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# *Salmonella enterica* Serovar Enteritidis *tatB* and *tatC* Mutants Are Impaired in Caco-2 Cell Invasion *In Vitro* and Show Reduced Systemic Spread in Chickens[▽]†

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***Salmonella enterica* subsp. *enterica* serovar Enteritidis is a leading causative agent of gastroenteritis in humans. This pathogen also colonizes the intestinal tracts of poultry and can spread systemically in chickens. Transfer to humans usually occurs through undercooked or improperly handled poultry meat or eggs. The bacterial twin-arginine transport (Tat) pathway is responsible for the translocation of folded proteins across the cytoplasmic membrane. In order to study the role of the Tat system in the infection and colonization of chickens by *Salmonella* Enteritidis, we constructed chromosomal deletion mutants of the *tatB* and *tatC* genes, which are essential components of the Tat translocon. We observed that the *tat* mutations affected bacterial cell morphology, motility, and sensitivity to albomycin, sodium dodecyl sulfate (SDS), and EDTA. In addition, the mutant strains showed reduced invasion of polarized Caco-2 cells. The wild-type phenotype was restored in all our *Salmonella* Enteritidis *tat* mutants by introducing episomal copies of the *tatABC* genes. When tested in chickens by use of a *Salmonella* Enteritidis Δ*tatB* strain, the Tat system inactivation did not substantially affect cecal colonization, but it delayed systemic infection. Taken together, our data demonstrated that the Tat system plays a role in *Salmonella* Enteritidis pathogenesis.**

*Salmonella* is a major concern in human health, because it is one of the leading causative agents of gastroenteritis. Humans usually become infected by ingestion of contaminated eggs and undercooked chicken meat (14).

In poultry, *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium are considered most important. *Salmonella* usually causes asymptomatic infection in birds, but outbreaks with high levels of mortality and symptomatic infection have occurred in birds less than 2 weeks old (20, 46). Infection of poultry is generally characterized by ingestion of the bacteria and colonization of intestinal mucosa. Those bacteria penetrate the intestinal mucosa and spread systemically (15, 21, 22, 37).

Adhesion and invasion of epithelial cells are complex multifactorial processes. Often, a number of different virulence factors contribute to the survivability and successful infection of a microorganism in a given host. This is particularly true for many enterobacteria, including *Salmonella*. Therefore, it is important to investigate new potential virulence determinants and to study their roles *in vitro* and *in vivo*.

In most bacteria, including the Gram-negative salmonellae, enzymatically active respiratory enzymes have to be transported across the impermeable cytoplasmic membrane. Since these respiratory enzymes often contain cofactors, they need to be translocated in a prefolded, sometimes oligomeric state. This can be accomplished by the twin-arginine transport (Tat) system, which recognizes and translocates into the periplasm only polypeptides with a specific N-terminal signal sequence containing a "twin-arginine" motif. The Tat system is well characterized in *Escherichia coli*. There an operon containing *tatABCD* genes is found with an additional gene, *tatE*, located elsewhere in the chromosome. Functionally, TatA, TatB, and TatC are found to form two separate complexes in the inner membrane (8, 65). For the export of a target protein, the twin-arginine motif in its signal sequence is recognized by TatC (1), followed by association of TatA with the complex and transport through a pore formed by TatA protomers (24, 48). Of the other two proteins, TatE is very similar to and functionally interchangeable with TatA (34), while TatD plays an unknown role, demonstrating some DNase activity and involvement in the degradation of misfolded FeS proteins (49, 53, 64, 74).

A number of studies regarding the function of the Tat system at the molecular level have demonstrated its importance in a wide variety of cellular functions (44, 63). However, there is limited information regarding the involvement of the Tat system in virulence. In *Pseudomonas aeruginosa*, the Tat system may affect virulence by the secretion of stress response- or pathogenesis-related factors (51). Another report finds the Tat system to be a virulence determinant in *Agrobacterium tumefaciens* (19). In *Escherichia coli* O157:H7, *tatABC* deletion resulted in a loss of motility on soft agar plates, which was considered to be due to impaired secretion of Shiga toxin 1 and H7 flagellin, both known as major virulence factors (56).

The present study aims to clarify the impact and possible role of the Tat system in *Salmonella* Enteritidis virulence. In order to accomplish this, Tat system mutants were subjected to

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TABLE 1. Primers used in this study

| Primer | Length (bp) | Nucleotide sequence |
|---|---|---|
| TatB FW[a] | 70 | 5′-GAAGCGAAAAAGGAAGACGCTAAAAGCCAAGATAAAGAGCAGGTATAATCCATATGAATATCCACCTTAG-3′ |
| TatB RV[a] | 70 | 5′-CAACTCGATCAGATGCGTGATAAGCGGTTGAGTATCTTCTACAGCCATGTGTGTAGGCTGGAGCTGCTTC-3′ |
| TatC FW[a] | 70 | 5′-GCTGCGCCTGTTGTCGAATCTTCCCCCTCGTCGAGTGATAAACCGTAAACCATATGAATATCCACCTTAG-3′ |
| TatC RV[a] | 70 | 5′-ATCAAACATGCTTGCCCCCATATGACAACCGCCCTGGCGGGCGGTTGTGTGTGTAGGCTGAGCTGTTC-3′ |
| TatB FW[b] | 20 | 5′-TAATGTGTATAATGCGGCC-3′ |
| TatB RV[b] | 20 | 5′-ACGACCAGACGACGCTCATG-3′ |
| TatC FW[b] | 20 | 5′-CCCTGCCGCCGCTGAAACAC-3′ |
| TatC RV[b] | 19 | 5′-TTTGCAAACTGGCTACTGG-3′ |
| *Eco*RITatA-C FW[c] | 23 | 5′-GAATTCTGGCTGGTTGGCTGGCG-3′ |
| *Hind*IIITatA-C RV[c] | 22 | 5′-AAGCTTAACGCCAATATCAAAC-3′ |
| *Eco*RITatA-D FW[c] | 22 | 5′-GAATTCTTACTCGTCAACCGCC-3′ |
| *Hind*IIITatA-D RV[c] | 24 | 5′-AAGCTTCGCGTTCGATGTTACTGC-3′ |

[a] λ red primer.
[b] Confirmatory primer.
[c] Primer used for amplification and cloning of the *tatABC* and *tatABCD* regions.

phenotypic assays and invasion assays using polarized human epithelial colorectal adenocarcinoma (Caco-2) cells and were also tested for their abilities to colonize chickens successfully. The results indicated that the Tat system plays an important role not only in cell invasion but also in the systemic spread of *Salmonella* Enteritidis in chickens.

## MATERIALS AND METHODS

**Search for putative Tat substrates by DNA sequence analysis.** The full genome sequence of *S.* Enteritidis strain Sal18, used in this study, is not available. However, we have evidence that the Sal18 genome is highly similar to the *S. enterica* serovar Enteritidis PT4 NCTC 13349 full-length sequence, provided by the Wellcome Trust Sanger Institute, United Kingdom. Previously, we have designed primers based on the published *S.* Enteritidis PT4 NCTC 13349 sequence in order to amplify >50 gene loci of interest from various chromosomal regions of Sal18 by PCR. So far, all tested primers resulted in the expected amplification products. A comparison of numerous sequenced fragments from Sal18 with the corresponding regions from PT4 resulted in 95 to 100% identity at the DNA level (data not shown). Therefore, the complete *S.* Enteritidis PT4 NCTC 13349 sequence was searched for candidate proteins to be translocated by the Tat system. Programs were run as provided by the TatP 1.0 server (Technical University of Denmark; http://www.cbs.dtu.dk/services/TatP) (6) and the TatFind server (http://signalfind.org/tatfind.html) (59).

**Construction of mutants.** The mutants generated in this study were derivatives of a *Salmonella* Enteritidis strain (Sal18) and were constructed using the lambda (λ) red system as described previously (17). Briefly, a PCR product was generated by using primers that complemented flanking regions of an antibiotic resistance cassette with an overhang of at least 50 nucleotides homologous to the region of interest in the Sal18 wild-type (WT) strain. The *S.* Enteritidis PT4 NCTC 13349 sequence was used as a reference. Plasmids pKD3 (17) and pBR322 (9), containing a chloramphenicol resistance and a tetracycline resistance cassette, respectively, were used as templates for the antibiotic resistance cassettes. The PCR conditions used were 94°C for 3 min; 35 cycles of 94°C (30 s), 53°C (45 s), and 72°C (1.5 min); and finally 72°C for 7 min. The primers used are listed in Table 1. The PCR product was transformed by electroporation into competent wild-type Sal18 cells containing plasmid pKD46, which expressed the λ red recombinase. Bacterial cells were resuspended in SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM $MgCl_2$, 20 mM glucose) and were allowed to grow for 3 h at 37°C with shaking. The bacteria were then plated onto Luria-Bertani (LB) broth agar plates containing 9 μg $ml^{-1}$ of chloramphenicol (or 7 μg $ml^{-1}$of tetracycline) and were incubated overnight at 37°C. The clones obtained were streaked onto LB agar plates containing 30 μg $ml^{-1}$of chloramphenicol (or 15 μg $ml^{-1}$of tetracycline) and were subjected to the same incubation conditions as in the previous step. The constructs were confirmed by PCR using a 2720 Thermal Cycler (Applied Biosystems) and by sequence analysis (3730 XL DNA Analyzer; Applied Biosystems). The primers used in this step are listed in Table 1. For removal of the antibiotic cassette, plasmid pCP20 was electroporated into a Δ*tatB* or Δ*tatC* strain with the chloramphenicol resistance cassette (LS37 and LS55, respectively). The strain was grown in SOC medium for 1 h at 30°C. The bacterial cells were then plated onto LB plates containing ampicillin at a concentration of 100 μg/ml and were incubated overnight at 30°C. One colony was picked and diluted in 1 ml of phosphate-buffered saline (PBS). Serial dilutions were then made, plated into LB agar plates, and incubated overnight at 42°C. Subsequently, 20 colonies were randomly picked among all dilutions plated and were restreaked onto LB agar plates, LB agar plates containing 100 μg/ml of ampicillin, or LB agar plates containing chloramphenicol at a concentration of 30 μg/ml. The positive clones (chloramphenicol and ampicillin sensitive) were then confirmed by PCR using the respective confirmatory primers listed in Table 1. The strains used in this study are listed in Table 2.

**Congo red binding assay.** *Salmonella* strains were tested for their abilities to bind Congo red dye. Briefly, 3 μl of a bacterial culture grown overnight in LB medium was diluted in an equal volume of distilled water. The mixture was spotted onto a Congo red agar plate (1% Bacto tryptone, 0.5% yeast extract, 40 μg $ml^{-1}$ Congo red dye, 1.5% agar) and incubated at 28°C for 24 h. In addition, the overnight culture of bacteria was also streaked onto Congo red plates and incubated under the same conditions (47, 58).

**Albomycin sensitivity assay.** Bacterial strains were tested for albomycin sensitivity as previously described (40). Briefly, 100 μl of an overnight bacterial culture was inoculated into 5 ml of 0.5% molten agar (45°C). The mixture was then immediately poured onto an LB agar plate. After solidification of the soft agar, a paper disc containing 10 μl albomycin solution at 1 mg/ml or serial dilutions thereof was placed on the plate, and the inhibition of growth was observed after 8 to 12 h of incubation at 37°C. Alternatively, the albomycin solution was spotted directly onto the bacterium-containing top agar.

**Motility assay.** To test for motility, 10 μl of an LB broth culture with an absorbance at 600 nm of 0.8 was placed in a stab in LB plates containing 0.3% agar. The plates were incubated at 37°C without being inverted, and the diameter of the hazy zone formed by the migrating bacteria was measured at 3 and 6 h of incubation.

**Cell morphology.** Cell morphology was determined by microscopic examination of a Gram stain (28) of bacteria that had been grown at 37°C in LB medium or on LB plates.

**Sensitivity assays.** Sensitivities to EDTA and sodium dodecyl sulfate (SDS) were determined for the Δ*tatB* and Δ*tatC* mutant strains and were compared to those of the wild-type strain Sal18. Briefly, 100 μl of an overnight culture was added to 5 ml of soft agar (0.5%). This mixture was then poured onto an LB plate. Filter paper discs containing 10 μl of SDS (5 or 10%) or EDTA (0.1 M, 0.02 M, or 0.004 M) were placed on the solidified medium and incubated overnight at 37°C without inversion. Alternatively, the solutions were spotted directly onto the bacterium-containing top agar. The inhibition of growth was assessed by measuring the clear zones around the discs. In addition, the SDS

TABLE 2. Strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant genotype and/or phenotype | Reference or source |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α | F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_K^-$ $m_K^+$) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1* | Invitrogen |
| MC4100 | F$^-$ *lacU169 araD139 rpsL150 relA1 ptsF rbs flbB5301* | 13 |
| B1LK0 | MC4100 *tatC* | 65 |
| *S.* Enteritidis | | |
| Sal18 | *S. enterica* serovar Enteritidis 301 PT13 | 55 |
| LS21 | Like Sal18, but ΔSPI-1::*cat* | 18 |
| LS25 | Like Sal18, but *att*Tn*7*::*cat* | This study |
| LS29 | Like Sal18, but *att*Tn*7*::*tet* | This study |
| LS37 | Like Sal18, but Δ*tatB*::*cat* | This study |
| LS39 | Like Sal18, but Δ*tatB*, *cat* removed | This study |
| LS55 | Like Sal18, but Δ*tatC*::*cat* | This study |
| LS106 | Like Sal18, but Δ*tatC*, *cat* removed | This study |
| LS107 | Like LS39, but with pLP196 | This study |
| LS108 | Like LS39, but with pLP198 | This study |
| LS114 | Like LS39, but with pLP199 | This study |
| LS117 | Like LS106, but with pLP198 | This study |
| LS118 | Like LS106, but with pLP199 | This study |
| Plasmids | | |
| pBR322 | Ap$^r$ Tc$^r$ ColElOri; 4,361 bp | 9 |
| pKD3 | $rep_{R6K\gamma}$ Ap$^r$FRT Cm$^r$FRT | 17 |
| pKD46 | $rep_{pSC101}$ts Ap$^r$ $P_{araBAD}$γβexo | 17 |
| pCP20 | Ap$^r$ Cm$^r$ *cI857* $P_R$ *flp* pSC101 oriTS | 17 |
| pLP196 | pBR322 + $tatA^+B^+C'$ (truncated *tatC*) | This study |
| pLP198 | pBR322 + $tatA^+B^+C^+$ | This study |
| pLP199 | pBR322 + $tatA^+B^+C^+D^+$ | This study |

sensitivities of the wild-type strain Sal18 and strain LS37 were assessed in a broth assay. An overnight culture of the bacteria was diluted 100 times in LB broth containing 5% SDS. The culture was incubated with shaking at 37°C, and the absorbance ($OD_{600}$) was determined for 7 h after inoculation.

**Caco-2 cell invasion assay. (i) Cell culture preparation.** Caco-2 cells, an immortalized line of human epithelial colorectal adenocarcinoma cells, were grown in 75-cm$^2$ flasks (BD Falcon), in HyClone medium (Dulbecco's modified Eagle medium [DMEM]/high glucose; Fisher Scientific) enriched with 10% inactivated fetal bovine serum (FBS; Seracare) and 1% essential amino acids (Invitrogen). The cells were incubated at 37°C under 5% $CO_2$ until they became confluent. Cells were harvested, and $2 \times 10^5$ cells were seeded in a 6.5-mm-diameter (pore size, 0.4 μm) transwell plate (Fisher Scientific) and grown until the transepithelial resistance (TER), determined using the Millicell-ERS (electrical resistance system) (Millipore), reached approximately 700 to 900 Ω, indicating that the cells had become polarized.

**(ii) Invasion assay.** The invasion assay has been described previously (18, 45). Bacterial cultures were grown overnight and inoculated into fresh LB broth at a 1/100 ratio. The bacterial cells were allowed to grow to an absorbance ($OD_{600}$) of 0.7. After a wash with DMEM, the bacteria were inoculated apically onto the polarized Caco-2 cells at a multiplicity of infection (MOI) of 100 and were incubated for 1 h at 37°C under 5% $CO_2$. The bacteria were removed, and the cell culture was washed with 200 μl of PBS (136 mM NaCl, 2.5 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$) three times. After the wash, DMEM containing 400 μg/ml of gentamicin was added, and the Caco-2 cells were incubated for 2 h under 5% $CO_2$. Cells were again washed with PBSA; 1% Triton X-100 was added; and the cells were incubated for 15 min at 37°C. Serial dilutions were plated onto LB agar alone or onto LB agar plus the appropriate antibiotic (5 μg/ml of tetracycline or 34 μg/ml of chloramphenicol) and were incubated overnight at 37°C. For competition experiments using bacterial strains expressing tetracycline or chloramphenicol resistance determinants, the lysed Caco-2 cells were plated on both tetracycline- and chloramphenicol-containing LB plates. Bacterial colonies were counted and CFU per milliliter calculated. The number of wild-type bacteria that invaded the polarized cells was considered to be 100%, and the invasiveness of the mutant strain was calculated as a percentage of that. In competition experiments, a competitive index of invasion (CII) was calculated. The CII is defined as the ratio (in colony-forming units) of the mutant wild type strains divided by their ratio in the input (initial mix added to the cell monolayer) (69). Three replicates of each bacterial strain were done per assay, and each assay was repeated at least three times.

**Cloning of the *tatABCD* genes.** To complement the chromosomal gene knockouts in strains LS37 (Δ*tatB*) and LS55 (Δ*tatC*) with the corresponding plasmid-encoded functional wild-type genes, a 1.9-kb EcoRI and HindIII PCR fragment containing $tatA^+B^+C'$ (*tatC* truncated due to frameshift), a 1.9-kb EcoRI and HindIII PCR fragment encoding $tatA^+B^+C^+$, or a 2.4-kb EcoRI and HindIII PCR fragment carrying $tatA^+B^+C^+D^+$ was cloned into vector pBR322 (9), resulting in plasmid pLP196, pLP198, or pLP199, respectively. Primers complementing the flanking regions of the *tatABCD* operon (according to the *S. enterica* serovar Enteritidis PT4 NCTC 13349 sequence provided by the Wellcome Trust Sanger Institute, United Kingdom) and also containing the appropriate restriction enzyme recognition sites were designed. The PCR product and pBR322 were digested with EcoRI and HindIII, purified, and ligated. The resulting plasmids were then electroporated into the mutant strains, yielding the complemented *tatB* and *tatC* mutants (Table 2).

**In vivo analysis of bacterial colonization and systemic spread. (i) *Salmonella* Enteritidis inocula.** A wild-type Sal18 *att*Tn*7*::*cat* strain (LS25) and a Δ*tatB* strain (LS37) were used. The bacteria were grown in LB broth overnight at 37°C with shaking and were used to seed the same medium at a 1/100 dilution. The strains were incubated using the previous condition until an absorbance ($OD_{600}$) of 0.7 was reached. The cells were harvested by centrifugation at 6,371 × *g* for 15 min at 4°C. The pellet was resuspended in PBS at a concentration of $5 \times 10^9$ CFU/ml. The viable cell counts were confirmed by serial dilution and plating on LB plates. An aliquot of the culture was used to perform PCR with specific primers to confirm that the bacteria had not reverted to wild type. At the conclusion of the experiment, PCR was performed on the bacteria recovered from chicken tissues and cecal contents to confirm the identities of the strains.

**(ii) Chickens.** Specific-pathogen-free (SPF) eggs were obtained from Charles River Laboratories and were incubated at the Poultry Science Building at the University of Saskatchewan, Saskatoon, Canada. Once hatched, the Leghorn

chickens were kept in isolation rooms at the Vaccine and Infectious Disease Organization (VIDO) animal care facility. When the chickens were 1 day old, cloacal swabs were obtained and plated on Brilliant Green Agar (BGA) to confirm that the birds were *Salmonella* free. The birds had access to water and food *ad libitum*. All procedures with animals were done according to the protocol approved by the University of Saskatchewan Committee on Animal Care.

**(iii) Experimental challenge of chickens with *Salmonella* Enteritidis.** Two animal trials were carried out. In each trial, three groups of 90 birds were used. Groups 1 and 2 were inoculated via oral gavage with $5 \times 10^9$ CFU/0.5 ml of strains LS25 and LS37, respectively, and group 3 received 0.5 ml of PBS. In trial 1, the birds were challenged at the age of 7 days, and in trial 2, they were challenged at the age of 4 days. In both trials, on days 1, 2, and 4 postchallenge, 10 birds from each group were euthanized per day. Cecum contents, livers, and spleens were collected in sterilized preweighed tubes containing saline (0.85% sodium chloride). The samples were then weighed, and the livers and spleens were homogenized. The resulting homogenates were then plated on BGA in serial dilutions and were incubated at 37°C for approximately 24 h. The CFU per gram of sample was calculated. In addition, 1 ml of each sample was inoculated into 5 ml of selenite broth and was incubated at 37°C for 24 h. After this, the enriched samples were streaked onto BGA plates and incubated at 37°C for 24 h.

**Statistical analysis.** For the Caco-2 coinfection and cell invasion assay, the calculated CII was the mean from three independent experiments ± standard error. Each CII was analyzed using Student's *t* test, and the null hypothesis was that each CII was not different from 1. Bacterial counts in the cecum, liver, and spleen following challenge with wild-type versus mutant strains were compared using the Mann-Whitney U test. The enrichment data were analyzed using the chi-square test, and comparison between groups was performed using Fisher's exact test. A $P$ value less than 0.05 was considered significant.

## RESULTS

**Putative substrates of the *Salmonella* Enteritidis Tat system.** In order to identify putative substrates of the Tat system, particularly in a search for candidate virulence factors translocated by this pathway, we applied the Tat prediction programs that are available online, TatP (6) and TatFind (59), to the *S.* Enteritidis PT4 NCTC 13349 sequence. The TatP program predicted 25 proteins with putative twin-arginine signal sequences, whereas the TatFind program predicted 27 "true" Tat substrates. Most candidates were detected by both programs. A substantial number of the Tat-transported candidates from *S*. Enteritidis represent proteins with high similarity to Tat substrates in *Escherichia coli* K-12. In *E. coli* 28 polypeptides are known or predicted to harbor N-terminal twin-arginine signal peptides (http://www.lifesci.dundee.ac.uk/groups/tracy_palmer/docs/signals.htm). The TatFind search in *S.* Enteritidis revealed 20 candidates with significant similarity to 10 proteins of the group from *E. coli*, namely, HyaA (2 copies), HybO, HybA, NapA, NapG, TorA, DmsA (3 copies), FdnG, FdoG, and YedY. Many of these proteins function in oxidation (hydrogen, formate) or reduction (nitrite, nitrate, dimethyl sulfoxide [DMSO], trimethylamine oxide [TMAO]) and are able to bind redox cofactors (e.g., molybdopterin, molybdopterin guanine dinucleotide, iron-sulfur clusters). In addition, polypeptides were predicted as Tat substrates that showed striking similarity to SufI, MdoD, AmiA, AmiC, FhuD, and YcbK of *E. coli*. A search using the TatP program gave the same picture with only two exceptions: first, an additional DmsA equivalent was predicted, and second, the periplasmic hydroxamate binding protein FhuD was not recognized as a putative Tat substrate.

The following *S*. Enteritidis proteins that had not been identified as Tat substrates in *E. coli* were predicted by TatP and/or TatFind: two citrate lyase beta chain proteins (corresponding accession numbers, YP_002242226.1 and YP_002242739.1), two putative sulfatases (YP_002242248.1 and YP_002242921.1), tetrathionate reductase subunit A (YP_002243762.1), a thiosulfate reductase precursor (YP_002244151.1), a putative colanic acid biosynthesis protein (YP_002244186.1), a putative inner membrane protein (YP_002245645.1), and a cytochrome *c*-type biogenesis protein (YP_002246083.1). Since none of these nine polypeptides suggested an obvious link to cell motility or virulence mechanisms, they were not investigated further by us.

**Sal18 *tat* mutants display altered cell morphology.** As previously discussed, TatA, TatB, and TatC are essential proteins for a fully functional Tat system. In order to study the possible role of the Tat system in invasion and infection, we concentrated on *tatB* and *tatC*, two loci that are known to be essential for a functional twin-arginine secretion system in *E. coli* (50, 52, 66, 73).

Cells of the wild-type Sal18 strain (LS25) and the Δ*tatB*::*cat* (LS37), Δ*tatB* (LS39), Δ*tatC*::*cat* (LS55), and Δ*tatC* (LS106) mutant strains were examined. In addition, strains LS39 and LS106 were also complemented with plasmid pLP198 ($tatA^+B^+C^+$) or pLP199 ($tatA^+B^+C^+D^+$). While the Sal18 wild-type cells presented the classic rod-like shape characteristic of most *Salmonella* strains, the Δ*tatB* (LS37, LS39) and Δ*tatC* (LS55, LS106) mutant cells were elongated in shape, often occurring in a chain-like formation. The complemented strains had restored the wild-type morphology, independently of the plasmid used for complementation (Fig. 1). The phenotypic analysis of bacterial cell morphology is summarized in Table 3.

**Mutations in *tatB* and *tatC* affect bacterial cell motility.** Under certain conditions, flagella are reported to contribute to the virulence of enteropathogenic bacteria, as they promote chemotaxis, host colonization, and host cell invasion (38, 61, 71). In order to analyze the impact of the Tat system on flagellar function in *Salmonella* Enteritidis, the motilities of strains LS37 (Δ*tatB*::*cat*), LS39 (Δ*tatB*, *cat* removed), LS55 (Δ*tatC*::*cat*), and LS106 (Δ*tatC*, *cat* removed) were assessed in comparison to those of the wild-type Sal18 and Sal18 *att*Tn*7*::*cat* (LS25) strains on soft LB agar plates. We compared the results for the strains with or without antibiotic cassettes in order to rule out any polar effect caused by the antibiotic cassette insertion. After 3 h of incubation, it was observed that the two wild-type strains had similar swimming zones with diameters of 12 mm and 11 mm, while the LS37 (Δ*tatB*::*cat*) and LS55 (Δ*tatC*::*cat*) mutants were less motile (swimming zones measuring 7 mm in diameter). Strains LS39 (Δ*tatB*, *cat* removed) and LS106 (Δ*tatC*, *cat* removed) displayed the same swimming zone diameters as the strains containing the antibiotic cassette (7 mm). The complemented strains LS39/pLP198 ($tatA^+B^+C^+$), LS39/pLP199 ($tatA^+B^+C^+D^+$), LS106/pLP198 ($tatA^+B^+C^+$), and LS106/pLP199 ($tatA^+B^+C^+D^+$) recovered the wild-type phenotype; their swimming zone diameters ranged from 11 mm to 13 mm. When the same plates were observed after 6 h of incubation, the wild-type strains Sal18 and LS25 produced swimming zones of 32 mm and 31 mm, respectively, while the motilities of LS37 (Δ*tatB*::*cat*) and LS55 (Δ*tatC*::*cat*) were restricted to swimming zones of 18 mm in diameter. Strains LS39 (Δ*tatB*, *cat* removed) and LS106 (Δ*tatC*, *cat* removed) presented the same motility as strains that contained an antibiotic cassette (18 mm). The complemented strains LS39/pLP198 ($tatA^+B^+C^+$), LS39/pLP199

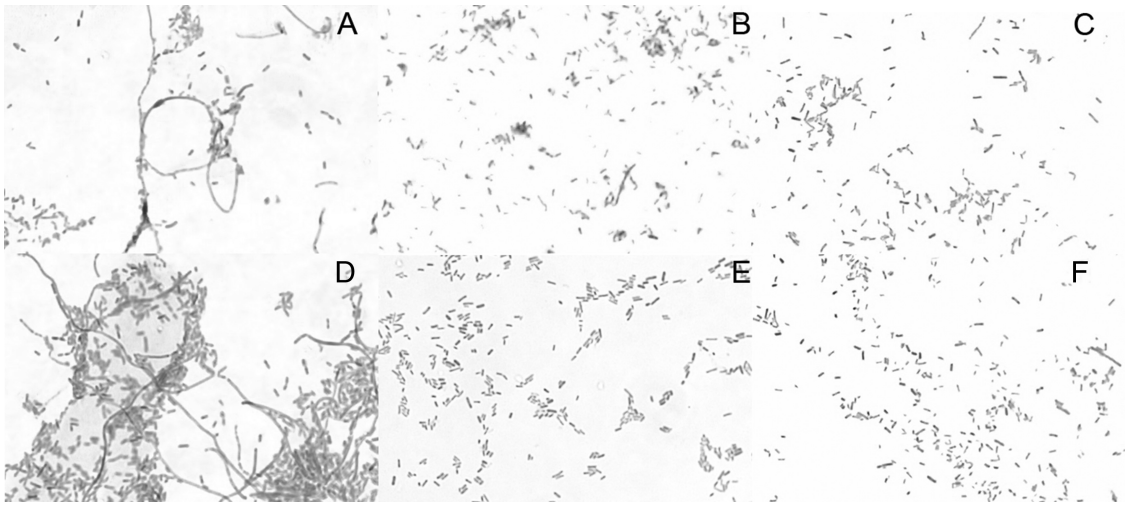


FIG. 1. The *Salmonella* Enteritidis wild-type strain Sal18 and its Δ*tatB* and Δ*tatC* derivatives were Gram stained, and the cell morphology was observed. The wild-type cells, as well as the plasmid-complemented cells, displayed the classic rod-like shape of Gram-negative bacteria, while the mutant cells showed an elongated shape and chain formation. (A) Δ*tatB*, *cat* removed (LS39); (B) Δ*tatB* pLP198 ($tatA^+B^+C^+$); (C) Δ*tatB* pLP199 ($tatA^+B^+C^+D^+$); (D) Δ*tatC*, *cat* removed (LS106); (E) Δ*tatC* pLP198 ($tatA^+B^+C^+$); (F) wild-type Sal18.

($tatA^+B^+C^+D^+$), LS106/pLP198 ($tatA^+B^+C^+$), and LS106/pLP199 ($tatA^+B^+C^+D^+$) recovered the wild-type phenotype; their swimming zone diameters were 28 mm, 28 mm, 27 mm, and 27 mm, respectively. The results are presented in Fig. 2. These data indicate that inactivation of *tatB* and *tatC* has an impact on cell motility. They also demonstrate that the insertion of an antibiotic cassette did not interfere with the expected phenotype, and therefore, it is unlikely that a polar effect on *tatD* or genes located downstream caused the altered motility in the mutated strain. Moreover, our data also show that *tatD* does not play an essential role for the functionality of the Tat system, since complementation of Δ*tatB* or Δ*tatC* with an episomal copy of the *tatA*, *tatB*, and *tatC* genes was enough to restore the wild-type phenotype.

**The Tat system does not influence the expression of curli.** Curli are proteinaceous, highly aggregative extracellular fibers present on the surfaces of enteric bacteria and are involved in biofilm formation and host colonization (4, 26, 27, 62). In theory, curli-mediated adhesion or motility may affect the invasiveness of *Salmonella* Enteriditis. In order to determine if the Tat system has an effect on the biogenesis of curli, the wild-type Sal18 strain (LS25) and the mutants LS37 (Δ*tatB*) and LS55 (Δ*tatC*) were tested for their abilities to bind Congo red dye. We were not able to observe any differences in the expression of curli on the samples tested.

**The *tatB* and *tatC* mutants show increased sensitivity to EDTA.** *Salmonella* Enteriditis strains Sal18, LS25 (WT *att*Tn*7*::*cat*), LS37 (Δ*tatB*), LS39 (Δ*tatB*), and LS106 (Δ*tatC*) and *E. coli* strain B1LK0 (*tatC*) were tested for their sensitivities to EDTA. The Δ*tatB* strain with the presence or absence of the antibiotic cassette was tested as a control to rule out any polar effect caused by the antibiotic cassette insertion in the bacterial chromosome. Filter paper discs containing different concentrations of EDTA (0.5 M, 0.1 M, 0.02 M, and 0.004 M) were placed on LB agar seeded with the different strains. The results are summarized in Table 3. We observed that *E. coli* strain B1LK0 (*tatC*) was more sensitive to EDTA than the Δ*tatB* (LS37, LS39) and Δ*tatC* (LS106) derivatives of *Salmonella* Enteritidis. However, we did not observe substantial differences in growth inhibition among strains LS37, LS39, and LS106.

TABLE 3. Phenotypic traits of *S*. Enteriditis wild-type, mutant, and complemented mutant strains

| Strain | Relevant genotype | Plasmid-encoded *tat* genes | Cell morphology | Inhibition[a] by EDTA at: | | | Albomycin sensitivity[b] |
|---|---|---|---|---|---|---|---|
| | | | | 0.5 M | 0.1 M | 0.02 M | |
| Sal18 | WT | No | Normal | T (2.7) | T (1.3) | | S (3.5) |
| LS25 | WT *att*Tn*7*::*cat* | No | Normal | T (2.7) | ND | ND | S (3.5) |
| LS37 | Δ*tatB*::*cat* | No | Elongated | C (2.0) | C (1.5) | C (1.0) | R |
| LS39 | Δ*tatB* | No | Elongated | C (2.7) | C (2.1) | T (1) | R |
| LS107 | Δ*tatB* | $tatA^+B^+C'$ | ND[c] | ND | ND | ND | (R) |
| LS108 | Δ*tatB* | $tatA^+B^+C^+$ | Normal | T (2.7) | T (2.1) | T (1.2) | S (3.2) |
| LS114 | Δ*tatB* | $tatA^+B^+C^+D^+$ | Normal | T (2.7) | T (2.1) | T (1.2) | S (3.2) |
| LS106 | Δ*tatC* | No | Elongated | C (3.1) | C (2.3) | T (1) | R |
| LS117 | Δ*tatC* | $tatA^+B^+C^+$ | Normal | T (2.7) | T (2.1) | T (1.2) | S (3.2) |
| LS118 | Δ*tatC* | $tatA^+B^+C^+D^+$ | Normal | T (2.7) | T (2.1) | T (1.2) | S (3.2) |

[a] C, clear inhibition zone; T, turbid, weak inhibition zone. Numbers in parentheses are diameters of the inhibition zone (in centimeters).
[b] R, resistant; (R), partially resistant; S, sensitive. Numbers in parentheses are diameters of the clear inhibition zone (in centimeters).
[c] ND, not determined.

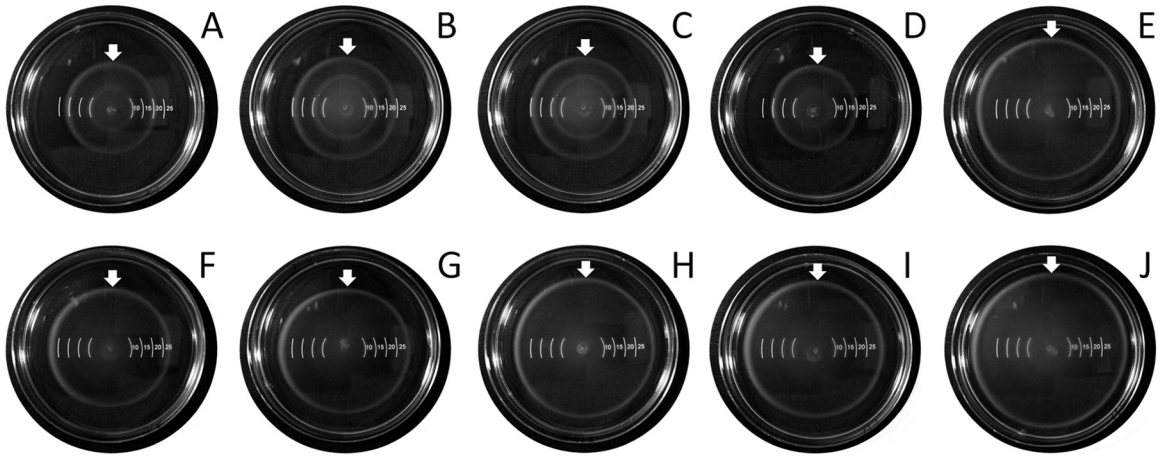


FIG. 2. Swimming motility results for wild-type *Salmonella* Enteritidis Sal18 and the different mutant derivatives and plasmid-complemented mutants in 0.3% agar after 6 h of incubation. (A) Δ*tatB*::*cat* (LS37); (B) Δ*tatB*, *Cm* removed (LS39); (C) Δ*tatC*::*cat* (LS55); (D) Δ*tatC*, *cat* removed; (E) Sal18 *att*Tn*7*::*cat* (LS25); (F) Δ*tatB* pLP198 ($tatA^+B^+C^+$); (G) Δ*tatB* pLP199 ($tatA^+B^+C^+D^+$); (H) wild-type Sal18; (I) Δ*tatC* pLP198 ($tatA^+B^+C^+$); (J) Δ*tatC* pLP199 ($tatA^+B^+C^+D^+$). Arrows point to the maximum radius of the swimming zone.

**Sensitivity to SDS.** Strains LS25 (wild type) and LS37 (Δ*tatB*) were tested for their sensitivities to SDS by two different approaches. In the first approach, the wild-type and Δ*tatB* strains were seeded in LB agar plates, and growth inhibition around the paper filter discs containing either 5 or 10% SDS was assessed. With both concentrations, we observed a more pronounced inhibition zone around the SDS-soaked discs on the plates seeded with the Δ*tatB* strain than on those seeded with the wild type. However, with both strains, some isolated colonies were observed within the halo of inhibition. In the second approach, the strains were grown in LB broth containing 5% SDS, and the absorbance ($OD_{600}$) was measured at every hour for 7 h after inoculation. Both strains grew exponentially until 4 to 5 h. After that, the absorbance ($OD_{600}$) decreased (Fig. 3). To further understand if there was a drop in bacterial viability, we plated the cells onto LB agar plates and examined them by microscopy. The Δ*tatB* bacterial cells formed aggregates, which is most likely the reason for the lower absorbance found with the Δ*tatB* mutant culture, but we did not find drastically reduced viability for the Δ*tatB* or wild-type strains when plated (after vigorous vortexing of the culture). Both the Δ*tatB* strain and the wild-type strain grew normally in the absence of SDS (Fig. 3) without forming aggregates.

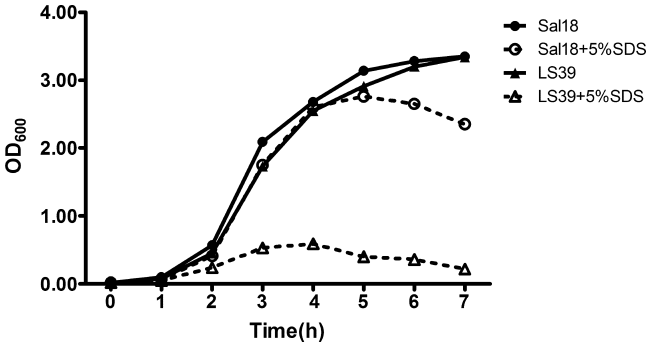


FIG. 3. Wild-type *Salmonella* Enteritidis Sal18 and LS37 (Δ*tatB*::*cat*) grown in LB broth without (solid lines) and with (dashed lines) 5% SDS. The absorbance was measured every hour after bacterial inoculation.

**Mutants lacking a functional Tat system are resistant to the antibiotic albomycin.** In the course of this study, we have developed a fast plate assay in our laboratory to determine if the Tat system is functional. Several years ago, the group of T. Palmer demonstrated that the *E. coli* FhuD protein is a substrate for the twin-arginine transport system (32). Our test is based on two observations: first, the translocation of the ferric-hydroxamate binding protein FhuD into the periplasm is strictly dependent on a functional Tat system; second, in order to be active, the antibiotic albomycin, which has structural homology to siderophores of the hydroxamate type, needs to be imported into the cytoplasm via an ABC transporter with FhuD as the periplasmic component. As a consequence, mutants with deletions in the *tatB* and *tatC* genes, which are unable to export FhuD into the periplasm, should be resistant to this antibiotic. This hypothesis was tested on LB agar plates with *E. coli* B1LK0 (*tatC*), which was resistant to albomycin, whereas the control strain, MC4100, displayed a zone of growth inhibition around a filter paper disc impregnated with albomycin, as outlined in Materials and Methods.

*Salmonella* Enteritidis strains Sal18 (wild type), LS37 (Δ*tatB*::*cat*), LS39 (Δ*tatB*, *cat* removed), and LS106 (Δ*tatC*, *cat* removed) were subjected to the albomycin sensitivity assay. The wild-type strain presented a clear halo of growth inhibition with a diameter of 7 mm around the impregnated filter paper disc. In contrast, the Δ*tatB* and Δ*tatC* mutants showed cell growth right up to the antibiotic-containing disc, demonstrating resistance to albomycin. Plasmid pLP196, expressing wild-type TatA and TatB and a C-terminally truncated TatC protein, mediated only a partial phenotypic complementation of the Δ*tatB* strain. Plasmids pLP198 and pLP199, which harbor the functional *tatABC* genes and the complete *tatABCD* operon, respectively, restored the wild-type phenotype in all *tatB* and *tatC* mutants (except for the *E. coli tatC* mutant, which

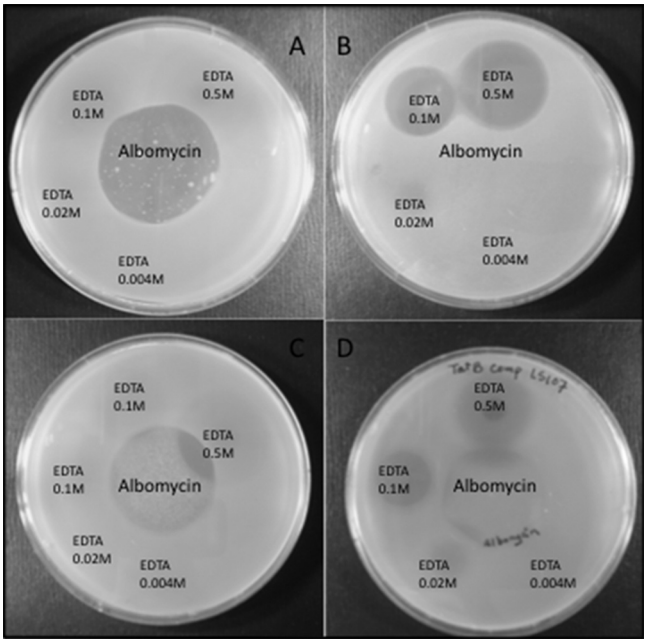


FIG. 4. Albomycin and EDTA sensitivity assay results. The center of the plate was loaded with albomycin surrounded by EDTA with concentrations of 0.5 M, 0.1 M, 0.02 M, and 0.004 M in the peripheries of the plates. The plates were seeded with either Sal18 (A), LS39 (Δ*tatB*) (B), LS39 (Δ*tatB*) plus pLP199 (*tatA*$^+$*B*$^+$*C*$^+$*D*$^+$) (C), or LS39 (Δ*tatB*) plus pLP196 (*tatA*$^+$*B*$^+$*C′* [truncated *tatC*]) (D).

remained albomycin resistant) (Table 3; Fig. 4). All phenotypic analysis is summarized in Table 3.

**A functional Tat system is important for invasion of polarized Caco-2 cells *in vitro*.** In order to assess their cell invasion capabilities, strains LS29 (WT *att*Tn*7*::*tet*) and LS37 (Δ*tatB*::*cat*) were used to infect polarized Caco-2 cells. In addition, the reference strain LS21 (ΔSPI-1::*cat*), previously used by our group and known to be defective in Caco-2 cell invasion (18), was used as an internal control to demonstrate that the assay was working as anticipated. *E. coli* DH5α, which is not able to invade cells, was used as a negative control in each experiment. In the single-infection experiments, the invasiveness of each strain was calculated as a percentage of the wild-type strain invasion. The Δ*tatB* mutant had an invasiveness of 23.23% of the wild-type strain invasion in polarized Caco-2 cells, while the ΔSPI-1 strain had an invasiveness of 4.82% of the wild-type strain invasion (Fig. 5). As another control of the experiment, the Δ*tatB* strain complemented with either plasmid pLP198 (*tatA*$^+$*B*$^+$*C*$^+$) or plasmid pLP199 (*tatA*$^+$*B*$^+$*C*$^+$*D*$^+$) was also tested and presented an invasiveness of 89.71% (Fig. 5) or 136% of the wild-type strain invasion.

A competitive assay was performed in which cells were coinfected with the Δ*tatB*, Δ*tatC*, or ΔSPI-1 mutant strain and the wild-type strain at a 1:1 ratio. In this type of assay, the wild-type Sal18 strain functions as an internal control, thus minimizing variations between assays and providing more-reproducible results (69). A CII (competitive index in invasion) was calculated as previously described (69). We observed CIIs of 0.010, 0.143, and 0.210 for the groups coinfected with the wild

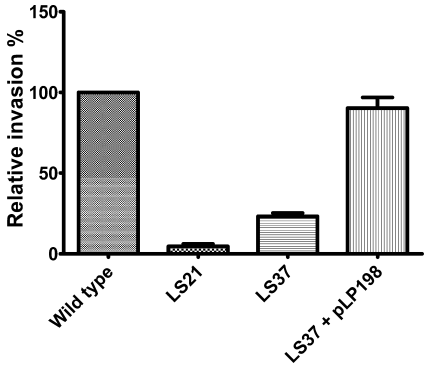


FIG. 5. Single infection of the Caco-2 polarized cell line with wild-type *S.* Enteritidis Sal18, LS37 (Δ*tatB*::*cat*), or LS37 (Δ*tatB*::*cat*) complemented with plasmid pLP198 harboring the *tatABC* genes. The ΔSPI::*cat* strain was used as a control. The invasiveness of the mutant strains is measured as a percentage of that of the wild-type strain. Each bar corresponds to an average of three experiments performed in triplicate.

type and the ΔSPI-1, Δ*tatB*, or Δ*tatC* strain, respectively (Fig. 6). The CII of each of the three different groups was statistically significantly different from 1 ($P$, ≤0.003).

**A functional Tat system is important for effective systemic spread of *Salmonella* Enteritidis in chickens.** In order to study the impact of the Tat system on the ability of *Salmonella* Enteritidis to colonize the intestines of chickens and invade their internal organs, 4-day old and 7-day old Leghorn chickens were challenged with $5 \times 10^9$ CFU of either LS25 (wild-type Sal18 *att*Tn*7*::*cat*) or LS37 (Δ*tatB*::*cat*). When cecal colonization was studied in birds challenged at the age of 4 days, no statistically significant difference was observed regardless of the time (postchallenge) of sampling ($P$, >0.05) (Fig. 7A).

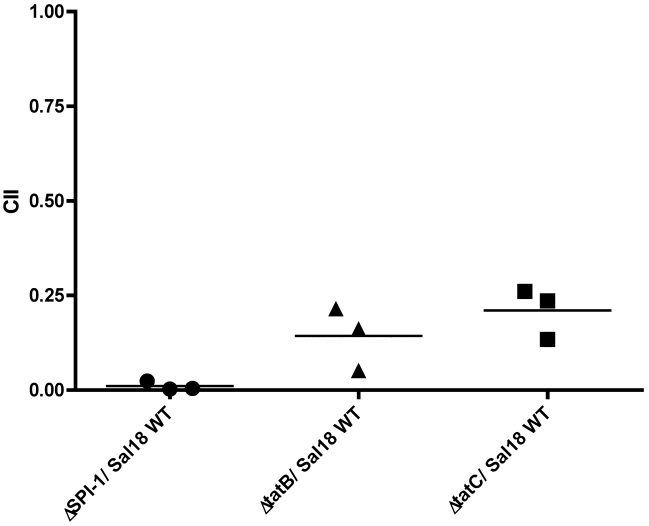


FIG. 6. Coinfection of polarized Caco-2 cells with the wild-type strain LS29 (Sal18 *att*Tn*7*::*tet*) and derivatives LS37 (Δ*tatB*::*cat*) and LS55 (Δ*tatC*::*cat*) using antibiotic markers to distinguish the strains. Values significantly different from 1 indicate impaired cell invasion. Strain LS21 (ΔSPI-1::*cat*) was used as a control. The values for each strain correspond to averages of three experiments performed in triplicate.

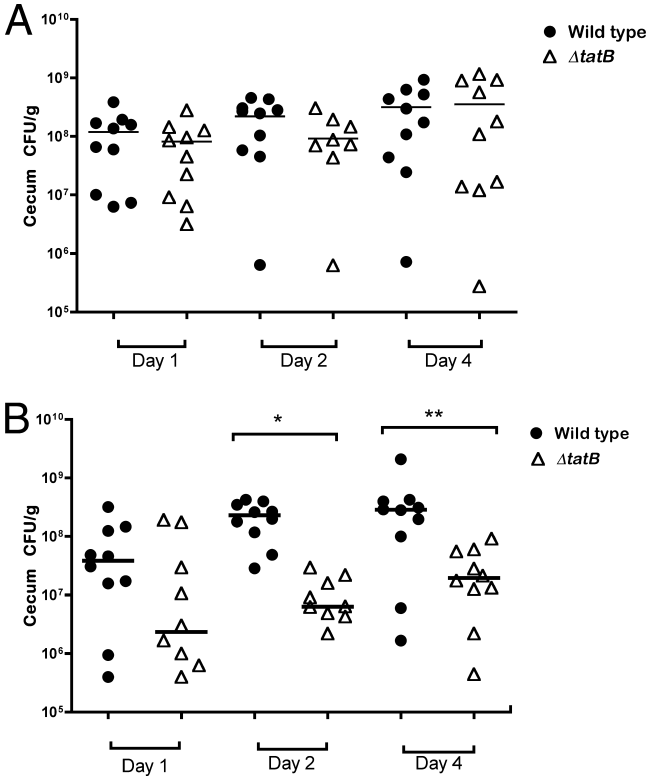


FIG. 7. Direct bacterial counts (as determined after plating on Brilliant Green Agar plates and incubation for 24 h at 37°C) in cecal contents on days 1, 2, and 4 postchallenge from birds challenged at the age of 4 days (A) or 7 days (B). Horizontal bars represent median values. Each symbol represents the CFU obtained from samples of an individual bird. The single asterisk indicates a significant statistical difference between the group challenged with wild-type LS29 (Sal18 *att*Tn*7*::*tet*) and that challenged with the Δ*tatB*::*cat* strain on day 2 ($P < 0.0001$); the double asterisk indicates a statistical difference between the two groups on day 4 ($P = 0.01$).

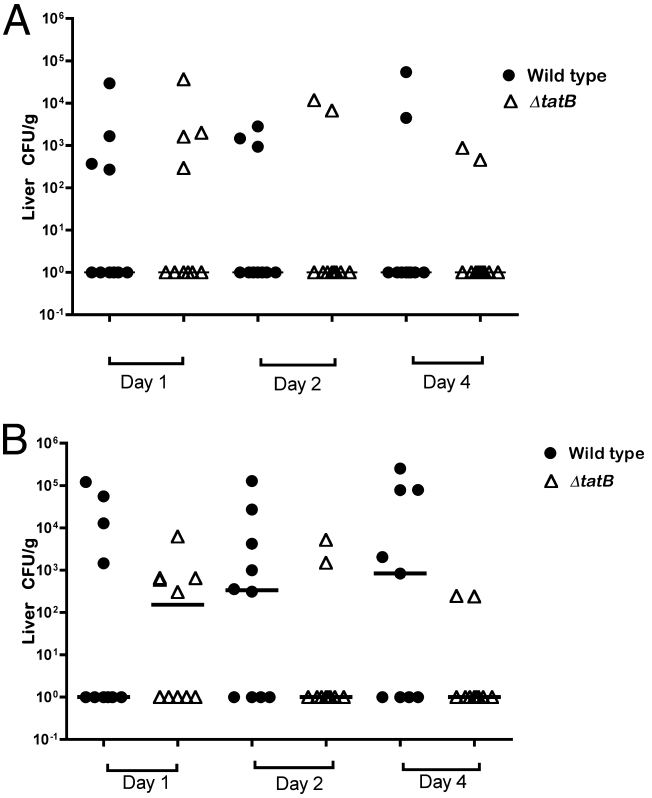


FIG. 8. Direct bacterial counts (as determined after plating on Brilliant Green agar plates and incubation for 24 h at 37°C) in the liver on days 1, 2, and 4 postchallenge from birds challenged at the age of 4 days (A) or 7 days (B). Horizontal bars represent median values. Each symbol represents the CFU obtained from samples of an individual bird. There is a trend for the group challenged with LS37 (Δ*tatB*::*cat*) to clear the infection faster on days 2 and 4 (*P*, 0.11 and 0.05, respectively).

However, when the experiment was repeated with 7-day-old birds, there was a statistically significant difference between colonization of the cecum by the wild type and that by the Δ*tatB* mutant on day 2 (*P*, <0.0001) and day 4 (*P*, 0.01) postchallenge (Fig. 7B), as determined by direct counts.

Initially, systemic infection was assessed by diluting the homogenized organ samples and plating for viable cell counts without enrichment. No statistically significant difference between the abilities of the wild-type Sal18 and Δ*tatB* mutant strains to colonize the livers of birds challenged at the age of 4 or 7 days was observed when the birds were sampled on day 2 or day 4 postchallenge (Fig. 8A and B). However, there appeared to be a trend for the Δ*tatB*-challenged group to clear the systemic infection faster, as demonstrated by the lower bacterial counts and the lower number of infected birds sampled on day 2 or day 4 postchallenge in both trials (Fig. 8A and B). Regarding the analysis of bacterial counts from the spleen, there was a statistically significant difference between the group challenged at the age of 7 days with the wild-type strain and the group challenged with the Δ*tatB* mutant when the birds were sampled on day 4 postchallenge (*P*, 0.004) (Fig. 9B). In addition, in birds challenged at the age of 7 days and sampled 2 days postchallenge. and in birds challenged at the age of 4 days and sampled 4 days after challenge, there was a trend for the Δ*tatB* mutant to be present in lower numbers than the wild-type strain in the spleens of the birds challenged on the same days (*P*, 0.08 and 0.10, respectively) (Fig. 9A and B). Systemic infection was also assessed after enrichment of liver and spleen samples in selenite broth. Throughout the course of the trial, in birds challenged at the age of 4 days, the number of spleens positive for the wild-type bacteria increased considerably, while the number of positive spleens in birds challenged with the Δ*tatB* mutant tended to decrease slightly (Fig. 10B). With respect to systemic spread, after enrichment of samples, the birds challenged at the age of 7 days showed a statistically significant difference between the group that received the wild-type bacteria and the group that received the Δ*tatB* mutant when sampled at 2 or 4 days postchallenge (*P*, 0.003 and 0.02) (Fig. 11). This was also observed for birds challenged at the age of 4 days and sampled 4 days postchallenge (*P*, 0.03) (Fig. 10). Taken together, these data indicated that the absence of a functional Tat system plays a role in *Salmonella* Enteritidis cecum colonization and is also important for effective and prolonged systemic infection of chickens.

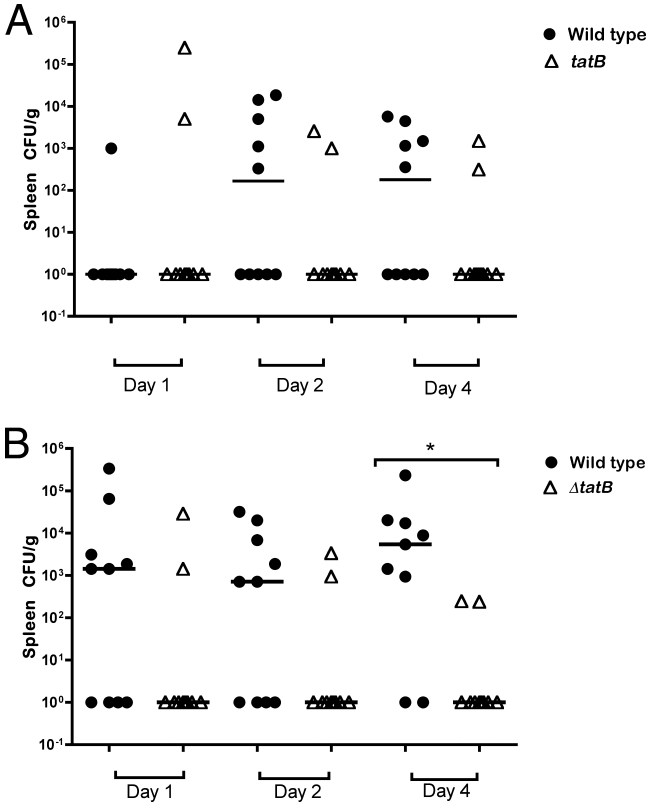


FIG. 9. Direct bacterial counts (as determined after plating on Brilliant Green Agar plates and incubation for 24 h at 37°C) in the spleen on days 1, 2, and 4 postchallenge from birds challenged at the age of 4 days (A) or 7 days (B). Horizontal bars represent the median values for different birds. There was a trend for the group challenged with LS37 (Δ*tatB*::*cat*) at the age of 4 days to clear the infection faster ($P$ = 0.10). Each symbol represents the CFU obtained from samples of an individual bird. The asterisk indicates a significant statistical difference between the groups challenged with wild-type LS29 (Sal18 *att*Tn7::*tet*) and LS37 (Δ*tatB*::*cat*) on day 4 ($P$ = 0.004). The group challenged with LS37 (Δ*tatB*::*cat*) at the age of 7 days presented a trend to clear systemic infection faster on day 2 ($P$ = 0.08).

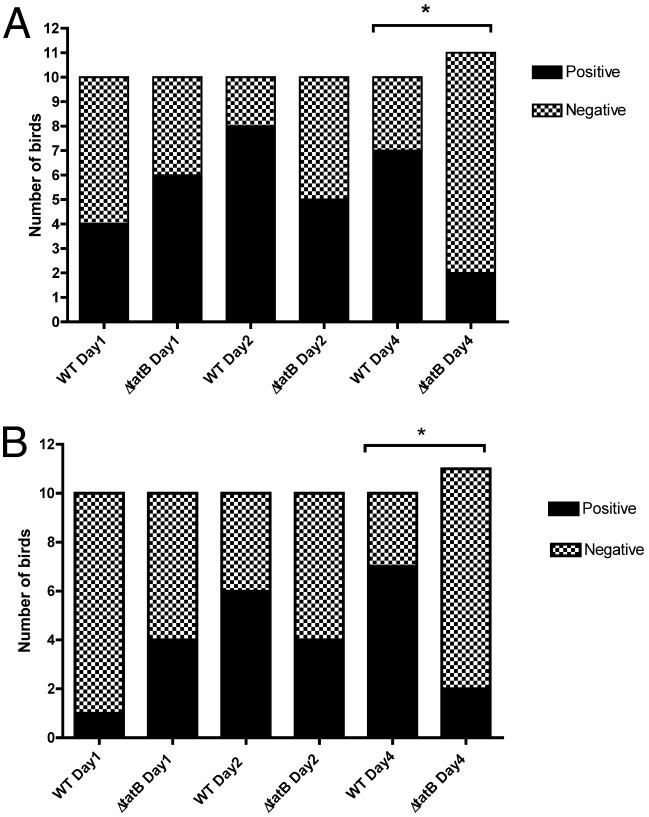


FIG. 10. (A) Enrichment results for livers and spleens from chickens challenged at the age of 4 days. The asterisk represents a significant statistical difference between the groups challenged with wild-type LS29 (Sal18 *att*Tn7::*tet*) versus LS37 (Δ*tatB*::*cat*) on day 4 postchallenge ($P$ = 0.03). (B) Enrichment results for spleens from chickens challenged at the age of 4 days. The asterisk represents a significant statistical difference between the groups challenged with wild-type LS29 (Sal18 *att*Tn7::*tet*) versus LS37 (Δ*tatB*::*cat*) on day 4 postchallenge ($P$ = 0.03).

## DISCUSSION

There has been a sustained interest in the identification of potential virulence factors in *Salmonella* Enteritidis in order to develop more-effective strategies to control this pathogen. The Tat system is a specialized secretion pathway in prokaryotes that is responsible for the transport of certain proteins in a folded conformation across the cytoplasmic membrane (7, 53). There are several reports demonstrating a link between the Tat system and virulence in various pathogens, such as *E. coli* O157, *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Agrobacterium tumefaciens* (12, 19, 25, 43, 51, 56). However, to our knowledge, there are no reports concerning the role of the *Salmonella* Enteritidis Tat system in infection and virulence. In this study we investigated that relationship.

The morphological changes in the *S.* Enteritidis Δ*tatB* and Δ*tatC* strains, including an elongated shape and chain formation, were similar to those observed in a previously characterized *E. coli tatC* mutant strain. The change in morphology could not be attributed to the antibiotic cassette present in both the Δ*tatB* (LS37) and the wild-type (LS25) strain, since the wild-type strain had the normal cell morphology. Further support for this conclusion was found in the observation that both Δ*tatB* mutants showed the same phenotype, independently of the presence or absence of the antibiotic cassette (LS37 and LS39). It has been shown previously that a disturbed Tat pathway can affect cell morphology, growth and biofilm formation (29), and cell wall integrity (19, 60). Cell wall amidases are responsible for cleaving the murein septum during cell division (30, 31), and a previous study of *E. coli tatABC* mutants demonstrated that the mislocalization of the cell wall amidases AmiA and AmiC led to the same morphotype as that observed in our study (33, 67). We did not specifically investigate the targeting of the cell wall amidases, but we found that AmiA, AmiC, and SufI, which is also assigned to play a role in cell division (66), were predicted as Tat substrates when we applied the TatP and TatFind programs to the *S.* Enteritidis genome.

The Δ*tatB* (LS37) strain was more sensitive to SDS than the wild-type strain when tested on LB plates and showed reduced growth compared to that of the wild-type strain in LB medium

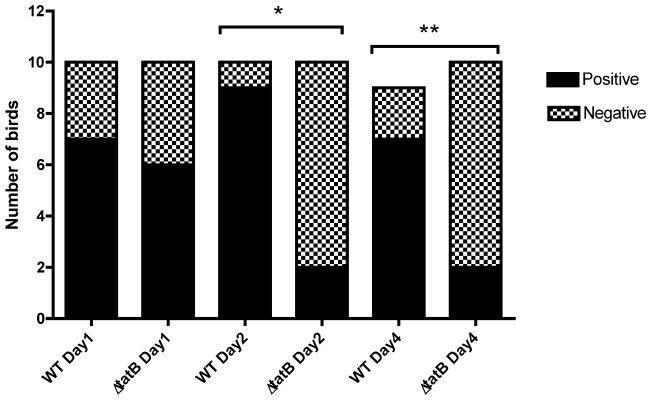


FIG. 11. Enrichment results for livers and spleens from chickens challenged at the age of 7 days. Asterisks represent significant statistical differences between the groups challenged with wild-type LS29 (Sal18 *att*Tn*7*::*tet*) versus LS37 (Δ*tatB*::*cat*) on days 2 (*) ($P = 0.003$) and 4 (**) ($P = 0.02$) postchallenge.

supplemented with 5% SDS (Fig. 3). Our results are in agreement with a previous report on *E. coli* that demonstrated the hypersensitivity of Δ*tatB* and Δ*tatC* mutant strains to SDS, although *Salmonella* Enteritidis was clearly less sensitive to SDS than *E. coli*. We also tested the different strains for EDTA sensitivity. While the wild-type *Salmonella* Enteritidis was resistant to EDTA, LS37 (Δ*tatB*::*cat*), LS39 (Δ*tatB* Δ*cat*), and LS106 (Δ*tatC* Δ*cat*) were sensitive proportionally to the EDTA concentration. The divalent cations $Ca^{2+}$ and $Mg^{2+}$ are important for the stability of the outer membrane; EDTA acts as a chelator of these cations, which in turn may affect the integrity of the outer membrane. The Δ*tatB* and Δ*tatC* mutants may be sensitive due to a compromised outer membrane structure. These results also demonstrated that the insertion of the chloramphenicol cassette did not influence the final phenotype, since the Δ*tatB* mutants with or without the chloramphenicol cassette were equally sensitive to EDTA. In addition, we also observed that *E. coli* strain B1LK0 (*tatC*) was more sensitive to EDTA than the *S.* Enteritidis *tat* mutants. This result is in agreement with a previous study that reported that this strain was hypersensitive to SDS (70).

In order to display its inhibitory activity, albomycin needs to be imported into the cytoplasm of bacteria and subsequently to be cleaved by peptidase N. One moiety of the molecule is the active part (thioribosyl pyrimidine derivative) of the antibiotic, whereas the remaining domain shows structural similarity to ferrichrome, a siderophore of the hydroxamate type. In *E. coli* and *Salmonella*, albomycin is actively imported by the ferrihydroxamate uptake (*fhu*) system (10, 23). Transport across the outer membrane is mediated by the TonB-dependent receptor FhuA, and the subsequent translocation into the cytoplasm involves FhuD in the periplasm, as well as a complex in the cytoplasmic membrane composed of FhuB and FhuC (11). The periplasmic location of the binding protein FhuD is essential for the uptake of ferric hydroxamates and albomycin (39, 41, 57). Two secretory pathways could be responsible for the export of FhuD into the periplasm: the Sec pathway, also called the general secretory pathway (GSP), which translocates unfolded polypeptide chains, and the Tat pathway, which transports folded proteins. Based on a previous study of *E. coli*, which demonstrated a Tat-dependent periplasmic localization of the ferrihydroxamate binding protein (32), we assumed that FhuD in *Salmonella* Enteritidis would be translocated into the periplasm via the Tat system. However, it was not known if FhuD was also able to enter the Sec pathway as an alternative route. In order to analyze this, we developed an albomycin sensitivity plate assay. Initially, *E. coli* strains MC4100 (wild type) and B1LK0 (MC4100 *tatC*) were tested. As anticipated, the wild type was sensitive and the *tatC* mutant was resistant to albomycin. We also tested the *S.* Enteritidis Δ*tatB* strain (LS37) and a Δ*tatB* strain with the chloramphenicol cassette removed (LS39), as well as the wild-type strain (LS25). The wild type was sensitive, while both *tat* mutants proved resistant to the antibiotic. These results confirm that the phenotype conferred on the LS37 strain was indeed the result of the *tatB* deletion and was not due to polar effects caused by the gene replacement or to the expression of the *cat* gene. More importantly, these data indicate that FhuD uses the Tat system exclusively to reach the periplasm of *Salmonella* Enteritidis. Amino acid sequences resembling the twin-arginine consensus motif were detected in the FhuD proteins of a number of Gram-negative and Gram-positive bacteria (W. Köster, unpublished data). It remains unclear why FhuD is the only known periplasmic siderophore binding protein to be exported through the Tat system. Other members of the siderophore binding protein family do not have an obvious Tat consensus region in their signal sequences, suggesting that they are exported by the general secretion pathway.

In addition, we tested the strains for their abilities to invade polarized Caco-2 cell monolayers. In the single-strain invasion assay, we compared the Δ*tatB* mutant, the ΔSPI-1 mutant, and *E. coli* DH5α with our wild-type *Salmonella* strain. Impaired invasion by the ΔSPI-1 mutant has been described by our group previously and was observed once again (18). The Δ*tatB* mutant also had decreased invasion capability (23.23%) in relation to the *Salmonella* Enteritidis wild-type strain (100%) (Fig. 5), suggesting that this mutation plays a role in Caco-2 cell invasion. To eliminate the variability of the cells within each experiment, polarized Caco-2 cells were coinfected with a Δ*tatB* or Δ*tatC* mutant and the wild-type Sal18 strain. In these competition assays, we observed a sharp decline in invasion by both Tat mutants (LS37 and LS39) (Fig. 6). These results confirm the decrease in invasiveness observed in the original assays. To our knowledge, this is the first time this assay has been used to analyze the invasiveness of Tat system mutants. Cell invasion has been associated with several bacterial factors, including the type 3 secretion system (T3SS), flagella, and fimbriae (18, 42, 54). We speculated that the decreased invasion presented by the Tat mutants could be an effect of the impairment of a phenotypic trait, such as curli-mediated adhesion or motility. Several *Salmonella enterica* serovars express, on their surfaces, thin adhesive fimbrial structures called curli (16). Curli have been shown to be involved in attachment to and invasion of host cells, as well as in biofilm formation, and are therefore considered a virulence determinant (5). We did not observe any differences in curli expression among the Δ*tatB* and Δ*tatC* mutants and the wild-type *Salmonella* strain, suggesting that the Tat system does not interfere with curli assembly. In addition, the Caco-2 invasion assays were performed under conditions (e.g., 37°C) that were not favorable

for optimal expression of curli. These facts eliminate an altered curli phenotype as a possible reason for the impaired invasiveness of Δ*tat* mutants in Caco-2 cells. In many Gram-negative bacteria, including *Salmonella* Typhimurium, motility is important for full virulence, since it enables the bacterium to cross the thick and viscous mucus layer in the intestinal epithelium and promotes the contact of the bacterium with the cell surface (35, 68). The *Salmonella* Enteritidis flagellum is considered a potential virulence factor in poultry (2, 3, 54). In our study, we observed that the Δ*tatB*::*cat* (LS37) and Δ*tatC*::*cat* (LS55) mutants had impaired motility in comparison to the wild-type Sal18 *att*Tn7::*cat* strain (LS25). This is in agreement with previous studies showing that an *E. coli* O157:H7 *tatABC* mutant was nonmotile (56) and that a Δ*tatC* mutant of *Pseudomonas aeruginosa* was less motile than the wild-type strain (51). No proteins involved in flagellar biogenesis that carry Tat signal sequences have been identified (56). Regarding our Caco-2 invasion experiments, Tat system impairment may play a direct or indirect role in the several systems responsible for bacterial invasion, such as SPI-1 and flagella. However, further studies are necessary to confirm this hypothesis.

For all *Salmonella tat* mutants tested in this study, the *tatABC* region expressed on a plasmid was both necessary and sufficient for the restoration of the phenotype to wild type with respect to morphology, motility, sensitivity to EDTA, SDS, and albomycin, and invasive properties in polarized Caco-2 cells. Expression of the complete plasmid-encoded *tatABCD* operon revealed the same effect, indicating that *tatD* does not play a major role in the assembly of the functional Tat system. The partial complementation of the Δ*tatB* mutant by pLP196 could be the result of low-level expression of chromosomally encoded TatC or could be due to a residual function of the plasmid-encoded truncated TatC. Moreover, it is noteworthy that inhibitory effects of over-stoichiometric TatB concentrations have been described previously (66).

Based on these findings, we conclude that the replacements of the chromosomal *tatB* and *tatC* genes by the chloramphenicol resistance cassette (and its subsequent removal) caused polar effects on *tat* genes located downstream, as one would anticipate. However, those effects seem to be restricted to the *tat* operon, and there is no evidence that the observed phenotypes of the *tat* mutant strains (LS37, LS39, LS55, and LS106) are caused by secondary mutations elsewhere in the chromosome.

In order to evaluate the effect of a nonfunctional Tat system on *Salmonella* Enteritidis infection in a poultry model, we challenged 4-day-old and 7-day-old SPF Leghorn chickens with the wild-type Sal18 and Δ*tatB* mutant strains administered via oral gavage. Younger chickens were used because they are more susceptible to *Salmonella* (72). We used a relatively high dose ($5 \times 10^9$ CFU/g) of *Salmonella* Enteritidis to ensure adequate levels of systemic spread by the wild-type bacteria. It is noteworthy that the plating efficiency obtained with Brilliant Green Agar (BGA) is only 40%, in contrast to that for other standard rich media such as LB medium (data not shown), which decreases the levels of *Salmonella* Enteritidis detection in the samples and therefore has an impact on our results. However, if BGA were not used as a selective medium, in many cases—notably cecal content counts—there would be a complete overgrowth of flora. We observed that the level of colonization of the ceca in chickens challenged at the age of 4 days did not differ between the wild-type Sal18- and Δ*tatB* mutant-treated groups on any of the days of sampling. In order to determine if the age of birds at the time of challenge had an effect on colonization, we challenged birds at the age of 7 days. In the older birds, we observed a difference between the group challenged with the wild type and the group challenged with Δ*tatB* bacteria. These differences were statistically significant 2 and 4 days postchallenge, indicating that the Tat system of *Salmonella* Enteritidis may play a role in cecum colonization in chickens (Fig. 7A and B). The ability of *Salmonella* Enteritidis to colonize the cecum and subsequently invade intestinal cells seems to be a synergistic process, since inactivation of single genes of important and recognized virulence systems in bacteria or even entire pathogenicity islands had little or no effect on intestinal colonization by *Salmonella* Enteritidis or other *Salmonella* species (18, 36).

For 4-day-old birds, we analyzed the systemic infection and observed that the spleens of birds challenged with the wild-type Sal18 strain had higher bacterial loads than those of birds challenged with the Δ*tatB* mutant when sampled 4 days postchallenge (Fig. 9A and B). Additionally, there was a tendency of the bacterial counts from the wild-type group to remain somewhat leveled, while the bacterial numbers in the Δ*tatB* group tended to decline throughout the period. Also, the wild-type Sal18 strain infected a higher number of birds than the Δ*tatB* mutant. This trend was confirmed by the systemic colonization enrichment data. Higher numbers of birds challenged at the age of 4 days were positive for the wild-type Sal18 strain on day 4 postchallenge (Fig. 9). In addition, among chicks challenged at the age of 7 days, higher numbers of birds given the wild-type Sal18 strain than of birds given the Δ*tatB* strain were positive on days 2 and 4 postchallenge (Fig. 10). These data indicate that the Tat system is involved in the processing of a protein that takes part in *Salmonella* interaction with the host during systemic spread of the bacterium, since we could clearly observe a delay in systemic infection presented by the Δ*tatB* mutants.

There have been several reports relating to the Tat system and virulence. In a rat model, a *Pseudomonas aeruginosa tatC* mutant did not cause chronic lung infection (51). A *tatC* mutant of *Yersinia paratuberculosis* showed impaired colonization of lymphoid organs in mice (43). Our data indicate that an impaired Tat system reduces cecal colonization, delays systemic infection, and decreases the efficiency of sustained systemic colonization. To our knowledge, this is the first report of the role of the *Salmonella* Enteritidis Tat system in both cecal colonization and systemic spread and persistence in chickens. These data provide evidence that the Tat pathway is important in virulence in yet another bacterial species and animal model. Since no obvious Tat-translocated virulence candidates were found with the help of the TatP and TatFind analyses, the exact mechanism by which a nonfunctional Tat system might interfere with cell adhesion, invasion, and survival in a host organism remains to be elucidated.

In conclusion we have demonstrated that a functional Tat pathway is essential for optimal cell division, motility, FhuD-mediated iron acquisition, and cell wall integrity in *Salmonella* Enteriditis. The Tat system plays an important role in the invasiveness of *Salmonella* Enteriditis for polarized Caco-2

cells, in the colonization of the cecum, and in sustaining systemic colonization in chickens.

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