

## REVIEW

# CsgD regulatory network in a bacterial trait-altering biofilm formation

Zhen Liu, Hua Niu, Shuyan Wu and Rui Huang

In response to the limited nutrients and stressful conditions of their habitats, many microorganisms including *Salmonella* form a biofilm by secreting a polymeric matrix to interweave individual cells and to build structural communities on an abiotic or living surface. The biofilm formation in *Salmonella* is tightly regulated by a regulatory network that involves multiple transcriptional regulators. As a master transcriptional regulator in biofilm formation, curli subunit gene D (*csgD*) functions by activating the biosynthesis of the extracellular polymeric matrix composed of exopolysaccharide cellulose, curli and biofilm-associated proteins (Baps), assisting bacterial cells in transitioning from the planktonic stage to the multicellular state. The expression of CsgD itself is affected by cell growth stage and environmental stimuli through the action of other transcriptional factors, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), regulatory small RNAs (sRNAs) and other elements. The formation of biofilm confers new physiological characteristics on the bacteria within, especially resistance against unfavorable environmental conditions. Herein, we summarize the CsgD regulatory network of *Salmonella* biofilm formation and the new traits acquired by *Salmonella* when within biofilm.

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## INTRODUCTION

*Salmonella enterica* is an intracellular facultative gram-negative pathogen that infects many hosts including humans and causes diseases ranging in severity from gastroenteritis and diarrhea to life-threatening systemic syndrome through food poisoning. When the nutrient supply is abundant and environment conditions are optimal, *Salmonella* reproduces exponentially and moves freely. As nutrients are consumed or when harsh conditions develop, *Salmonella* grows more slowly and secretes extracellular polymers to encase itself in an interwoven structure called biofilm. Biofilm formation is important for the spread of *Salmonella* because this biofilm is resistant to disinfectants, environmental stresses, antibiotics and the host immune system, consequently promoting bacterial dispersal and survival and enhancing its virulence.<sup>1-3</sup> Thus, biofilm formation is associated with the outbreak of salmonellosis and persistent infections in patients.<sup>4-7</sup> Although a fitness advantage is provided by biofilm for bacterial survival, biofilm formation is an energy-consuming process. Therefore, biofilm formation is tightly regulated in response to environmental changes. Previous studies identified a variety of environmental factors affecting *Salmonella* biofilm formation such as temperature, pH value and osmolarity.<sup>8</sup> Recent studies revealed underlying molecular mechanisms that link biofilm formation with environmental stresses by showing that multiple transcriptional regulators including sigma factor RpoS and response regulators of two-component systems, bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) and regulatory small RNAs (sRNAs) relay signals from the extracellular environment to a master regulator of biofilm formation, curli subunit gene D (*csgD*).<sup>9</sup> CsgD integrates these signals and synchronizes the synthesis of several important constituents of the extracellular

polymeric matrix to form biofilm.<sup>10</sup> Once *Salmonella* biofilm is formed, new traits emerge in the bacteria within these multicellular aggregates such as increased resistance to unfavorable conditions, enhanced virulence and altered metabolism.

## CsgD REGULATES *Salmonella* BIOFILM FORMATION

The main constituents of the extracellular matrix of *Salmonella* biofilm are cellulose, curli fimbriae, biofilm-associated protein (Bap),<sup>10</sup> O-antigen-capsule (O-Ag-capsule), lipopolysaccharides and extracellular DNA. Cellulose, a  $\beta$ -1-4-D-glucose polymer, is an important biofilm-associated extracellular polymeric substance (EPS) that interweaves with other extracellular components to form three-dimensional structures and to promote cell-cell interactions and protect cells from unfavorable environments. Its synthesis requires genes encoded in the *bcsABZC-bcsEFG* operons. Curli fimbria, an amyloid-like proteinaceous substance, is another major component of the extracellular matrix in biofilm and is encoded by the *csgBAC-csgDEFG* operons.<sup>11</sup> BapA is a large proline-threonine-rich protein that is essential for bacterial aggregation and subsequent pellicle formation at the air/liquid interface in Luria-Bertani medium.<sup>12</sup> BapA is encoded by *bapA* and is secreted to the cell surface by a genetically linked BapBCD type I secretion system. The O-Ag-capsule is crucial for *S. Typhimurium* and *S. Typhi* gallstone biofilm formation.<sup>13</sup> The *yihU-yshA* and *yihVW* operons are responsible for O-Ag-capsule assembly and translocation.

With the decline of nutrient availability, *Salmonella* cells begin their transition from an exponential growth phase to a stationary phase and begin to form biofilm. The synthesis of the extracellular matrix components of *Salmonella* biofilm is regulated by a highly complex

regulatory network that has many components including sigma factors, transcriptional factors, several nucleotide messengers and sRNAs. As a master transcriptional regulator, CsgD is major control and integration unit for *Salmonella* biofilm formation due to its regulation of the expression of *Salmonella* biofilm-associated extracellular matrix substances including curli and cellulose.<sup>14</sup> Curli production is directly activated by the binding of the transcription factor CsgD to the promoter of the *csgBAC* operon,<sup>15</sup> which encodes the major curli subunit CsgA as well as the nucleator protein CsgB. Recently, Zakikhany *et al.*<sup>16</sup> revealed the mechanism in *Escherichia coli* by which unphosphorylated CsgD directly binds to an 11 bp variant and thus activates *csgBAC* transcription. CsgD also regulates cellulose synthesis at a post-transcriptional level by controlling the activity of several cellulose synthases including BcsB through the synthesis of c-di-GMP by AdrA. Bearing a GGDEF domain, AdrA synthesizes c-di-GMP as diguanylate cyclase, and its transcription is under the control of CsgD. The c-di-GMP binds to the PilZ domain in cellulose synthases and increases their enzyme activity via the allosteric change of the protein conformation.<sup>17</sup>

CsgD is considered to be a regulator for BapA expression in *Salmonella* because a special binding motif, which is very similar to the CsgD-binding sequence in the *adrA* promoter, is present in the *bapA* promoter. Thus, CsgD may influence *bapA* transcription or expression.<sup>10,18</sup> However, in one controversial report, Zakikhany *et al.* did not find any evidence that CsgD regulates *bapA* transcription or expression using a combined bioinformatics and global transcription approach.<sup>16</sup>

As a component of EPS in *S. enteritidis* biofilm, the O-Ag-capsule is assembled and translocated by proteins encoded by the divergently oriented operons *yihU-yshA* and *yihVW*. A gene expression assay revealed that CsgD regulates the *yih* operons, although the detailed mechanism of this is unknown.<sup>19</sup>

Although CsgD is essential for biofilm formation, an individual bacterium may not express CsgD at levels similar to that in the multicellular community. Grantcharova *et al.*<sup>20</sup> identified the bistable characteristic of CsgD expression: a subpopulation of bacteria in biofilm has a high level of CsgD expression, whereas in another subpopulation of bacteria, the expression of CsgD is low. The bacteria with upregulated CsgD expression are responsible for generating the extracellular matrix and for forming the biofilm structure that encases the low CsgD-expressing bacteria. Because bistable CsgD expression saves energy in biofilm production, it may confer additional advantages to the entire bacterial community while maintaining the developmental potential of the population.

## THE INTERACTION BETWEEN CsgD AND OTHER REGULATORS IN BIOFILM FORMATION

It has long been known that environmental factors (temperature, nutrients and starvation, ethanol, oxygen tension, osmolarity, iron and pH) have important effects on biofilm EPS production and their composition. These environmental factors affect *Salmonella* biofilm formation by acting on CsgD expression through RNA polymerase sigma factor RpoS, multiple transcriptional factors and small nucleotides.<sup>21</sup> The intergenic region between *csgBAC* and *csgDEFG* operons is 582 bp, providing the binding sites with multiple transcriptional factors and making it a hub for integrating different environmental signals.<sup>9</sup> When *Salmonella* cells enter the stationary growth phase, sigma factor RpoS becomes dominant in competition with sigma factors. RpoS joins RNA polymerase to form holoenzyme, binding to the promoter sequences of a set of stationary phase and stress-induced

genes and initiating mRNA transcription, which is assisted by other transcriptional regulators and accessory proteins.<sup>22</sup> RpoS-containing RNA polymerase has a low activity until it binds to accessory protein Crl, which also accumulates in the stationary phase. Crl is more stable at low temperatures (28 °C) than at high temperatures (37 °C). Biofilm forms at 28 °C, indicating that Crl serves as a temperature sensor in biofilm formation.<sup>23</sup>

Multiple transcriptional factors bind to the promoter sequence of *csgD* and regulate its transcription, including OmpR, integrating host factor (IHF), the histone-like nucleoid-structuring protein and the MerR-like regulator (MlrA). OmpR, a response regulator in the EnvZ/OmpR two-component regulatory system, is phosphorylated in response to environmental stimuli such as osmolarity and pH and binds to the *csgD* promoter to enhance its expression. OmpR-binding sites in the *csgD* promoter were identified using a combination of genetic studies and *in vitro* experimentation.<sup>24</sup> Similar to the response to osmolarity, EnvZ/OmpR is involved in the ethanol-inducing upregulation of *csgD*.<sup>2</sup> IHF is a key architectural protein involved in a variety of cellular processes and binds to consensus sequences in the *csgD* promoter to facilitate the activation or repression of *csgD* transcription.<sup>25</sup> Mutants in IHF subunits showed altered and reduced biofilm morphotypes on Congo Red agar plates. Because the same phenotypes were observed at different temperatures, IHF appears to be uninvolved in temperature-dependent biofilm morphotype regulation. Histone-like nucleoid-structuring protein is a DNA structural protein that plays a significant role in integrating a wide range of environmental stimuli such as osmolarity and temperature.<sup>26</sup> Histone-like nucleoid-structuring protein was demonstrated to bind AT-rich sites in the intergenic *csgBAC-csgDEFG* region and has indirect effects on *csgD* expression.<sup>9</sup> MlrA, the expression of which is regulated by RpoS, binds upstream of the *csgD* promoter and induces the transcription of the *csgD* gene. MlrA specifically acts on the transcription of operon *csgDEFG* primarily in response to metal ions.<sup>27</sup> Recent evidence in *E. coli* found that MlrA directly binds to a 33 bp palindromic sequence between IHF- and OmpR-binding sites in the upstream sequence of the *csgD* promoter.<sup>28</sup> These three positive regulators of *csgD* expression (IHF, OmpR and MlrA) are found to bind to the same site of *csgD* promoter and function independently without strong cooperation.<sup>28</sup>

c-di-GMP is a newly identified message molecule that naturally occurs in bacteria and is responsible for regulating many biological functions including biofilm formation, motility, virulence, the cell cycle, differentiation and other processes.<sup>29</sup> Due to the importance and prevalence of c-di-GMP, the interactions between the second message and biofilm have drawn significant attention. It was demonstrated that increased c-di-GMP enhances *csgD* expression. We have previously mentioned that AdrA, the expression of which is promoted by CsgD, was the first identified diguanylate cyclase involving in the production of c-di-GMP. The produced c-di-GMP also provides positive feedback for *csgD* expression in some strains. However, phosphodiesterase degrades c-di-GMP, thereby inhibiting *csgD* expression.

sRNAs are involved in the regulation of biofilm formation.<sup>30</sup> Most sRNAs are 80–200 nt long and directly bind to their target mRNAs in the 5'-UTR or in the open reading frame to execute their regulation activity by changing the exposure of the SD sequence of mRNAs to ribosomes or altering the stability of mRNAs.<sup>31</sup> Chaperone protein Hfq protects sRNAs from degradation and facilitates their binding to the target mRNAs. Recently, Hfq-dependent sRNAs (ArcZ and SdsR) were found to positively affect *csgD* expression and biofilm formation.<sup>32</sup> The mRNA of *csgD* forms a relatively long and stable

stem-loop structure in its 5'-UTR. In *E. coli*, *csgD* mRNA is the target of McaS, RprA or OmrA/OmrB sRNAs. McaS sRNA binds *csgD* mRNA, which results in *csgD* mRNA degradation. RprA and OmrA/OmrB sRNAs interfere with *csgD* mRNA translation.<sup>33</sup>

Lytic transglycosylases were found to have effects on the production and regulation of *Salmonella* biofilm through the *csgD* pathway. Lytic transglycosylases are a group of ubiquitous bacterial enzymes involved in the turnover of the bacterial cell wall constituent peptidoglycan. Among these, two lytic transglycosylases (MltE and MltC) are able to uniquely affect biofilm production by mediating *csgD* expression.<sup>34</sup> The mRNA level of *csgD* in *Salmonella*  $\Delta$ mltE and  $\Delta$ mltC double mutant was found to be approximately 47% of that of the wild type, indicating its role in the regulation of *csgD* expression. Although RpoS and OmpR were indicated to mediate several factors to affect *Salmonella* biofilm production,<sup>15</sup> RpoS and OmpR were not found to participate in the process of MltE- and MltC-mediated regulation of *csgD* expression. However, a high concentration of the second messenger c-di-GMP inside the cells can reverse the change of adar (for red, dry and rough) morphotypes and *csgD* production in the  $\Delta$ mltE and  $\Delta$ mltC double mutant, suggesting that c-di-GMP signaling acts downstream of MltE and MltC.

Taken together, CsgD is a major biofilm formation regulator that controls the production of the extracellular matrix of biofilm. Meanwhile, environment factors regulate *csgD* expression through the action of sigma factors, transcription factors, c-di-GMP and sRNAs, thereby determining biofilm formation (Figure 1).

## BIOFILM FORMATION REGULATED BY CsgD COINCIDES WITH A METABOLIC CHANGE

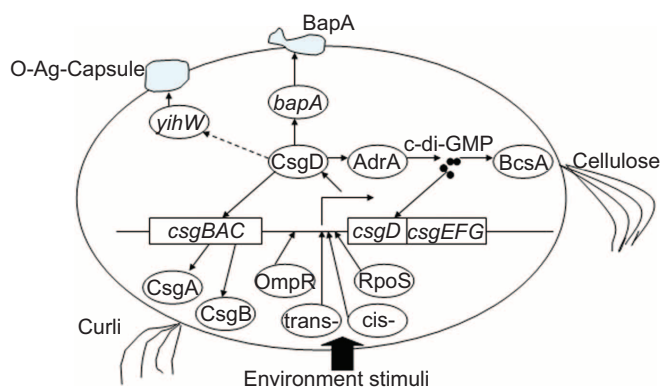
A number of studies demonstrated that the switch from the planktonic state to the biofilm mode in bacteria can cause a global shift in metabolism.<sup>15,35</sup> White *et al.*<sup>36</sup> used metabolomics to compare *Salmonella* cells in rdar colony biofilms to isogenic *csgD* deletion mutants and found that there are 25 detected metabolites at significantly different concentrations between the two populations. In wild-type colonies, many end products and important intermediates of gluconeogenesis are detected at higher levels than in colonies formed by *csgD* deletion mutants. Three of the major osmoprotectants used by *S. Typhimurium* trehalose, as well as glycine betaine (betaine) and glutamate, were found at higher concentrations in wild-type colonies. Other metabolites more abundant in wild-type colonies included nicotinamide adenine dinucleotide, octanoic acid, pyroglutamate and glutathione. However, the upper tricarboxylic acid cycle (cycle) intermediates, adenosine monophosphate and polyamine compounds

were detected in *csgD* deletion mutant colonies. Thus, the hypothesis that the accumulation of tricarboxylic acid cycle intermediates and polyamine compounds would block the gluconeogenesis in *csgD* mutant cells is reasonable. Adenylate kinase catalyzes the reaction of ATP+adenosine monophosphate into two ADP molecules and is known to buffer ATP levels during periods of rapid ATP consumption.<sup>37</sup> Adenylate kinase expression was upregulated in wild-type cultures compared with *csgD* mutant cultures, suggesting that cells living in biofilm have increased ATP requirements. These results could explain the increased adenosine monophosphate levels detected in *csgD* mutant colonies.<sup>35</sup> *speA*, *speB*, *speC* and *cadBA*, the main genes encoding polyamine biosynthesis enzymes, had similar magnitudes of expression in wild-type and *csgD* mutant cells. These results suggest that the accumulated polyamines in *csgD* mutant cells are caused by the reduced carbon flux of gluconeogenesis and other biosynthetic pathways.

## CsgD REGULATING BIOFILM RESISTANCE TO STRESS FACTORS

*Salmonella* is profoundly adapted to live in non-host environments, with biofilm formation acting as a primary contributor. *Salmonella* biofilm has been shown to confer resistance to desiccation, disinfectants and antibiotics and has drawn significant attention in the food and medical fields. White *et al.*<sup>38</sup> demonstrated that *S. Typhimurium* cells in biofilm formed in ATM have a survival rate of 10% after 9 months of storage and 68% after 3 months of storage. Several studies have shown that *Salmonella* biofilms on the surface of various materials are significantly more resistant to the sanitizers chlorine, hypochlorite, iodine, glutaraldehyde and cationic tensides.<sup>39</sup> Further studies showed that curli pili, cellulose and other CsgD-regulated compounds play a key role in enhanced the long-term survival and persistence of *Salmonella* in biofilm under stressful conditions. After the knockout of the *csgD*, *csgA* and *bcsA* genes, the biofilm's resistance to stress factors was significantly reduced.<sup>38</sup> Similar to the *csgD* mutant, O-Ag-capsule mutants (*yihP*, *yihQ* and *yihO*) have reduced survival ability against desiccation stress, demonstrating the role of the O-Ag-capsule in desiccation tolerance.<sup>19</sup>

Doctors usually treat bacterial infections with antibiotics that have been validated via drug susceptibility tests. However, the antibiotic sensitivity of bacteria in the planktonic state is different from that in a biofilm state, which can also cause infections in patients. This may explain why the therapeutic effect *in vivo* is inconsistent with drug susceptibility tests *in vitro*. Studies were performed to compare the effects of several antibiotics (enrofloxacin, gentamicin, erythromycin, tilmicosin, ampicillin, oxytetracycline and trimethoprim-sulfadoxine) on planktonic cells and the established biofilm of clinical *Salmonella Typhimurium* and *Salmonella Bredeney* isolates on polystyrene plates.<sup>40</sup> With the exceptions of erythromycin and tilmicosin, planktonic cells were found to be sensitive to all antibiotics, whereas *Salmonella* in biofilm was only sensitive to enrofloxacin and ampicillin. Furthermore, Tabak *et al.*<sup>41</sup> discovered that *Salmonella* biofilms are up to 2000-fold more resistant to ciprofloxacin compared with planktonic cells. This is of great significance as ciprofloxacin together with a third-generation cephalosporin is commonly used to treat nontyphoid *Salmonella* infections. Recently, Papavasileiou *et al.*<sup>43</sup> investigated 194 strains of *Salmonella enterica* isolated from patients for their ability to form biofilm on silicon disks and compared the biofilm status of strains with their corresponding planktonic form regarding their susceptibility to nine antibiotic types. The cells in biofilm showed increased antimicrobial resistance to all antimicrobial agents



**Figure 1** Regulation of *Salmonella* biofilm formation by CsgD.



compared with the planktonic bacteria, with the highest resistance rates for gentamicin (90%) and ampicillin (84%).

At present, research into bacterial biofilm resistance mechanisms is very active. There are several types of resistance mechanisms: (i) bacteria in a biofilm state have lowered metabolic and growth rates, especially those deep within the biofilm, which decreases their sensitivity to drugs;<sup>44</sup> (ii) EPS acting as an adsorbent prevents antibiotic contact with bacteria;<sup>45</sup> (iii) the physiological characteristics of biofilm-state bacteria are different from those in a planktonic state;<sup>46</sup> and (iv) biofilm bacteria often express special protective factors such as multidrug efflux pumps and stress response regulon.<sup>47</sup> Additionally, c-di-GMP may participate in antibiotic resistance in bacteria within biofilm. Chua *et al.*<sup>48</sup> found that *P. aeruginosa* cells with a low level of c-di-GMP are more resistant to colistin than cells with high c-di-GMP. This finding contradicts the current view that dispersed cells are more susceptible to antibiotics than their sessile counterparts.

### CsgD MEDIATING BIOFILM FORMATION INFLUENCES BACTERIAL INVASION AND VIRULENCE

Crull *et al.*<sup>49</sup> created a mouse tumor model by inoculating *Salmonella* through the tail vein after injecting CT26 cells into the mouse body. The surface of the tumor was covered by *Salmonella Typhimurium* biofilm, but the bacteria were not able to invade into the tumor cells, which contradicts the *in vitro* observation that *Salmonella Typhimurium* can invade CT26 cells. To further investigate this finding, the researchers used the *Salmonella csgD* knockout mutant to repeat the experiments in a mouse tumor model and found that the mutant was detected intracellularly. *csgD* is a major control and integration unit for *Salmonella* biofilm formation and regulates the expression of some important *Salmonella* biofilm-associated matrix compounds. A *csgD* mutant lacked any multicellular behavior, as visualized via a morphotype on Congo red agar plates.<sup>50</sup> These tests confirmed the important role of *csgD* in the process of bacteria invasion *in vivo*, but the detailed mechanism remains to be explored.

Further studies indicate that c-di-GMP can control the bacterial invasion ability through *csgD*.<sup>51</sup> Intracellular c-di-GMP was regulated by a series of proteases containing GGDEF or EAL domains, which involve the catalytic activity of guanylate cyclase and phosphodiesterase, respectively. To date, more than 20 protease types with diguanylate cyclase or phosphodiesterase activity have been discovered in *Salmonella*. Using gene mutant and gene overexpression methods, Ahmad *et al.*<sup>52</sup> found that STM3611 and STM4264 proteins significantly promote the activity of *Salmonella* in invading human colon cancer cells (HT-29). Both proteins contain EAL domain and exert phosphodiesterase catalytic activity to degrade the c-di-GMP. To confirm the involvement of *csgD* in the process of the high level of intracellular c-di-GMP inhibiting bacterial invasion, the Ahmad team performed further studies on *csgD* and *bcsA*. The STM4264 mutant of *Salmonella Typhimurium* had reduced invasion due to the high level of c-di-GMP. However, the STM4264 mutant with knocked-out *csgD* or *bcsA* restored the ability to invade HT-29. CsgD and BcsA appear to play a role downstream of STM4264. This finding suggests that the c-di-GMP degraded by STM4264 restrains the invasive ability of *S. Typhimurium* through CsgD and BcsA. In the background of the STM3611 mutant, knocking out *csgD* or *bcsA* has little effect on *Salmonella* invasion.<sup>49</sup> Other genes may be involved in the pathway of this mutant. *csgD* expression inhibits the functions of effector proteins SopE2 and SipA of *Salmonella* T3SS-1 (type III secretion system-1).<sup>30</sup> In the STM3611 mutant or STM4264 mutant, the production of effector protein SipA was increased when the gene *csgD* was knocked

out. The increased SipA in *Salmonella* with *csgD* and the STM3611 double mutant obviously failed to reverse the invasive ability of *Salmonella*.

Biofilm formation has been considered to be related with chronic infections. It has been found that the expression of biofilm-related genes is apparently able to increase the expression of bacterial virulence genes in *E. coli*.<sup>53</sup> In some bacteria, the expression of some virulence genes under biofilm conditions is higher than that in a planktonic state.<sup>34</sup> In *Salmonella*, there are many essential virulence genes such as the *sipA* gene in *S. Typhimurium*, which encodes an outer-membrane protein of the *Salmonella* pathogenicity island-2 type III secretion system essential for host infection.<sup>54</sup> It has been demonstrated that knocking out the *sipA* gene can impair the ability of bacteria to form biofilm, which indicates that virulence is closely related with the ability of bacteria to form biofilm.<sup>55</sup> A further study found that in biofilm conditions, both wild-type *S. Typhimurium* and the *sipA* mutant *S. Typhimurium* are more adhesive than in planktonic conditions *in vitro*; moreover, in a biofilm state, *Salmonella* has more proliferative capacity in macrophages than in a planktonic state.<sup>55</sup> The strong evidence presented above shows that biofilm formation promotes bacterial virulence, but the mechanism of the interaction between virulence and the formation of biofilm remains to be elucidated. The studies on biofilm affecting virulence primarily focus on the molecular level and have been performed *in vitro*; this phenomenon should be further investigated *in vivo*. The *csgD* gene could become a novel target for the future control of *Salmonella* infection.

Biofilm formation is an efficient mechanism that bacteria utilize to evade recognition and elimination by the host immune system. Biofilm may play a key role in bacteria adaptation to adverse environments in a way that makes them both more persistent and less invasive.<sup>56,57</sup> Biofilm increases the resistance of bacteria and makes them less conspicuous to the immune system due to hidden antigens and repressed key ligands.<sup>58</sup> Additionally, it has been reported that bacterial cells isolated from chronically infected patients lack flagella, pili and motility. In *in vitro* experiments, *P. aeruginosa* living in mature biofilm lost flagella, pili and motility.<sup>59,60</sup> These adaptations contribute to the change of virulence of bacteria living in biofilm.

### CONCLUSION

Taken together, biofilm formation in *Salmonella* is controlled by a regulatory network composed of multiple transcriptional factors, secondary messengers, and sRNAs. The formation of biofilm offers a fitness advantage to *Salmonella* and the ability to survive in unfavorable conditions. Biofilm formation is related with *Salmonella* spread and its pathogenesis. Thus, further studies are needed to clearly understand the relationship between biofilm and microbial physiological functions in *Salmonella* in an effort to develop new tools and strategies to prevent and treat biofilm-related diseases.

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