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RESEARCH ARTICLE

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

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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 Δ*csgA* strains did not produce curli fimbriae and Δ*bcsA* mutants had decreased cellulose production. The Δ*csgA* strains were unable to form biofilms. The Δ*csgA* strains also showed decreased adhesion and invasion to HeLa cells and reduced intracellular proliferation in HD11 macrophages. The Δ*bcsA* mutants had similar adhesion, invasion, and proliferation as compared to the wild-type strains. The Δ*csgA* 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*.

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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-bcsEFG* 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 (Datsenko 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 100µl of each bacterial suspension was added to a 96-well U-bottomed polystyrene microtiter-plate (Corning, New York, NY, USA). The Optical Density (OD_{550}) nm was measured using a micro-plate reader (Bio-Tek, Winooski, VT, USA).

To assay BF on glass tube surfaces, 2ml of overnight cultures of each strain diluted 1:100 in 1/10 TSB were added to borosilicate glass test tubes and incubated at 28ºC for 48h as similar as mentioned previously (Kim *et al*., 2007; Crawford *et al*., 2008). To assay for curli production, 10µl 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 28ºC 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 50ml LB and shaken at 37ºC 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 Δ*csgA* and Δ*bcsA* 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 Δ*bcsA* strains stained violet, the Δ*csgA* mutant of both strains was colorless (Fig. 2A). Complementation of the Δ*csgA* strains restored the WT phenotypes. In biofilm assays as assessed by crystal violet staining, the OD_{550} of the S016 Δ*csgA*, S016 Δ*bcsA* and S025 Δ*csgA* 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 Δ*bcsA* strains was weaker than that formed by wild-type strains. The Δ*csgA* strains did not form colored rings on the glass tube surface (Fig. 2C).

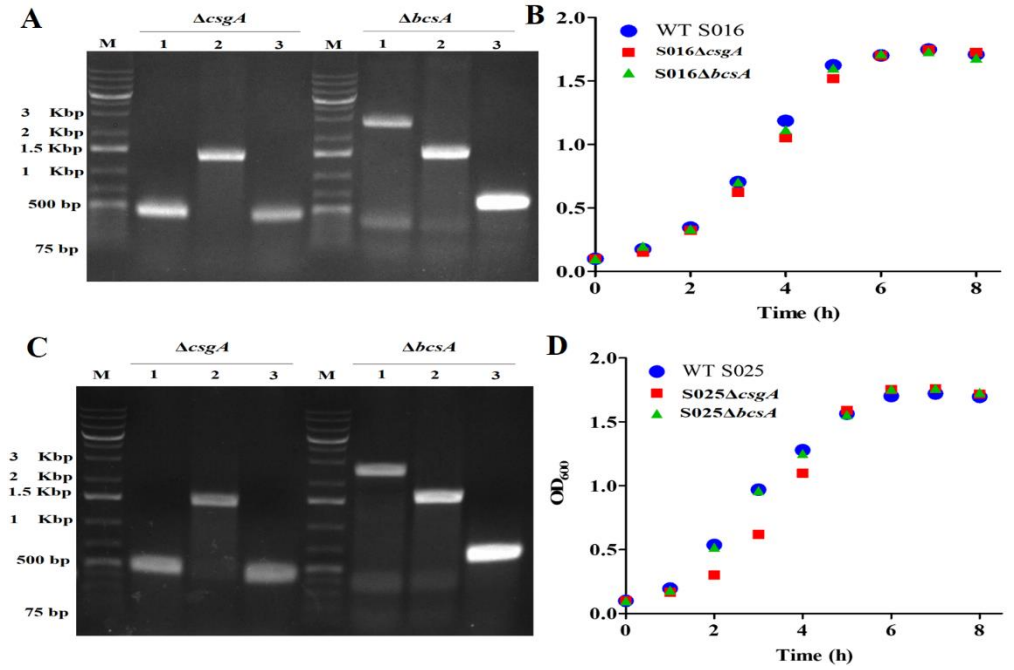

Fig. 1: Construction and characterization of Δ*csgA* and Δ*bcsA* mutants in *S. Typhimurium* strains S016 and S025. **A, C.** Mutant validation. M, DNA Marker; 1, Fragments amplified from WT strains; 2, *cat* gene insertion; 3, Amplified fragments without *cat* gene. **B, D.** Growth curves of WT strains, Δ*csgA*, and Δ*bcsA* mutants.

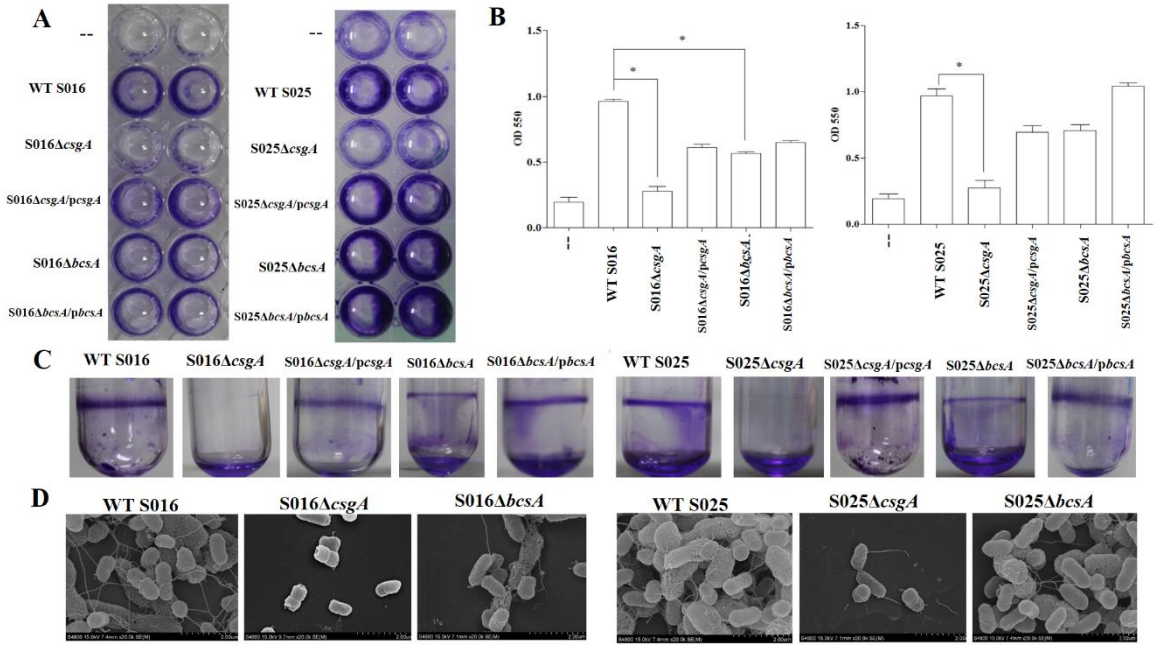

Fig. 2: Biofilm assays. **A.** Crystal violet (CV) staining of bacteria grown on 96-well plates. **B.** CV staining quantification by measuring optical density (OD_{550}). Values represent the mean±SD. Asterisk indicates significant difference, one-way ANOVA, $P<0.05$. **C.** Biofilm formation in glass tubes incubated at 28°C for 48h. **D.** Field emission scanning electron microscope (FESEM) analysis of bacteria grown on coverslips at 28°C for 24h. Scale bar = 2.00µm.

In (FESEM) analyses, both WT S016 and S025 strains had meshwork-like structures surrounding the bacteria, whereas both Δ*csgA* mutant strains produced thin and smooth cell surface structures, while Δ*bcsA* 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 Δ*csgA* strains appeared as pink/white colonies, indicating a lack of curli production, while both Δ*bcsA* strains appeared as brown/white colonies, indicating a lack of cellulose production (Fig. 3A). In calcofluor staining assays, both Δ*bcsA* strains exhibited reduced fluorescence, perhaps due to reduced cellulose production (Fig. 3B).

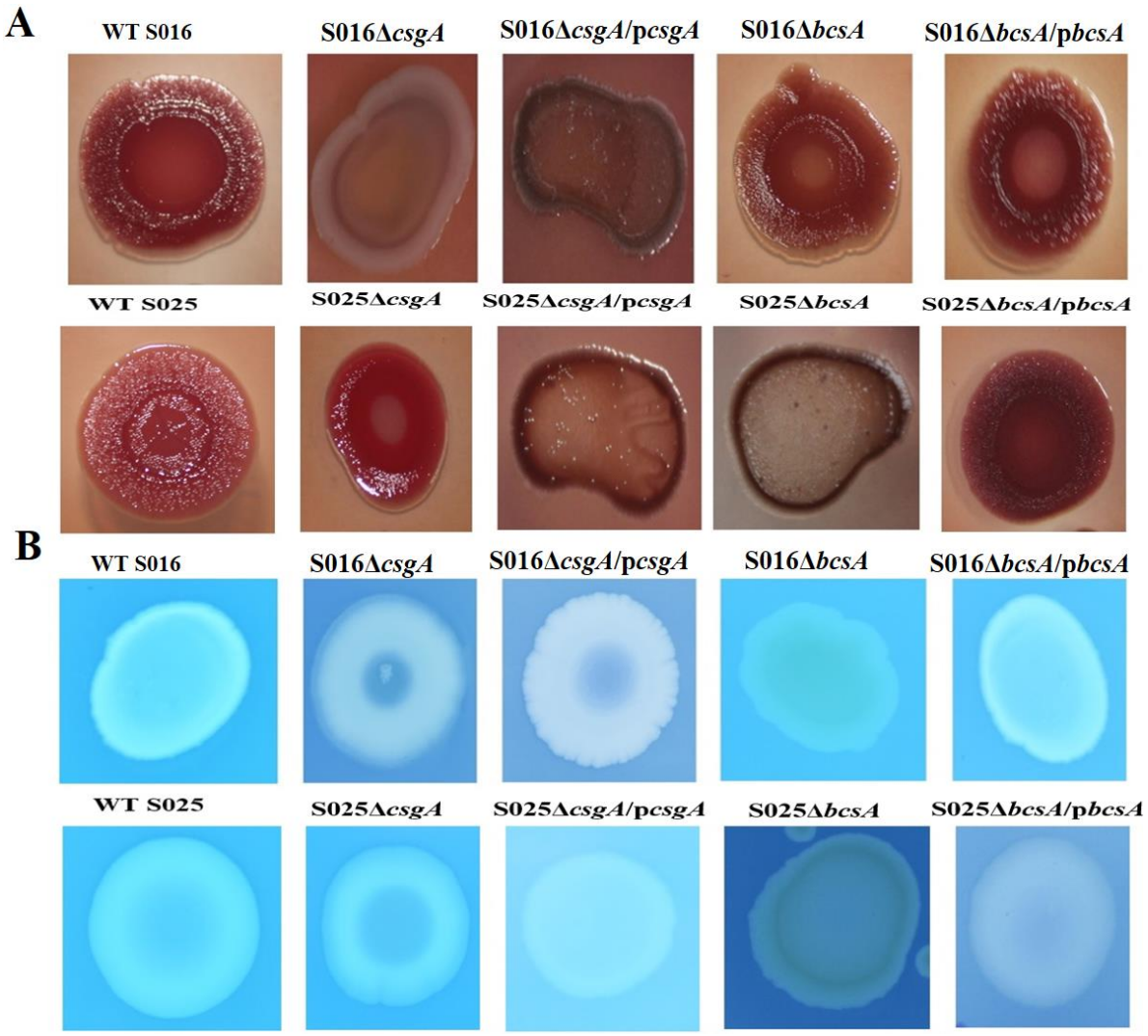

Fig. 3: Biofilm components of *S. Typhimurium* mutants. **A**. Congo Red phenotypes. **B.** Calcofluor morphotypes.

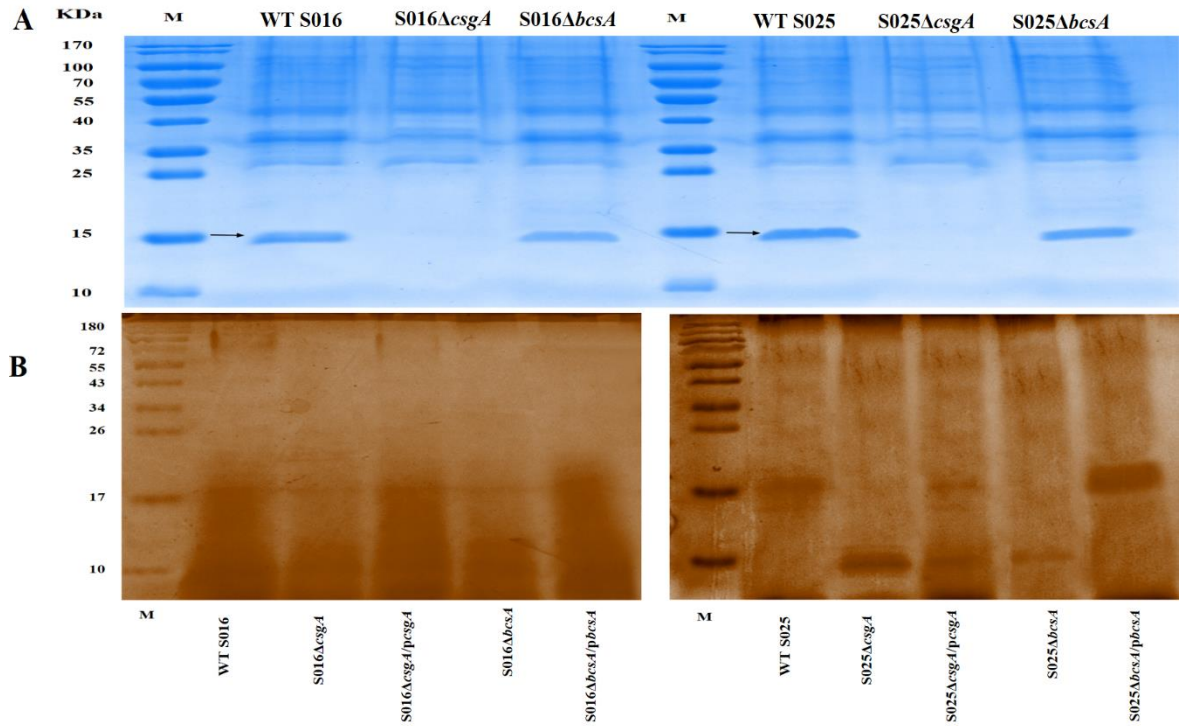

Fig. 4: Protein and LPS analysis of *S. Typhimurium* mutants. **A.** Curli protein (arrows) expression detected using SDS-PAGE. **B.** Analysis of purified LPS from WT and mutant strains.

Table 1: Proliferation of *S. Typhimurium* mutants in HD11 cells, Values are mea± SD

Strain	Bacterial Inoculum (CFUs)	Bacterial Count (CFUs) at 3 h post-infection	Percentage (%) invasion (3h)	Bacterial Count (CFUs) at 23 h post-infection	Percentage (%) proliferation (23h)	Proliferation ratio (23h/3h)
WT S016	1.92×10^{6}	5.15×10^{4}	2.682±0.335	6.30×10^{4}	3.281±0.085	1.22
Δ*csgA*	1.90×10^{6}	2.40×10^{4}	1.263±0.200	2.35×10^{4}	1.236±0.225	0.96[a]
Δ*bcsA*	1.94×10^{6}	4.55×10^{4}	2.345±0.235	5.50×10^{4}	2.835±0.225	1.20
WT S025	1.82×10^{6}	6.10×10^{4}	3.342±0.230	5.55×10^{4}	3.041±0.360	0.90
Δ*csgA*	1.81×10^{6}	6.00×10^{4}	3.305±0.100	5.00×10^{4}	2.754±0.225	0.833[a]
Δ*bcsA*	1.80×10^{6}	5.80×10^{4}	3.222±0.202	5.50×10^{4}	3.055±0.210	0.94

[a]Significantly different from the WT strain (P<0.05).

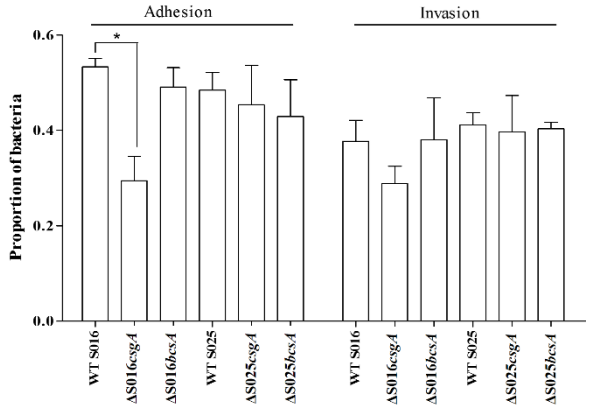

Fig. 5: Adhesion and invasion assays of *S. Typhimurium* mutants. Adhesion to and invasion of HeLa cells. Values represent the mean±SD. Asterisk indicates significant difference, one-way ANOVA, P<0.05.

Table 2: Determination of LD_{50} in one-day-old SPF chickens after oral challenge of *S. Typhimurium* mutants

Strain	Group number	Challenge dose (CFUs)	Chicks (n)	Mortality rate (%)	LD_{50}
	1	1.46×10^{8}	6	3/6 (50.0)	
WT S016	2	1.46×10^{7}	6	2/6 (33.3)	$10^{7.81}$
	3	1.46×10^{6}	6	2/6 (33.3)	
	4	1.53×10^{8}	6	1/6 (16.7)	
Δ*csgA*	5	1.53×10^{7}	6	0/6 (0.0)	$10^{8.40a}$
	6	1.53×10^{6}	6	0/6 (0.0)	
	7	1.41×10^{8}	6	2/6 (33.3)	
Δ*bcsA*	8	1.41×10^{7}	6	1/6 (16.7)	$10^{8.18}$
	9	1.41×10^{6}	6	1/ 6 (16.7)	
	10	2.75×10^{8}	6	4/6 (66.7)	
WT S025	11	2.75×10^{7}	6	3/6 (50.0)	$10^{7.58}$
	12	2.75×10^{6}	6	1/6 (16.7)	
	13	2.76×10^{8}	6	2/6 (33.3)	
Δ*csgA*	14	2.76×10^{7}	6	1/6 (16.7)	$10^{8.56a}$
	15	2.76×10^{6}	6	0/6 (0.0)	
	16	2.75×10^{8}	6	3/6 (50.0)	
Δ*bcsA*	17	2.75×10^{7}	6	2/6 (33.3)	$10^{7.97}$
	18	2.75×10^{6}	6	1/6 (16.7)	
PBS	19	-	6	0/6 (0.0)	-

[a]Significantly 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 Δ*bcsA* strains produced a 15.3-kDa curli protein, but Δ*csgA* strains did not (Fig. 4A). Silver staining of purified LPS components showed that the molecular masses of LPS from Δ*csgA* and Δ*bcsA* strains were smaller than that from the WT S016 and S025 strains (Fig. 4B).

Adhesion, invasion and proliferation of *S. Typhimurium* mutants: The Δ*csgA* 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 Δ*csgA* mutants from *S. Typhimurium* S016 and S025 strains showed significantly higher LD_{50} ($10^{8.40}$ and $10^{8.56}$) than the WT strains ($10^{7.81}$ and $10^{8.18}$). The Δ*bcsA* mutants showed similar LD_{50s} ($10^{7.58}$ and $10^{7.97}$) 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 *csgBAC* 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 *bcsABZC-bcsEFG* (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 Δ*csgA* strains were defective in curli production while the Δ*bcsA* 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 osmolality (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 Δ*csgA* and Δ*bcsA* 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 fimbriae 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 Δ*csgA* strains had significantly reduced adhesion and invasion into HeLa cells, while Δ*bcsA* strains were not significantly different from their WT counterparts. In HD11 macrophages, both Δ*bcsA* 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 Δ*csgA* mutant of *S. Typhimurium* was not attenuated during murine infection (White *et al*., 2008). However, the Δ*csgA* mutant in *S. Pullorum* showed attenuated virulence in 1-day-old chickens (El Hag *et al*., 2017). Here we found that the Δ*csgA* mutants were attenuated for virulence in a chicken oral challenge model, whereas the Δ*bcsA* mutant strains showed similar virulence as the wild-type strains S016 and S025. Δ*csgA* 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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