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Molecules and Cells

A Mutation in *tdcA* Attenuates the Virulence of *Salmonella enterica* Serovar Typhimurium

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The *Salmonella tdc* operon encodes enzymes belonging to a metabolic pathway that degrades L-serine and L-threonine. The upregulation of the *tdc* operon and increased virulence of *Salmonella* grown under oxygen-limiting conditions prompted us to investigate the role of the *tdc* operon in the pathogenesis of *Salmonella* Typhimurium. A *Salmonella* strain carrying a null mutation in *tdcA*, which encodes the transcriptional activator of the *tdc* operon, was impaired in mice infected intraperitoneally with the bacterium. In addition, the *Salmonella tdcA* mutant showed reduced replication compared with the parental strain in cultured animal cells, although their growth rates were similar in various culture media. To understand the function of TdcA in pathogenesis, we performed two-dimensional gel electrophoresis and found that flagellar and PhoP-regulated proteins were affected by the *tdcA* mutation. The results of β-galactosidase assays and FACS analysis showed that, among the four PhoP-dependent genes tested, the expression of *ssaG*, which is located in *Salmonella* pathogenicity island 2 (SPI2), was reduced in the *tdcA* mutant, especially in the intracellular environment of macrophages. Taken together, our data suggest that *tdcA* plays an important role in the pathogenesis of *Salmonella*.

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; Heßlinger et al., 1998). The *tdc* operon is composed of a regulatory gene (*tdcA*), which encodes a LysR family of transcription factor containing a helix-turn-helix DNA-binding motif (Ganduri et al., 1993), and six structural genes (*tdcB* to *tdcG*), which encode enzymes that catalyze steps in the production of energy-rich keto acids, which are subsequently catabolized to produce ATP *via* substrate-level phosphorylation (Heßlinger et al., 1998; Sawers, 1998).

The expression of the *tdc* operon is induced under anaerobic conditions (Sawers, 1998). The requirement for an anaerobic environment is presumably linked to intracellular levels of cyclic AMP (cAMP) (Shizuta and Hayaishi, 1970). When an exponentially growing aerobic culture was made anaerobic, internal cAMP levels increased significantly, leading to *tdc* expression (Phillips et al., 1978). We also found that the expression of the *Salmonella tdc* operon was dramatically increased during the transition from aerobic to anaerobic conditions, even though the *Salmonella tdc* operon lacks *tdcR*, which encodes a positive transcriptional regulator of the *E. coli tdc* operon (Kim et al., 2008).

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a facultative intracellular pathogen that cause disease, normally limited to gastroenteritis in humans but causing typhoid-like disease in mice (Jones and Falkow, 1996). During *Salmonella* infection, the growth conditions and phase are known to influence attachment, invasion, and regulation of the pathogen's virulence genes (Mekalanos, 1992). One of the conditions encountered by *S.* Typhimurium during invasion of the intestinal epithelium, as well as in host tissues during systemic infection, is a low-oxygen environment (Altier, 2005; Lee and Falkow, 1990). Compared with cells grown under aerobic conditions, *S.* Typhimurium grown under limited-oxygen conditions is more invasive and virulent against mammalian cells (Lee and Falkow, 1990; Lucas and Lee, 2000). In addition, *S.* Typhimurium grown under limited oxygen causes cytoskeletal rearrangements and morphological changes in infected HEp-2 cells, which may contribute to increased pathogenicity of the organism by allowing deeper penetration into tissues and thus avoidance of host defense mechanisms (Galán, 1996; Pace et al., 1993). *Salmonella* might have evolved specific mechanisms to adapt to limited-oxygen environments, thereby increasing its survival and pathogenesis within a host. The upregulation of the *tdc* operon in *Salmonella* grown under limited-oxygen conditions suggest that *Salmonella tdc* genes participate in these specific mechanisms.

We previously reported that a mutation in *tdcA* made the *S.* Typhimurium less invasive owing to defects in flagellar biosynthesis (Kim et al., 2009). In the present study, we used a murine model of intraperitoneal infection to investigate the effect of the same *tdcA* mutation on systemic infection and found that the mutant was defective in virulence. In addition, the *tdcA* mutation resulted in reduced expression of *ssaG*, which encodes a component of the type III secretion system (TTSS)

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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
EG10237	14028s *mgtA*::*MudJ*, Km^R	Groisman E.A.
P4P70	SL1344 *ssaG*::*lacZ*, Km^R	This study
SR3264	SL1344 *mgtA*::*MudJ*, Km^R	This study
SR3266	SL1344 *sseA*::*lacZ*, Km^R	This study
SR3278	SL1344 *pagD*::*lacZ*, Km^R	This study
SR3501	SL1344 Δ*tdcA*	Kim et al. (2008)
SR3512	SR3501 *mgtA*::*MudJ*, Km^R	This study
SR3514	SR3501 *pagD*::*lacZ*, Km^R	This study
SR3517	SR3501 *ssaG*::*lacZ*, Km^R	This study
SR3518	SR3501 *sseA*::*lacZ*, Km^R	This study
Plasmids		
pKD46	*bla* P_{BAD} *gam beta exo* rep_{pSC101}^{ts}	Datsenko and Wanner (2000)
pKD4	*bla* FRT *ahp* FRT PS1 PS2 $rep_{R6K\gamma}$	Datsenko and Wanner (2000)
pKD13	*bla* FRT *ahp* FRT PS1 PS4 $rep_{R6K\gamma}$	Datsenko and Wanner (2000)
pCP20	*bla cat cI*857 λP_R *flp* rep_{pSC101}^{ts}	Datsenko and Wanner (2000)
pCE70	*ahp* FRT *lacZY*$^+$ t_{his} oriR6K	Merighi et al. (2005)
pFMI10	*ssaG*::*gfp* in pFPV25, Amp^R	Valdivia and Falkow (1997)
pACYC184	cloning vector, Cm^R Tet^R p15A	Jones B.D.
pMJ-2	pACYC184 with *tdcA* and *tdcA* promoter	Kim et al. (2009)

produced by *Salmonella* pathogenicity island 2 (SPI2); this gene product is essential for intracellular replication *in vivo* (Hensel, 2000).

MATERIALS AND METHODS

Bacterial strains

The bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella* Typhimurium SL1344 was used as wild type. Strain SR3264 was constructed by the P22 transduction of an *mgtA*::*MudJ* allele from strain EG10237 (a gift from Dr. E. A. Groisman, Washington University School of Medicine, USA) into strain SL1344. To introduce the *lacZ* reporter gene into SL1344, the one-step gene inactivation method (i.e., λ red and FLP-mediated site-specific recombination system) was used (Datsenko and Wanner, 2000; Merighi et al., 2005). Briefly, a kanamycin cassette from pKD4 or pKD13 was amplified using the forward and reverse primers listed in Table 2, and the resulting PCR product (FRT-kan-FRT) was introduced into the corresponding chromosomal gene by homologous recombination. Three PCRs were performed using locus-specific primers with the common test primers (Table 2) as previously described (Datsenko and Wanner, 2000) to ensure integration of the products into the correct chromosomal location. The antibiotic resistance cassette was removed using the temperature-sensitive plasmid pCP20, which carries the gene encoding FLP recombinase. FLP-mediated excision of the *kan* cassette left a single FRT site, which was used for site-specific integration of the *lacZ* fusion plasmid pCE70 (Merighi et al., 2005). Strains containing transcriptional *lac* fusions to *ssaG*, *sseA*, and *pagD* were constructed as previously described (Merighi et al., 2005). Finally, each *lacZ* fusion was transferred from wild type into the *tdcA* mutant strain SR3501 by P22 transduction (Table 1).

Bacterial growth conditions

Bacteria were cultivated at 37°C in Luria-Bertani (LB) broth containing 1% tryptone, 0.5% yeast extract, and 1% NaCl. A stationary-phase culture that had been grown overnight with shaking was inoculated into fresh LB broth at a 1:100 dilution and grown aerobically to exponential phase. The culture was then shocked anaerobically (i.e., static culture conditions for 30 min) to maximally induce *tdcA* expression (Kim et al., 2008). Upon the shift from aerobic to anaerobic conditions, the dissolved oxygen concentration decreased from about 4 to 0.3 ppm. Minimal media were used as follows: N minimal medium, pH 7.7 [50 mM Tris·HCl, 50 mM Bis-Tris, 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, and 1 mM KH_2PO_4] was supplemented with 0.1% casamino acids, 38 mM glycerol, and 50 μM $MgCl_2$; and M9 minimal medium, pH 5.8 (13 μg/L $Na_2HPO_4 \cdot 7H_2O$, 3 g/L KH_2PO_4, 0.5 g/L NaCl, and 1 g/L NH_4Cl) was supplemented with 2 mM $MgSO_4$, 10 mM glucose, and 0.004% L-histidine. When necessary, antibiotics were used at the following concentrations: 50 μg/ml ampicillin, 50 μg/ml kanamycin, and 25 μg/ml chloramphenicol.

Mouse infection experiments

All animal experiments were conducted according to the principles set forth in the *Policy and Regulation for the Care and Use of Laboratory Animals* in a laboratory accredited by Seoul National University (permission no. SNU-060908-1). 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 stationary phase in LB medium, harvested by centrifugation, and suspended in sterile PBS for in-

Table 2. Oligonucleotides used in this study

Primer name	Sequence (5′→3′)	Purpose
pagD P1(F)[a]	tct acg att ttg gta gta aaa ccc cgc aac cac cta caa a*tg tag gct gga gct gct tcg*	λ Red deletion of *pagD*; *lacZY* insertion
pagD P2(R)[a]	atg ggt ttt gtc gtc ggg cag gac ggt gaa cta atc tgc c*at tcc ggg gat ccg tcg acc*	
ssaG P1(F)[a]	ata ttt att aat tac gaa agt tca ctg atc *gtg tag gct gga gct gct tc*	λ Red deletion of *ssaG*; *lacZY* insertion
ssaG P2(R)[a]	aat aaa att ttc gcg gct ttt agc ggc tca *atg gga att agc cat ggt cc*	
sseA P1(F)[a]	tag tta gca cgt taa tta tct atc gtg tat atg gag ggg a*gt gta ggc tgg agc tgc ttc*	λ Red deletion of *sseA*; *lacZY* insertion
sseA P2(R)[a]	ttt gac ttc ccc ata aga tgt ttc ctg aag aca tta tgc t*at ggg aat tag cca tgg tcc*	
pagD-F1	cag gag gtg cta tga aac at	Verification of mutation
pagD-R1	ggg tgg cta aaa gta gtc gt	
ssaG-F1	ccg gat gtt cat tgc ttt cta aat t	
ssaG-R1	aac ccc tca ttt ggt gca gga aa	
sseA-F1	ccc agg ttc aaa atg gca agt aa	
sseA-R1	ata ccc ttc ggc aaa cgg att t	
k1[b]	cag tca tag ccg aat agc ct	
k2[b]	cgg tgc cct gaa tga act gc	

[a]P1 and P2 priming sites in pKD4 and pKD13 are shown in italics
[b]The common test primers adapted from Datesenko and Wanner (2000)

fection. Groups of five female BALB/c mice were infected by intraperitoneal injection with ~10^2 *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.). For analysis of bacterial colonization of the liver and spleen, groups of five mice were infected intraperitoneally with 10^2 colony forming units (CFU) of SL1344 and SR3501. On day 5 p.i., the mice in each group were euthanized with a mixture of ketamine and xylazine; their spleens and livers were removed aseptically and placed in 1 ml of PBS. The organs were then homogenized and serially diluted. Bacterial loads were determined by plating the samples on MacConkey agar plates containing 50 μg/ml streptomycin. For statistical analysis of the bacterial load, a Mann-Whitney test was performed using Graphpad Prism, version 4.0.

Replication assay (gentamicin protection assay)

Survival of the opsonized strains in J774A.1 macrophages was determined as previously described (Lim et al., 2006). Opsonization was performed differentially as follows: (i) Aerated cultures grown to stationary phase were opsonized with aeration for 30 min in PBS containing 20% normal mouse serum; or (ii) To induce *tdcA* expression, cultures grown aerobically to mid-log phase were statically opsonized. Briefly, J774A.1 cells (2×10^5) were cultured in 24-well plates for 24 h prior to infection. Bacterial cultures were applied to the cell monolayers at a multiplicity of infection of about 10:1. After 1 h, the cells were washed three times with 1 ml of PBS and incubated for 1 h in DMEM supplemented with100 μg/ml gentamicin. After washing three times with PBS, the cells were lysed in 1% Triton X-100 for 10 min and then diluted with PBS. Dilutions of the suspension were placed on LB agar to enumerate the CFU. To assess the survival of the *Salmonella* strains, the monolayer was lysed at 18 h p.i. and the intracellular bacteria were counted as described above.

Two-dimensional gel electrophoresis

Wild-type *S.* Typhimurium wild-type (SL1344) and *tdcA* mutant (SR3501) cells were harvested, washed with PBS, and then resuspended in lysis buffer (8 M urea, 2 M thiourea, 2% CHAPS, 1% Pharmalyte, 1% DTT, and protease inhibitor). After 5 min of incubation at room temperature, cellular debris was removed by centrifugation (10,000 × *g* for 3 min at 4°C), and the clear supernatant was precipitated with 100% acetone for 3 h at -20°C. After centrifugation (12,000 × *g* for 10 min at 4°C), the protein pellet was resuspended in solubilization buffer [9 M urea, 2% β-mercaptoethanol, 2% Pharmalyte (pH 3-10), and 8 mM PMSF] and subjected to two-dimensional gel electrophoresis using a previously described protocol (Yoon et al., 2003). In brief, isoelectric focusing in the first dimension using 3-10 IPG strips (Amersham Pharmacia Biotech, UK) with a Multiphor™ II electrophoresis system (GE Healthcare, USA) was followed by 12% SDS-PAGE in the second dimension using a Hoefer SE600 Chroma vertical unit (USA). For comparative analysis, three protein profiles from three different biological replicates were analyzed using Bio-Rad's PDQuest 2-D analysis software. Spots showing more than 2-fold difference in intensity between SL1344 and SR3501 were subjected to Student's *t*-test, with $P < 0.05$ considered significant. For mass spectrometric analysis, in-gel digestion with trypsin was performed according to the published method (Yoon et al., 2003). Proteins were identified by peptide mass fingerprinting with the search programs MS-FIT (UCSF Mass Spectrometry Facility, at http://prospector.usuf.edu) and Mascot (Matrix Science, UK, at http://www.matrixscience.com) and the NCBInr and Swiss-Prot databases. The searches were performed with a mass tolerance of 100 ppm.

Measurement of β-galactosidase activity *in vitro*

S. Typhimurium strains containing chromosomal *lacZ* transcriptional fusions were assayed by determining the level of β-galactosidase activity according to the standard method (Maloy et al., 1996). Increased expression of the *mgtA*::*lacZ* and *pagD*::*lacZ* fusions was induced by a shift from a high to a low Mg^{2+} concentration, as described previously (Shin et al., 2006). Briefly, overnight cultures in N medium were diluted 1:100 in N medium containing a high Mg^{2+} concentration (2 mM $MgCl_2$). The bacteria were then grown with agitation for 4 h and washed twice with PBS before resuspension in N medium containing a low Mg^{2+} concentration (50 μM $MgCl_2$) for 30 min. For the induction of SPI2 genes, strains containing the *ssaG*::*lacZ* and *sseA*::*lacZ* fusions were grown with shaking overnight in M9 minimal medium (Kim and Falkow, 2004). These cultures were then re-inoculated into M9 minimal medium (pH 5.8) at a 1:100 dilution and grown aerobically to stationary phase (~8 h).

Assay of β-galactosidase activity in *S.* Typhimurium recovered from macrophages

The bacterial strains used for infection were grown with shaking overnight to stationary phase in LB medium. Following opsonization (20% mouse serum for 30 min), the bacteria were diluted in DMEM and used to infect monolayers of J774A.1 cells as described in the section titled "Replication assay." After 18 h, the cells were washed twice with 1 ml of PBS, and the macrophages were lysed with 1 ml of 1% Triton X-100 in PBS for 10 min. The released bacteria were collected by centrifugation, and dilutions of each sample were plated on LB agar to determine the number of intracellular bacteria. The β-galactosidase activity assay was performed as described previously (Walthers et al., 2007). The arbitrary units were calculated per CFU of intracellular bacteria in each sample.

Fluorescence-activated cell sorter (FACS) analysis

Salmonella containing the pFMI10 plasmid, which encodes GFP under control of the *ssaG* promoter, was statically opsonized and used to infect monolayers of J774A.1 cells as described in the section titled "Replication assay." After 2 and 18 h, the macrophages were washed twice with 1 ml of PBS and lysed with 1 ml of 1% Triton-X 100 in PBS for 10 min. The intracellular bacteria were harvested, labeled with anti-*S.* Typhimurium lipopolysaccharide antibodies, and fixed with paraformaldehyde as described previously (Hautefort et al., 2003). The fixed bacteria were detected as described previously (Hautefort et al., 2003; Lim et al., 2006). Briefly, 10,000 bacterium-sized particles were collected for each experiment, and the fluorescence intensity of each particle was detected using a FACSCalibur™ flow cytometer (BD Biosciences, USA) equipped with a 15-mW air-cooled argon ion laser as the excitation light source (488 nm). For analysis of the bacterial cells released from the macrophages, the samples were gated for *Salmonella*-like particles using the orange fluorescence of the anti-*Salmonella* label to identify bacterial cells and to exclude mammalian cell debris and background noise. Data for all parameters were collected using amplification gains set on the LOG mode. The GFP fluorescence intensity values were analyzed using CELLQUEST 3.3 software (Becton Dickinson).

RESULTS

A mutation in *tdcA* attenuates the virulence of *Salmonella* in a mouse model

The effects of a *tdcA* mutation on *Salmonella* virulence were studied using a mouse model. To monitor survival kinetics, two groups of five streptomycin-pretreated mice were infected intraperitoneally with 10^2 CFU per strain and monitored daily for morbidity. The survival curves (Fig. 1A) show a significant delay in mortality in the mice inoculated with the *tdcA* mutant strain compared with the SL1344-infected mice, and two of the five mice survived until at least day 21 (P = 0.0231). This indicates that *tdcA* is required for *Salmonella* to cause a systemic infection in mice. Therefore, we examined the mutant more carefully in a mouse model. The spleens and livers of mice infected with 10^2 CFU of *Salmonella* cells *via* an intraperitoneal route were assayed for colonization on day 5 p.i. The number of bacterial colonies in all sampled tissues of the SR3501-infected mice was lower than that in mice infected with SL1344. For example, the mean SR3501 bacterial loads in the spleen were 1.3×10^3 on day 5 p.i., whereas the mean CFU/g spleen for the SL1344-infected mice was 1.5×10^5 (Fig. 1B). These results strongly suggest that *tdcA* contributes to the pathogenesis of *Salmonella* in mice.

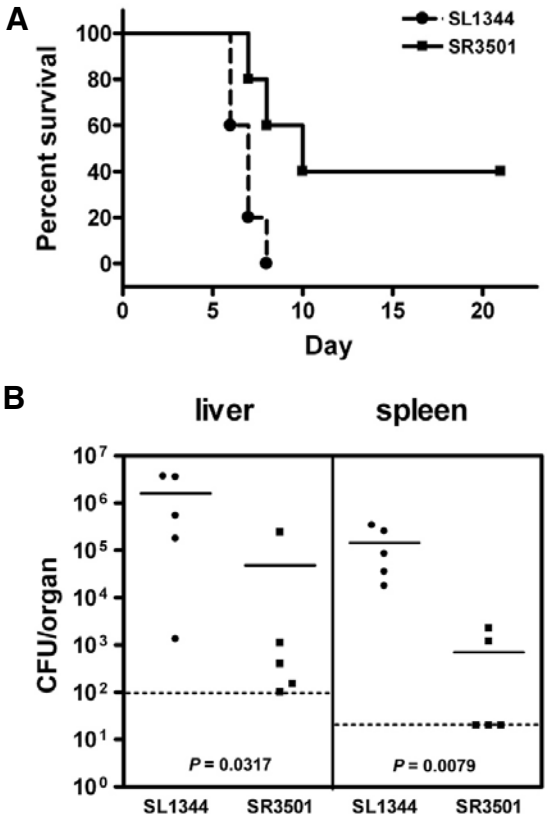

Fig. 1. Survival of mice and systemic bacterial loads following *Salmonella* infection. BALB/c mice were challenged with 10^2 CFU of SL1344 (wild type) or SR3501 (*tdcA* mutant). A survival assay was performed twice, using groups of five mice per strain, and survival was recorded for 3 weeks. Shown are the results of one of the two experiments, which gave similar results (A). Groups of five mice infected intraperitoneally with 10^2 CFU of SL1344 or SR3501 were euthanized 5 days after infection, and bacterial loads in the liver and spleen were determined. The number of bacteria per organ is given on the *y*-axis. Each black circle (SL1344) and square (SR3501) represents data from a single mouse; the horizontal bar shows the average number of CFU per group. The dashed line indicates the limit of detection, and the P values for SR3501 were derived from comparisons with SL1344 (B).

Replication of the *S.* Typhimurium *tdcA* mutant is reduced in macrophages

Given that the colonization of the liver and spleen by *S.* Typhimurium following intraperitoneal infection was reduced by a mutation in *tdcA* (Fig. 1B), we compared the abilities of SL1344 and SR3501 to replicate within J774A.1 macrophages. The number of CFU recovered from each strain at 2 h p.i. was designated as the reference point for replication. Opsonized bacteria grown to stationary phase under aerobic conditions have been used extensively as a model for *Salmonella* replication (Cirillo et al., 1998). The number of intracellular SR3501 was increased about 10-fold at 18 h following infection; however, this level was about 2-fold lower than the levels of SL1344 (~22-fold) and was restored by the introduction of an intact version of *tdcA* (Fig. 2A). When opsonized strains following anaerobic shock were used in replication assays (Fig. 2B), limited intracellular replication of SL1344 was observed compared with that shown in Fig. 2A, due to cytotoxicity of the

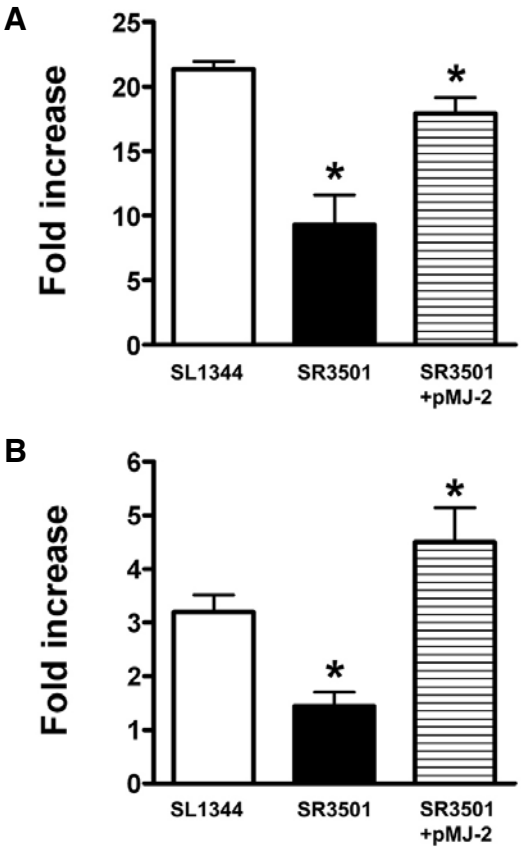

Fig. 2. Effect of the *tdcA* mutation on the replicative ability of *Salmonella* in macrophages. J774A.l macrophage-like cells were infected with opsonized strains grown to stationary phase under aeration (A). For the induction of *tdcA*, *Salmonella* grown with aeration to mid-exponential phase were transferred to static culture conditions and simultaneously opsonized (B). At 2 and 18 h post-infection (p.i.), cells were lysed and cultured for enumeration of the viable intracellular bacteria. The values are the means and standard deviations of three independent experiments performed with duplicate samples. Values of P were calculated by Student's *t*-test. The P-values for SR3501 and SR3501 containing pMJ-2 were derived from comparisons with SL1344 and SR3501, respectively (*, $P < 0.01$).

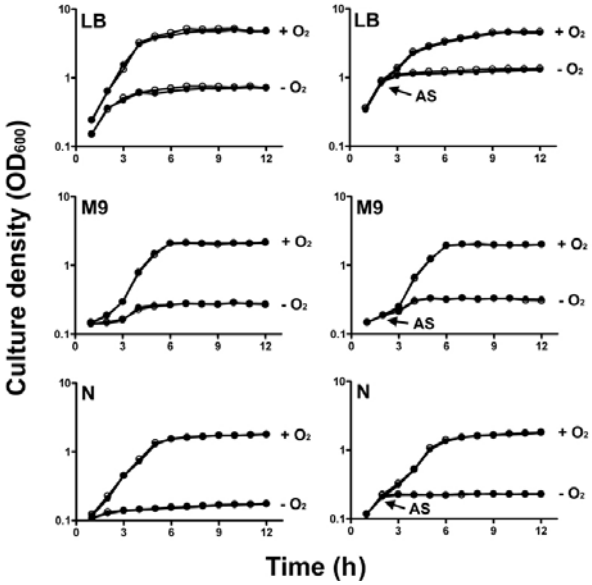

Fig. 3. Effect of the *tdcA* mutation on the growth rate of *Salmonella* in various culture media. The optical density at 600 nm of cultures of wild-type (SL1344, ●) and *tdcA* mutant (SR3501, ○) strains were monitored over time. *Salmonella* were grown in LB rich medium and in M9 and N minimal media under aerobic (+ O_2) or anaerobic conditions (– O_2). To induce *tdcA* expression, strains grown aerobically for 2 h were transferred to static culture conditions for 30 min [i.e., anaerobic shock (AS)] and then cultivated under aerobic (+ O_2) or anaerobic conditions (– O_2). The data are presented as mean values of two independent experiments.

highly invasive bacteria (Cirillo et al., 1998). In contrast, SR3501 showed a severe defect in replication, as the increase was only about 1.5-fold. We also monitored the growth of SL1344 and SR3501 in LB rich medium and in M9 and N minimal media, which mimic intracellular conditions. Because the growth rate of SR3501 was comparable to that of SL1344 regardless of the medium used or growth conditions tested (Fig. 3), the growth defect of the *tdcA* mutant inside macrophages does not seem to be related to impaired nutrient availability, but rather to impairment of specific virulence factors.

Changes in protein profiles of the *tdcA* mutant

We next compared the protein profiles of the wild-type (SL1344) and *tdcA* mutant (SR3501) strains, to survey the mechanisms underlying the reduced invasion and replication of the *tdcA* mutant. Whole-cell proteins were prepared from *Salmonella* subjected to an anaerobic shock, i.e., transition from aerobic to anaerobic growth to induce *tdcA* expression (Kim et al., 2008). Protein identification by two-dimensional gel electrophoresis was accomplished using MS-Fit and MASCOT. The expression levels of 20 protein spots showed significant changes with the *tdcA* mutation: 13 spots showed increased expression in the wild-type strain, whereas seven spots showed increased expression in SR3501 (Fig. 4). The *tdcA*-induced proteins could be divided into several groups, depending on their cellular function; excluding the *tdcA* regulon (*tdcE*), these groups included proteins involved in flagellar biosynthesis (*fljB*, *fliC,* and *flgE*), translation (*tufA*), and energy production and conversion (*purA* and *prpC*). Among seven spots repressed by *tdcA*, keto-hydroxyglutarate-aldoase (*eda*), maltose ABC transporter (*malE*), and dTDP-D-glucose-4,6-dehydratase (*rfbB*) are related to sugar catabolism, and DnaK, a molecular chaperone, is a stress response protein (Table 3).

The negative effect of the *tdcA* mutation on SPI2 expression is evident inside macrophages

Of the proteins differentially expressed in the *tdcA* mutant strain (Fig. 4 and Table 3), TktA, AtpA, and MalE have been previously identified as a PhoP-dependent regulon in *E. coli* and *S.* Typhimurium (Monsieurs et al., 2005). The PhoP/PhoQ two-component regulatory system is a master regulator of *S.* Typhimurium virulence functions (Groisman, 2001) and controls the expression of SPI2 (Bijisma and Groisman, 2005), which is essential for the replication of *Salmonella* within macrophages (Hensel, 2000). To further investigate the association between *tdcA* with *phoP*, we constructed chromosomal *lacZ* fusion strains of PhoP-regulated (*mgtA* and *pagD*) and SPI2 genes (*ssaG* and *sseA*) (Navarre et al., 2005). Under *tdcA*-inducing conditions, the levels of transcription were slightly reduced (~10-30%) except for *mgtA*, but the extent of the reduction was

Table 3. Identification of possible proteins induced and repressed by *tdcA*

Spot	Protein*	Gene	M.W. (kDa)	pI
Proteins induced by *tdcA*				
1	Pyruvate formate lyase I	*tdcE*	85.32	5.75
2	Phosphopyruvate hydratase	*purA*	45.63	5.25
3	EF-Tu	*tufA*	43.39	5.30
4	Transketolase	*tktA*	72.37	5.42
5	Phase-2 flagellin	*fljB*	52.45	4.73
6	Flagellar hook protein	*flgE*	42.18	4.87
7	Pyrophosphate-phospho hydrolase (PPase)	*ppa*	19.78	5.01
8	Aspartate chemoreceptor protein	*tarH*	59.31	5.10
9	Citrate synthase	*prpC*	43.20	6.2
10	Transfer protein	*trhC*	71.67	5.3
11	Putative DnaK suppressor protein	*ybiI*	9.90	5.3
12	Phosphoenolpyruvate carboxykinase	*pckA*	59.82	5.67
13	Phase-1 flagellin	*fliC*	51.19	4.8
Proteins repressed by *tdcA*				
14	Keto-hydroxyglutarate-aldolase	*eda*	22.34	5.92
15	ATP synthase subunit alpha	*atpA*	55.24	5.8
16	Maltose ABC transporter periplasmic protein	*malE*	43.15	5.9
17	Arginine-binding periplasmic protein 1 precursor	*artI*	27.09	7.66
18	Molecular chaperone DnaK	*dnaK*	69.27	4.83
19	Putative merR family bacterial regulatory protein	*arcR*	22.10	5.01
20	dTDP-D-glucose-4,6-dehydratase	*rfbB*	40.70	5.56

*Mass finger prints of numbered protein obtained by MALDI-TOF were analyzed with MS-Fit or MASCOT. MW and p*I* mean theoretical values depicted in NCBI database.

not as great as expected (Figs. 5A and 5D). We presume that under these growth conditions (anaerobic shock in LB media), TdcA had minimal influence on the expression of the genes tested, because PhoP-activated and SPI2 genes are induced at low Mg^{2+} concentrations and in acidic minimal media, respectively (Groisman, 2001; Kim and Falkow, 2004).

To test the possibility that the effect of the *tdcA* mutation was increased under inducing conditions for PhoP-activated genes, *Salmonella* cultures were shifted from N minimal medium containing a repressing Mg^{2+} concentration (2 mM) to that containing an activating Mg^{2+} concentrations (50 μM) (Fig. 5B). However, no large differences were observed in the expression of *mgt* (SR3512) and *pagD* (SR3514) when monitored using the β-galactosidase fusions in the *tdcA* mutant compared with the parental strains. In addition, the *tdcA* mutation had little effect on the expression of *ssaG* or *sseA*, even under SPI2-inducing conditions, such as in M9 minimal medium at pH 5.8 (Fig. 5E). TdcA-dependent gene expression was then monitored after growth of the bacteria inside macrophages for 18 h. As shown in Fig. 5C, the expression of *mgtA* and *pagD* did not differ much between the *tdcA* mutant and wild type. However, *ssaG* expression was decreased by about 2-fold compared to that in the wild type (Fig. 5F).

We also verified the effect of the *tdcA* mutation on *ssaG* expression in macrophages by measuring the fluorescence of intracellular bacteria carrying the plasmid pFMI10, which contains the *ssaG* promoter fused to *gfp* (Valdivia and Falkow, 1997). Bacteria were released from the cells at 2 and 18 h p.i., and GFP fluorescence intensities in SL1344 and SR3501 were compared. Interestingly, SR3501 showed a 2- to 3-fold reduction in *ssaG* expression inside the macrophages compared with wild type, and SR3501 harboring pMJ-2 achieved levels of *ssaG* expression comparable to those in SL1344 (Fig. 6). These results suggest that *tdcA* is required to activate the expression of SPI2 genes when the bacteria are inside macrophages, but that such conditions were not met in the culture media or under the conditions tested in this study.

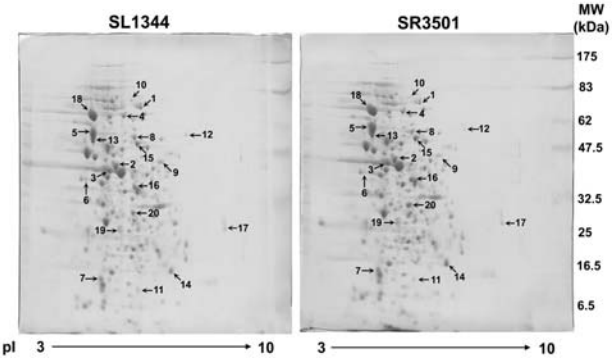

Fig. 4. Two-dimensional electrophoresis protein profiles of SL1344 (wild-type) and SR3501 (*tdcA* mutant) strains. Bacteria were grown aerobically to exponential phase and then exposed to anaerobic shock. Isoelectric focusing was performed with IPG strips ranging from pH 3-10, and separation in the second dimension was accomplished using 12% polyacrylamide gels. Proteins whose expression was upregulated or downregulated more than 2-fold by *tdcA* are designated with arrows. The identities of the proteins indicated by numbered arrows are shown in Table 3.

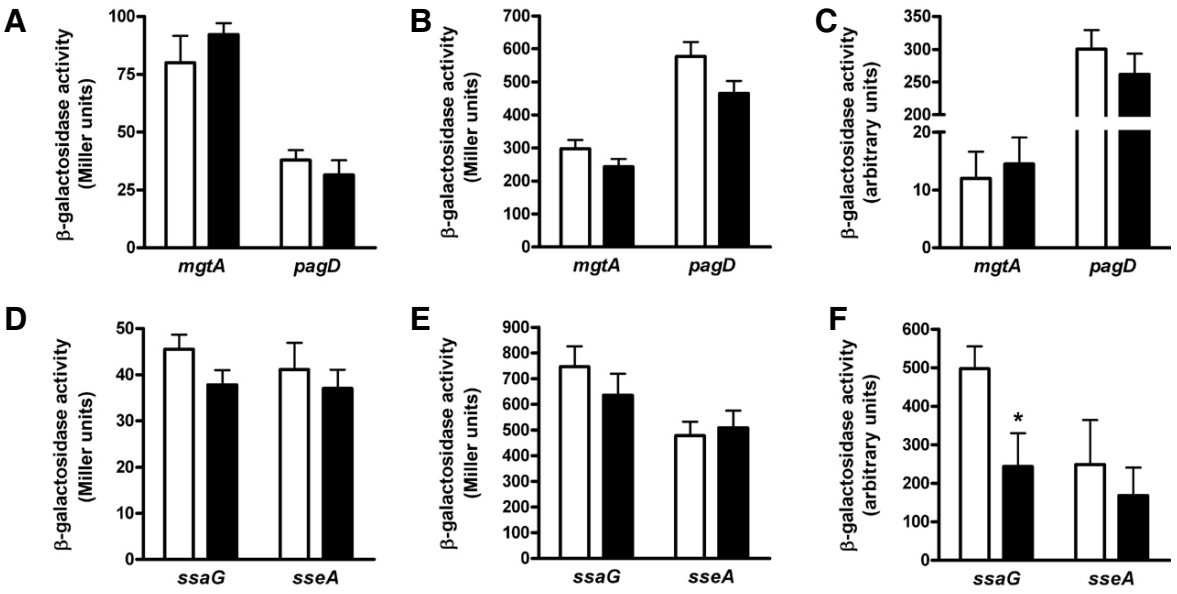

Fig. 5. β-Galactosidase assay of chromosomal *mgtA*::*lacZ*, *pagD*::*lacZ*, *ssaG*::*lacZ*, and *sseA*::*lacZ*. Cultures of wild-type (SL1344 containing the fusion, white bars) or *tdcA* mutant (SR3501 containing the fusion, black bars) cells were grown in LB medium and then subjected to anaerobic shock (A and D). The strains were also grown aerobically in N minimal medium containing 2 mM Mg^{2+} and then shifted to medium containing 50 μM Mg^{2+} (B), or grown to stationary phase in M9 minimal medium at pH 5.8 (E). Expression was determined by measuring β-galactosidase activity as described in the "Materials and Methods" (A, B, D, and E). Cells containing each *lacZ* fusion were grown in LB medium to stationary phase and were used to infect macrophages after opsonization. *Salmonella* growing in the macrophages were isolated at 18 h p.i., and β-galactosidase activity was calculated in arbitrary units, using the intracellular *Salmonella* numbers in place of the OD_{600} values (C and F). The data represent the averages of three independent experiments with duplicate samples. The P-value for SR3501 was derived from a comparison with SL1344 (*, $P < 0.05$).

DISCUSSION

Salmonella infections are usually acquired after the consumption of contaminated food or water. After surviving the harsh acidic environment of the stomach, *Salmonella* causes disease by adhering to and invading the epithelial cells of the small intestine (Ohl and Miller, 2001). During the pathogenic lifecycle of *Salmonella*, a transition from aerobic growth to anaerobic conditions may occur *in vivo*, and this induces the *Salmonella tdc* operon (Kim et al., 2008).

When administered intraperitoneally in mice, the *S*. Typhimurium *tdcA* mutant was less virulent than wild type in mice (Fig. 1A), which suggests that *tdcA* is required during the intracellular stages of the *Salmonella* life cycle. In fact, colonization of the liver and spleen was significantly lower with the *tdcA* mutant strain than with a wild-type strain (Fig. 1B), consistent with the results of gentamicin protection assays (Fig. 2). Based on the fact that some proteins altered in the *tdcA* mutant were regulated by *phoP* (Table 3; Monsieurs et al., 2005), we speculate that *phoP* acts as an intermediary, displaying the replicative function of *tdcA*, because the inactivation of *phoP* renders *S*. Typhimurium less virulent in mice and unable to proliferate in phagocytic cells (Groisman, 2001; Groisman and Mouslim, 2006). The four PhoP-dependent genes tested were not strongly downregulated by the *tdcA* mutation in cultures, whereas the gene encoding the SPI2-associated apparatus protein SsaG, which was induced by about 400-fold in the intracellular environment (Valdivia and Falkow, 1997), showed significantly reduced expression in the *tdcA* mutant strains inside macrophages (Figs. 5F and 6). Although the role of the PhoP/Q regulatory system implicated in the expression of SPI2 genes is somewhat controversial, it was recently reported that PhoP/Q is essential for the transcription of *spiC* located within SPI2 in macrophages (Bijisma and Groisman, 2005). Our results suggest that TdcA affects SPI2 gene regulation via the PhoP/Q system inside macrophages.

SPI2 gene expression is directly regulated by an SsrAB two-component system encoded within SPI2, and the expression of SsrAB is in turn regulated by the OmpR/EnvZ two-component system by direct binding to the *ssrAB* promoter (Feng et al., 2003). SlyA exhibits partial functional redundancy with OmpR/EnvZ and contributes to the expression of SPI2 genes through the SsrAB two-component system (Linehan et al., 2005). We do not yet know why the expression of *ssaG* was unchanged by the *tdcA* mutation our the culture medium (Fig. 5), but it is likely that these redundant and multiple regulatory pathways mask the effect of *tdcA* on the expression of SPI2 genes in culture. In particular, none of the SPI2-related proteins appeared to be altered in the protein profiles (Table 3). However, the protein samples analyzed in our two-dimensional assay were obtained under anaerobic-shock conditions, which are not optimal for SPI2 expression (Löber et al., 2006). In addition, the low abundance of the SPI2 proteins restricted a direct analysis by proteomic approaches (Deiwick et al., 2002). Thus, it is not surprising that the product of *ssaG* was not identified in our proteomic experiment. Our two-dimensional results indicate that a number of proteins belonging to various functional groups (e.g., flagellar biosynthesis, energy conservation, and sugar metabolism) were differentially expressed. Although flagella were not required for the expression of virulence in a mouse model of typhoid fever or for survival in mouse macrophages *in vitro* (Josenhans and Suerbaum, 2002; Schmitt et al., 2001), these proteins may be involved in the decreased virulence of the *tdcA* mutant. Nevertheless, it is clear that one reason for the reduced virulence of the *tdcA* mutant is the down

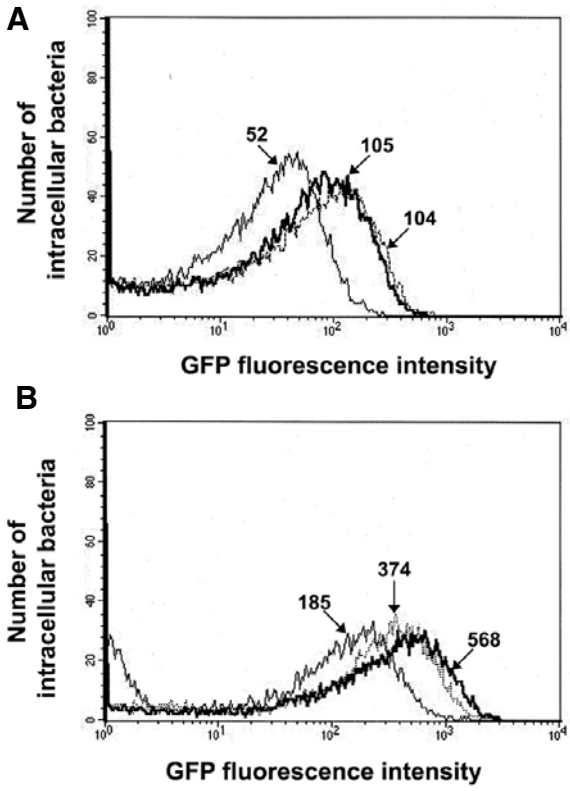

Fig. 6. FACS analysis of *ssaG* expression inside macrophages. J774A.1 cells were infected with wild-type (SL1344) and *tdcA* mutant (SR3501) strains bearing a reporter plasmid, pFMI10, and then subjected to anaerobic shock. Intracellular bacteria were released from the infected macrophages at 2 (A) or 18 h (B) after infection. The histograms show GFP expression in SL1344 (thick lines) and SR3501 (thin lines), and in SR3501 with pMJ-2 (dotted lines). The peak fluorescence intensity is indicated for each sample.

regulation of SPI2 genes.

Interestingly, *tdcB*, which encodes a threonine dehydratase, was upregulated about 4- to 10-fold at all time points after bacterial uptake by macrophages (Eriksson et al., 2003), and the protein product of *tdcC* has been detected *in vivo*, although it is classified as dispensable for virulence because of its absence in *Salmonella* Typhi (Becker et al., 2006). These data indicate that the *tdc* operon can be induced in an intracellular compartment. Despite a lack of conclusive information, we propose, based on these previous reports, that intraphagosomal conditions allow the expression of *tdc* genes together with the relaxation of *Salmonella* DNA in macrophages (Marshall et al., 2000). It has been proposed that once inside macrophages, *S.* Typhimurium uses gluconate and related carbohydrates as principal carbon sources, but the intracellular bacteria appear to experience nutritional stress (Eriksson et al., 2003). Therefore, we hypothesize that during intracellular growth, when energy levels in *Salmonella* are low, induced synthesis of the dehydratase may be needed for the metabolic conversion of threonine and serine to more usable sources of energy, and the subsequent sequence of reactions could be used for ATP production. Considering that some PhoP-regulated genes such as *pag* loci and *phoN*, which encodes a nonspecific acid phosphatase, are maximally induced under starvation conditions (Behlau and Miller, 1995; Kier et al., 1997), it is reasonable to conclude that induced synthesis of the *tdc* genes inside macrophages affects the expression of a subset of PhoP-dependent genes such as SPI2.

In conclusion, together with the recent report that a *Salmonella* strain carrying a null mutation in *tdcA* exhibited a defect in flagellar biosynthesis and decreased virulence when administered orally to mice (Kim et al., 2009), our data suggest that *tdcA* has a pleiotropic effect on the pathogenesis of *S.* Typhimurium. Additional studies are required to elucidate the mechanism by which mutations in *tdcA* decrease SPI2 expression.

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REFERENCES

Altier, C. (2005). Genetic and environmental control of *Salmonella* invasion. J. Microbiol. *43*, 85-92.

Becker, D., Selbach, M., Rollenhagen, C., Ballmaier, M., Meyer, T.F., Mann, M., and Bumann, D. (2006). Robust *Salmonella* metabolism limits possibilities for new antimicrobials. Nature *440*, 303-307.

Behlau, I., and Miller, S.I. (1995). A *phoP*-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. J. Bacteriol. *175*, 4475-4484.

Bijisma, J.J.E., and Groisman, E.A. (2005). The PhoP/PhoQ system controls the intramacrophage type three secretion system of *Salmonella enterica*. Mol. Microbiol. *57*, 85-96.

Cirillo, D.M., Valdivia, R.H., Monack, D.M., and Falkow, S. (1998). Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Micriobiol. *30*, 175-188.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA. *97*, 6640-6645.

Deiwick, J., Rappl, C., Stender, S., Jungblut, P.R., and Hensel, M. (2002). Proteomic approaches to *Salmonella* pathogenicity island 2 encoded proteins and the SsrAB regulon. Proteomics *2*, 792-799.

Eriksson, S., Lucchini, S., Thompson, A., Rhen, M., and Hinton, J.C. (2003). Unravelling the biology of macrophage infection by gene expression profiling of intracellular *Salmonella enterica*. Mol. Micriobiol. *47*, 103-118.

Feng, X., Oropeza, R., and Kenney, L.J. (2003). Dual regulation by phospho-OmpR of *ssrA/B* gene expression in *Salmonella* pathogenicity island 2. Mol. Micriobiol. *48*, 1131-1143.

Galán, J.E. (1996). Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. *20*, 263-271.

Ganduri, Y.L., Sadda, S.R., Datta, M.W., Jambukeswaran, R.K., and Datta, P. (1993). TdcA, a transcriptional activator of the *tdcABC* operon of *Escherichia coli*, is a member of the LysR family of proteins. Mol. Gen. Genet. *240*, 395-402

Goss, T.J., Schweizer, H.P., and Datta, P. (1988). Molecular characterization of the *tdc* operon of *Escherichia coli* K-12. J. Bacteriol. *170*, 5352-5359.

Groisman, E.A. (2001). The pleiotropic two-component regulatory system PhoP-PhoQ. J. Bacteriol. *183*, 1835-1842.

Groisman, E.A., and Mouslim, C. (2006). Sensing by bacterial regulatory systems in host and non-host environments. Nat. Rev. Microbiol. *4*, 705-709.

Hautefort, I., Proença, M.J., and Hinton, J.C.D. (2003). Single-copy green fluorescent protein gene fusions allow accurate measurement of *Salmonella* gene expression *in vitro* and during infection of mammalian cells. Appl. Environ. Microbiol. *69*, 7480-7491.

Hensel, M. (2000). *Salmonella* pathogenicity island 2. Mol. Microbiol. *36*, 1015-1023.

Heßlinger, C., Fairhurst, S.A., and Sawers, G. (1998). Novel keto acid formate-lyase and propionate kinase enzymes are compo-

nents of an anaerobic pathway in *Escherichia coli* that degrades L-threonine to propionate. Mol. Microbiol. *27*, 477-492.
Jones, B.D., and Falkow, S. (1996). Salmonellosis: host immune responses and bacterial virulence determinants. Annu. Rev. Immunol. *14*, 533-561.
Josenhans, C., and Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. Int. J. Med. Microbiol. *291*, 605-614.
Kier, L.D., Weppelman, R., and Ames, B.N. (1977). Regulation of two phosphatases and a cyclic phosphodiesterase of *Salmonella typhimurium*. J. Bacteriol. *130*, 420-428.
Kim, C.C., and Falkow, S. (2004). Delineation of upstream signaling events in the *Salmonella* pathogenicity island 2 transcriptional activation pathway. J. Bacteriol. *186*, 4694-4704.
Kim, M., Lim, S., and Ryu, S. (2008). Molecular analysis of the *Salmonella* Typhimurium *tdc* operon regulation. J. Microbiol. Biotechnol. *18*, 1024-1032.
Kim, M., Lim, S., Kim, D., Choy, H.E., and Ryu, S. (2009). A *tdcA* mutation reduces the invasive ability of *Salmonella enterica* serovar Typhimurium. Mol. Cells *28*, 389-395.
Lee, C.A., and Falkow, S. (1990). The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proc. Natl. Acad. Sci. USA *87*, 4304-4308.
Lim, S., Kim, B., Choi, H.-S., Lee, Y., and Ryu, S. (2006). Fis is required for proper regulation of *ssaG* expression in *Salmonella enterica* serovar Typhimurium. Microb. Pathog. *41*, 33-42.
Linehan, S.A., Rytkönen, A., Yu, X.-J., Liu, M., and Holden, D.W. (2005). SlyA regulates function of *Salmonella* pathogenicity island 2 (SPI-2) and expression SPI-2-associated genes. Infect. Immun. *73*, 4354-4362.
Löber, S., Jäckel, D., Kaiser, N., and Hensel, M. (2006). Regulation of *Salmonella* pathogenicity island 2 genes by independent environmental signals. Int. J. Med. Microbiol. *296*, 435-447.
Lucas, R.L., and Lee, C.A. (2000). Unraveling the mysteries of virulence gene regulation in *Salmonella typhimurium*. Mol. Microbiol. *36*, 1024-1033.
Maloy, S.R., Stewart, V.J., and Taylor, R.K. (1996). Genetic analysis of pathogenic bacteria: A Laboratory Manual, (Plainview, NY: Cold Spring Harbor Laboratory Press).
Marshall, D.G., Bowe, F., Hale, C., Dougan, G., and Dorman, C.J. (2000). DNA topology and adaptation of *Salmonella typhimurium* to an intracellular environment. Phil. Trans. R. Soc. Lond. B. *355*, 565-574.
Mekalanos, J.J. (1992). Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. *174*, 1-7.
Merighi, M., Ellermeier, C.D., Slauch, J.M., and Gunn, J.S. (2005). Resolvase-in vivo expression technology analysis of the *Salmonella enterica* serovar Typhimurium PhoP and PmrA regulons in BALB/c mice. J. Bacteriol. *187*, 7407-7416.
Monsieurs, P., De Keersmaecker, S., Navarre, W.W., Bader, M.W., De Smet, F., McClelland, M., Fang, F.C., De Moor, B., Vanderleyden, J., and Marchal, K. (2005). Comparison of the PhoPQ regulon in *Escherichia coli* and *Salmonella typhimurium*. J. Mol. Evol. *60*, 462-474.
Navarre, W.W., Halsey, T.A., Walthers, D., Frye, J., McClelland, M., Potter, J.L., Kenney, L.J., Gunn, J.S., Fang, F.C., and Libby, S.J. (2005). Co-regulation of *Salmonella enterica* genes required for virulence and resistance to antimicrobial peptides by SlyA and PhoP/PhoQ. Mol. Microbiol. *56*, 492-508.
Ohl, M.E., and Miller, S.I. (2001). *Salmonella*: A model for bacterial pathogenesis. Annu. Rev. Med. *52*, 259-274.
Pace, J., Hayman, M.J., and Galán, J.E. (1993). Signal transduction and invasion of epithelial cells by *S. typhimurium*. Cell *72*, 505-514.
Phillips, A.T., Egan, R.M., and Lewis, B. (1978). Control of biodegradative threonine dehydratase inducibility by cyclic AMP in energy-restricted *Escherichia coli*. J. Bacteriol. *35*, 828-840.
Sawers, G. (1998). The anaerobic degradation of L-serine and L-threonine in enterobacteria: networks of pathways and regulatory signals. Arch. Microbiol. *171*, 1-5.
Schmitt, C.K., Ikeda, J.S., Darnell, S.C., Watson, P.R., Bispham, J., Wallis, T.S., Weinstein, D.L., Metcalf, E.S., and O'Brien, A.D. (2001). Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of *Salmonella enterica* serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis. Infect. Immun. *69*, 5619-5625.
Shin, D., Lee, E.-J., Huang, H., and Groisman, E.A. (2006). A positive feedback loop promotes transcription surge that jump-starts *Salmonella* virulence circuit. Science *314*, 1607-1609.
Shizuta, Y., and Hayaishi, O. (1970). Regulation of biodegradative threonine deaminase synthesis in *Escherichia coli* by cyclic adenosine-3′,5′-monophosphate. J. Biol. Chem. *245*, 5416-5423.
Valdivia, R.H., and Falkow, S. (1997). Fluorescence-based isolation of bacterial genes expressed within host cells. Science *277*, 2007-2011.
Walthers, D., Carroll, R.K., Navarre, W.W., Libby, S.J., and Fang, F.C., and Kenney, L.J. (2007). The response regulator SsrB activates expression of diverse *Salmonella* pathogenecity island 2 promoters and counters silencing by the nucleoid-associated protein H-NS. Mol. Microbiol. *65*, 477-493.
Yoon, H., Lim, S., Heu, S., Choi, S., and Ryu, S. (2003). Proteome of *Salmonella enterica* serovar Typhimurium *fis* mutant. FEMS Microbiol. Lett. *226*, 391-396.