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CHARACTERISTICS AND REGULATION OF BIOFILM FORMATION IN *SALMONELLA*

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Abstract: The ability to from 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.

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 multistage 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

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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 (heteropolisaccharides). 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 *Samonella* 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

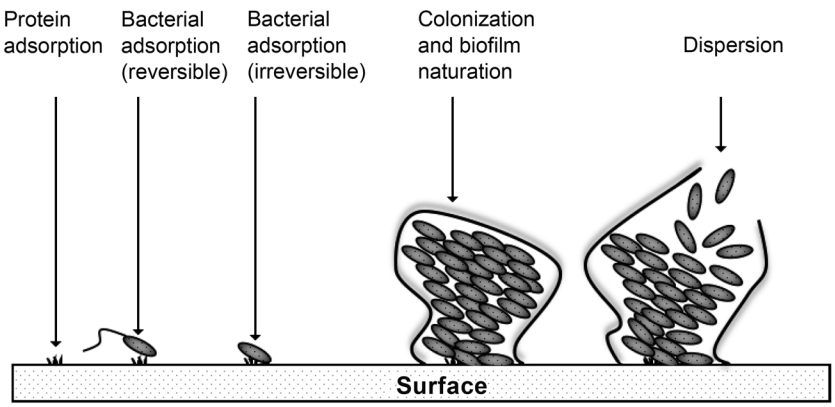

Fig. 1. Formation of bacterial biofilm structures

Adsorption of extracellular adhesive proteins to the biotic or abiotic surfaces; reversible adsorption: bacteria-protein interactions, electrostatic forces, pH, hydrophobicity, bacteri-surface interactions; irreversible adsorption: dipol:dipol interactions, ionic and covalent bonds, H bonds; Colonization and biofilm maturation: fimbrial structues and autotransporter proteins, exopolymeric matrix, microcolony organisation; Dispersion: Exopolymeric matrix degredation by physical, chemical agents and enzymes.

formed by the expression of the two main matrix compotents, 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* promotor 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-conservative 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 are emphasizes 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, act 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 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]. Beside 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. sRNA's

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

[44]. To date, at least six sRNAs (*arcZ, sroC, csrB, dsrA, oxyS,* and *rprA*) associated with biofilm formation in *Salmonella* and its closely related bacteria have been identified. These can be divided into two groups as positively regulating biofilm formation and negatively regulating biofilm formation. *arcZ, sroC* and *csrB* are sRNAs that positively regulate biofilm formation and were observed to be significantly down-regulated in anaerobiosis. However, in microaerobiosis, no significant difference of these three sRNAs was observed. Additionally, *dsrA, oxyS* and *rprA* are sRNAs that negatively regulate biofilm formation. These three exhibited differences in transcription patterns, both based on atmospheric oxygen level and culture medium [43, 45, 46].

The function of sRNAs in regulating biofilm formation occurs through two general mechanisms: sRNAs that act via base pairing with other RNAs and protein binding. Protein binding sRNAs mimic the protein binding sequences found in various mRNAs, antagonizing and separating their cognate regulatory proteins. Base pairing sRNAs are categorized as cis or trans in their position within the bacterial genome relative to their mRNA targets. The sRNAs that are copied from the DNA strand directly opposite the mRNA targets are called cis-encoded sRNAs. cis-encoded sRNAs generally add extensive complementarity to their targets. In contrast, trans-coded sRNAs reside elsewhere on the genome, function as transduced molecules and add only limited (10–25 bp) complementarity to base pairing interactions [43, 47, 48].

2.6. *dam* and *seqA*

DNA methylation status in specific GATC sequences in promoters of some genes besides the *dnaA* gene can activate or suppress transcription by affecting the binding of RNA polymerases or transcription factors [49–51]. It has been determined that the SeqA protein regulates the transcription of some genes in bacteria, just like the Dam methylase enzyme. It performs this function through a GATC methylation or by acting as a co-activator [52]. It has been found that Dam and SeqA activity is involved in the regulation of different genes in *Salmonella*, and in mutants of the *dam* and *seqA* genes, attachment to host cells and especially host cell invasion is significantly reduced [53–57]. However, few studies have been conducted describing the effect of Dam methylation on biofilm formation in *Salmonella* [58, 59]. Aya Castaneda et al. (58) found that DNA methylation in *S.* Enteritidis increased the expression of biofilm production factors such as cellulose and pleated fimbria by modifying *csgD* expression. However, only at one study it was found that *seqA* genes are not effective in biofilm formation [59].

Uğur et al. [60] found that the biofilm forming ability on steel and polystyrene surfaces in *dam* gene mutants of different *Salmonella* serovars significantly decreased compared to wild type strains. When the *dam* gene is cloned into a pBAD24 vector containing a promoter induced in the presence of arabinose, recovery of the biofilm-forming ability to the same mutants gives certainty to the findings of the *dam* mutation. On the other hand, for the first time in this study, it was determined that the SeqA protein played a role in the regulation of biofilm formation in *Salmonella* serovars. The same results were obtained when the verification tests of biofilm formation on steel and polystyrene surfaces were performed with *seqA* mutants using the pBAD24 vector mentioned above. In the light of these findings, it has been suggested that the *dam* and *seqA* genes carry out their biofilm regulation activities by changing the activities of RNA polymerase or transcription factors in the promoter regions. Studies carried out in wild strain *S.* Typhimurium 14028 and its dam and *seqA* mutants have proven that these genes are effective in the regulation of many genes related to biofilm formation, virulence and motility (Akçelik, M. unpublished data)

2.7. *marT*

The MarT protein, a close homologue of the ToxR-like regulatory protein family, was first identified by Tükel et al. [61] as a positive regulator of the misL autotransporter protein in *S.* Typhimurium. Later as a result of microarray studies conducted by Akkoç et al. [62], using *S.* Typhimurium 14028 wild type strain and *marT* mutant, it was determined that the gene in question could be a positive regulator of many properties related to bacterial physiology. In the latest sudy, Eran et al. [63] determined that the *marT* gene is a positive regulator of 14 genes in *Salmonella*, called *fimA, fimD*, *fimF*, *fimH*, *stjB*, *stjC*, *csgA*, *csgD*, *ompC*, *sthB*, *sthE*, *rmbA*, *fliZ* and *yaiC*. As a result of QRT-PCR studies, it has also been proven that the protein encoded by the *marT* gene is an autoregulator that positively regulates its own promoter. All these data indicate that the MarT protein not only regulates *misL* gene expression but also acts as a global regulator in *Salmonella*. When the participation of these genes subjected to *marT* gene regulation to biofilm formation on polystyrene surfaces was examined, it was determined that the biofilm production capacity in mutant strains for each gene was statistically significantly decreased ($p = 0.05$). These results showed that all genes tested were associated with biofilm production.

3. Conclusion

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 disinfection applications, are necessary to developt these effective antibiofilm strategies.

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