Role of StdA in adhesion of *Salmonella enterica* serovar Enteritidis phage type 8 to host intestinal epithelial cells

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**Abstract**

**Background:** *Salmonella* is often implicated in foodborne outbreaks, and is a major public health concern in the United States and throughout the world. *Salmonella enterica* serovar Enteritidis (SE) infection in humans is often associated with the consumption of contaminated poultry products. Adhesion to epithelial cells in the intestinal mucosa is a major pathogenic mechanism of *Salmonella* in poultry. Transposon mutagenesis identified *stdA* as a potential adhesion mutant of SE. Therefore, we hypothesize StdA plays a significant role in adhesion of SE to the intestinal mucosa of poultry.

**Methods and results:** To test our hypothesis, we created a mutant of SE in which *stdA* was deleted. Growth and motility were assayed along with the *in vitro* and *in vivo* adhesion ability of the Δ*stdA* when compared to the wild-type SE strain. Our data showed a significant decrease in motility in Δ*stdA* when compared to the wild-type and complemented strain. A decrease in adhesion to intestinal epithelial cells as well as in the small intestine and cecum of poultry was observed in Δ*stdA*. Furthermore, the lack of adhesion correlated to a defect in invasion as shown by a cell culture model using intestinal epithelial cells and bacterial recovery from the livers and spleens of chickens.

**Conclusions:** These studies suggest StdA is a major contributor to the adhesion of *Salmonella* to the intestinal mucosa of poultry.

**Keywords:** *Salmonella*, Adhesion, StdA, Poultry

**Background**

*Salmonella* is a significant foodborne bacterium associated with enteric disease outbreaks in humans due to the consumption of contaminated food. *Salmonella* serovars, like *Salmonella enterica* serovar Enteritidis (SE), are the leading cause of death among the major foodborne pathogens [1]. SE phage type (PT) 8 is one of the most common PTs associated with egg-associated outbreaks in the United States while SE PT4 is the most common in Europe [2,3]. Therefore, the identification and evaluation of *Salmonella* virulence factors could help develop new ways to control salmonellosis in the farm to fork food processing cycle.

A hallmark of *Salmonella* virulence is its ability to invade host intestinal epithelial cells [4]. This is a multi-step process mediated by a type 3 secretion system (T3SS) encoded within *Salmonella* pathogenicity island-1 (SPI-1) [5,6]. The first step in the invasion process is the adhesion of *Salmonella* to the host intestinal epithelial cells. Several pathogenic factors have been implicated in adhesion to host cells. The best characterized are the fimbrial adhesins which include type 1, plasmid-encoded, long polar, and thin aggregative fimbriae [7-10]. A further study has suggested that the T3SS itself can mediate host cell adhesion by showing that SipB, SipC, and SipD are required for the intimate association of *Salmonella* with mammalian cells [11]. Inhibition of *Salmonella* adhesion at the initial stages of infection is potentially the most effective strategy for controlling salmonellosis in production animals which could result in reduced contamination of our food supply [12].

In this study, we identified *stdA* as an adhesion mutant of SE by transposon mutagenesis. The *stdA* deletion mutant (Δ*stdA*) displayed a normal growth profile when compared to the wild-type (WT) SE PT8 and complemented

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strains. A motility assay showed a significant decrease in motility for ΔstdA. Adhesion and invasion assays showed ΔstdA was deficient in cell culture models of Salmonella adhesion and invasion. Furthermore, ΔstdA was deficient in a poultry model of Salmonella adhesion and invasion with the systemic infection deficiency most likely due to the decreased adhesion. Taken together, these data indicated a major role for StdA in the adhesion ability of SE host cells.

Results
Analysis of ΔstdA
The chromosomal stdA gene was replaced by a kanamycin resistance gene (KnR) cassette using the lambda Red recombination system. Deletion of stdA from the chromosome of SE was confirmed by PCR analysis. The primer set K2/K5 was used to amplify the KnR cassette, while the primer set F2/R3 was used to confirm the absence of stdA.

To ensure correct orientation of the KnR cassette, the primer set K3/K5 was used to amplify the KnR cassette, while the primer set F3/K5 was used to amplify the upstream stdA flanking sequence along with the KnR cassette, while R3/K5 was used to amplify the downstream stdA flanking sequence along with the KnR cassette. Overall, these results indicated that a stdA deletion mutant of SE PT8 was successfully created.

StdA does not affect Salmonella growth
Growth curve analysis was conducted for the WT, ΔstdA, and complemented strains in order to determine the relevance of StdA on Salmonella growth. All three strains displayed nearly identical growth profiles suggesting StdA does not play a significant role in SE growth (Figure 1).

ΔstdA is deficient in motility
A motility assay was conducted to see if StdA has a role in SE motility. Measurement of the motility plates displayed a significantly reduced migration from the inoculation site to the periphery of the plate for ΔstdA (19 mm) when compared to the WT (68 mm) and complemented (64 mm) strains (Figure 2).

ΔstdA is attenuated in adhesion and invasion in vitro
Inoculation of intestinal epithelial cells displayed a significant decrease in adhesion ability in ΔstdA (3.6 logs) when compared to the WT (6.0 logs) and complemented strains (5.9 logs) (Figure 3A). Furthermore, ΔstdA displayed the same significant decrease in the ability to invade T84 intestinal epithelial cells (Figure 3B). The adhesion and invasive ability of ΔstdA was restored after complementation, suggesting StdA plays a role in adhesion and invasion of intestinal epithelial cells by SE. It is logical to conclude that the invasion defect seen in ΔstdA is an effect of the adhesion deficiency.

Deletion of stdA attenuates SE adhesion and invasion in chickens
A chicken model of infection was used to determine the role of StdA in the adhesion ability of SE to the intestinal mucosa of chickens. At 16 hours post-infection, the bacterial counts of WT SE from the small intestine were 7.1 logs compared to 0.75 logs for ΔstdA. At day 7 post-infection, bacterial counts from the small intestine were 4.1 logs for the WT with no bacteria recovered for ΔstdA (Figure 4A). For the cecum, bacterial counts from chickens infected with the WT SE strain were 8.7 and 9.1 logs at the 16 hour and day 7 time points, respectively compared to 5.8 and 6.1 logs for ΔstdA (Figure 4B). These data suggest StdA plays a role in the adhesion ability of SE to the intestinal mucosa of chickens.

We also assayed the bacterial counts from the livers and spleens to see if the adhesion deficiency displayed by ΔstdA affected systemic infection in chickens. At the 16 hour time point, there was no bacteria recovered from the livers and spleens of chickens infected with the WT and ΔstdA strains. At the day 7 time point, bacterial counts in the liver were 7.2 and 4.5 logs comparing the WT and ΔstdA, respectively (Figure 5A). Furthermore, bacterial counts in the spleen were 7.8 and 5.0 logs comparing the WT and ΔstdA, respectively (Figure 5B). These data suggest that the adhesion deficiency displayed by ΔstdA contributes to an overall reduction in systemic infection by SE in poultry.

Discussion
In this study, an adhesion mutant of SE was created and characterized. Transposon mutagenesis identified StdA as a potential adhesion mutant of SE. A ΔstdA strain of SE was created using the lambda Red recombination system, and was deficient in adhesion in both cell culture and chicken models of infection. Additionally, this adhesion
defect lead to a deficiency in invasion of T84 intestinal epithelial cells, and decreased overall systemic infection ability in a poultry model as evidenced by reduced bacterial counts in the livers and spleens of chickens inoculated with ΔstdA. These data indicated that StdA plays a significant role in bacterial infection and pathogenesis.

Figure 2 Motility assay of the WT, ΔstdA, and complemented strains. Bacteria were spotted onto soft agar, and migration of the bacteria was measured from the inoculation point to the periphery of the plate. (A) Images showing the migration of each SE strain. (B) Graph displaying the migration of each SE strain. The actual P values are given displaying a statistically significant difference between ΔstdA and the WT strain.

Figure 3 Cell culture assays using T84 intestinal epithelial cells. (A) Adhesion assay and (B) invasion assay with the WT, ΔstdA, and complemented strains. The actual P values are given displaying a statistically significant difference between ΔstdA and the WT strain.

Figure 4 Determination of adhesion ability in poultry. Bacterial counts in (A) small intestine and (B) cecum of chickens inoculated by oral crop gavage with 1 × 10^7 CFU of WT and ΔstdA. The actual P values are given displaying a statistically significant difference between ΔstdA and the WT strain.
role in the adhesion ability of SE to the intestinal mucosa of poultry.

StdA is a 19-kDa fimbrial protein that is part of the std operon which was originally identified during sequence analysis of the Salmonella enterica serovar Typhi CT18 strain [13]. It was later found to be in other serovars of Salmonella including Salmonella enterica serovar Typhimurium (STM) [14-17]. In STM, the Std fimbriae play a role in Salmonella adhesion to specific sections of the intestinal mucosa as evidenced by std operon deletion mutants having reduced intestinal persistence in mice [18,19]. This correlates to the data observed in our study, where deletion of stdA significantly alerted the adhesion ability of SE in the intestinal mucosa of poultry.

The synthesis of Std fimbriae is tightly regulated, but the mechanisms involved in std expression are unclear. In the study by Balbontin et al., gene expression profiling of a dam mutant of STM demonstrated that transcription of the std operon is repressed by Dam methylation [20]. In another study, Jakomin et al. showed that uncontrolled expression of Std fimbriae contributes to the attenuated virulence observed in dam mutants of STM [21]. They also described a regulatory role for SeqA as a repressor of the std operon and HdfR as an activator of std expression whose activity may be antagonized by SeqA [21]. Further regulatory evidence was displayed in the study by Chessa et al. which identified RosE as a novel transcriptional regulator of Std fimbrial expression in STM [18]. Further investigation into the regulation of stdA, and how it affects Salmonella adhesion to the intestinal mucosa of poultry will be conducted in our laboratory.

An interesting observation in our study is that ΔstdA displayed a significant decrease in motility. Motility is hypothesized to be a pathogenic mechanism because it promotes contact with the surface of epithelial cells by allowing the bacterium to penetrate the thick mucus layer covering the intestinal mucosa [22,23]. Some studies suggest a role for flagella in bacterial adhesion to host tissue [24,25]. The study by Erdem et al. suggests a role for FliC in E. coli adhesion to bovine intestinal tissue while the study by Olsen et al. suggests a role for FliC in Salmonella binding to intestinal epithelial cells [24,25]. Further studies will be needed to determine how StdA affects Salmonella motility and if this motility reduction contributes to the adhesion and invasion defect seen in ΔstdA.

Additional studies will also be needed in order to gauge the level of attenuation of the ΔstdA SE strain in chickens. Depending on the outcome of these studies, further studies could be conducted to determine if ΔstdA is a good candidate for use in a live-attenuated poultry vaccine.

**Conclusions**

Transposon mutagenesis identified StdA as a potential adhesion mutant of SE. A ΔstdA strain of SE was created using the lambda Red recombination system, and was deficient in adhesion both in vitro and in vivo. Additionally, this lack of adhesion lead to a deficiency in invasion of T84 intestinal epithelial cells, and decreased overall systemic infection ability in a poultry model as evidenced by reduced bacterial counts in the livers and spleens of chickens inoculated with ΔstdA. Overall, our data suggest StdA plays a role in the adhesion ability of Salmonella to the intestinal mucosa of chickens, and could be an important factor in the early stages of Salmonella infection in poultry.

**Methods**

**Bacterial strains, plasmids, and cell lines**

The WT SE PT8 E2627 strain was isolated from an egg-associated outbreak in the United States [26]. All Salmonella strains were grown in either Luria-Bertani (LB) medium or on Salmonella-Shigella (SS) plates. Additionally, all homogenates from the in vivo experiments were incubated in Selenite-F broth (BD, Sparks, MD). Nalidixic acid (100 µg/ml), kanamycin (50 µg/ml), tetracycline (15 µg/ml), and ampicillin (100 µg/ml) were added to the media as necessary. A complete list of the bacterial strains and plasmids used in this study is shown in Table 1. T84 intestinal epithelial cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA), and were subsequently

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**Figure 5** Systemic infection ability of the WT and ΔstdA SE strains. Bacterial counts in (A) liver and (B) spleen of chickens inoculated by oral crop gavage with 1 × 10⁷ CFU of WT and ΔstdA. There was no bacterial recovery at the 16 hour time point from either the liver or spleen of chickens inoculated with the WT or ΔstdA strain, so only the day 7 data is shown. The actual P values are given displaying a statistically significant difference between ΔstdA and the WT strain.
grown and maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F12 (DMEM/F12) medium supplemented with 10% fetal bovine serum (FBS), and incubated at 37°C with 5% CO2.

Construction of the mutant and complemented strains

The transposon binding screening which identified ΔstdA as a potential binding mutant of SE is described in [27]. The ΔstdA strain was created using the lambda Red recombination system as previously described [28]. Briefly, WT SE PT8 was transformed with the pKD46 plasmid that carries the lambda Red recombinase genes [28]. Arabinose-induced WT SE carrying pKD46 was cultured and used to generate the electrocompetent cells. The kanamycin resistance gene (KnR) was PCR amplified from the pKD4 plasmid using primer set LF/LR [28]. The 5′-end of the LF primer carries 40 extra bases homologous to the upstream Salmonella stdA gene while the 5′-end of the LR primer carries 40 bases homologous to the downstream stdA flanking sequence. The PCR product was purified and electroporated into the WT-pKD46 electrocompetent cells. After transformation, colonies growing on LB plates supplemented with kanamycin were selected as candidates for stdA mutants of SE. To confirm deletion of the stdA gene, the selected mutants were subjected to PCR analysis using primer sets K3/K5 and F2/R2 to show the presence of the KnR and the absence of stdA.

The stdA complemented strain was constructed by amplifying a DNA fragment containing stdA from the WT SE strain using primer set F3/R3. The DNA fragment was blunt-ended using a PCR polishing kit (Stratagene, Santa Clara, CA) and ligated into the blunt-ended ScaI restriction enzyme digested pBR322 vector. The recombinant plasmid was transformed into the stdA mutant by electroporation. A complete list of the primers used in this study is shown in Table 2.

Growth analysis

Growth curve profiles were constructed in order to determine the significance of StdA on SE growth. An equal number of cells from the WT, ΔstdA, and complemented strains were inoculated in LB and grown at 37°C. The optical densities at 600 nm were recorded each hour.

Motility assay

The motility assay was performed as previously described [29]. Briefly, soft agar (LB medium with 0.3% agar) was

Table 1 Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| Serovar Enteritidis phage type 8 E2627 | Isolated from an egg-associated outbreak in the United States | [26] |
| ΔstdA | Mutant of serovar Enteritidis in which stdA was deleted using lambda Red; KnR | This study |
| ΔstdA/pBRstdA | ΔstdA complemented with a copy of the stdA gene via pBR322; KnR, TcR | This study |
| **E. coli** | | |
| DH5a | Used for recombinant DNA methods | Lab stock |
| **Plasmids** | | |
| pKD46 | lambda Red recombinase genes; ApR | Lab stock |
| pBR322 | ApR, TcR | Lab stock |
| pKD4 | KnR gene cassette | Lab stock |
| pBRstdA | stdA gene cloned into pBR322 at the ScaI site | This study |

Table 2 Sequence and purpose of primers used in this study

| Primer name and sequence | Purpose |
|--------------------------|---------|
| LRS4-CCGGAGGCGTCTTCCCGTTGTTATTTACCGCGTGAAAACATATGAATATCCTCCTTAG-3′ | Reverse primer for amplification of the KnR gene cassette and downstream stdA flanking sequence |
| LRS4-CCGGAGGCGTCTTCCCGTTGTTATTTACCGCGTGAAAACATATGAATATCCTCCTTAG-3′ | Reverse primer for amplification of the KnR gene cassette and downstream stdA flanking sequence |
| F2*R-CTACCGTCAGTACGTGTTACGTCAC-3′ | Forward primer for amplification of the stdA gene |
| R2*S-CTAGGATATCGCTACGGC-3′ | Reverse primer for amplification of the stdA gene |
| F3*ATTCATATGGGCTTACCGCTGTTATTACCGCGG-3′ | Forward primer for amplification of stdA for complementation |
| R3*S-AGACTCAGTCAGTATCTCAGG-3′ | Reverse primer for amplification of stdA for complementation |
| Kp 5*-AACGCCAAGGCAGGCTGTTCTA-3′ | Forward primer for amplification of the KnR gene cassette |
| Kp 5*-CGCTGAGGCTGCTGCTT-3′ | Reverse primer for amplification of the KnR gene cassette |
used to characterize the motility phenotype of the WT, $\Delta stdA$, and complemented SE strains. Overnight cultures of each Salmonella strain were adjusted to the same optical density. Equal numbers of CFU ($1 \times 10^6$) were spotted onto 0.3% LB agar. The plates were incubated at 37°C, and motility was determined by examining the migration of the bacteria from the center of the inoculation point to the periphery of the plate.

Adhesion assay
The adhesion assay was performed as previously described [30]. Briefly, $5 \times 10^5$ T84 intestinal epithelial cells were seeded per well in a 24-well tissue culture plate and incubated overnight at 37°C with 5% CO2. The following day, cells were infected with the WT, $\Delta stdA$, and complemented strains at a multiplicity of infection (MOI) of 10:1. The plate was briefly centrifuged, and incubated for 30 minutes at 37°C with 5% CO2. Unbound bacteria were aspirated; the wells washed six times with phosphate buffered saline (PBS), and the cells lysed with 0.1% Triton X-100 (TX-100). Dilutions of the cell lysates were plated on SS agar for enumeration of bacteria.

Invasion assay
The invasion assay was performed as previously described [30]. Briefly, $5 \times 10^5$ T84 intestinal epithelial cells were seeded per well in a 24-well plate and incubated overnight at 37°C with 5% CO2. The cells were infected with the WT, $\Delta stdA$, and complemented strains at an MOI of 10:1, and briefly centrifuged so that the bacterial cells would be in direct contact with the T84 cells. After incubation for 30 minutes at 37°C with 5% CO2, the cells were washed three times with PBS and incubated for an additional 45 minutes with gentamicin-containing medium (100 μg/ml) to kill extracellular bacteria. Following incubation, the gentamicin-containing medium was removed, the wells were washed six times with PBS, and the cells were lysed with 0.1% TX-100. The lysate was diluted and plated out on SS agar plates for colony-forming unit (CFU) determination.

Chicken experiments
One-week-old specific-pathogen-free (SPF) White Leghorn chickens were purchased from Charles River (Wilmington, MA). Groups of 11 birds were infected by oral crop gavage with $1 \times 10^7$ CFU of the WT or $\Delta stdA$ SE strains. Another group of birds (n = 4) was inoculated by oral crop gavage with 100 μl sterile PBS to serve as a control. At 16 hours and 7 days post-infection, 5 birds from each group were euthanized using CO2. Portions of the liver, spleen, small intestine, and cecum were removed from each bird. The individual organs were pooled and 1 gram was homogenized in 10 ml PBS. One ml from each homogenate was incubated in 10 ml Selenite-F broth at 36°C for 18 hours. Direct plating of the organ homogenates was done in parallel with plating from the enrichment cultures [31–34]. Enumeration of bacteria was performed by serial dilution from the Selenite-F broth and plating on SS agar.

Statistical analysis
Wherever appropriate, the data were analyzed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA) and a Student’s t test. P values of ≤ 0.05 were considered significant. Unless otherwise stated, experiments were repeated two times and data were expressed as arithmetic means with standard deviations.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
DS carried out the in vivo and in vitro experimental work, performed the statistical analysis, and drafted the manuscript. NE performed the transposon screening and identified the adhesion mutants. DM created the stdA mutant strain. AF designed and coordinated the study, and edited the manuscript. All authors read and approved the final manuscript.

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