell to surface interactions work together [13].

One of the most important features that play a role in Salmonella virulence is the biofilm forming properties of the serovarieties of this genus. Therefore, it is extremely important to define the genetic, physiological and biochemical properties and microbial community characteristics of the biofilm structures in question. In this review article, it is aimed to summarize the information available in the literature on Salmonella biofilms and to define future perspectives.

2. Regulation of Biofilm Formation in Salmonella

2.1. csgD

Salmonella members show a morphotype called “rdar” because of its red, dry and rough structure on agar containing Congo Red [17]. This biofilm form is
formed by the expression of the two main matrix components, cellulose and curli fimbria, of *Salmonella* [18–21]. The transcriptional regulator CsgD protein is the main regulator of the “rdar” morphotypes [13]. CsgD regulates the transcription of the csgBAC operon encoding the structural subunits of the curli fimbria and indirectly contributes to cellulose production by activation of adrA transcription [13, 22]. AdrA protein is a diguanylate cyclase that binds to cellulose and synthesizes secondary messenger cyclic diguanosine monophosphate (cyclic diguanosine monophosphate, c-di-GMP) that activates cellulose. C-di-GMP regulates the bcsABZC operon encoding genes, transcribed during the cellulose biosynthesis, in the post-transcriptional phase by changing the concentration of c-di-GMP [13, 23].

CsgD is an integral part of the curli fimbria biosynthesis system, which is created by different transcribing of csgBAC and csgDEFG operons. The CsgD transcriptional regulator contains an acceptor N-terminal region. There is a preserved aspartate (D59) in this region. The csgD mutant strains exhibit a “saw” (smooth and white; plain and white) phenotype in the Congo Red (CR) agar medium. Point mutations that may occur in the csgD promoter region (in the 521 bp region between the csgB and csgD genes) can convert the protected promoter region from a highly regulated form to a semi-conservatory form [13]. As a result of passivating this gene in csgD insertion mutants, strains cannot form a pellicle structure in Luria Bertani (LB) broth, while ATM (adhesion test medium) can [20]. At the nucleotide and protein level, the high similarity of *S. Typhimurium* and *E. coli* curli fimbriae indicates that these genes evolved from a common ancestor. Comparative genetic analysis performed in the region between the csgD-csgB genes showed a high degree of similarity in all *Salmonella* members, with the exception of *S. bongori* strains. This is an indication that changes in the csgD-csgB intermediate region are caused by natural mutations caused by genetic drift. These mutations are observed more frequently in strains adapted to laboratory conditions and as a result of possible mutational effects, “rdar” morphotype is lost. This change can be seen as a result of passage of *Salmonella* strains in rich nutrient media and laboratory conditions for long generations and the “rdar” morphotype can be lost. In wild type strains, these mutational changes are seen less frequently [24].

There is a strong relationship between activation of csgD and STM2123 and STM3388 (proteins containing complex GGDEF / EAL domain, respectively) proteins. STM2123 is a component needed for activation of csgD at the first step of biofilm formation. STM3388 protein, on the other hand, was found to have contributed positively to the formation of the biofilm since the stage when the biofilm began to mature. Proteins containing four other important EAL domains found in *S. Typhimurium* (STM1703, STM1827, STM3611 and STM4264) show similar activity in the expression of csgD. In some studies with the mutants of these proteins, a significant increase was also detected in the expression of csgD due to the increase in c-di-GMP at the cellular level was determined. In this context, the view has arisen that cellular c-di-GMP levels can control different targets in regulation of these proteins and biofilm formation [23].

These data emphasize that c-di-GMP plays an important role in virulence and mobility in biofilm formation due to its role in curli fimbria and cellulose biosynthesis via csgD [20].

### 2.2 BarA / SirA and Csr system

The BarA / SirA system is a widely conserved system in gamma-proteobacteria [25]. SirA is a response regulator that is a member of the FixJ family proteins. BarA, on the other hand, acts as a sensor kinase specific to SirA. It is known that bile salts and short chain fatty acids in the environment affect the BarA / SirA system in *Salmonella*. The SirA protein has also been found to be responsible for the transcriptional activation of csrB and csrC sRNAs, which are regulators of *Salmonella* invasion. This indicates that sirA controls host cell invasion of *Salmonella* [26–28].

In the study carried out by Teplitski et al. [25]; it was determined that sirA, fimI, csrB and csrC binary mutants could not perform biofilm formation on plastic surfaces. On the other hand *flhDC* mutants could form much more biofilm. In this study, the regulatory roles of SirA at the transcriptional level and the post-transcriptional level of the Csr system on the expressions of flagellar or type I fimbrial components that positively or negatively contribute to biofilm formation were clarified. Phosphorylated SirA-P activates csrB and csrC, fim operon and hilA at the transcriptional level. Increased csrB / csrC level inhibits CsrA activity. Reduced CsrA activity promotes biofilm formation by causing a decrease in expressions of factors that can inhibit biofilm formation, such as FlhDC and HilA proteins. CsrA also reduces film expression. The decrease in the activity of CsrA allows for more type I fimbria biosynthesis to be realized in this context and to have more biofilm production.

### 2.3. PhoPQ and RstA

*Salmonella* PhoPQ system is a binary system consisting of the cytoplasmic response regulator PhoP and the sensor kinase PhoQ localized in the inner membrane [29]. As a result of PhoP activation, LPS modification is controlled by direct or indirect expression of more than 120 genes associated with many functions such as magnesium transport, invasion of epithelium cells and survival within macrophages [29, 30]. It is
known that the phoP mutant strains of S. Typhimurium produce better biofilm compared to wild type strains. This mutation is also capable of increasing biofilm production on glass slides. These data clearly show that the PhoPQ system suppresses biofilm formation in S. Typhimurium. It was also found that prgH may be associated with PhoPQ dependent biofilm regulation and determined that mutant Salmonella strains in terms of prgH gene could not form mature biofilms on gallstones and glass surfaces [29].

Another factor contributing to PhoPQ dependent biofilm regulation is the indirect regulation of RpoS by the PhoPQ system. As mentioned before, besides biofilm formation RpoS also regulates the synthesis of CsgD and mobility-related elements at the transcriptional level. PhoP can stabilize RpoS by acting as a transcriptional activator of iraP. iraP provides stabilization of RpoS by encoding a product that interacts with RssB [31]. PhoP also activates RstA's expression [32]. This protein indirectly induces the breakdown of RpoS by the ClpXP-SsrB proteolytic pathway. RstA is the response regulator of the RstA / RstB binary system. The opposite effects of IraP and RstA play an active role in regulating RpoS's expression based on extracellular signals. Activation of RstA by PhoP may offer other alternatives to PhoPQ dependent biofilm regulation. Unlike its effects on RpoS, RstA also affects the expression of bapA. High expression of RstA in E. coli leads to negative regulation by connecting RstA to the csgD promoter [33]. The presence of RstA's binding motif in the csgD operon in Salmonella proves that RstA directly inhibits the expression of csgD [34].

2.4. The interaction of cells in the biofilm structures through signal molecules

Biofilm forming is not a random event where bacteria only get together, attach to significant surface then adhere there and maintain their lives together with the other species on that surface. Many organisms give signals to each other to coordinate their activities, use little signal molecules. With the process called quorum sensing (QS) which is an important mechanism in biofilm forming, bacteria can measure the signal molecule density they produce, sense the amount of other microorganisms around them and enable to transfer this data to other bacteria [35]. In another words with QS, bacteria determine the bacterial population in their environment. As increasing the amount of bacteria attaching to the surface, this signal's local concentration increase and with this increase, a number of processes direct begining of biofilm forming. So, bacteria in the structure of biofilm contact to each other through the low molecular weighted messengers. QS also has some important regulative roles at synthesizing antibiotic, virulence factor formation, reproducing, spore forming, cell separation and pathogen bacterial infections [36]. This mechanism which provides cellular interaction is regulated by auto-inducer (AI) molecules [37].

The reason why QS molecules are expressed as auto-inducer since they show regulative effect on the cell metabolism where they are produced [38]. Some microorganisms use more than one different QS molecule. QS takes place in two ways as between species and inner species. Gram negative bacteria use N-acyl homoserine lactone (AHL, AHLS, acyl-HSL or HSL), Gram positive bacteria mostly use oligo-peptides as an auto-inducer in QS mechanism [39]. Besides this, the usage characteristics of auto-inducer signal molecules in QS system of Gram negative and positive bacteria are mutual. In the studies conducted on QS systems it was determined that S. enterica has actualized the cellular interaction through auto-inducer signals [38].

Besides the formation of single and multi-species biofilm structures; symbiosis also plays an important role in the control of other social / physiological behaviors such as the formation of spore, bacteriocin production, genetic competence, programmed cell death, and virulence [40]. This intracellular communication process was first described in the marine bacteria Vibrio fischeri, which produces bioluminescence. In this system, bacteria communicate by producing, detecting, and responding to small diffusable signal molecules called autoinducers. The bacterial QS system is generally divided into three types: 1) The LuxI / LuxR system in which Gram-negative bacteria use acyl homoserine lactones (AHL) as signal molecules 2) Two-component-oligopeptide system at which Gram-positive bacteria use small peptides as oligopeptide signaling molecules and 3) Autoinducer-2 (AI-2) system, encoded by luxS, common in both Gram-negative and Gram-positive bacteria. Each signal system type is detected and responded by the correct sensing element and regulatory control [41, 42].

2.5. sRNAs

Small RNAs (sRNA) are non-coding RNA molecules produced by bacteria that can be 50 to 250 nucleotides in length. Different studies have found that biofilm formation is influenced by the production of sRNA molecules in various S. enterica serovar Typhimurium mutants [43]. The sRNA is encoded in the same region as the QS syntase (LuxS). MicA is a family of highly preserved small RNA molecules in some Enterobacteriaceae members. It has been determined that members of this small RNA family are a regulatory mechanism for biofilm formation in many bacterial species and play a critical role in the development of mature Salmonella biofilms by adjusting the level of balanced expression

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Biofilms are the main cause of persistent contaminations, which evoke serious economic losses and hygienic problems in the food industry and medicine.
Improperly cleaned food production surfaces contribute to biofilm formation for different food spoilage bacteria and food-borne pathogens such as *Salmonella*, possessing high adhesive characteristics for biotic and abiotic materials. Detached cells from biofilms, yielding by the effects of the aerosols from contaminated equipments and products flow from contaminated surfaces, create cross contamination. Thus, development of effective strategies to prevent biofilm formation and to eradicate mature biofilm forms from food producing environments are crucial for food industry and human health. Understanding the molecular patterns of biofilm formation and determining the biofilm behavior under different environmental conditions and disinfecction applications, are necessary to develop these effective antibiofilm strategies.

References