The Role of csgA and bcsA Genes on Biofilm Formation and Virulence in *Salmonella enterica* Serovar Typhimurium

Muhanad El Hag¹,²,³,⁴,⁵, Zheng Feng¹,²,³, Yangyang Su¹,²,³, Tao Qin¹,²,³, Sujuan Chen¹,²,³, Daxin Peng¹,²,³,⁴ * and Xiufan Liu¹,²,³

¹College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu, PR China; ²Jiangsu Co-Innovation Center for the Prevention and Control of Important Animal Infectious Disease and Zoonoses, Yangzhou, Jiangsu, PR China; ³Jiangsu Research Centre of Engineering and Technology for Prevention and Control of Poultry Disease; Yangzhou, Jiangsu, 225009, P.R. China; ⁴Joint International Research Laboratory of Agriculture and Agri-Product Safety of Ministry of Education, Yangzhou, Jiangsu, 225009, P.R. China; ⁵Faculty of Public and Environmental Health, University of Khartoum, Sudan

*Corresponding author: pengdx@yzu.edu.cn

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csgA  
*Salmonella Typhimurium*  
Virulence

**ABSTRACT**

Biofilm formation can increase bacterial resistance to adverse conditions. However, limited information is available regarding the roles of the csgA and bcsA genes involved in biofilm formation and virulence for *Salmonella enterica* serovar Typhimurium. Here we deleted the csgA and bcsA genes in *S. Typhimurium* strains S016 and S025 and assessed several aspects of biofilm formation and virulence. The ΔcsgA strains did not produce curli fimbriae and ΔbcsA mutants had decreased cellulose production. The ΔcsgA strains were unable to form biofilms. The ΔcsgA strains also showed decreased adhesion and invasion to HeLa cells and reduced intracellular proliferation in HD11 macrophages. The ΔbcsA mutants had similar adhesion, invasion, and proliferation as compared to the wild-type strains. The ΔcsgA strains were significantly attenuated in the virulence in assays involving oral challenge of one-day-old chickens. These findings clarify the respective roles of csgA and bcsA in biofilm formation and pathogenicity of *S. Typhimurium*.


**INTRODUCTION**

*Salmonella enterica* serovar *Typhimurium* (*S. Typhimurium*) is a common zoonotic bacterial pathogen. *Salmonella* infections can occur through direct contact with infected animals, through indirect contact with animal environments, or through consumption of food or liquids prepared in contaminated environments (Anderson et al., 2016; Bloomfield et al., 2017).

*S. Typhimurium* causes a significant disease burden in human in China, A laboratory-based surveillance of *Salmonella* infections showed that the most frequent serotype isolated from patients with diarrhea were *S. Typhimurium* (n=352, 45%), which also have high multidrug resistance (Deng et al., 2012). Another study confirmed that *Salmonella Typhimurium* (n=523, 29.65%) was one of the most common serovars causing infant salmonellosis (Ke et al., 2014).

*Salmonella* infections also cause systemic disease in young chickens, resulting in growth retardation, blindness, twisted necks, and lameness, with some mortality, especially in chicks less than 2 weeks of age (El-Sharkawy et al., 2017). The prevalence of *Salmonella* in the poultry in Henan, China was found to be high and represented by a different serotypes such as *S. Typhimurium, S. Enteritidis, S. Hadar* and *S. Indiana* (Bai et al., 2015).

Bacterial biofilms are complex communities composed of microorganisms embedded in a self-produced extracellular matrix. Microbes grow on either biotic or abiotic surfaces, attaching to the surface, and confer resistance to both immunity-related as well as antimicrobial agents (Römling, 2005). *S. Typhimurium* biofilm formation (BF) is described as a red-dry and rough (rdar) morphotype when grown on adverse condition (Zogaj et al., 2003; Römling, 2005).
The regulation of BF in *Salmonella* relies on the biofilm master gene regulator protein CsgD. This protein activates the production of curli by transcriptional activation of the csgBAC operon that encodes the structural genes of curli (Römling et al., 1998; Gerstel and Römling, 2003). AdrA regulates cellulose by activating the basABZC-bscSEFG operons (Zogaj et al., 2001; Solano et al., 2002). We previously studied the function of CsgA and BcsA in *Salmonella Pullorum* BF (El Hag et al., 2017). However, limited information is available regarding the roles of the csgA and bcsA genes in BF and virulence for *S. Typhimurium*. Here we constructed csgA and bcsA deletion mutants in *S. Typhimurium*, and evaluated their ability to forms biofilms and infect chickens.

**MATERIALS AND METHODS**

The bacterial strains, plasmids and primers used in this study were similar as previously described (El Hag et al., 2017). Strains were routinely cultured in Luria-Bertani (LB; Difco, Sparks, MD, USA) broth and LB agar medium with antibiotic supplementation as needed. Tryptic soy broth (TSB; Difco) diluted 1:10 (1/10 TSB) was used as for biofilm assays. The csgA and bcsA genes of *S. Typhimurium* strains S016 and S025 were deleted by using lambda red-mediated mutagenesis (Dat senko and Wanner, 2000). PCR products were confirmed using DNA sequencing (Huada Gene Sequencing Company, Shanghai, China). Complementation strains were constructed by expressing each gene from the pEASY plasmid using the TA cloning vector One Step Cloning Kit (Promega Biotech Co., Ltd, Beijing, China). Growth assays were performed as described previously (Lu et al., 2012).

**Biofilm assays:** BF was measured in 1/10 TSB as previously described (Kim et al., 2007; Crawford et al., 2008). Overnight broth cultures of each strain were diluted 1:100 in 1/10 TSB and 100 µl of each bacterial suspension was added to a 96-well U-bottomed polystyrene microtiter-plate (Corning, New York, NY, USA). The Optical Density (OD$_{595}$) nm was measured using a micro-plate reader (BioTek, Winooski, VT, USA).

To assay BF on glass tube surfaces, 2 ml of overnight cultures of each strain diluted 1:100 in 1/10 TSB were added to borosilicate glass test tubes and incubated at 28°C for 48h as similar as mentioned previously (Kim et al., 2007; Crawford et al., 2008). To assay for curli production, 10 µl of overnight broth cultures were added to LB plates lacking NaCl and supplemented with 40 µg/ml Congo red (Sigma, St. Louis, MO, USA) and 20 µg/ml brilliant blue (Sigma). After incubation at 28°C for 4 days, colony morphologies were assessed (Anriany et al., 2006). Cellulose production was monitored by supplementing LB agar plates with 200 µg/ml calcofluor (Sigma) as a fluorescent brightener, and then cellulose production was detected by assaying for fluorescence under ultraviolet light (Anriany et al., 2006).

**Field emission scanning electron microscope (FESEM):** Biofilm morphology was also determined and observed with FESEM-S4800 (Hitachi, Tokyo, Japan), as previously described (Anriany et al., 2006; Lu et al., 2012).

**Detection of curli protein:** Curli protein was determined as described previously (Anriany et al., 2006; El Hag et al., 2017).

**Lipopolysaccharide (LPS) profiles:** Each strain was inoculated into 50 ml LB and shaken at 37°C for 18h. Bacterial suspensions were subjected to phenol-water extraction (Wigley et al., 2001). Purified LPS samples were resolved using SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining (Tsai and Frasch, 1982).

**Adhesion and invasion assay:** Adhesion and invasion assays were carried out according to standard methods (Peng et al., 2005). HeLa cells were used to determine the adhesion and invasion assays of bacterial strains. The avian macrophage cell line HD11 was used for macrophage infection assays (Mu et al., 2013).

** Determination of LD$_{50}$ in chickens:** All chicken experiments were approved by the Jiangsu Administrative Committee for Laboratory Animals and complied with the guidelines of laboratory animal welfare and ethics (Permission number: SYXKSU-2007-0005). One-day-old specific pathogen free (SPF) chickens were used. Chickens were randomly assigned into 19 groups (n=6/group) and inoculated orally. Mortality rates were monitored over a two-week period. The 50% lethal dose (LD$_{50}$) was calculated using the Reed-Muench method (Reed and Muench, 1938).

**Statistical analysis:** Assays were monitored three times, using duplicate samples in each independent assay. All data are expressed as mean and standard deviations. All statistical analyses were performed using IBM SPSS Statistics 22. P values less than 0.05 were considered significant, when using one-way analysis of variance (ANOVA) to test for differences among groups.

**RESULTS**

**Characterization of csgA and bcsA mutants of *S. Typhimurium:*** The csgA and bcsA genes were deleted from *S. Typhimurium* strains S016 and S025 by using lambda red recombination (Fig. 1A and C). The growth rates of ΔcsgA and ΔbcsA were similar to that of the wild-type (WT) strains (Fig. 1 B and D). BF was examined by crystal violet staining of bacterial strains grown on 96-well polystyrene plates. Whereas the WT (S016 and S025) and ΔbcsA strains stained violet, the ΔcsgA mutant of both strains was colorless (Fig. 2A). Complementation of the ΔcsgA strains restored the WT phenotypes. In biofilm assays as assessed by crystal violet staining, the OD$_{590}$ of the S016 ΔcsgA, S016 ΔbcsA and S025 ΔcsgA mutants were significantly lower than that of their wild-type strains (Fig. 2B). In glass test tubes assays, the intensity of the color ring at the liquid-air interface formed by the ΔbcsA strains was weaker than that formed by wild-type strains. The ΔcsgA strains did not form colored rings on the glass tube surface (Fig. 2C).
In (FESEM) analyses, both WT S016 and S025 strains had meshwork-like structures surrounding the bacteria, whereas both ΔcsgA mutant strains produced thin and smooth cell surface structures, while ΔbcsA strains showed reduced meshwork-like structures of extracellular substances (Fig. 2D), indicating that expression of csgA mainly contributes to biofilm formation in S. Typhimurium strains S016 and S025.

Identification of biofilm components of S. Typhimurium mutants: Colony morphologies and colors were further determined by growing the strains on Congo red plates. The WT S016 and S025 strains showed red, dry, and rough colonies, in contrast to the smooth colonies produced by the mutant strains. Both ΔcsgA strains appeared as pink/white colonies, indicating a lack of curli production, while both ΔbcsA strains appeared as brown/white colonies, indicating a lack of cellulose production (Fig. 3A). In calcofluor staining assays, both ΔbcsA strains exhibited reduced fluorescence, perhaps due to reduced cellulose production (Fig. 3B).
Fig. 3: Biofilm components of *S. Typhimurium* mutants. **A.** Congo Red phenotypes. **B.** Calcofluor morphotypes.

Fig. 4: Protein and LPS analysis of *S. Typhimurium* mutants. **A.** Curli protein (arrows) expression detected using SDS-PAGE. **B.** Analysis of purified LPS from WT and mutant strains.
Table 1: Proliferation of S. Typhimurium mutants in HD11 cells. Values are mean ± SD

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bacterial Inoculum (CFUs)</th>
<th>Bacterial Count (CFUs) at 3 h post-infection</th>
<th>Percentage (%) invasion (3h)</th>
<th>Bacterial Count (CFUs) at 23 h post-infection</th>
<th>Percentage (%) proliferation (23h)</th>
<th>Proliferation ratio (23h/3h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT S016</td>
<td>1.92x10^7</td>
<td>5.15x10^7</td>
<td>2.68±0.33</td>
<td>6.30±0.10</td>
<td>3.28±0.085</td>
<td>1.22</td>
</tr>
<tr>
<td>ΔcsgA</td>
<td>1.90x10^7</td>
<td>2.40x10^7</td>
<td>2.16±0.20</td>
<td>2.35±0.10</td>
<td>1.23±0.225</td>
<td>0.96*</td>
</tr>
<tr>
<td>ΔbcsA</td>
<td>1.9±10^7</td>
<td>4.55x10^7</td>
<td>2.24±0.35</td>
<td>5.50±1.04</td>
<td>2.83±0.225</td>
<td>1.20</td>
</tr>
<tr>
<td>WT S025</td>
<td>1.62±10^7</td>
<td>6.11x10^7</td>
<td>3.34±0.230</td>
<td>5.55±1.04</td>
<td>3.01±0.360</td>
<td>0.90</td>
</tr>
<tr>
<td>ΔcsgA</td>
<td>1.61x10^7</td>
<td>3.05±0.06</td>
<td>3.05±0.220</td>
<td>5.50±1.04</td>
<td>2.75±0.225</td>
<td>0.833*</td>
</tr>
<tr>
<td>ΔbcsA</td>
<td>1.61x10^7</td>
<td>5.00±1.04</td>
<td>3.05±0.210</td>
<td>5.50±1.04</td>
<td>0.94</td>
<td></td>
</tr>
</tbody>
</table>

1Significantly different from the WT strain (P<0.05).

Fig. 5: Adhesion and invasion assays of S. Typhimurium mutants. Adhesion to and invasion of HeLa cells. Values represent the mean ± SD. Asterisk indicates significant difference, one-way ANOVA, P<0.05.

Table 2: Determination of LD50 in one-day-old SPF chickens after oral challenge of S. Typhimurium mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group number</th>
<th>Challenge dose (CFUs)</th>
<th>Chicks (n)</th>
<th>Mortality (%)</th>
<th>LD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT S016</td>
<td>1</td>
<td>1.46±10^7</td>
<td>6</td>
<td>3/6 (50.0)</td>
<td>10^7.81</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.46±10^7</td>
<td>6</td>
<td>2/6 (33.3)</td>
<td>10^7.81</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.5±10^8</td>
<td>6</td>
<td>6/6 (100)</td>
<td>10^6.40</td>
</tr>
<tr>
<td>ΔcsgA</td>
<td>5</td>
<td>1.5±10^7</td>
<td>6</td>
<td>0/6 (0.0)</td>
<td>10^6.40</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.5±10^7</td>
<td>6</td>
<td>0/6 (0.0)</td>
<td>10^6.40</td>
</tr>
<tr>
<td>ΔbcsA</td>
<td>8</td>
<td>1.41±10^7</td>
<td>6</td>
<td>1/6 (16.7)</td>
<td>10^6.18</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.41±10^7</td>
<td>6</td>
<td>1/6 (16.7)</td>
<td>10^6.18</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2.75±10^4</td>
<td>6</td>
<td>4/6 (66.7)</td>
<td>10^7.58</td>
</tr>
<tr>
<td>WT S025</td>
<td>11</td>
<td>2.75±10^4</td>
<td>6</td>
<td>3/6 (50.0)</td>
<td>10^6.18</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.75±10^4</td>
<td>6</td>
<td>1/6 (16.7)</td>
<td>10^6.18</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>2.75±10^4</td>
<td>6</td>
<td>2/6 (33.3)</td>
<td>10^6.18</td>
</tr>
<tr>
<td>ΔcsgA</td>
<td>14</td>
<td>2.76±10^4</td>
<td>6</td>
<td>1/6 (16.7)</td>
<td>10^6.56</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.76±10^4</td>
<td>6</td>
<td>0/6 (0.0)</td>
<td>10^6.56</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>2.75±10^4</td>
<td>6</td>
<td>3/6 (50.0)</td>
<td>10^6.56</td>
</tr>
<tr>
<td>ΔbcsA</td>
<td>17</td>
<td>2.75±10^4</td>
<td>6</td>
<td>2/6 (33.3)</td>
<td>10^6.97</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2.75±10^4</td>
<td>6</td>
<td>1/6 (16.7)</td>
<td>10^6.97</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>6</td>
<td>0/6 (0.0)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1Significantly different from the WT strain (P<0.05).

Protein patterns and LPS profiles of S. Typhimurium mutants: Comparative examination of protein patterns on SDS-PAGE revealed that both WT strains and ΔbcsA strains produced a 15.3-kDa curli protein, but ΔcsgA strains did not (Fig. 4A). Silver staining of purified LPS components showed that the molecular masses of LPS from ΔcsgA and ΔbcsA strains were smaller than that from the WT S016 and S025 strains (Fig. 4B).

Adhesion, invasion and proliferation of S. Typhimurium mutants: The ΔcsgA mutant from S. Typhimurium S016 exhibited significantly reduced adhesion to HeLa cells, as compared to the WT strain (Fig. 5). In HD11 macrophages, both csgA mutants from S. Typhimurium S016 and S025 showed significantly lower proliferation as compared to their WT strains (Table 1).

Virulence assays in chickens after oral challenge of S. Typhimurium mutants: When one-day-old SPF chickens were orally challenged with different amounts of WT or mutant strains, both the ΔcsgA mutants from S. Typhimurium S016 and S025 strains showed significantly higher LD50 (10^7.84 and 10^6.66) than the WT strains (10^6.31 and 10^5.71). The ΔbcsA mutants showed similar LD50s (10^7.58 and 10^7.58) as the WT strains (Table 2).

DISCUSSION

S. Typhimurium is a common zoonotic pathogen that forms biofilms on a wide variety of surfaces (Korber et al., 1997; Römling et al., 2003). The csgA gene is associated with curli formation and its expression is regulated by CsgD, which activates the csgRAB operon. Cellulose is also a component of the biofilm extracellular matrix; its production is activated by the adRA gene at the post-transcriptional level through direct interaction with one or more of the gene products of bacterial cellulose synthesis operons cbsABZC-bcsEF (Zogaj et al., 2001; Gerstel and Römling, 2003).

To determine whether the csgA and bcsA genes contribute to biofilm formation or virulence in S. Typhimurium, we constructed knockout mutants in these genes. We found that ΔcsgA strains were defective in curli production while the ΔbcsA strains were defective in production of cellulose. Both deficiencies resulted in reduced biofilm formation to different extents in in vitro assays. Consistent with our previous study of S. Pullorum (El Hag et al., 2017), the csgA gene plays a critical role in biofilm formation in S. Typhimurium, whereas the bcsA gene has lesser influence.

LPS is also involved in biofilm formation. Deletion mutants of the ddHC and waaG genes in S. Typhimurium, which are involved in LPS synthesis, had opposing effects on biofilm formation, depending on osmolarity (Anriany et al., 2006). The rfaG (also known as waaG) and rfbH (also known as ddHC) deletions in Salmonella Pullorum altered LPS profiles but not BF (Lu et al., 2012). In our study, the LPS molecules of the ΔcsgA and ΔbcsA in both S. Typhimurium strains were smaller than that of wild-type strains, consistent with our previous study of S. Pullorum (Lu et al., 2012; El Hag et al., 2017).

Curli are adherent fibrillae of Salmonella enterica and Escherichia coli (E. coli). In enterohemorrhagic and enteropathogenic E. coli, deletions in either csgA or bcsA do not significantly alter bacterial adherence to human colonic HT-29 epithelial cells, while a double csgA/bcsA mutant was significantly less adhesive, indicating a synergistic role of curli and cellulose in cell adherence (Saldaña et al., 2009). Similarly, cellulose-deficient S. Enteritidis mutants showed no difference in bacterial adherence and invasion assays of eukaryotic cells (Solano et al., 2002). However,
mutations of csgA and csgB in *S. Enteritidis* affected attachment to alfalfa sprouts differently (Barak et al., 2005). Here we found that the ΔcsgA strains had significantly reduced adhesion and invasion into HeLa cells, while ΔbcsA strains were not significantly different from their WT counterparts. In HD11 macrophages, both ΔbcsA strains exhibited similar proliferation as WT strains, which was different from previous studies that shows that blocking cellulose production promotes bacterial proliferation in macrophages (Pontes et al., 2015; Ahmad et al., 2016; El Hag et al., 2017). So further trials are needed to understand the exact reasons for this variation that may be related to *Salmonella* serovars.

It was reported that the ΔcsgA mutant of *S. Typhimurium* was not attenuated during murine infection (White et al., 2008). However, the ΔcsgA mutant in *S. Pullorum* showed attenuated virulence in 1-day-old chickens (El Hag et al., 2017). Here we found that the ΔcsgA mutants were attenuated for virulence in a chicken oral challenge model, whereas the ΔbcsA mutant strains showed similar virulence as the wild-type strains S016 and S025. ΔcsgA attenuation may be due to reduced adhesion and invasion of epithelial cells or due to decreased intracellular proliferation in macrophages. Overall, these data illustrate the respective functions of csgA and bcsA in *S. Typhimurium* biofilm formation and virulence.

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Authors contribution: El Hag conceived, designed and performed the experiments and also compiled to the writing manuscript; Su and Feng participated in sample processing; Qin analyzed the data; Chen, Peng and Liu critically revised article for additional intellectual input and approved the present manuscript. All authors read and approved the final version of the manuscript.

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