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Biofilm Formation of *Salmonella*

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Abstract

Salmonella spp. may form biofilm, and bacteria in biofilm are more resistant to drug, chemical, physical and mechanical stresses, and host immune system. The progress on biofilm research will be helpful for the development of new tools and strategies to prevent biofilm-related disease and decontaminate biofilm-derived *Salmonella* in food production. In this review, we present a comprehensive overview of biofilm formation in *Salmonella*, included that (1) the component of *Salmonella* biofilm, (2) the detection methods for biofilm, (3) the identification of biofilm-formation-associated genes, (4) the regulation mechanism of biofilm formation, and (5) virulence or resistance of *Salmonella* in biofilm.

Keywords: *Salmonella*, biofilm, component, mechanism, gene, pathogenicity, drug resistance

1. Introduction

Salmonella enteric is an intracellular gram-negative pathogen that infects various hosts, which is classified into more than 2500 serovars [1]. Many serovars, such as those most commonly associated with human infections, including *Salmonella enteritidis*, *Salmonella typhimurium*, have a broad host range [2]. In contrast, other serovars, such as *Salmonella typhi*, *Salmonella paratyphi*, *Salmonella gallinarum*, *Salmonella choleraesuis*, *Salmonella abortusovis*, and *Salmonella dublin*, have restricted host ranges and are associated primarily with one or a few hosts [3]. *Salmonella* can cause disease in domestic animals, ranging in severity of asymptomatic, diarrhea and enteritis to systemic syndrome, and result in a huge economic loss in pig and poultry industry. Salmonellosis is also a growing public health concern in both the developed and developing countries, since nontyphoidal *Salmonella* disease, a major cause of diarrheal disease globally, is estimated to cause 93 million enteric infections and 155,000 diarrheal deaths each year [4]. The

illnesses and outbreaks are most commonly attributed to exposure to contaminated food, and the eggs, broiler chickens, and pigs are among the top sources [5]. *Salmonella* often exist not only as planktonic cells but also as sessile, multicellular forms such as biofilms attached to surfaces. Biofilm formation is important for the spread of *Salmonella* because bacteria in the biofilm are resistant to disinfectants and chemical, physical, and mechanical stresses [6–8]. The biofilm formation is also contributed to *Salmonella* virulence, since bacteria in the biofilm are more resistant to antibiotics and host immune system, resulting in a chronic infection and the development of *Salmonella* carrier state [9, 10]. In our review, we present a comprehensive overview of biofilm formation in *Salmonella*.

2. The component of *Salmonella* biofilm

The biofilm formation is a multistep developmental process that always has several distinguishable steps: (a) attachment to the carrier surface, reversible, (b) irreversible attachment, binding to the surface with the participation of adhesions or exopolysaccharides, (c) the development of microcolonies, a distinct mushroom shape, (d) the maturation of biofilm architecture [11, 12], (e) under favorable conditions, the synthesis of matrix compounds decreases and the matrix is enzymatically cleaved, leading to biofilm dispersion [13]. In natural environments, *Salmonella* forms biofilms on plant [14], abiotic surfaces, including plastics, metal and glass [15–17], meat and meat-processing environments [18, 19]. In addition, *Salmonella* can colonize gallstones under laboratory conditions [20], and the *Salmonella* biofilm can be directly visualized by confocal micrographs of extracellular matrix on the surface of human cholesterol gallstones [21]. They can also form biofilms on chicken intestinal epithelium [22] or HEp-2 cells that are suspended in once-flow-through continuous culture conditions [23].

The extracellular matrix of *Salmonella* biofilm is majorly composed of curli (amyloid fimbriae), cellulose [24, 25], biofilm-associated protein (Bap) [26], O-antigen capsule [14, 27], extracellular DNA [28, 29]. The expression pattern of the biofilm is serovar specific and correlates with contact surface [30]. Curli were first discovered in the late 1980s on *Escherichia coli* strains that caused bovine mastitis, and they are mainly involved in adhesion to surfaces, cell aggregation and biofilm formation. Curli also mediate host cell adhesion and invasion, and they are potent inducers of the host inflammatory response [12]. The curli protein is encoded by the divergently transcribed *csgBAC* (*agfBAC*) and *csgDEFG* (*agfDEFG*) operons [31, 32]. The *csgBAC* operon encodes the major structural subunit, CsgA, and the surface-exposed nucleator protein CsgB. A third gene, *csgC*, is in the *csgBAC* operon, but no transcript for *csgC* has been detected in curli biogenesis [32]. The other study shows that both CsgC and CsgE facilitate extracellular thin aggregative fimbriae synthesis in *Salmonella enteritidis* [33]. The *csgDEFG* operon encodes accessory proteins required for curli assembly. The *csgD* gene encodes a transcriptional regulator belonging to the LuxR family, CsgD, for active transcription of *csgBAC* promoter [24]. Although Giaouris et al. [34] found that CsgF was expressed in biofilm growth when compared with planktonic and biofilm cells of *Salmonella enteritidis* on stainless steel surface, the function of *csgF* and *csgG* genes has not been re-

solved in *Salmonella*. Cellulose is a polysaccharide composed of $\beta(1 \rightarrow 4)$ -linked D-glucose units [35], which is an important exopolysaccharide normally synthesized in the *Salmonella* biofilms. The production of cellulose and curli by *Salmonella* leads to a matrix of tightly packed cells covered in a hydrophobic network. The operons, *bcsABZD* and *bcsEFG*, are required for cellulose biosynthesis [36]. Cellulose biosynthesis is positively regulated by CsgD, which stimulates the transcription of AdrA that harbours a cytoplasmic GGDEF domain. AdrA activates cellulose production on the post-transcriptional level either by direct interaction with *bcs* operons or indirect interaction with bis-3'-5'-cyclic dimeric guanosine monophosphate (c-di-GMP) [25, 37, 38]. BapA, a large cell-surface protein required for biofilm formation, is encoded by *bapA* gene and secreted through a type-I protein secretion system (*bapBCD* operons) situated downstream of the *bapA* gene. The expression of *bapA* is coordinated with that of genes encoding curli fimbriae and cellulose, through the action of *csgD* [26, 39]. The *bapA* gene is also highly conserved in *Salmonella* [40]. *Salmonella* produces an O-antigen capsule coregulated with the fimbria- and cellulose-associated extracellular matrix. The operons *yihU-yshA* and *yihVW* are responsible for capsule assembly and translocation [41] and regulated by CsgD. Although the O-antigen capsule do not appear to be important for multicellular behavior, they play an important role in attachment and environmental persistence [14]. However, the O-antigen capsule is required for biofilm formation of *Salmonella typhimurium* and *Salmonella Typhi* on cholesterol gallstones, and the operons are regulated in a *csgD*-independent manner [42]. Extracellular DNA is shown to be a matrix component of *Salmonella* biofilms cultivated in flow chambers and on glass surfaces [28]. However, the presence of extracellular DNA plays an inhibitive and destabilizing effect during biofilm development of *Salmonella* on abiotic surfaces [29].

3. The detection methods for biofilm

3.1. Quantification of biofilm formation

Biofilm formation of *Salmonella* can be quantitated by microplate-based crystal violet staining [43]. Briefly, the overnight broth cultures of bacterium are diluted 1:100 in the diluted tryptic soy broth (TSB). One hundred μ l of bacterial suspension is added into 96-well U-bottomed polystyrene microtiter plates. Plates are incubated at 28°C for 24 h under static conditions. Then, non-adherent bacteria are removed and the wells are washed gently three times with 200 μ l of distilled water. One hundred μ l of 0.4% crystal violet (v/v) is added into each well and stained for 20 min. After discard of staining liquid, all loosely adhering bacteria and dye are gently washed off with distilled water for three times. The dye bound to the adherent cells is solubilized with 100 μ l of anhydrous ethanol per well. The optical density (OD) is measured at 590 nm, and OD value of biofilm-formation strain is significantly higher than that of negative control. It provides more reproducible results with an addition of a fixation step (80°C for 30 min) prior crystal violet staining [19]. Combined with resazurin assay, the number of metabolically active cells is able to be evaluated [44]. With wheat germ agglutinin-Alexa Fluor 488 conjugate, which selectively binds to N-acetylglucosamine residues in

biofilms, the spectrofluorometric assay provides a more sensitive method for quantification and characterization of bacterial biofilms [45].

3.2. Biofilm formation in glass tube

The overnight cultures of bacteria are diluted 1:100 in the diluted TSB. Two milliliters of each bacterial suspension are added into borosilicate glass tubes and incubated at 28°C for 48 h. Then, the liquid is decanted and the tubes are washed gently three times with distilled water. Two ml of 0.4% crystal violet (v/v) are added into each tube and stained at room temperature for 20 min. The stained biofilm is observed at the liquid–air interface on the glass test tube walls or at the bottom of the tube [46]. The glass tubes may also be incubated at 37°C at 200 rpm by using an orbital shaker, and biofilm is observed at interphase without staining [47].

3.3. Congo red/carbol fuchsin staining

The overnight culture (1:100 diluted in TSB) is inoculated into 3 ml of fresh TSB in a 6-well plate containing sterile polystyrene coverslip (20 × 20 mm). After incubation at 28°C for 24 or 48 h without agitation, the coverslips are removed carefully, treated with cetylpyridinium chloride (10 mM) for 30 s, rinsed with distilled water and air dried for 20–30 min. After fixation by gentle heating, the coverslips are stained with a mixture of saturated aqueous Congo red solution and 10% Tween-80 (2:1, V/V) for 30 min and rinsed with distilled water. After staining with 10% (v/v) Ziehl carbol fuchsin for 6 min and rinsing in distilled water, the coverslips are air dried and mounted on slides [48]. Under a light microscope, bacterial cells on slides show purple staining, while the exopolysaccharides of biofilm show pink staining [46].

3.4. Field emission scanning electron microscopy

The coverslips with cultured bacteria are fixed in 3% glutaraldehyde in 0.1 M phosphate-buffered saline at 4°C for 2 h. The samples are then dehydrated with increasing concentrations of ethanol (50, 70, 80, 90, and 100%) followed by isoamyl acetate (100%), each for 15 min. The samples are critical point dried for 5 h, coated with gold palladium alloy, and observed under a field emission scanning electron microscope [49]. The biofilm-formation strain exhibits increased clusters of bacteria cells with curli fimbriae and has meshwork-like structures surrounding the cell surfaces.

3.5. Congo red and calcofluor plates

LB agar plate without salt supplemented with 40 mg/L Congo red and 20 mg/L brilliant blue is used to determine the Congo red-binding property of the colonies. LB agar plate supplemented with 200 mg/L calcofluor (fluorescent brightener) is used to determine the cellulose production by comparing the fluorescence of the test strains under UV light [49]. Biofilm of *Salmonella* is mainly composed of curli and cellulose, and *Salmonella* strains were grouped into distinct morphotypes according to Congo red binding: (a) red, dry, and rough indicating curli and cellulose production (RDAR), (b) brown, dry, and rough, indicating a lack of cellulose synthesis (BDAR), (c) pink, dry, and rough, indicating a defect in curli expression (PDAR), (d)

smooth, brown, and mucoid, indicating a lack of cellulose synthesis but overproduced capsular polysaccharide (SBAM), and (e) smooth and white, indicating a lack of both curli and cellulose production (SAW) [19, 31, 50].

3.6. Confocal laser microscopy

Bacteria cultured on coverslips, dish, or microplate are stained by 0.1 M phosphate-buffered saline (pH 7.2) containing SYTO 9 and propidium iodide. After 10 min incubation in the dark at room temperature, stained samples are examined using a confocal scanning laser microscopy. Fluorochromes are excited using an argon laser source at 488 nm. Images are collected in two channels, 490–515 and 620–640 nm, corresponding to the emission maxima for SYTO 9 and propidium iodide, respectively. Optical sections approximately 1 μ m in height are collected starting from below the focal plane to upward through the entire biofilm. The biofilm cells are clearly observed in a multilayer community [20, 51].

4. Identification of biofilm-formation-associated genes

The most common biofilm-formation-associated genes are the genes encode adhesins. The best characterized of the *Salmonella* fimbriae is type-1 fimbriae. This fimbrial type is encoded by the fim gene cluster and is assembled by the chaperone–usher system [52]. The fimA gene encodes the major structural subunit, while the fimH gene encodes the adhesin protein that is located at the tip of the assembled fimbrial structure and mediates binding to the receptor. The FimH adhesin is involved in biofilm formation on HEP-2 tissue culture cells, murine intestinal epithelium, and chicken intestinal epithelium [22, 23]. The long polar fimbriae (Lpf) are encoded by the *lpfABCDE* genes and have been implicated in the colonization of the murine intestinal mucosa [53, 54]. Plasmid-encoded fimbriae (Pef) are encoded on the 90-kb *Salmonella* virulence plasmid and are majorly encoded by *pefBCD*, *orf5*, and *orf6* genes. Both Lpf and Pef contribute to the early steps of biofilm formation [55]. *Salmonella enteritidis* produce a variety of potentially adherent fimbrial types including SEF14 (SefA), SEF17 (CsgA), SEF18 (SefD), and SEF21 (type I, FimA), the role of each fimbrial in biofilm formation is different. The SEF17 encoded by *csgA* gene stabilize cell–cell interactions during biofilm formation, while SEF21 fimbriae may involve cell surface adherence [56]. SadA is trimeric autotransporter adhesin of *Salmonella typhimurium*, the expression of SadA resulted in cell aggregation, biofilm formation, and increased adhesion to human intestinal Caco-2 epithelial cells [57]. *Salmonella* may persist on post-harvest lettuce during cold storage, the genes *stfC*, *bcsA*, *misL*, and *yidR*, encoding a fimbrial outer membrane usher, a cellulose synthase catalytic subunit, an adhesin of the autotransporter family expressed from the *Salmonella* pathogenicity island-3, and a putative ATP-/GTP-binding protein, respectively, have a role in persistence of the pathogen. The *bcsA*, *misL*, and *yidR* knockout mutants are impaired in attachment and biofilm formation, suggesting that these functions are required for biofilm formation [58].

Salmonella flagella are not required for the formation of the multicellular morphotype on plates. However, the global behavior of the bacterial community on air–liquid, surface–liquid, or cell–

liquid interfaces is changed in the absence of flagella. In a mutant lacking flagella and thin aggregative fimbriae, the contribution of the latter to the multicellular morphotype is dominant [59]. Biofilm formation of an *flgK* mutant in meat and poultry broths and their attachment on surfaces of stainless steel and glass are significantly reduced compared with that of the wild-type strain, suggest that expression of flagella could be involved in biofilm formation and attachment of *Salmonella* on contact surfaces [60]. The presence of the flagellar filament enhances binding and biofilm formation in the presence of bile, while flagellar motility and expression of type-1 fimbriae were unimportant in biofilm formation on cholesterol gallstones [61].

Pathogenicity islands accommodate large clusters of genes that contribute to a particular virulence phenotype. *Salmonella* possess at least seven *salmonella* pathogenicity islands (SPIs). Among these, SPI1 is primarily required for bacterial motility and invasion of host cells. *Salmonella typhimurium* cultures containing cloned SPI-1 display an adherent biofilm and cell clumps in the media. This phenotype was associated with hyper-expression of SPI-1 type-III secretion functions. Surprisingly, mutations in genes essential for known bacterial biofilm pathways (*bcsA*, *csgBA*, *bapA*) did not affect the biofilms formation, indicating that this phenomenon is independent of established biofilm mechanisms [62]. *Salmonella* biofilm cells exposed to superheated steam show decreased transcription of flagella and SPI-1 genes, respectively, whereas increased transcription of SPI-2 genes, important for bacterial survival and replication inside host cells, is detected [63]. In contrast, when compared biofilms of *Salmonella typhimurium* with planktonic cells, the most highly downregulated genes in the biofilm are located on SPI-2 and that a functional SPI2 secretion system regulator (*ssrA*) is required for *Salmonella typhimurium* biofilm formation. Genes involved in tryptophan (*trp*) biosynthesis and transport are upregulated in the biofilm. Deletion of *trpE* results in decreased bacterial attachment and biofilm formation, indicating that aromatic amino acids make an important contribution to biofilm formation [64]. The *aro* mutants of *Salmonella* are frequently used as live vaccines for the oral vaccination of domestic animals, and they are unable to synthesize chorismate, which is a key intermediate in the synthesis of aromatic amino acids. The *aro* mutants exhibit a decreased production of cellulose, N-acetyl-D-glucosamine, or N-acetylneuraminic acid-containing capsular polysaccharide and fimbriae, which explains their inability to form biofilms [65].

Lipopolysaccharide (LPS) synthesis also involves the biofilm formation of *Salmonella*. Two Tn5 insertion mutations in genes that are involved in *ddhC* and *waaG* result in diminished expression of colony rugosity. Both mutants have impaired biofilm formation when grown in rich medium with low osmolarity, they constitutively form larger amounts of biofilms when the growth medium was supplemented with either glucose or a combination of glucose and NaCl [49]. The *rfaA* gene also involve in lipopolysaccharide biosynthesis. Biofilm formation by the *rfaA* mutant in meat and poultry broths and their attachment on surfaces of stainless steel and glass is significantly reduced [60]. Using transposon mutagenesis, the genes *metE*, *ompR*, *rpoS*, *rfaG*, *rfaJ*, *rfaK*, *rfaP*, *rfaH*, *rhlE*, *spiA*, and *steB* are found to be associated with biofilm formation of *Salmonella enteritidis* [66, 67]. When eight mutants with knockout of genes *ompR*, *rpoS*, *rfaG*, *rfaH*, *rhlE*, *metE*, *spiA*, or *steB* from the *Salmonella pullorum* are constructed. Only the

ompR mutant showed a complete loss of production of curli and biofilm formation. The other mutants showed a modified production of curli and cellulose with less effect related to biofilm formation [68]. Therefore, an integral LPS, at both the O-antigen and core polysaccharide levels, are important in the modulation of curli protein and cellulose production, as well as in biofilm formation.

5. Regulation mechanism of biofilm formation

Biofilm formation is majorly regulated by CsgD protein, a regulator belonging to the LuxR family [69]. CsgD has an N-terminal receiver domain with a conserved aspartate (D59) as a putative target site for phosphorylation and a C-terminal LuxR-like helix-turn-helix DNA binding motif. The unphosphorylated CsgD directly binds the *csgBA* and *adrA* promoter regions to activate transcription [70]. Multiple factors bind to the promoter sequence of *csgD* and regulate its transcription, such as OmpR, RpoS, RpoE, integration host factor (IHF), histone-like nucleoid structuring protein (H-NS), and MlrA. OmpR is one of first discovered to be required for *csgD* transcription [71]. Six binding sites (D1–D6) for OmpR are identified in *csgD* promoter regions. Binding of OmpR-P to D2 centered immediately upstream of D1 is proposed to repress promoter activity. IHF competes with OmpR-P for binding at its upstream site IHF1, which overlaps with D3–D6 and thereby activate the transcription of *csgD* [72]. The mutant of *ompR* in *Salmonella enteritidis* and *Salmonella pullorum* has inability to produce cellulose, curli, and biofilm [68, 73]. RpoS, encodes an alternative sigma factor of RNA polymerase, is critical for bacterial endurance under the most-stressful conditions, including stationary-phase entrance and host adaptation. RpoS is required for transcriptional activation of the *csgD* promoter in *Salmonella typhimurium* strains that rdar morphotype are normally expressed at low temperature [31]. However, in two *Salmonella typhimurium* strains, spontaneous mutants are found forming rdar colonies independent of temperature, the regulation of *csgD* is independent of *rpoS* [71]. Partially independent of *rpoS* for regulation of *csgD* is observed in *Salmonella enteritidis*. The *rpoS* mutant in *Salmonella pullorum* also shows similar biofilm forming ability as the wild-type strain [68], suggests that another sigma factor may recognize the *csgD* promoter. RpoE is an another regulator in the expression of thin aggregative fimbriae in *Salmonella* [74], since the *rpoE* deletion mutant shows significantly reduced amounts of *csgD* expression and modulated biofilm formation. Compared the expression of six different Sigma factors during biofilm formation in a *rpoS*-independent biofilm-formation strain, the expression of *rpoE* gene was the highest, and the *rpoE* mutant could not produce biofilm [75]. Therefore, RpoE acts as a regulator for *csgD* expression. IHF is a histone-like heterodimeric protein composed of two homologous subunits. IHF interacts with a define DNA sequence that has a supportive A-tract upstream of the consensus sequence by binding to the minor groove of the DNA. The *ihf* mutants show altered and reduced biofilm morphotypes on Congo Red agar plates [72]. H-NS prefers to bind AT-rich sites in the intergenic *csgBAC* and *csgDEFG* regions and causes moderate activation of *csgD* promoter. The inactivation of *hns* gene result in reduced expression of the rdar morphotype on agar plate [72]. MlrA (MerR-like regulator) acts directly or indirectly on the *csgD* promoter, the *mlrA* mu-

tants of *Salmonella typhimurium* no longer produce curli or rugose colony morphology. However, inactivation of *mlrA* did not affect curli production and aggregative morphology in an upregulated curli producing *Salmonella typhimurium* derivative containing a temperature- and RpoS-independent *csgD* promoter region. Therefore, MlrA acts as a positive regulator of RpoS-dependent curli and extracellular matrix production by *Salmonella typhimurium* [76].

c-di-GMP is recognized as a ubiquitous bacterial second messenger and a key regulator in bacterial transition from a motile and planktonic to a sessile and biofilm lifestyle. High intracellular c-di-GMP levels promote extracellular matrix production and subsequent biofilm formation and repress motility, whereas low intracellular c-di-GMP levels suppress matrix production and promote single-cell motility [77]. The synthesis/degradation of c-di-GMP depends on diguanylate cyclase/phosphodiesterase enzymatic activities. The cyclase activity, which converts two molecules of GTP to c-di-GMP, is encoded in the GGDEF protein domain, while phosphodiesterase activity, which hydrolyzes c-di-GMP to linear 5'-pGpG or two GMP molecules, is encoded in the EAL and HD-GYP domains. For example, Adar, containing a GGDEF domain, encodes diguanylate cyclase synthesizing c-di-GMP, is required for cellulose production and biofilm formation. In another seven GGDEF family (GcpA-G), only GcpA and GcpE are critical for biofilm formation [37]. The EAL domain protein STM4264, STM3611, and the GGDEF-EAL domain protein STM1703 play a determinative role in the expression level of multicellular behavior of *Salmonella typhimurium* [78, 79]. In contradiction, the EAL-like protein STM1697, neither degrade nor bind c-di-GMP, promotes biofilm formation and *CsgD* expression through interaction with proteins that regulate flagella function [80]. High intracellular amounts of c-di-GMP in *Salmonella typhimurium* inhibited invasion and abolished induction of a pro-inflammatory immune response in the colonic epithelial cell line HT-29. Inhibition of the invasion and IL-8 induction phenotype by c-di-GMP requires the major biofilm activator *CsgD* and/or *BcsA*. Therefore, c-di-GMP signaling is at least equally important in the regulation of *Salmonella*-host interaction as in the regulation of biofilm formation at ambient temperature [81].

CsgD synthesis is also regulated at the post-transcriptional level by sRNA. sRNAs have emerged as a diverse group of trans- or cis-encoded regulatory molecules of approximately 50–250 nt in size. The RNA chaperone Hfq protects sRNAs from degradation and facilitates their binding to the target mRNAs. All these sRNA may negatively regulate *csgD* gene expression by binding to the overlapping 5'-region of the transcript, masking the ribosome binding site, resulting in the inhibition of translation or the degradation of mRNA [82]. In *Escherichia coli*, sRNAs, OmrA/B, McaS, RprA, and GcvB are identified, which downregulate *CsgD* translation [83]. In *E. coli* and *Salmonella*, RydC's 5'-domain interacts with *csgD* mRNA translation initiation signals to prevent initiation, stimulation of RydC expression reduces biofilm formation by impairing curli synthesis [84]. Surprisingly, two Hfq-dependent sRNAs (ArcZ and SdsR) are responsible for positively regulation of *rdar* morphotype expression in *Salmonella typhimurium* [85]. *Salmonella* biofilm development depends on the phosphorylation status of RcsB. The unphosphorylated RcsB is essential to activate the expression of the biofilm matrix compounds. The inhibition of biofilm development by phosphorylated RcsB is

due to the repression of *CsgD* expression, through a mechanism dependent on the accumulation of the sRNA RprA [86].

Many gram-negative bacteria utilize N-acyl-L-homoserine lactones (AHLs) to bind to transcriptional regulators leading to activation or repression of target genes. *Salmonella* do not synthesize AHLs but do contain the AHL receptor, SdiA. The *Salmonella sdiA* gene regulates the *rck* gene, which mediates its adhesion and invasion of epithelial cells and the resistance of the organism to complement [87]. The *rck* gene is located on the virulence plasmid of pRST98, AHLs increase *rck* expression in pRST98-carrying strains, thereby enhancing bacterial adherence, serum resistance, and bacterial biofilm formation [88].

6. Virulence or resistance for biofilm

Biofilm formation may involve in the virulence of *Salmonella*. *Salmonella enteritidis* strains isolated from either the environment, dairy products, or infected patients are divided into two groups on the basis of their virulence (50% lethal dose) in chickens infected intraperitoneally. Only the virulent strains produce aggregates and formed visible filaments attached to the glass tube [47]. Further study confirms that the virulence of the biofilm-producing strain in infected chickens increases proportionally to the amount of stored glycogen, suggesting a possible role of the glycogen depot in the virulence of *Salmonella enteritidis* [89]. When tested for infection in Caco-2 cells and HEp-2 cells, the more virulent strains of *Salmonella enteritidis*, which are biofilm producers in adherence test medium, are able to disrupt monolayers. In contrast, the low-virulence strains of *Salmonella enteritidis*, which do not produce biofilms in adherence test medium, have no effect on the same cells. The high-virulence *Salmonella enteritidis* strains incubated under optimum biofilm-forming conditions may release a soluble factor, which enables the disruption of the integrity of Caco-2 monolayers [90]. The relationship between biofilm-forming ability and the pathogenicity is also evaluated in *Salmonella pullorum*. Although the virulence of *Salmonella pullorum* strains is independent of their ability of biofilm formation, prior growth as a biofilm for a biofilm producer of *Salmonella pullorum* leads to enhanced virulence in chickens, suggested that biofilm formation may be one of important virulence factor for *Salmonella pullorum* infection [46].

The *csgBAC* operon is required for curli biosynthesis in *Salmonella*. The *csgA* mutation is not reduced in ability to attach or colonize alfalfa sprouts, whereas the *csgB* mutation is reduced. Thus, *csgB* alone can play a role in attachment of *Salmonella* to plant tissue [91]. Competitive infection experiments in mice shows that *csgA* mutant cells outcompeted *rdar*-positive wild-type cells, indicating that aggregation via the *rdar* morphotype is not a virulence adaptation in *Salmonella typhimurium*. Furthermore, in vivo imaging experiments show that thin aggregative fimbriae genes are not expressed during infection but are expressed once *Salmonella* was passed out of the mice into the feces [92]. However, *Salmonella typhimurium* strains isolated from water buffalo calves affected by lethal gastroenteritis are tested in vivo in a mouse model of mixed infection. The most pathogenic strain is characterized by a high number of virulence factors and the presence of the locus *csgA*, coding for a thin aggregative fimbria [93].

The *bcsABZC* and *bcsEFG* operons are required for cellulose biosynthesis in *Salmonella*. Bacterial adherence and invasion assays of eukaryotic cells and in vivo virulence studies of cellulose-deficient mutants of *bcsC* and *bcsE* genes indicate that the production of cellulose is not involved in the virulence of *Salmonella enteritidis*. However, cellulose-deficient mutants are more sensitive to chlorine treatments, suggesting that cellulose production and biofilm formation may be an important factor for the survival of *S. enteritidis* on surface environments [36]. *Salmonella typhimurium* makes cellulose when inside macrophages. An attenuated mutant lacking the *mgtC* gene exhibits increased cellulose levels due to increased expression of the cellulose synthase gene *bcsA* and of cyclic diguanylate, the allosteric activator of the BcsA protein. Inactivation of *bcsA* restore wild-type virulence to the *Salmonella mgtC* mutant, indicating that *Salmonella* promotes virulence by repressing cellulose production [94].

BapA, a large cell-surface protein, is required for biofilm formation by *Salmonella*. Studies on the contribution of BapA to *Salmonella enteritidis* pathogenesis reveal that orally inoculated animals with a *bapA*-deficient strain survived longer than those inoculated with the wild-type strain. Also, a *bapA* mutant strain showed a significantly lower colonization rate at the intestinal cell barrier and consequently a decreased efficiency for organ invasion compared with the wild-type strain [26]. Osmoregulated periplasmic glucans (OPGs) are major periplasmic constituents of Gram-negative bacteria. An *opgGH* mutant strain in *Salmonella typhimurium*, which is defective in OPG biosynthesis, severely impairs biofilm formation. The *opgGH* mutant strain poorly colonizes mouse organs when introduced orally along with the wild-type strain [95].

Besides, the constitutional components of biofilm, there are many regulation proteins involved in both biofilm formation and virulence. An *ompR* mutant of *Salmonella enteritidis* has no ability to produce cellulose, curli, and biofilm and shows similar adherence percentage to and invasion percentage of epithelial cells as wild-type strain. Intraperitoneal challenge of bacteria in BALB/c mice reveals that the *ompR* mutant strain is significantly attenuated [73]. A *spiA* gene mutant shows reduced biofilm formation and significantly decreased curli production, and reduced intracellular proliferation of macrophages during the biofilm phase. In addition, the *spiA* mutant was attenuated in a mouse model in both the exponential growth and biofilm phases [67]. Deletion of genes *ompR* and *spiA* in *Salmonella pullorum* strains contribute to attenuation of virulence in 1-day-old chickens [68]. DksA is a conserved gram-negative regulator that binds directly to the RNA polymerase secondary channel. In *Salmonella typhimurium*, expression of the *dksA* gene is induced during the logarithmic phase and DksA plays an important role in motility and biofilm formation. DksA positively regulates the *Salmonella* pathogenicity island 1 and motility-chemotaxis genes and is necessary for *Salmonella typhimurium* invasion of human epithelial cells and uptake by macrophages. The *dksA* gene is induced at the midcecum during the early stage of the infection and required for gastrointestinal colonization and systemic infection in a colitis mouse model [96].

Salmonella in biofilm is resistant to antibiotic. One of key mechanisms of antibiotic resistance is efflux. There are five families of multidrug resistance (MDR) efflux pumps, in which the AcrAB–TolC efflux system is the best characterized MDR system. Ten mutants of *Salmonella typhimurium* lacking MDR efflux systems, such as *tolC*, *acrB*, *acrD*, *acrEF*, *mdtABC*, *mdsABC*,

emrAB, *mdfA*, *mdtK*, and *macAB* are compromised in their ability to form biofilms. The mutants expressed significantly less *csgB* or *csgD* than wild type, indicating that loss of all multidrug resistance efflux pumps of *Salmonella typhimurium* results in impaired ability to form a biofilm [97]. Further study confirms that mutants of *Salmonella typhimurium* that lack TolC or AcrB, but surprisingly not AcrA, are compromised in their ability to form biofilms. The biofilm defect results from transcriptional repression of curli biosynthesis genes and consequent inhibition of production of curli. Therefore, the inhibition of efflux is a promising antibiofilm strategy [98]. However, recent studies offer contradictory findings about the role of multi-drug efflux pumps in bacterial biofilm development. When no selective pressure is applied, *Salmonella typhimurium* is able to produce biofilms even when the AcrAB efflux pumps are inactivated. Upon exposure to chloramphenicol, the formation of biofilms on solid surfaces as well as the production of curli are either reduced or delayed more significantly in both AcrA and AcrAB mutants, implying that the use of efflux pump inhibitors to prevent biofilm formation is not a general solution and that combined treatments might be more efficient [99]. Triclosan is a potent biocide that is included in a diverse range of products. *Salmonella* biofilm-derived cells are more resistant to Triclosan. Within biofilms, triclosan upregulate the transcription of *acrAB*, *marA*, *bcsA*, and *bcsE* genes. Thus, *Salmonella* within biofilms could experience reduced influx, increased efflux and enhanced exopolysaccharides production. The data suggest that tolerance of *Salmonella* towards triclosan in the biofilm is attributed to low diffusion through the extracellular matrix, while changes of gene expression might provide further resistance to triclosan and to other antimicrobials [100].

In summary, *Salmonella* biofilm formation is major controlled by CsgD regulatory network and regulated by multiple transcriptional factors, c-di-GMP, and sRNAs. More and more genes are found to be associated with both biofilm formation and virulence. Dissection of their function and relationship will helpful for development of new tools and strategies to prevent biofilm-related disease and decontaminate biofilm-derived *Salmonella* in food production.

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