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Molecules and Cells

A *tdcA* Mutation Reduces the Invasive Ability of *Salmonella enterica* Serovar Typhimurium

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We previously observed that the transcription of some flagellar genes decreased in *Salmonella* Typhimurium *tdcA* mutant, which is a gene encoding the transcriptional activator of the *tdc* operon. Since flagella-mediated bacterial motility accelerates the invasion of *Salmonella*, we have examined the effect of *tdcA* mutation on the invasive ability as well as the flagellar biosynthesis in *S.* Typhimurium. A *tdcA* mutation caused defects in motility and formation of flagellin protein, FliC in *S.* Typhimurium. Invasion assays in the presence of a centrifugal force confirmed that the defect of flagellum synthesis decreases the ability of *Salmonella* to invade into cultured epithelial cells. In addition, we also found that the expression of *Salmonella* pathogenicity island 1 (SPI1) genes required for *Salmonella* invasion was down-regulated in the *tdcA* mutant because of the decreased expression of *fliZ*, a positive regulator of SPI1 transcriptional activator, *hilA*. Finally, the virulence of a *S.* Typhimurium *tdcA* mutant was attenuated compared to a wild type when administered orally. This study implies the role of *tdcA* in the invasion process of *S.* Typhimurium.

INTRODUCTION

In *Escherichia coli*, the anaerobically-regulated *tdcABCDEFG* operon is implicated in the transport and metabolism of L-threonine and L-serine (Goss et al., 1988; Sawer, 1998). The *tdc* operon is composed of one regulatory gene, *tdcA,* and six structural genes, *tdcB* to *tdcG*. The expression of the *tdc* operon is very complex and is affected by at least five transcription factors including the cyclic AMP (cAMP) receptor protein (CRP), integration host factor (IHF), histone-like protein (HU), and the operon-specific regulators TdcA and TdcR (Wu and Datta, 1995; Wu et al., 1992). TdcA is a member of the LysR family of transcription factors, which contain a helix-turn-helix DNA-binding motif (Guaduri et al., 1993). TdcR, which is located upstream of the *tdc* operon and is transcribed in the opposite orientation, encodes a small protein that is essential for the efficient transcription of the *E. coli tdc* operon (Hagewood et al., 1994; Schweizer and Datta, 1989). Sequence analysis has shown that the sequence of the *tdc* operon of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is about 80% identical to that of *E. coli,* with the exception of *tdcF* and *tdcR*, which are absent from the *Salmonella tdc* operon (Kim et al., 2008). Maximum induction of the *tdc* operon is seen when *E. coli* are grown under anaerobic conditions (Heßlinger et al., 1998; Sawer, 1998), while the expression of the *Salmonella tdc* operon is dramatically increased only during the transition from aerobic growth to anaerobic conditions, even though the *Salmonella tdc* operon lacks the *tdcR* gene (Kim et al., 2008).

Flagellar biogenesis in *S. enterica* is controlled by a regulatory cascade, which is initiated by the production of FlhDC. These regulatory proteins induce expression of the class 2 flagellar genes, including *fliA*, which encodes an alternative sigma factor required for transcription of the class 3 flagellar genes (Chilcott and Hughes, 2000; Soutourina and Bertin, 2003). The bacterial flagellum is the most important organelle of motility in bacteria and plays a key role in many bacterial lifestyles, including virulence. Especially, the role of flagella in *S.* Typhimurium invasiveness has been extensively studied *in vitro* and *in vivo*. *S.* Typhimurium strains carrying a null mutation in the flagellar genes were less able to attach and enter various cultured epithelial cells (Jones et al., 1992), and had a reduced capacity to get near the intestinal epithelium in an oral infection model (Schmitt et al., 2001; Stecher et al., 2004). Therefore, flagella seem to play a role mostly in the initial phases of the infection and are not needed later once the infection has been established (Josenhans and Suerbaum, 2002). Interestingly, we have previously found that the expression of flagellar genes such as *fljA*, *fljB*, and *flgN* was reduced two- to three-fold by the *tdcA* mutation by using a cDNA microarray analysis (Kim et al., 2008).

Here, we investigated the effect of a mutation in the *tdcA* gene encoding the transcriptional activator of the *tdc* operon on entry into epithelial cells and virulence of *Salmonella* Typhimurium as well as flagellar gene expression and found that *tdcA* mutation reduced the invasive ability of *S.* Typhimurium.

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Table 1. The bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
SL1344	wild type serovar Typhimurium *xyl rpsL hisG*	Lab stock
AK01	SL1344 *flhD*$^+$ Φ(*flhD*::*lacZY*) integrant	Kelly et al. (2004)
EE658	SL1344 *hilA*:: Tn*5lacZY*, Tet^R	Bajaj et al. (1996)
EE639	SL1344 *invF*::Tn*5lacZY*, Tet^R	Bajaj et al. (1996)
SR3501	SL1344 Δ*tdcA*	Kim et al. (2008)
SR3553	SR3501 Φ(*flhD*::*lacZY*)	This study
SR3574	SR3501 *invF*::Tn*5lacZY*	This study
SR3578	SR3501 *hilA*:: Tn*5lacZY*	This study
Plasmids		
pACYC184	cloning vector, Cm^R Tet^R p15A	Lab stock
pMJ-2	pACYC184 with *tdcA* and *tdcA* promoter	This study

MATERIALS AND METHODS

Bacterial growth conditions

Bacteria were routinely cultivated at 37°C in LB broth overnight with shaking and used as seed cultures at a 1:100 dilution. The cells were grown with aeration to the exponential phase for 2 h. This culture was then subjected to an anaerobic shock, i.e., static culture conditions for 30 min, to maximally induce *tdcA* expression (Kim et al., 2008). For anaerobic culture conditions, the cells were grown to the exponential phase for 4 h without shaking in a 15 ml Falcon tube containing 15 ml of LB. Antibiotics at the following concentrations were used when necessary: kanamycin 50 μg ml^{-1}, chloramphenicol 25 μg ml^{-1}.

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *S.* Typhimurium SL1344 was used as a wild type strain in this study. Chromosomal *lacZ* fusion strains of SR3501 were obtained through P22HT-mediated transduction (Lim et al., 2002; Maloy et al, 1996). The plasmid pMJ-2 for overexpression of *tdcA* was constructed by inserting the DNA fragment containing the *tdcA* promoter and structural region into the *Hin*dIII and SphI sites of the pACYC184 plasmid. An approximately 1,450-bp PCR fragment of the *tdcA* region was amplified using tdcA-com(F) (5′-gaa gtg aaa agc ttt cga ttt ctt tca-3′) and tdcA-com(R) (5′-ctg cgt ctg cat gca tta tat gat gaa ta-3′), which introduced the HindIII and SphI sites (underlined), respectively. The clone was verified by DNA sequencing.

Motility assay

Bacteria were subjected to an anaerobic shock, and a 10 μl aliquot of each cell culture was spotted onto semi-solid (0.35%) LB agar plates which were then incubated at 37°C. The diameter of the growth halo was measured after 7 h and 24 h on three different LB agar plates for each strain.

Analysis of protein secretion

The supernatants of cultures were prepared and the analysis of secreted proteins was performed as previously described (Komoriya et al., 1999; Song et al., 2004). Bacterial cultures were grown with aeration for 2 h and then subjected to an anaerobic shock, and an equivalent amount of sample was obtained from the supernatant of each strain. The proteins in the resulting cell-free supernatant were precipitated with trichloroacetic acid and separated by 10% SDS PAGE. FliC was identified by tandem mass spectra obtained by LTQ ion trap mass spectrometer (Thermo Finnigan) with the search programs SEQUEST. Images were taken with the ChemiPro system equipped with a high-resolution CCD camera (Roper scientific) and the mean values of signal intensity of FliC were then analyzed using MetaMorph image analysis software version 7.0 (Universal Imaging Corp., USA).

β-galactosidase assay

S. Typhimurium strains containing the chromosomal *lacZ* transcriptional fusion were assayed by determining β-galactosidase activity according to the standard method (Maloy et al., 1996).

Invasion assay

Invasion assay was performed as described previously (Lim et al., 2005; 2007). Briefly, HEp-2 epithelial cells (2×10^5) were cultured in 24-well plates for 24 h prior to infection. Bacterial cultures were applied onto the cell monolayer at a multiplicity of infection (MOI) of about 10 to 1. When indicated, a mild centrifugal force (500 × *g* for 5 min) was applied to the 24-well tissue culture plates at the start of the 2-h infection period.

Real-time PCR analysis

Total RNA was prepared as described previously (Choi et al., 2007; Lim et al., 2006). *Salmonella* strains were grown to *tdcA* inducing condition and the total RNA was isolated by using an RNeasy® mini kit (Qiagen). For exclusion of contaminated genomic DNA, the total RNA was treated with the TURBO DNA-free Kit (Ambion) according to the manufacturer's instructions. For RT-PCR analysis, cDNA was synthesized using the Omniscript Reverse Transcription Kit (Qiagen) and random hexamers (Invitrogen). Quantification of cDNA was carried out using 2XiQ SYBR green Supermix (Bio-Rad), and real-time amplification of PCR product was analyzed by using iCycler real-time detection system. A relative amount of cDNA was calculated by using a standard curve obtained from PCR on serially diluted genomic DNA as templates. The mRNA expression level of the target gene was normalized to the 16S rRNA expression level. The sequences of the primers used are as follows; fliZF (5′-ccg ctg aag gtg taa tgg at-3′) and filZR (5′-ccg cat tta ata acc cga tg-3′) and rrsF (5′-cgg gga gga agg tgt tgt g-3′) and rrsR (5′-cag ccc ggg gat ttc aca tc-3′).

Animal experiments

Six-week-old female BALB/c mice were purchased from the

Institute of Laboratory Animal Resources at Seoul National University. Bacterial cultures were grown to the stationary phase in LB medium, and suspended in sterile PBS for infections. Groups of five mice were infected by oral injection with ~10^7 *Salmonella* cells in 100 μl of PBS. Water and food were withdrawn 8 h before infection and were provided again at 2 h post infection (p.i.). Mice were monitored for death for up to 3 weeks. For comparison of survival curves, the statistical analysis was performed using Graphpad Prism, version 4.0.

RESULTS

Inactivation of *tdcA* affects flagella biosynthesis and motility

We found that the expression of genes involved in flagellar biosynthesis decreased in a *tdcA* deletion mutant compared to a wild type (Kim et al., 2008). To further investigate this phenotype, first, the effect of *tdcA* on the motility phenotype was surveyed using tests on semi-solid agar plates. The growth halo of the *tdcA* mutant strain SR3501 after 24 h of incubation was smaller (32 mm) than that of the wild-type strain SL1344 (59 mm), indicating SR3501 was less motile than SL1344 (Fig. 1A). We also analyzed the protein profile of the culture supernatant after anaerobic shock (Komoriya et al., 1999), comparing between SL1344 and SR3501. The proteins in the resulting cell-free supernatant were precipitated with trichloroacetic acid and separated by SDS-PAGE. As shown in Fig. 1B, the culture supernatant protein profile of SR3501 showed lower levels of the flagellin subunit FliC. In addition, SR3501 containing pMJ-2, which expresses *tdcA* by its native promoter, secreted increased amounts of FliC into the culture supernatant. Finally, to know whether *tdcA* mutation can affect the entire flagellar operon, we assayed the expression of *flhD*, a master activator of flagellar biosynthesis, using chromosomal *lacZ* fusion strain of *flhD*. When cells were grown continuously with aeration, the expression level of *flhD* was almost unaffected by *tdcA* mutation (Fig. 1C). However, when cells subjected to an anaerobic shock were returned to aerobic culture conditions, *flhD* expression in SR3501 was lower than SL1344. Under anaerobic conditions, *tdcA* mutation reduced of *flhD* expression (~80% of wild-type levels; data not shown). These results strongly suggest that the *tdcA* gene contributes to flagella biosynthesis and motility.

Reduced invasion of the *S.* Typhimurium *tdcA* mutant is associated with reduced motility

To determine the role of *tdcA* in the invasive process of *Salmonella*, we compared the invasiveness of the wild-type (SL1344) and the *tdcA* null mutant (SR3501) of *Salmonella* grown to exponential phase anaerobically into HEp-2 epithelial cells. SL1344 was used as the wild-type control, and the invasiveness of this strain was arbitrarily set as 100% in each assay. Under oxygen-limiting conditions, which increase the adherence and invasiveness of *S.* Typhimurium (Lee and Falkow, 1990), the invasiveness of SR3501 in epithelial cells was about one-fourth compared to that of SL1344 (Fig. 2). The fully invasive phenotype was restored when the *tdcA* lesion was complemented *in trans* using a plasmid (pMJ-2) containing a copy of the functional *tdcA* gene (Fig. 2A). Transfer of *Salmonella* into static culture conditions following aeration for 2 h, which initiated anaerobic shock to activate the expression of *tdcA* (Kim et al., 2008), resulted in a three-fold reduction in the invasiveness of SR3501 (Fig. 2B). Because the growth rate of SR3501 was comparable to that of SL1344 regardless of oxygen tension (aerobic and anaerobic) or medium used (LB and M9), the invasion defect of the *tdcA* mutant seems not to be

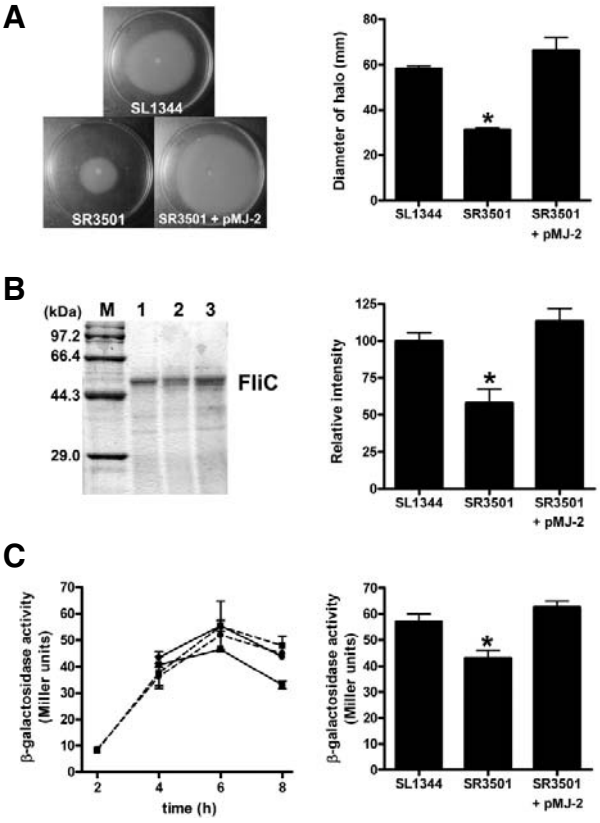

Fig. 1. Effects of *tdcA* mutation on the expression and biosynthesis of flagella. (A) SL1344 (wild type) and SR3501 (*tdcA* mutant) were compared for motility. Equal numbers of bacteria were used to inoculate the centers of semi-solid swarming agar (0.35% LB agar) plates and incubated at 37°C for 24 h. (B) Culture supernatants were collected from the wild-type strain (lane 1), the *tdcA* mutant strain (lane 2), or the *tdcA* mutant strain with a plasmid-borne copy of *tdcA*, pMJ-2 (lane 3). Molecular weights of standard proteins (lane M) are shown on the left. Proteins were visualized by Colloidal blue. Mean values of FliC intensity (n = 3) were shown on the right panel. (C) Cultures of the wild-type (SL1344 containing the *flhD*::*lacZ* fusion, ●) or the *tdcA* mutant (SR3501 containing the *flhD*::*lacZ* fusion, ■) strain were grown aerobically in LB medium (dashed lines). After an anaerobic shock for 30 min, cultures of both strains were transferred into aerobic conditions and grown continuously with shaking (solid lines). Expression level of *flhD* was also determined in SR3501 harboring *tdcA* complementation plasmid pMJ-2 at 6 h post inoculation and compared to each β-galactosidase activity in SL1344 and SR3501 (right panel). Data presented represent the means of three independent experiments with duplicate samples. The *P*-value was calculated by Student's *t*-test; *, $P < 0.005$ for SR3501 in comparison with SL1344.

based on the difference of bacterial growth between SL1344 and SR3501 (data not shown). It has been reported that non-motile *Salmonella* strains are deficient in their ability to gain access to cultured host cells (Jones et al., 1992; Liu et al., 1988). In the case of *S.* Typhimurium, such a defect can be overcome and the entry into host cells can be restored to wild-type levels if a mild centrifugal force is applied during the internalization process because the effect of bacterial motility is minimized (Jones et al., 1981; Tomita and Kanegasaki, 1982). To test whether a lower level of invasion caused by the *tdcA* mutation is related to

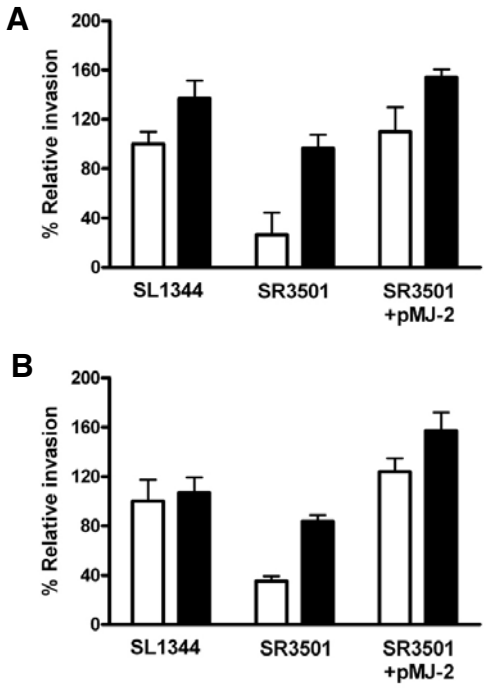

Fig. 2. Effect of *tdcA* mutation on the invasiveness of *S.* Typhimurium. The *tdcA* mutant strain (SR3501) was tested for their ability to enter cultured epithelial cells in the absence (white bars) or presence of a mild centrifugal force (black bars). HEp-2 epithelial cells were infected with *Salmonella* grown statically to mid-exponential phase (A) or subjected to an anaerobic shock (B). Each value has been standardized to the level of internalization of the wild-type strain without centrifugation, which was considered 100%. SR3501 was complemented with pMJ-2 harboring *tdcA* driven by its promoter. The values represent the means with standard deviations from two independent experiments performed with triplicate samples.

a defect in the motility of *Salmonella*, SR3501 was tested for its ability to enter cultured epithelial cells in the absence or presence of a mild centrifugal force. In this experiment, the application of centrifugal force restored the invasion level of SR3501 to that observed in SL1344 without centrifugation (Fig. 2). These data suggest that *tdcA* is necessary for invasiveness of *Salmonella*.

The expression of SPI1 genes is reduced in the *tdcA* mutant strain

Upon mild centrifugation, there was still a reduction in the capacity of SR3501 to enter HEp-2 cells compared to SL1344 (Fig. 2), suggesting the reduced invasiveness of SR3501 is not solely due to mobility defect. Many of the *Salmonella* proteins responsible for the initial invasion steps, cytoskeleton rearrangement, and membrane ruffling of the infected cells are encoded within a virulence gene cluster termed *Salmonella* pathogenicity island 1 (SPI1) (Lostroh and Lee, 2001). The class 2 flagella gene encoding the regulator of class 2 operon, *fliZ*, is positive regulators of *hilA*, which plays an important role in SPI1 regulation (Iyoda et al., 2001; Lucas et al., 2000). Thus, we analyzed the expression of two SPI1 regulatory genes (*hilA* and *invF*), along with *fliZ* after both anaerobic growth (AN) and anaerobic shock (AS) conditions. The *tdcA* knockout mutation resulted in an approximately two-fold reduction of *fliZ* expression, especially under the anaerobic shock conditions (Fig. 3). The expression of the SPI genes tested was also reduced by 30% to 40% in SR3501 as compared to SL1344 (Fig. 3). Moreover, *tdcA* complementation restored wild type levels of all genes tested, *filZ*, *hilA* and *invF* (Fig. 3). Considering the 2-fold reduction in *hilA* and *invF* expression in the *fliZ* mutant strain (Eichelberg and Galán, 2000; Iyoda et al., 2001), it is reasonable for *hilA* and *invF* to decrease slightly in *tdcA* mutant. Conclusively, these results show that a concomitant reduction in SPI1 expression stemmed from the reduced *fliZ* expression (Fig. 3) causes the slight defect in invasiveness of the *tdcA* mutant, which was not restored completely even though the application of the centrifugal force (Fig. 2).

Virulence of a *tdcA* mutant is attenuated

Finally, the effect of a mutation in *tdcA* on *Salmonella* virulence was studied using a mouse model after oral inoculation. To monitor survival kinetics, two groups of five streptomycin-pretreated mice were infected orally with 10^7 CFU of the wild-type strain SL1344 or *tdcA* mutant strain SR3501 grown aerobically to stationary phase. The mice were monitored daily for morbidity, and survival curves were plotted (Fig. 4). In the case of wild type, three of the five mice died by day 8, and then remaining two mice died by day 11 after oral infection. Although the *tdcA*-infected mice were dead by day 9 (two) and 12 (one) of infection, respectively, but two of the five mice survived at least until day 21. This result suggests that the *tdcA* mutant is attenuated in its ability to kill mice.

DISCUSSION

During the pathogenic lifecycle of *Salmonella*, oxygen concentrations vary greatly between the lumen of the bowel and perfused tissue, and low oxygen stimulates *Salmonella* invasion from the gastrointestinal tract (Guiney, 1997; Lee and Falkow, 1990). *Salmonella* is therefore required to switch from aerobic to an anaerobic metabolism, in which the *Salmonella tdc* operon is likely to be induced (Kim et al., 2008). A *S.* Typhimurium strain carrying a mutation in the *tdcA* gene exhibited a defect in invasion of cultured epithelial cells (Fig. 2) and decreased virulence when administered to mice via the oral route (Fig. 4). Flagella play a role in the attachment to and invasion of intestinal cells, as *S.* Typhimurium strains carrying null mutations in flagella genes are less able to enter epithelial cells (Jones et al., 1992; Stecher et al., 2004). In the current study, we found that a mutation in *tdcA* caused a detrimental effect on flagellar biosynthesis and the motility phenotype of *Salmonella* (Fig. 1). This suggests that an impediment in flagellar biosynthesis may be the major reason for the decreased invasiveness of the *tdcA* mutant, since the application of centrifugal force restored the invasive ability of a strain carrying a mutation in *tdcA*, but not completely (Fig. 2). The result that upon mild centrifugation the *tdcA* mutant still had a defect in invasiveness is consistent with the decreased expression of SPI1 shown in Fig. 3, implying that the reduced invasiveness and attenuation of virulence after oral inoculation can be attributed, at least in part, to the decreased expression of SPI1 genes through the reduction in *fliZ* expression.

When bacteria experience certain stresses, such as an upshift in osmolarity or a transition from aerobic to anaerobic growth, gyrase activity is altered and the result is a change in the level of supercoiling in the DNA (Dorman et al., 1988; Higgins et al., 1988). Because gyrase requires ATP to negatively supercoil DNA, the negative supercoiling activity of gyrase decreases as ATP levels fall and ADP levels rise (Drlica, 1992; Gellert et al., 1976). Shifting bacteria from aerobic to anaerobic

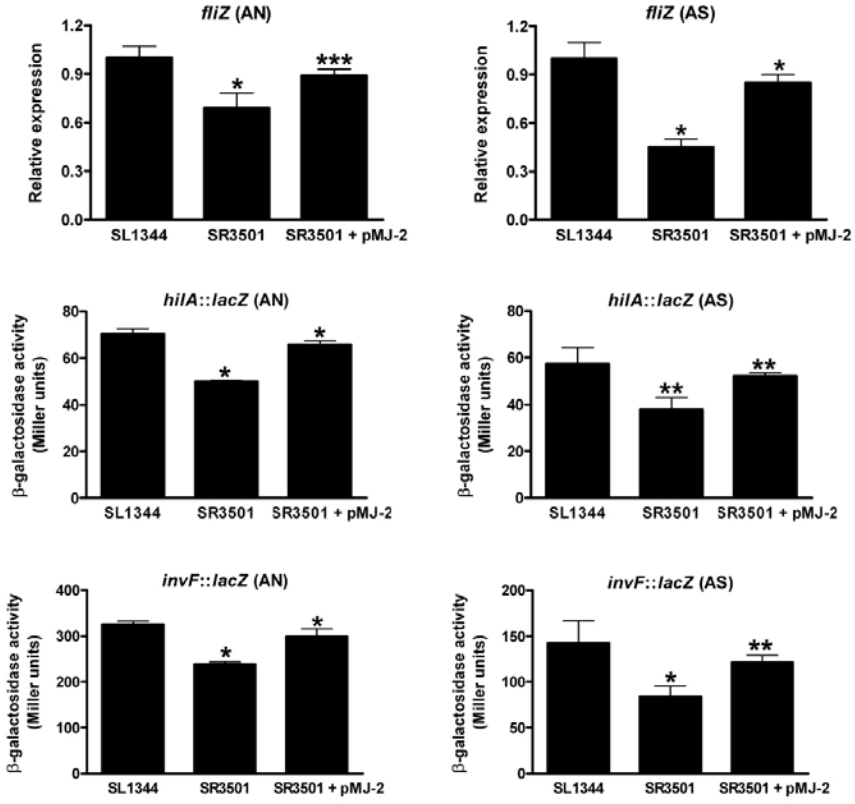

Fig. 3. Effects of *tdcA* mutation on the expression of SPI1 genes. The mRNA level of *fliZ* was determined using RT-PCR. Total RNAs were isolated from SL1344, SR3501, and SR3501 containing *tdcA* complementation plasmid, pMJ-2, after both 4-h anaerobic incubation (AN) and anaerobic shock (AS) conditions. Expression levels of the target genes were normalized to those of the 16S rRNA gene. Strains harboring the transcriptional *lacZ* fusions with SPI1 genes (*hilA* and *invF*) were grown under both AN and AS conditions. Expression levels of each gene were determined by measuring β-galactosidase activity. The means with standard deviations of three independent experiments in duplicate are shown. The *P*-value was calculated by Student's *t*-test. *P* values for SR3501 and SR3501 containing pMJ-2 are derived from comparisons to SL1344 and SR3501, respectively; (*, $P < 0.005$; **, $P < 0.01$; ***, $P < 0.05$).

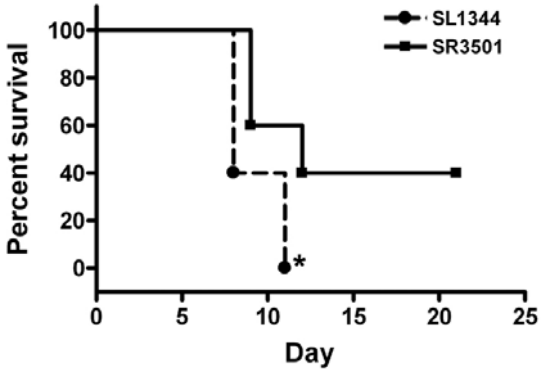

Fig. 4. Survival of mice following *Salmonella* infection. BALB/c mice were challenged orally with 10^7 CFU of *Salmonella* strains. The survival assay was performed twice with groups of five mice per strain, and survival was recorded for 3 weeks. Shown is the result of one of the two experiments, which gave similar results. An asterisk denotes $P < 0.05$ when comparing the percent survival of mice infected with SL1344 (wild type) to that of mice infected with SR3501 (*tdcA* mutant).

growth conditions causes a decrease in the ratio of [ATP]/[ADP] so that negative supercoiling of chromosomal DNA decreases, depending on gyrase activity (Hsieh et al., 1991). On the other hand, mutational analyses of the genes encoding DNA gyrase and topoisomerase I revealed that relaxation of supercoiled DNA significantly enhances *tdc* transcription (Sumantran et al., 1989). Conversely, a small histone-like protein, HU, which bends and compacts chromosomal DNA, drastically reduces *tdc* transcription (Wu and Datta, 1995). It has been well known that the *tdc* enzymes expressed under anaerobic conditions are involved in energy metabolism, which is largely dependent on TdcA, rather than in threonine or serine degradation *per se* (Heßlinger et al., 1998; Sawers, 1998). Based on these observations, a hypothesis can be proposed to explain the down-regulation of the transcription of flagellar genes in the *tdcA* mutant. When *Salmonella* are subjected to anaerobic shock, it is probable that the reduced degree of DNA supercoiling increases the transcription of the *tdc* operon, and this in turn can restore gyrase activity through the concomitant generation of ATP by *tdc* enzymes. Considering the decreased flagellar gene transcription by relaxation of DNA and concomitant reduction of bacterial motility (Shi et al., 1993), it is likely that a little defect of gyrase activity in *tdcA* mutant may lead to an insufficient activation of flagellar expression.

Although the mechanism of motility control in response to environmental factors remains largely unknown, recent experimental data suggest that this mechanism requires an alteration to the specific DNA topology of the entire *flhDC* region in concert with various regulatory proteins (Soutourina and Bertin, 2003). It is worthy to be considered that the expression of a master activator of flagellar biosynthesis, *flhD*, was reduced by a *tdcA* mutation (Fig. 1C). In this respect, the control of flagellum biosynthesis in anaerobic conditions might be partially mediated by the *tdc* operon, which could affect the DNA conformation through ATP generation. This hypothesis is supported by a previous report, in which an increase in the production of flagella was observed under limited-oxygen conditions (Landini and Zehnder, 2002). The possibility still also remains that TdcA can serve as a transcriptional activator of *flhDC*, like other LysR-type regulators such as HdfR and LrhA (Ko and Park, 2000; Lehnen et al., 2002). In conclusion, a *tdcA* mutation reduced the ability of *S.* Typhimurium to enter into epithelial cells

due to a defect of flagellar biosynthesis, which caused the virulence attenuation of *tdcA* mutant in mice.

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