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Potassium transport of *Salmonella* is important for type III secretion and pathogenesis

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Intracellular cations are essential for the physiology of all living organisms including bacteria. Cations such as potassium ion (K^+), sodium ion (Na^+) and proton (H^+) are involved in nearly all aspects of bacterial growth and survival. K^+ is the most abundant cation and its homeostasis in *Escherichia coli* and *Salmonella* is regulated by three major K^+ transporters: high affinity transporter Kdp and low affinity transporters Kup and Trk. Previous studies have demonstrated the roles of cations and cation transport in the physiology of *Escherichia coli*; their roles in the virulence and physiology of pathogenic bacteria are not well characterized. We have previously reported that the *Salmonella* K^+ transporter Trk is important for the secretion of effector proteins of the type III secretion system (TTSS) of *Salmonella* pathogenicity island 1 (SPI-1). Here we further explore the role of *Salmonella* cation transport in virulence *in vitro* and pathogenesis in animal models. Impairment of K^+ transport through deletion of K^+ transporters or exposure to the chemical modulators of cation transport, gramicidin and valinomycin, results in a severe defect in the TTSS of SPI-1, and this defect in the TTSS was not due to a failure to regulate intrabacterial pH or ATP. Our results also show that K^+ transporters are critical to the pathogenesis of *Salmonella* in mice and chicks and are involved in multiple growth and virulence characteristics *in vitro*, including protein secretion, motility and invasion of epithelial cells. These results suggest that cation transport of the pathogenic bacterium *Salmonella*, especially K^+ transport, contributes to its virulence in addition to previously characterized roles in maintaining homeostasis of bacteria.

INTRODUCTION

Living organisms must fulfil basic physiological needs in order to grow and replicate. Critical among them is the need to maintain a stable intracellular environment, usually within a strict range that is unique to each organism. Bacteria, like any other living organism, maintain a homeostasis of pH, osmolarity and ion concentrations. The physiologically important cations for bacteria include potassium ions (K^+), sodium ions (Na^+) and protons (H^+), and their intracellular concentrations are controlled by multiple transporters and channels. Among the intracellular cations, K^+ is the most abundant and its concentration is regulated by K^+ efflux pumps and transporters (Kem & Trachewsky, 1983). K^+ transport is best characterized in the Gram-negative bacterium *Escherichia coli*, which has three major K^+ transport systems – Trk, Kdp and Kup. Trk is a low affinity, rapid transport system that is the main K^+ transporter at neutral or alkaline pH (Bossemeyer *et al.*, 1989; Epstein, 2003; Trchounian & Kobayashi, 2000). Kdp is a high affinity K^+ transport system that is induced in low K^+ environments of 5 mM or less (Epstein, 2003; Frymier *et al.*, 1997). Kup has similar affinity to K^+ as Trk, and is believed to be the major K^+ transport system under acidic conditions (Trchounian & Kobayashi, 1999; Zakharyan & Trchounian, 2001).

A subset of Gram-negative bacteria are important human pathogens, such as pathogenic *E. coli* and *Salmonella* species (Murray, 2003). K^+ transport in the pathogenic species of Gram-negative bacteria is expected to be important but has not been characterized extensively so far. Pathogenic bacteria face many more challenges than their non-pathogenic counterparts. Not only do they need to carry out the activities necessary for all living organisms, such as metabolism and reproduction, they additionally need to produce virulence factors to enable them to invade, survive and proliferate in their hosts (Gal-Mor & Finlay, 2006; Groisman & Ochman, 1997; Jones & Falkow, 1996). Whether and how cations and

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Abbreviations: PMF, proton motive force; SPI-1, *Salmonella* pathogenicity island 1; TTSS, type III secretion system.

cation transport are involved in pathogenesis has not been extensively characterized. We have previously reported that the K^+ transporter Trk is involved in regulating the expression and secretion of the effector proteins SipA, SipC and SopB of the type III secretion system (TTSS) of *Salmonella* pathogenicity island 1 (SPI-1) of *Salmonella*, suggesting the existence of a link between the physiological state of *Salmonella* and its virulence characteristics (Su *et al.*, 2009). Here we report the characterization of roles of the three major K^+ transporter systems, Kdp, Kup and Trk, in the pathogenesis of *Salmonella*. We present evidence that modulation of *Salmonella* K^+ transport, either through deletion of K^+ transporters or through exposure to chemical modulators of cation transport, leads to lowered secretion of effector proteins of the TTSS of SPI-1 and reduced virulence in animal models of infection.

METHODS

Reagents. All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. Reagents for Western blot analyses were from Bio-Rad and reagents for PCR were from Invitrogen. Restriction and modifying enzymes for recombinant DNA were obtained from New England Biolabs. Custom oligonucleotides used for generating and complementing *Salmonella* mutants were from Sigma Genosys. Bacteria and cell culture media were obtained from BD Diagnostics and Invitrogen, respectively.

Bacterial strains, culture conditions and growth curves. *Salmonella enterica* serovar Enteritidis isolate SE2472 (clinical isolate) was used as the WT parental strain for all experiments (Table 1) (Lu *et al.*, 1999, 2002, 2003). SE2472 was virulent in mouse and chick infection, can survive in macrophages and was resistant to a number of stress conditions including reactive oxygen and nitrogen intermediates and the antimicrobial activities of chicken egg albumin (Lu *et al.*, 1999, 2002, 2003). *E. coli* DH5α was used for constructing recombinant plasmid DNA (Table 1). Unless otherwise stated, all bacteria were cultured in Luria–Bertani (LB) broth (BD Diagnostics) at 37 °C with shaking at 225 r.p.m. (Ausubel *et al.*, 1997). LB broth contains 8 mM K^+ as measured by a K^+-selective electrode (Denver Instruments). A minimal K^+ medium (MKM) consisting of 6.78 g Na_2HPO_4 l^{-1}, 2.9587 g $NaH_2PO_4 . H_2O$ l^{-1}, 10 g NaCl l^{-1}, 1 g NH_4Cl l^{-1}, 4 g glucose l^{-1}, 2 mM $MgSO_4$ and 0.2 mM $CaCl_2$ (pH 7) was used to examine the role of K^+ in bacterial growth. Although no

Table 1. Bacterial strains and plasmids

Bacterial strain	Characteristics	Source or reference
Strains		
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA–arg*F) *U169deoRrecA1endA1 hsdR17*(r_K^- m_K^+) *phoAsupE44*λ^- *thi-1gyrA96relA1*	Invitrogen
Salmonella enterica serovar Enteritidis		
SE2472	Clinical isolate	Lu *et al.* (1999)
Δ*trkA*	SE2472 Δ*trkA*::*kan*	Su *et al.* (2009)
Δ*trkA*-comp	SE2472 Δ*trkA*::*kan* transformed with pRB3-trkA	Su *et al.* (2009)
Δ*kdpA*	SE2472 Δ*kdpA*::*cm*	This study
Δ*kup*	SE2472 Δ*kup*::*cm*	This study
Δ*kdpA*/Δ*kup*	SE2472 Δ*kdpA*::*cm* Δ*kup*	This study
Δ*kdpA*/Δ*trkA*	SE2472 Δ*kdpA*::*cm* Δ*trkA*::*kan*	This study
Δ*kup*/Δ*trkA*	SE2472 Δ*kup*::*cm* Δ*trkA*::*kan*	This study
SipA(HF)	SE2472 *sipA*::His-FLAG, $SipA^+$	Su *et al.* (2009)
SipC(HF)	SE2472 *sipC*::His-FLAG, $SipC^+$	Su *et al.* (2009)
SipA(HF)Δ*trkA*	SipA(HF) Δ*trkA*::*kan*	Su *et al.* (2009)
SipA(HF)Δ*trkA*-comp	SipA(HF) Δ*trkA*::*kan* transformed with pRB3-trkA	Su *et al.* (2009)
SipC(HF)Δ*trkA*	SipC(HF) Δ*trkA*::*kan*	Su *et al.* (2009)
SipC(HF)Δ*trkA*-comp	SipC(HF) Δ*trkA*::*kan* transformed with pRB3-trkA	Su *et al.* (2009)
SipA(HF) Δ*kdpA*/Δ*kup*	SipA(HF) Δ*kdpA*::*cm* Δ*kup*	This study
SipC(HF) Δ*kdpA*/Δ*kup*	SipC(HF) Δ*kdpA*::*cm* Δ*kup*	This study
SipA(HF) Δ*kdpA*/Δ*trkA*	SipA(HF) Δ*kdpA*::*cm* Δ*trkA*::*kan*	This study
SipC(HF) Δ*kdpA*/Δ*trkA*	SipC(HF) Δ*kdpA*::*cm* Δ*trkA*::*kan*	This study
SipA(HF) Δ*kup*/Δ*trkA*	SipA(HF) Δ*kup*::*cm* Δ*trkA*::*kan*	This study
SipC(HF) Δ*kup*/Δ*trkA*	SipC(HF) Δ*kup*::*cm* Δ*trkA*::*kan*	This study
Plasmids		
pRB3-273C	Ap^r, low to medium copy number plasmid for *Salmonella*	Berggren *et al.* (1995)
pRB3-trkA	Derivative of pRB3-273C containing *trkA*	Su *et al.* (2009)
pLN	Derivative of pRB3-273C containing pHluorin	This study
pKD3	Ap^r Cm^r, $oriR_\gamma$	Datsenko & Wanner (2000)
pKD4	Ap^r Kan^r, $oriR_\gamma$	Datsenko & Wanner (2000)
pKD46	Ap^r, containing the Red recombinase of λ phage	Datsenko & Wanner (2000)
pCP20	Ap^r, *cat* cl857 λPr *flp* pSC101 oriTS	Datsenko & Wanner (2000)

K^+ was added to MKM, it contained approximately 0.2 mM K^+ as measured by a K^+-selective electrode, likely from K^+ contaminants in the salts used (Su *et al.*, 2009). Antibiotics were added as appropriate.

Growth curves of *Salmonella* in various media were determined by plating as previously described (Lu *et al.*, 1999). *Salmonella* was cultured overnight in LB broth and was diluted 1 : 100 in LB broth, MKM or MKM supplemented with 100 mM KCl. Diluted cultures were incubated at 37 °C with shaking at 225 r.p.m.; an aliquot was collected after various incubation periods and serial dilutions of each bacterial culture were plated onto LB agar plates to determine the bacterial concentration (c.f.u. ml^{-1}). Growth curves were constructed by plotting bacterial concentration against the incubation period.

Construction of K^+ transporter mutants and complementation of the mutants. Single K^+ transporter mutants of *Salmonella* were generated using the one-step mutagenesis method of Datsenko and Wanner (Datsenko & Wanner, 2000). The deletion mutant Δ*trkA* of *Salmonella* was reported previously, in which the coding sequence of *trkA* was replaced by a kanamycin resistance cassette (Kan^R) (Su *et al.*, 2009). The deletion mutants Δ*kdpA* and Δ*kup* were generated similarly using oligonucleotides listed in Table 2. The mutagenesis primer pairs (kdp5KO/kdp3KO and kup5KO/kup3KO) were used to amplify the chloramphenicol resistance cassette (Cm^R) from plasmid pKD3 (Table 1). The PCR products were then used to generate deletion mutations in *kdpA* and *kup*, respectively, through homologous recombination. Each mutant was verified with the corresponding verification primer pair (kdpA5/kdpA3 or kup5/kup3) in combination with primers in the Cm^R cassette. All mutations were transduced into fresh *Salmonella* by general transduction using phage P22, and phage-free colonies were used for further analysis (Maloy *et al.*, 1996). Double mutants were generated by transducing a second deletion mutation into single mutants using general transduction by phage P22. Since Cm^R was used as the selection marker for both the Δ*kdpA* and the Δ*kup* mutants, the Cm^R cassette was first removed from the chromosome of the Δ*kup* mutant by transformation of plasmid pCP20 before the Δ*kdpA* mutation was transduced into the Δ*kup* mutant to generate the Δ*kdpA*/Δ*kup* double mutant (Datsenko & Wanner, 2000). The Δ*trkA* mutant of *Salmonella* was complemented by the plasmid pRB3-trkA (Su *et al.*, 2009). Vector pRB3-273C was also transformed into the mutants as a control for any possible effect of plasmid transformation alone and no effect was observed in any assay (Lu *et al.*, 2002).

Quantification of protein levels of culture supernatant. *Salmonella* strains were cultured in LB broth overnight at 37 °C with shaking at 225 r.p.m. Antibiotics were added to mutant *Salmonella* strains as appropriate. An aliquot of each culture was serially diluted and plated onto LB agar plates to determine the bacterial c.f.u. ml^{-1}. Three millilitres of each bacterial culture was then spun down at 13 000 r.p.m. for 10 min and 2.4 ml of culture supernatant was transferred to a fresh tube without disturbing the bacterial pellet. Proteins were purified from culture supernatant by TCA precipitation as described by Komoriya *et al.* (1999). Briefly, 2.4 ml of culture supernatant was mixed with 0.8 ml 25 % TCA and incubated on ice for 30 to 60 min. After incubation, the culture supernatant and TCA mix was spun down at 13 200 r.p.m. for 10 min at 4 °C and the supernatant was then removed. The protein pellet was washed three times vigorously with 2 ml acetone, dried briefly and resuspended in a urea sample buffer (8 M urea, 2 % CHAPS and 10 mM Tris pH 8.0). Protein concentration from each *Salmonella* strain was determined by DC protein assay (Bio-Rad) and normalized against the bacterial concentration of the culture (c.f.u. ml^{-1}).

Analysis of protein levels of SipA and SipC in culture supernatant and whole-cell lysate. *Salmonella* strains containing tandem epitope tags of FLAG and six histidines (6 × His) in SipA or SipC were constructed and described previously (Su *et al.*, 2009). The resulting strains, SipA(HF) and SipC(HF), were used to monitor the levels of the respective protein in the culture supernatant and the bacterial lysate (Table 1). All bacterial strains were cultured in LB broth at 37 °C overnight with shaking. The OD_{600} of bacterial cultures was measured and the cultures of all bacterial strains were adjusted to the same density before being used for analysis of SipA and SipC expression and secretion. Bacterial whole-cell lysate was prepared by spinning down bacterial cultures, washing bacteria twice in ice-cold PBS, resuspending bacterial pellets in an SDS sample buffer (50 mM Tris/HCl pH 6.8, 100 mM DTT, 2 % SDS, 10 % glycerol, 0.1 % bromophenol blue) followed by boiling for 10 min (Sambrook & Russell, 2001). Secreted proteins were prepared by using TCA precipitation as described by Komoriya *et al.* (1999) and protein concentrations were determined by DC protein assay. Five micrograms of secreted proteins from SipA-tagged strains or 15 μg of secreted proteins from SipC-tagged

Table 2. Oligonucleotides used for mutagenesis and complementation of mutants

Oligonucleotide	Used for	Sequence (5′→3′)
kdpA5KO	Mutagenesis of *kdpA*	caaggatttttactgatcgccagtttttactgattttattggtactggcgaaaccgttgggttcgggcctggcaaggc tcatcgccgccgttccgttgcgtgtaggctggagctgcttc
kdpA3KO	Mutagenesis of *kdpA*	taccgggccaagcgccagggcaggaataaacgtcagcgcgccgaccagcagtaccgtgccgatgagcagccca ataaatagcgcgccgtgggtcgccagccatatgaatatcctccttag
kup5KO	Mutagenesis of *kup*	ataataagcaatcgttgcctgcgattaccctcgcagctattggggttgtctacggtgatattggtaccagcccgcttt atacgcttcgtgaatgtttgtcgtgtaggctggagctgcttc
kup3KO	Mutagenesis of *kup*	taactcaatcacgcggttaggcgggatctcgaactgatccggcgcgcgcagggcgttgcgttgcaacaacaaata cagcttgccacgcagacgcaaataccatatgaatatcctccttag
kdpA5	Confirmation of *kdpA* mutation	tatgccctgattaatgcgga
kdpA3	Confirmation of *kdpA* mutation	atcaatgtactccgcatcgc
kup5	Confirmation of *kup* mutation	gtgtataaacgaaagatgag
kup3	Confirmation of *kup* mutation	tgttgatgggaggttaaatc
LNHd5	Expression of pHluorin	agaccaggaagcttgatgagtaaaggagaagaactt
LNHd3	Expression of pHluorin	atagcaacaagcttttatttgtatagttcatccat

strains were used for Western blot analysis to compare the relative level of SipA or SipC in the total secreted proteins. Fifteen micrograms of secreted proteins were used for Western blot analysis for SipC because the SipC level in secreted proteins was much lower than the SipA level. The equal loading of protein samples was further confirmed by Coomassie blue staining of SDS-PAGE gels following electrophoresis. Following electrophoresis, SipA or SipC was detected by Western blot analysis as described previously (Su *et al.*, 2009). A monoclonal anti-FLAG antibody (Sigma-Aldrich) was used at 1 : 1000 dilution as the primary antibody and a horseradish peroxidase-conjugated sheep anti-mouse IgG (GE Healthcare) was used at 1 : 2000 dilution as the secondary antibody. Hybridization signal was visualized by the enhanced chemiluminescence method (GE Healthcare). The exposure time was adjusted according to the signal level for each protein.

Exposure of *Salmonella* to cation transport modulators. Cation transport modulators gramicidin, valinomycin and nigericin were obtained from Sigma-Aldrich. All modulators were dissolved in DMSO and subsequently diluted in bacterial culture to the concentrations indicated. To determine the effect of the cation transport modulators on the growth and the TTSS of *Salmonella*, overnight culture of *Salmonella* in LB broth was diluted 1 : 100 in 3 ml of fresh LB broth supplemented with various concentrations of cation transport modulators and cultured for 16 to 20 h at 37 °C with shaking. The levels of SipA and SipC in the culture supernatant and in bacterial cells were analysed as described above.

Measurement of intrabacterial pH. The pH inside bacteria was measured using a pH-sensitive green fluorescent protein, pHluorin, that displays a pH-dependent ratio of fluorescence emission at 510 nm with excitation at 375 nm to emission at 510 nm with excitation at 405 nm (Miesenböck *et al.*, 1998). Primers LNHd5 5′-AGACCAGGAAGCTTGATGAGTAAAGGAGAAGAACTT-3′ and LNHd3 5′-ATAGCAACAAGCTTTTATTTGTATAGTTCATCCAT-3′ were used to amplify the pHluorin sequence from plasmid pGEX2T (gift of Dr Sabine Ehrt, Weill Cornell Medical College) by PCR. Amplified DNA was digested with *Hin*dIII and cloned into the *Hin*dIII site of pRB3-273C (Berggren *et al.*, 1995). The resulting plasmid, pLN, contained pHluorin under the control of the *lac* promoter. The plasmid pLN was transformed into *Salmonella* strains for measuring intrabacterial pH as described by Vandal *et al.* (2008). Bacterial culture was spun down and the pellet was resuspended in PBS at 1/10 volume of the original culture. Three hundred microlitres of resuspended bacteria was transferred to a 96-well black plate (USA Scientific) and the fluorescence was detected with a SpectraMax II plate reader (Molecular Devices). Fluorescence was detected at emission of 510 nm (Em510) with excitation of 375 nm (Ex375 nm) or 405 nm (Ex405 nm), and the ratio of excitation of Em510-Ex375 nm to Em510-Ex405 nm was calculated.

For each experiment, a standard curve of pHluorin was prepared for determining the intrabacterial pH of *Salmonella*. Whole-cell lysate of *Salmonella* transformed with plasmid pLN was prepared and diluted to 50 μg ml^{-1} in a series of sodium phosphate buffers at defined pH from 6.6 to 8.0. Em510 was measured with a SpectraMax II plate reader at Ex375 nm or Ex405 nm and the ratio of Em510-Ex375 nm to Em510-Ex405 nm was plotted against the pH of the phosphate buffer. The intrabacterial pH of whole-cell *Salmonella* was determined by plotting the ratio of Em510-Ex375 nm to Em510-Ex405 nm on the standard curve prepared for each experiment.

Measurement of bacterial ATP and K^+. For measuring bacterial ATP, *Salmonella* strains were grown overnight at 37 °C with shaking at 225 r.p.m. in LB broth with appropriate antibiotics. Overnight cultures were diluted 1 : 100 in fresh LB or LB supplemented with various concentrations of cation transport modulators. Aliquots were harvested at various time points and serially diluted bacterial cultures were plated to determine bacterial concentration (c.f.u. ml^{-1}) after overnight incubation. Bacteria were then collected by centrifugation at 13 200 r.p.m. for 5 min and bacterial extracts were prepared by perchloric acid extraction (Bagnara & Finch, 1972). Two hundred microlitres of bacterial culture was mixed with 100 μl 1.2 M perchloric acid pre-cooled on ice and vortexed for 10 s. The mixture was incubated on ice for 15 min and spun down at 132 000 r.p.m. for 5 min at 4 °C. Two hundred microlitres of supernatant was transferred to a fresh tube and mixed with 100 μl neutralizing solution containing 0.72 M KOH and 0.16 M $KHCO_3$. Neutralized extract was spun down at 132 000 r.p.m. for 5 min and supernatant was transferred to a fresh tube for ATP assay using a BacTiter-Glo microbial cell viability assay (Promega) following the manufacturer's procedure.

Luminescence was read in a SpectraMax M2 plate reader (Molecular Devices). A standard curve was prepared in each assay using ATP standard solutions prepared from adenosine 5-triphosphate disodium salt hydrate (A2383; Sigma-Aldrich) and ATP quantity in bacterial samples was determined by comparing to the standard curve.

Bacteria K^+ content was determined as described by Yan *et al.* (1996) with modifications. *Salmonella* strains were grown overnight at 37 °C with shaking at 225 r.p.m. in LB broth with appropriate antibiotics. An aliquot of each culture was serially diluted and plated to determine bacterial concentration (c.f.u. ml^{-1}) after overnight incubation. Overnight culture of *Salmonella* was spun down at 10 000 r.p.m. for 2 min and the supernatant was removed. Bacterial pellet was resuspended in an equal volume of 1 % nitric acid and the mixture was incubated at room temperature for 1 h to lyse bacteria. The reaction mixture was spun down at 13 200 r.p.m. for 5 min and supernatant containing lysed bacteria was neutralized with 10 M NaOH. The K^+ concentration in neutralized bacterial lysate was measured using a K^+-selective electrode and calculated by comparing to a K^+ standard curve. Each measurement was carried out three times and the mean was used for data analysis.

Invasion of HeLa cells by *Salmonella*. HeLa cell invasion assay was performed as described (Lu *et al.*, 1999). All *Salmonella* strains were cultured in LB broth overnight at 37 °C without shaking. Antibiotics were added as appropriate. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco by Life Technologies) supplemented with 10 % FBS that has a K^+ concentration of approximately 5 mM as measured by a K^+-selective electrode. Overnight culture of bacteria was added to HeLa cells at an m.o.i. of 5 : 1 to 10 : 1, and intracellular bacteria were quantified after one or two hours of invasion followed by an incubation period in the presence of 50 μg gentamicin ml^{-1} to kill extracellular bacteria. The invasiveness of *Salmonella* was measured by the ratio of intracellular bacteria to the input bacteria, which was calculated as (number of intracellular bacteria/number of input bacteria) × 100 %.

Motility of the K^+ transporter mutants of *Salmonella*. *Salmonella* strains were grown overnight at 37 °C with shaking at 225 r.p.m. in LB broth with appropriate antibiotics. Motility agar plates were prepared using 25 ml motility agar (BD Diagnostics) in each Petri dish. Overnight culture of bacteria was inoculated in the centre of a Petri dish using an inoculation needle and each strain was analysed in triplicate. After overnight incubation at 37 °C, the diameter of the zone of bacterial growth on each Petri dish was measured. To study the effect of K^+ or Na^+ on the motility of the K^+ transporter mutants of *Salmonella*, *Salmonella* strains were grown in LB broth supplemented with 100 mM KCl or NaCl and assayed on motility agar plates supplemented with the same concentration of the corresponding salt.

Mouse and chick infection of *Salmonella*. *Salmonella* cultured overnight in LB broth at 37 °C with shaking at 225 r.p.m. was used in all animal infection experiments. Six- to eight-week-old BALB/c mice (Jackson Laboratory) were infected intragastrically and the LD_{50} was determined by infecting groups of five mice with 10-fold dilutions of bacteria in PBS (Lu *et al.*, 1999; Reed & Muench, 1938).

For infection of chicks, specific pathogen-free eggs were obtained from Charles River SPAFAS (North Franklin, CT) and incubated in an incubator/hatcher (G.F.Q. Manufacturing) with automatic turning. After 18 days of incubation, eggs were moved to the hatching tray of the incubator until hatched. Newly hatched chicks were left in the incubator to dry for 4–6 h and then moved to a cage. Within 24 or 36 h after hatching, 10^9 c.f.u. of *Salmonella* suspended in 250 μl sterile PBS was administered to the crop of each chick via a feeding tube. Infected chicks (in groups of 10) were monitored twice daily for 14 days and survival was recorded.

RESULTS

Construction of K^+ transporter mutants of *Salmonella*

Since K^+ is the most abundant cation in all living cells and plays an important role in maintaining the electrical gradient in bacteria, we generated mutations in K^+ transporters and determined if K^+ transporters are important for the pathogenesis of *Salmonella*. We have previously reported the construction and analysis of a Δ*trkA* mutant of *Salmonella* that contained a deletion mutation of the low affinity K^+ transport system Trk (Su *et al.*, 2009). The Δ*trkA* mutant was found to be defective in the expression and secretion of the effector proteins of the TTSS of SPI-1, invasion of epithelial cells and virulence in mouse infection (Su *et al.*, 2009). In this analysis, we constructed additional mutants of the low affinity K^+ transport system Kup and the high affinity K^+ transport system Kdp in the virulent clinical *Salmonella* strain SE2472 (Lu *et al.*, 1999). We generated double mutants Δ*kdpA*/Δ*kup*, Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* for *in vitro* and *in vivo* analyses (Table 1).

All K^+ transporter mutants of *Salmonella* displayed the same colony morphology as the WT *Salmonella* on LB agar, except that the Δ*trkA*-containing mutants (Δ*trkA*, Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA*) formed smaller colonies (data not shown). The growth properties of the K^+ transporter mutants were determined in LB broth, MKM and MKM supplemented with a high concentration (100 mM) of KCl (Fig. 1). Using a K^+-selective electrode, the concentration of K^+ in LB broth was determined to be approximately 8 mM. The MKM was prepared without any K^+ salt; however, it contained approximately 0.2 mM K^+, likely from K^+ contamination in salts used for the medium. In LB broth, all mutants grew to the same concentration after 24 h of incubation (Fig. 1a). In the MKM, mutants with deletions in Kdp failed to reach the same concentration as those strains with an intact Kdp system (Fig. 1b) ($P=0.00008$ and 0.00007 for Δ*kdpA*/Δ*kup* and Δ*kdpA*/Δ*trkA*, respectively at 24 h, Student's *t*-test). The double mutant Δ*kdpA*/Δ*trkA* was especially severely affected and displayed little or no growth over 24 h of incubation (Fig. 1b). In the presence of a high K^+ concentration (100 mM), the growth of all K^+ transporter mutants was relatively normal and reached the same bacterial concentration after 24 h of incubation as the WT parental strain (Fig. 1c). The Δ*trkA*-containing double mutants displayed a slight growth delay during mid- to late-exponential phase ($P=0.002$ for both Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* at 6 h, Student's *t*-test), but eventually reached the same bacterial concentration at the stationary phase as the WT *Salmonella* (Fig. 1c).

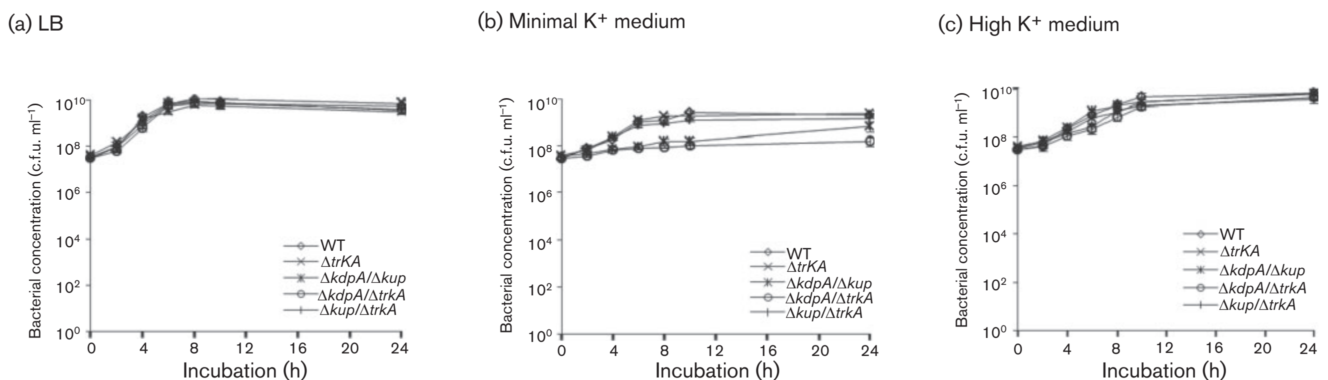

Fig. 1. Growth properties of the K^+ transporter mutants of *Salmonella* in culture media. The WT *Salmonella* (◇) and Δ*trkA* (×), Δ*kdpA*/Δ*kup* (*), Δ*kdpA*/Δ*trkA* (○) and Δ*kup*/Δ*trkA* (|) mutants were cultured in LB broth (a), minimal K^+ medium (b) and minimal K^+ medium supplemented with 100 mM KCl (c). Bacterial concentrations were determined by plating. At least three experiments were performed and results from a representative experiment performed in triplicate are shown. Error bars indicate SD.

K^+ transporters are important for the secretion of the effector proteins of the TTSS of SPI-1

Since the K^+ transporter Trk was shown to be involved in the secretion of effector proteins of the TTSS of SPI-1 (Su *et al.*, 2009), we tested how other transporters, Kdp and Kup, affect the secretion of the effector proteins SipA and SipC of the TTSS of SPI-1, factors that have been previously shown to play important functions in host-cell invasion and in the modulation of the host cytoskeleton to facilitate *Salmonella* infection (Jepson *et al.*, 2001; Kaniga *et al.*, 1995; Raffatellu *et al.*, 2005). We used tagged strains SipA(HF) and SipC(HF) that contained a tandem tag of $6\times$His and FLAG epitope fused in-frame to the C-terminus of the corresponding protein in the chromosome (Su *et al.*, 2009; Uzzau *et al.*, 2001). The Δ*kdpA*, Δ*kup* and Δ*trkA* mutations were transduced into each tagged strain either singularly or in combinations (Table 1) and the levels of the tagged SipA or SipC protein in the whole bacterial lysate and the culture supernatant were analysed by Western blot using an anti-FLAG antibody (Fig. 2).

Secreted proteins were purified from culture supernatant of the WT and K^+ transporter mutant *Salmonella* and equal quantities of secreted proteins from each strain were used for Western blot analysis (Fig. 2a). Since no secreted protein has been shown to be constitutive and can thus serve as an internal control, a duplicate gel of secreted proteins from SipA-tagged K^+ transporter mutant strains was stained to visualize the protein content in the culture supernatant (Fig. 2b). The Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* double mutants were severely defective in SipA and SipC secretion (Fig. 2a) and also had altered compositions of total secreted proteins (Fig. 2b). Compared with the double mutants, the Δ*trkA* single mutant had a less severe defect in SipA and SipC secretion and the defect was rescued by complementation with plasmid pRB3-trkA, which carries a WT allele of the *trkA* gene (Fig. 2a). The SipA and SipC levels in the Δ*kdpA*/Δ*kup* mutant were close to those in the WT *Salmonella* (Fig. 2a).

In contrast to changes of the levels of SipA and SipC in culture supernatant of K^+ transporter mutants, the levels of SipA and SipC in bacterial lysate were not significantly affected by the deletion of any of the K^+ transporters (Fig. 2c), suggesting that the lowered levels of SipA and SipC in the culture supernatant of K^+ transporter mutants were not due to a lack of their expression. The levels of SipA and SipC were slightly lower in the Δ*trkA* mutant (Fig. 2c), but not in the Δ*trkA*-containing double mutants (Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA*), possibly because more SipA and SipC were retained in bacteria in the double mutants instead of being secreted (Fig. 2c).

Cation transport modulators affect the TTSS of *Salmonella*

Since analysis of K^+ transporter mutants indicated that K^+ transport may be involved in the TTSS, we next tested if chemical reagents that modulate cation transport (including K^+ transport) would affect the TTSS of *Salmonella*. The cation transport modulators we used included valinomycin, nigericin and gramicidin. Valinomycin is an ionophore for K^+ and allows transport of K^+ along the K^+ gradient. Gramicidin increases permeability to all cations instead of specifically to K^+. Nigericin mediates the exchange of K^+ and H^+. The cation transport modulators were first tested for their effect on the growth of *Salmonella*. None was found to have any effect on the growth of *Salmonella* at the highest soluble concentrations tested (data not shown).

Salmonella was treated with the cation transport modulators to determine if they affect the TTSS using SipA and SipC proteins as markers. *Salmonella* strains with the FLAG epitope tag at SipA or SipC were exposed to various concentrations of the cation transport modulators and the levels of SipA and SipC in the culture supernatant and the bacterial cell lysates were determined by Western blot analysis (Fig. 3). Valinomycin and gramicidin inhibited the secretion of both SipA and SipC, while nigericin had no effect at all concentrations tested (Fig. 3). None of the cation transport modulators inhibited the expression of SipA or SipC and the levels of both proteins were comparable to those in the WT *Salmonella* (Fig. 3). DMSO was used as a solvent for all cation transport modulators and it had no effect on the expression or secretion of SipA or SipC (Fig. 3).

The cation transport modulator gramicidin increases the permeability of bacterial cell membranes to cations and exposure to gramicidin inhibits the TTSS in *Salmonella* (Fig. 3c, d). We reasoned that the inhibition of the TTSS by gramicidin could be due to a loss of intrabacterial K^+ along the gradient since intracellular K^+ concentration is much higher than that in the medium ($>$100 mM vs 8 mM). To test this hypothesis, we exposed the WT *Salmonella* to a low concentration of gramicidin in the presence or absence of 100 mM KCl or NaCl, two of the most physiologically relevant cations. The partial inhibition of SipA and SipC secretion by a low concentration of gramicidin was relieved by KCl supplementation but not by NaCl (Fig. 4a). This result suggests that the rescue of gramicidin inhibition of the TTSS by KCl is not simply due to the osmotic pressure KCl provides since NaCl provides the same osmotic pressure as KCl but failed to restore the TTSS in gramicidin-treated *Salmonella* (Fig. 4a).

The cation transport modulator nigericin does not affect the TTSS of the WT *Salmonella* (Fig. 3e, f). We next treated the K^+ transport mutants of *Salmonella* with nigericin to determine if this affects the TTSS when K^+ transport is impaired. Similar to that observed with the WT *Salmonella*, exposure to nigericin did not affect the growth and survival of the single or double K^+ transporter mutants (data not shown). Only the single mutant Δ*trkA* and the double mutant Δ*kdpA*/Δ*kup* were included in the analysis since

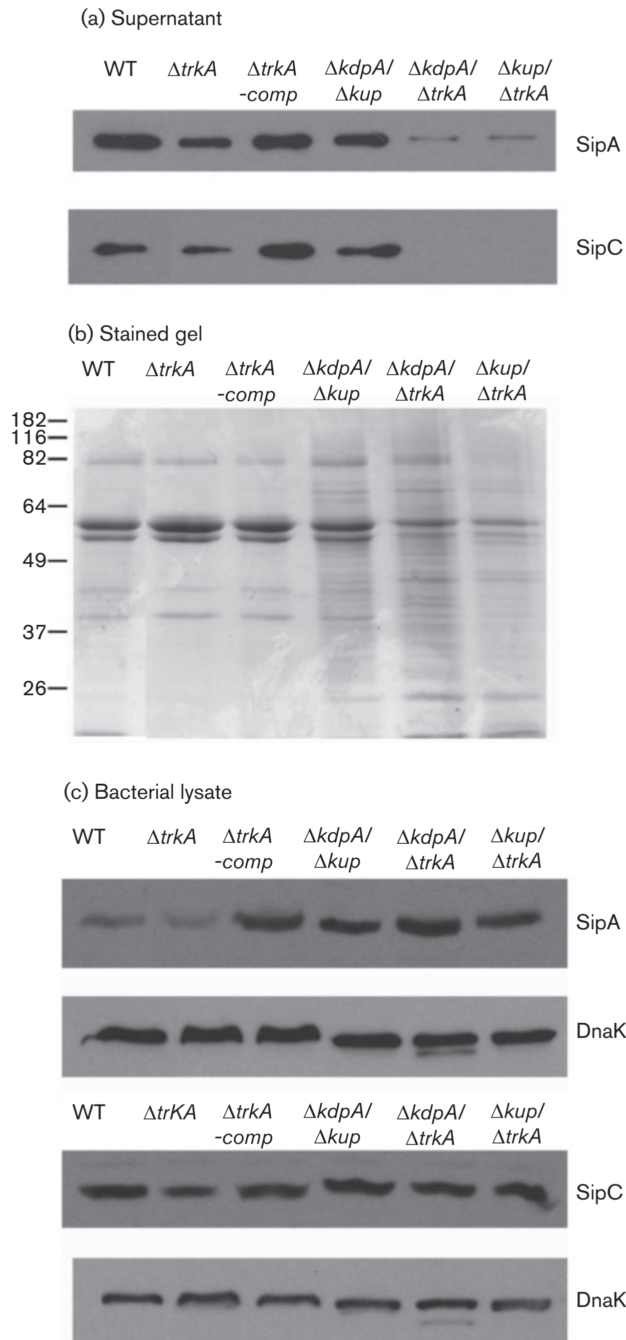

Fig. 2. Secretion and expression of effector proteins SipA and SipC of the SPI-1-encoded TTSS by K^+ transporter mutants of *Salmonella*. (a) Western blot analysis of SipA and SipC levels in culture supernatant. Epitope-tagged strains SipA(HF) and SipC(HF) with the WT K^+ transporters, one or two K^+ transporter mutant alleles (Δ*trkA*, Δ*kdpA*/Δ*kup*, Δ*kdpA*/Δ*trkA* or Δ*kup*/Δ*trkA*), or complemented single mutant Δ*trkA*-comp were cultured in LB broth. Secreted proteins were purified from culture supernatant by TCA precipitation, quantified by DC protein assay and equal quantities of secreted proteins from each strain were used for each gel. SipA and SipC levels in the culture supernatant were determined by Western blot analysis. The bacterial strain used for each sample is labelled above each lane. The effector protein analysed in each panel is labelled on the right. (b) A duplicate gel of secreted proteins from SipA-tagged strains was included to show the total secreted proteins from each strain. (c) Western blot analysis of SipA and SipC levels in whole-cell lysates. DnaK was used as a control for equal loading. The effector protein analysed in each panel or DnaK control is labelled on the right. At least three experiments were performed and results from a representative experiment are shown.

the double mutants Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* were defective in SipA and SipC secretion without nigericin treatment (Fig. 3). As shown in Fig. 4(b), SipA secretion was inhibited in the nigericin-treated Δ*kdpA*/Δ*kup* double mutant, and SipC secretion was inhibited in both the Δ*trkA* mutant and the Δ*kdpA*/Δ*kup* double mutant treated with nigericin. Nigericin treatment affected SipC more than SipA in the Δ*trkA* mutant likely because the SipC level is much lower than SipA in secreted proteins and is thus more easily disturbed.

TTSS defect observed in the K^+ transport mutant *Salmonella* and *Salmonella* treated with cation transport modulators was not due to a failure in maintaining intrabacterial pH

We have shown above that cation transport is important for the TTSS and interference with K^+ and H^+ transport compromises the TTSS of SPI-1. Since both K^+ and H^+ are important intrabacterial cations that are major components of proton motive force (PMF), it is possible that K^+ transport affects the TTSS because the disturbance of K^+ transport causes a distubance in PMF. To evaluate this possibility, we measured the intrabacterial pH in the K^+ transporter mutant or cation transport modulator-treated *Salmonella* to determine if the suppression of the TTSS correlates with a failure in regulating intrabacterial pH. We took advantage of the pH-sensitive green fluorescent protein, pHluorin, to measure the intrabacterial pH (Miesenböck *et al.*, 1998; Vandal *et al.*, 2008). A plasmid, pLN, was constructed that expresses pHluorin in *Salmonella* and transformed into the WT or K^+ transport mutant *Salmonella*. Overnight culture of *Salmonella* was diluted in LB broth and intrabacterial pH was measured after 3 or 16 h of growth. The Δ*trkA* single mutant and Δ*trkA*-containing double mutants displayed a generally lower intrabacterial pH as they grew from log to stationary phase (Fig. 5a). The difference between the Δ*trkA*-containing single or double mutants and the WT *Salmonella* was small, but statistically significant (P=0.01, 0.009 and 0.005 at 16 h for Δ*trkA*, Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA*, respectively, Student's *t*-test). However, the intrabacterial pH in the Δ*trkA* single mutant was indistinguishable from those of the Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* double mutants, suggesting that intrabacterial

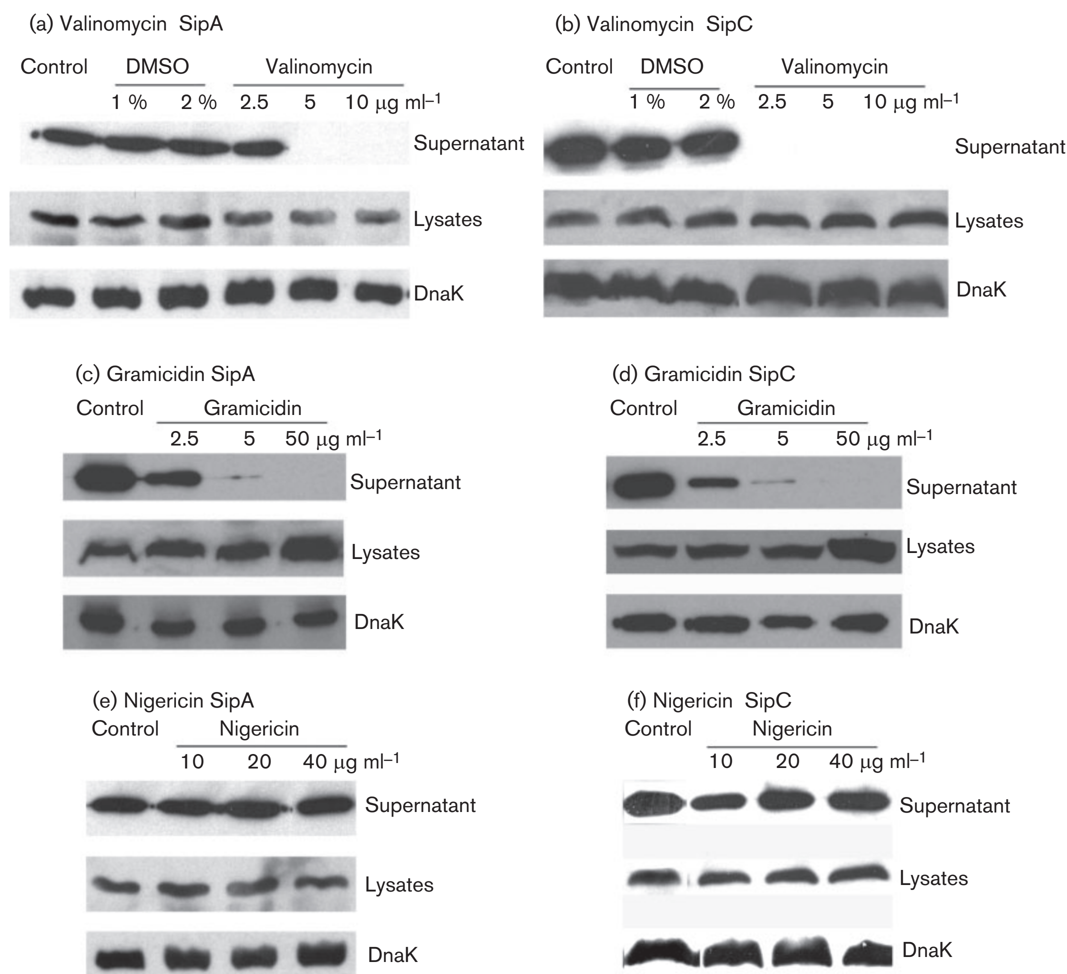

Fig. 3. Secretion and expression of effector proteins SipA and SipC of the SPI-1-encoded TTSS in *Salmonella* exposed to cation transport modulators. Epitope-tagged strains SipA(HF) and SipC(HF) were grown in LB broth and exposed to various concentrations of the cation transport modulators valinomycin (a, b), gramicidin (c, d) or nigericin (e, f). DMSO was used as a solvent for all cation modulators and was included as a control. The levels of SipA (a, c, and e) and SipC (b, d, and f) in the culture supernatant and whole-cell lysates were determined by Western blot analysis and compared to those of untreated *Salmonella*. Proteins were isolated from culture supernatant by TCA precipitation and quantified by DC protein assay. Equal quantities of proteins from culture supernatant were loaded in each lane in each experiment. DnaK was used as a control for equal loading of bacterial lysates. At least three experiments were performed and results from a representative experiment are shown.

pH did not correlate with the TTSS level as SipA and SipC secretion was much lower in the Δ*trkA*-containing double mutants compared to the Δ*trkA* single mutant (Fig. 2a). *Salmonella* mutant Δ*yafD* (Lu *et al.*, 2003), a deletion mutant of the *yafD* gene that was shown to be involved in the survival of *Salmonella* in egg albumin and has no known role in the TTSS, was included as a control for possible influence of mutagenesis or antibiotic resistance markers on intrabacterial pH. The intrabacterial pH of the Δ*yafD* mutant was similar to that of the WT *Salmonella*, suggesting that the slightly lowered pH observed in the Δ*trkA* mutants was not due to the mutagenesis procedure itself (Fig. 5a).

The intrabacterial pH of *Salmonella* exposed to cation transport modulators was determined as described for K^+ transport mutants. WT *Salmonella* transformed with pLN was exposed to gramicidin (25–100 μg ml^{-1}), nigericin (10–40 μg ml^{-1}) or valinomycin (2.5–10 μg ml^{-1}) and bacteria were harvested after 3 or 16 h of exposure. The intrabacterial pH of treated *Salmonella* was determined and compared to the untreated *Salmonella* or *Salmonella* treated with the solvent DMSO. The results of the highest concentrations tested are shown in Fig. 5b. Treatment with gramicidin, nigericin or valinomycin did not affect the intrabacterial pH, as pH in the treated *Salmonella* was indistinguishable from that of the untreated *Salmonella* or *Salmonella* treated with the solvent DMSO (Fig. 5b).

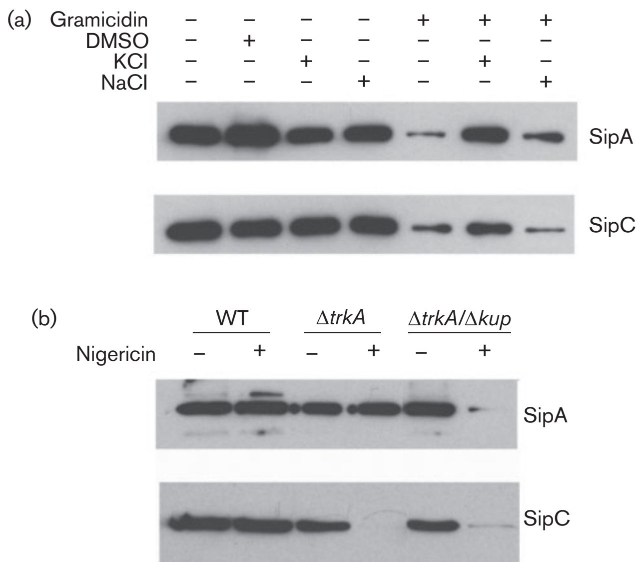

Fig. 4. Ions, cation transport modulators and K^+ transporters interact to affect the TTSS of SPI-1. (a) Epitope-tagged strains SipA(HF) and SipC(HF) were grown in LB broth in the presence of the cation transporter modulator gramicidin and cation supplementation as marked. (b) Epitope-tagged strains SipA(HF) and SipC(HF) containing mutations in K^+ transporters were cultured in the presence or absence of the cation transport modulator nigericin. Proteins were isolated from culture supernatant and equal quantities of proteins from culture supernatant were loaded in each lane. The levels of SipA and SipC were determined by Western blot analysis. At least three experiments were performed and results from a representative experiment are shown.

TTSS defect observed in the K^+ transport mutant *Salmonella* or cation transport modulator-treated *Salmonella* was not due to a failure in maintaining intrabacterial ATP level

Intracellular ATP levels were measured in the K^+ transporter mutants to determine if the defect in the TTSS was due to a lack of intracellular ATP. All strains were cultured in fresh medium and aliquots of each culture were harvested after various incubation periods to quantify bacterial concentration (c.f.u. ml^{-1}) and total ATP levels. The total ATP levels were normalized against bacterial concentration and plotted against the incubation period (Fig. 6a). ATP levels in all bacterial cultures were high during early exponential phase of culture (at 3 h) and subsequently decreased with incubation period. Compared with the WT *Salmonella*, the K^+ transporter mutants displayed comparable or higher ATP levels, suggesting that the defect in the TTSS cannot be explained by a lack of intracellular ATP (Fig. 6a).

We next determined the energy level of cation modulator-exposed *Salmonella* by measuring the ATP level in the treated bacteria. Overnight culture of *Salmonella* was diluted in fresh LB, or LB supplemented with the solvent DMSO, gramicidin (25–100 μg ml^{-1}), nigericin (10–40 μg ml^{-1}) or valinomycin (2.5–10 μg ml^{-1}). Bacterial culture was harvested after various periods of incubation and bacterial concentration and the ATP level in each sample were determined. Results from treatment with the highest concentration of gramicidin (100 μg ml^{-1}), nigericin (40 μg ml^{-1}) or valinomycin (10 μg ml^{-1}) are shown in Fig. 6(b). No difference was observed between *Salmonella* treated with cation transport modulators and untreated or DMSO-treated *Salmonella*. The lack of effect of cation transport modulators on intrabacterial ATP levels suggests that the inhibition by gramicidin and valinomycin of the TTSS of SPI-1 was not likely due to a lack of intracellular ATP.

K^+ transporters are necessary for protein secretion and motility of *Salmonella* and its invasion of epithelial cells

We examined the K^+ transporter mutants for their protein secretion, motility and invasion of epithelial cells to determine how K^+ transporters are involved in these processes. We first determined if the K^+ transporters affect the overall protein secretion in addition to their effects on the TTSS of SPI-1 (Fig. 2). An equal volume of culture supernatant was collected from each of the single mutant Δ*trkA* and the double mutants Δ*kdpA*/Δ*kup*, Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA*, and total proteins in the culture supernatant were isolated, quantified and normalized against their bacterial c.f.u. ml^{-1}. The quantity of total proteins from the culture supernatant of the WT *Salmonella* was set as 100 % and the quantity of proteins from each mutant was expressed as a relative value to that of the WT *Salmonella* (Fig. 7a). Mutation in Trk decreased protein secretion by approximately 50 % compared to that of the WT *Salmonella* and the protein secretion was restored in the complemented mutants (Fig. 7a). The difference between the Δ*trkA* mutant and the WT *Salmonella* was statistically significant ($P=0.04$, Student's *t*-test). Consistent with this result, the double mutants Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* also displayed lower protein levels in culture supernatant compared to the WT *Salmonella* ($P=0.001$ and 0.002, respectively, Student's *t*-test). The double mutant Δ*kdpA*/Δ*kup* secretes less protein than the WT *Salmonella*; however, the difference was not statistically significant ($P>0.05$, Student's *t*-test) (Fig. 7a).

Since some of the K^+ transporter mutants of *Salmonella* have altered protein secretion compared to the WT parental strain, we tested the motility of the mutants because flagellar proteins involved in motility are exported through the flagellar secretion apparatus and may be affected in the K^+ transporter mutants (Aizawa, 1996;

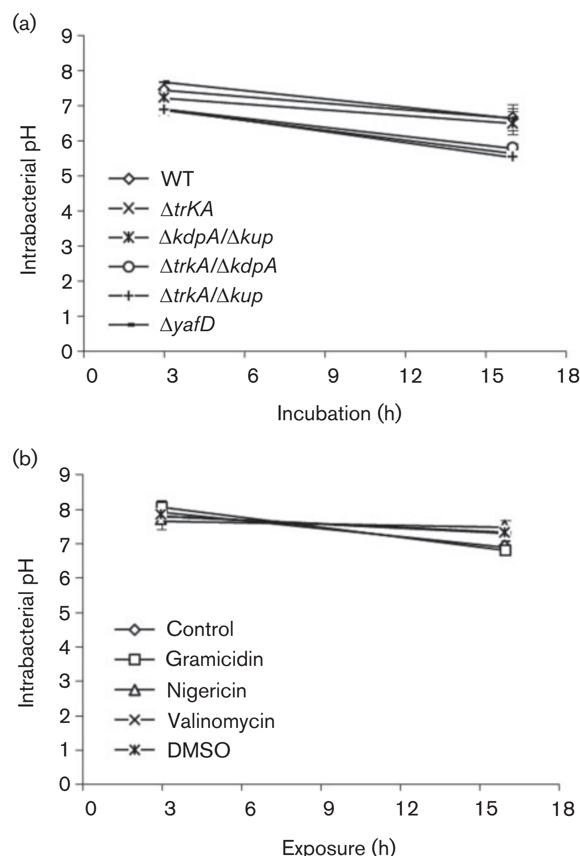

Fig. 5. Intrabacterial pH of K^+ transporter mutants of *Salmonella* and *Salmonella* exposed to cation transport modulators. (a) WT and K^+ transporter mutants of *Salmonella* transformed with PHluorin-expressing plasmid pLN were cultured in LB broth. (b) *Salmonella* transformed with pLN was cultured in LB broth with or without exposure to cation transport modulators. The fluorescence from each culture was measured and intrabacterial pH was determined by comparing to a standard curve. At least three experiments were performed, and results from a representative experiment performed in triplicate are shown. Error bars indicate SD.

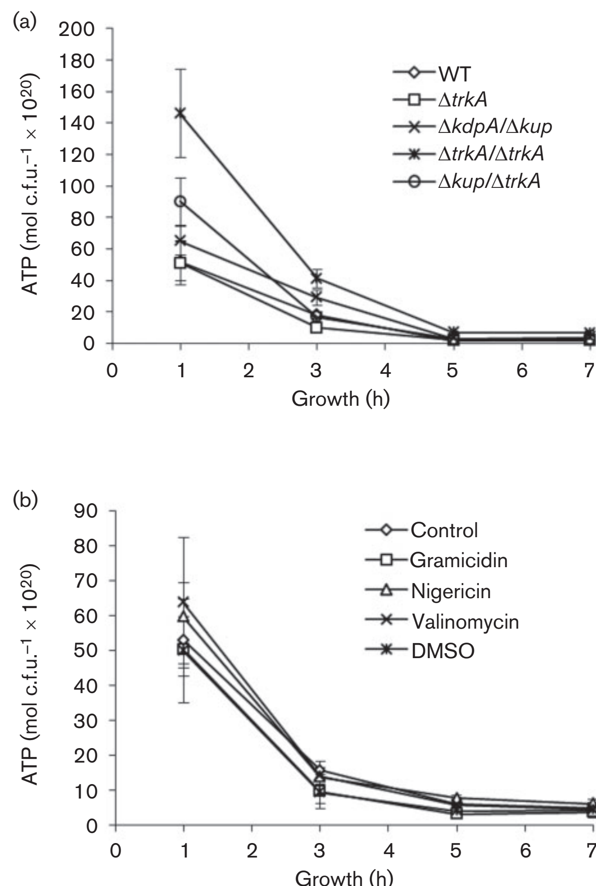

Fig. 6. Intrabacterial ATP level of K^+ transporter mutants of *Salmonella* and *Salmonella* exposed to cation transport modulators. (a) ATP level of the WT and K^+ transporter mutants of *Salmonella*. *Salmonella* was cultured in LB broth. (b) ATP level of *Salmonella* exposed to cation transport modulators. *Salmonella* was cultured in LB broth supplemented with gramicidin (100 μg ml^{-1}), nigericin (40 μg ml^{-1}), valinomycin (10 μg ml^{-1}) or the solvent DMSO. ATP level was determined at various time points of incubation and normalized against bacterial c.f.u. ml^{-1}. At least three experiments were performed, and results from a representative experiment performed in triplicate are shown. Error bars indicate SD.

Fraser & Hughes, 1999; Journet *et al.*, 2005). The overnight culture of each single and double K^+ transporter mutant was inoculated onto motility agar plates and the diameter of the zone of growth for each strain was measured after overnight incubation. All mutants displayed a significant defect in motility ($P<0.05$, Student's *t*-test) and *Salmonella* lacking two of the K^+ transporters, Kdp, Kup or Trk, were more severely affected than that lacking only Trk (Fig. 7b). The results suggest that at least two of the three major K^+ transporters, Kdp, Kup and Trk, are needed for a relatively normal level of motility. Supplementation of KCl or NaCl partially restored the motility of the double mutants (Fig. 7b). The rescue was especially effective for the Δ*kdpA*/Δ*trkA* double mutant supplemented with KCl. KCl supplementation restored the motility of the mutant to close to the level of the WT parental strain. In contrast, the same concentration of NaCl only slightly increased the motility of the Δ*kdpA*/Δ*trkA* double mutant (Fig. 7b).

We then tested the importance of the K^+ transporters in epithelial cell invasion. The invasiveness of the single K^+ transporter mutant Δ*trkA* and the double mutants Δ*kdpA*/Δ*kup*, Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* was determined in a HeLa cell invasion assay and the ratio of intracellular bacteria to the input bacteria after one or two hours of invasion was measured for all mutant strains and compared to the WT *Salmonella*. The ratio of intracellular bacteria for the WT *Salmonella* at 1 h post infection was set as 100 % and the ratio of intracellular bacteria for the other samples was expressed as a relative value. All mutants, with

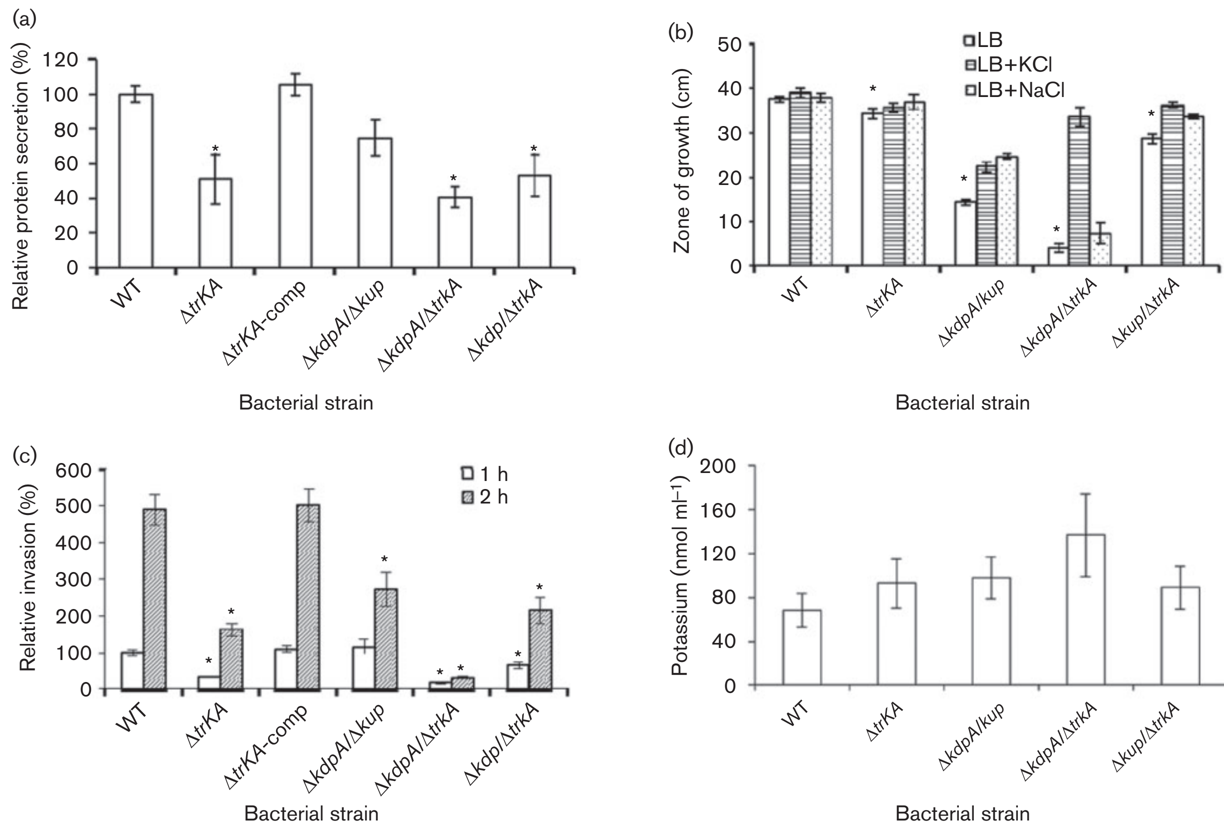

Fig. 7. K^+ transporter mutants of *Salmonella* display defective phenotypes in characteristics related to pathogenesis. WT *Salmonella*, K^+ transporter Δ*trkA* mutant, complemented Δ*trkA* mutant (Δ*trkA*-comp) and double K^+ transporter mutants Δ*kdpA*/Δ*kup*, Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* were analysed for total protein secretion (a), motility (b) and epithelial cell invasion (c). (a) Protein secretion by the WT and K^+ transporter mutants of *Salmonella*. *Salmonella* strains were grown in LB broth. Secreted proteins were purified from culture supernatant, quantified by DC protein assay and normalized against the c.f.u. ml^{-1} of each bacterial culture. The level of protein secretion from the WT *Salmonella* was set as 100 % and the level of protein secretion from each K^+ transporter mutant *Salmonella* strain was expressed as a relative value to that of the WT *Salmonella*. (b) Motility of the WT and K^+ transporter mutants of *Salmonella*. Overnight LB broth culture of each *Salmonella* strain was inoculated in the centre of a motility agar plate with or without supplement of KCl or NaCl. The zone of growth on each plate was measured after overnight incubation and plotted. (c) Epithelial cell invasion of the WT and K^+ transporter mutants of *Salmonella*. *Salmonella* strains were growth in LB broth and used to infect HeLa cells and the ratio of intracellular bacteria to the input bacteria was determined for each *Salmonella* strain. The ratio of intracellular bacteria for the WT *Salmonella* at 1 h post infection was set as 100 % and the ratio for the other samples was expressed as a relative value. All results are the mean of three independent experiments. Error bars indicate SD. $*P<0.05$ compared to the WT, Student's *t*-test. (d) K^+ content of *Salmonella* strains. WT and K^+ transporter mutants of *Salmonella* were cultured overnight in LB broth and total K^+ level in each strain was determined. At least three experiments were performed and results from a representative experiment performed in triplicate are shown. Error bars indicate SD.

the exception of Δ*kdpA*/Δ*kup* at 1 h post infection, displayed a significant defect in invasion ($P<0.05$, Student's *t*-test) (Fig. 7c).

Since intracellular K^+ plays important physiological functions and K^+ transporters are responsible for maintaining K^+ balance, it is possible that K^+ transporter mutants are defective in the TTSS because of a K^+ deficiency that affects their basic physiology. We measured the total K^+ content and bacterial c.f.u. ml^{-1} in the overnight culture of each *Salmonella* strain and quantified intracellular K^+ levels per c.f.u. (Fig. 7d). The results showed that K^+ transporter mutants did not have K^+ deficiency. Instead, the Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* double mutants appeared to have a higher K^+ content than the WT *Salmonella* and the Δ*kdpA*/Δ*kup* mutant (Fig. 7d).

K^+ transporters of *Salmonella* are involved in infection of mice and chicks

As we have shown that K^+ transporters contribute to several virulence characteristics of *Salmonella* (e.g. protein

Table 3. LD_{50} of the WT and K^+ transporter mutants of *Salmonella* in intragastric infection of mice

Results are the means ± SD of three experiments.

Strain	LD_{50} (c.f.u.)
WT	$2.4 \pm 1.0 \times 10^3$
Δ*trkA*	$2.0 \pm 0.9 \times 10^4$*
Δ*trkA*-comp	$1.4 \pm 0.8 \times 10^3$
Δ*kdpA*/Δ*kupA*	$1.2 \pm 0.5 \times 10^6$*
Δ*kdpA*/Δ*trkA*	$2.4 \pm 1.1 \times 10^7$*
Δ*kup*/Δ*trkA*	$2.8 \pm 1.4 \times 10^8$*

*$P<0.05$, Student's *t*-test.

secretion, motility and epithelial cell invasion), we reasoned that K^+ transporters may contribute to the pathogenesis in a host. We tested the virulence of the single K^+ transporter mutant Δ*trkA* and the double mutants Δ*kdpA*/Δ*kup*, Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* in the mouse model of *Salmonella* infection. Each strain of *Salmonella* was used for intragastric infection and the LD_{50} was measured. All mutants displayed a significant attenuation compared to the WT *Salmonella* (Table 3; $P<0.05$, Student's *t*-test). The single mutant Δ*trkA* displayed an attenuation in virulence of approximately 10-fold and the attenuation was rescued by a plasmid expressing *trkA*. The double mutants Δ*kdpA*/Δ*kup*, Δ*kdpA*/Δ*trkA* and Δ*kup*/Δ*trkA* displayed a much higher degree of attenuation, up to 100 000-fold (Table 3).

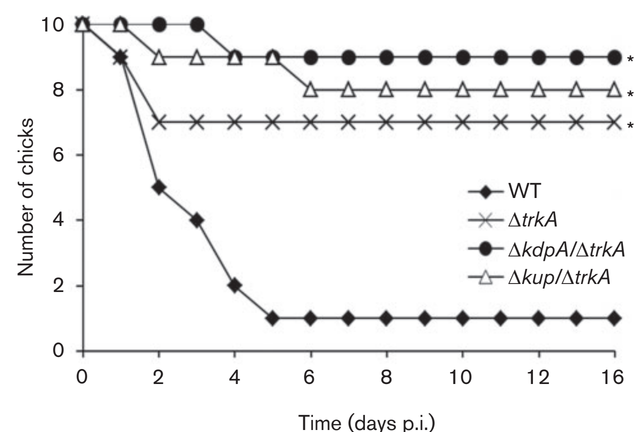

Fig. 8. Virulence of the WT and K^+ transporter mutants of *Salmonella* in infection of chicks. Day-old chicks were orally inoculated with overnight culture of the WT *Salmonella* (◆) or K^+ transporter mutants Δ*trkA* (×), Δ*kdpA*/Δ*trkA* (●) and Δ*kup*/Δ*trkA* (△) grown in LB broth. The number of surviving chicks was recorded for each group for two weeks following infection. The experiment was performed twice and results from a representative experiment are presented. *$P<0.05$ compared with the WT *Salmonella* (Kaplan–Meier analysis).

In addition to the mouse model of *Salmonella* infection, the K^+ transporter mutant *Salmonella* strains were also used to infect chicks, a natural host of *Salmonella* that is important for its transmission to humans. Day-old chicks were infected orally with *Salmonella* and the survival of infected chicks was monitored for 2 weeks. Similar to what was observed in the mouse infection, the virulence of the K^+ transporter mutants was attenuated in the chick infection (Fig. 8; $P<0.05$, Kaplan–Meier analysis).

DISCUSSION

Cations and cation transport have long been recognized as essential for maintaining the normal physiology of all living organisms including bacteria. In this report, we provide evidence that cation transport is also important for virulence characteristics of pathogenic *Salmonella*. Chemical modulators of cation transport inhibit the TTSS of SPI-1 (Fig. 3). Mutations in transporters of the most abundant cation, K^+, led to deficiencies in the TTSS of SPI-1 (Fig. 2) as well as protein secretion, motility and epithelial cell invasion (Fig. 7). In addition, K^+ transporter mutants of *Salmonella* are attenuated in infection of both mice and chicks (Table 3 and Fig. 8). These results suggest that cation transport plays important roles beyond maintaining the physiology of *Salmonella*.

K^+ is reported to play important functions in many basic cellular processes, such as maintaining cell turgor and homeostasis, adaptation of cells to osmotic conditions and activation of cytoplasmic enzymes (Bossemeyer *et al.*, 1989; Epstein, 2003). K^+ transporters are responsible for maintaining intracellular K^+ within the normal range regardless of the extracellular K^+ concentration. K^+ transporter mutants of *Salmonella* grow relatively normally except at extreme K^+ concentrations (Fig. 1); however, they are impaired in carrying out activities contributing to virulence *in vitro* and are attenuated in mice and chicks (Fig. 8, Table 3). The defects of the K^+ transporter mutants were not due to a K^+ deficiency since intracellular K^+ levels were at or above that of the WT *Salmonella* (Fig. 7d). Therefore *Salmonella* is capable of maintaining intracellular K^+ levels necessary for growth and survival without one or more of the K^+ transporters, although other processes such as those involved in pathogenesis can be severely affected. These results suggest that active cation transport, especially K^+ transport, is important for *Salmonella* as a pathogenic bacterium to carry out its full range of activities contributing to host colonization and transmission.

The TTSSs of *Salmonella* pathogenicity islands have been extensively studied since their discovery more than a decade ago. However, their energy source has not been identified. In their report on the TTSS of flagella, Paul *et al.* (2008) proposed that the TTSS of flagella was driven by the electrical component of the PMF. They showed that the TTSS of flagella was independent of intracellular ATP levels, similar to what we observed for the TTSS of SPI-1.

They had also demonstrated that at an external pH of 7, the TTSS of flagella was not affected by acetate treatment that eliminates the proton concentration across the cell membrane (Paul *et al.*, 2008), which is consistent with our findings that intracellular pH does not correlate with the TTSS of SPI-1 (Figs 5, 6). Taken together, these results suggest that the TTSS of SPI-1 is likely driven by the electrical potential across the bacterial cell membrane.

Many conditions, such as contact with host cells, salts (Mizusaki *et al.*, 2008; Su *et al.*, 2009), acidic pH (Bajaj *et al.*, 1996), oxygen (Bajaj *et al.*, 1996; Ibarra *et al.*, 2010) and metabolic products (Van Immerseel *et al.*, 2004a, b), have been shown to regulate the TTSS. The regulation pathways of these systems are extremely complex. Using the TTSS of SPI-1 as an example, research by several groups has demonstrated that SPI-1 is controlled by a large number of protein factors including HilA (Bajaj *et al.*, 1995, 1996; Boddicker *et al.*, 2003; Thijs *et al.*, 2007), HilC (Akbar *et al.*, 2003; Golubeva *et al.*, 2012; Schechter & Lee, 2001), HilD (Akbar *et al.*, 2003; Boddicker *et al.*, 2003; Schechter & Lee, 2001), HilE (Baxter *et al.*, 2003; Ellermeier & Slauch, 2007), RtsA (Ellermeier & Slauch, 2003, 2004), PhoP/Q (Aguirre *et al.*, 2006; Behlau & Miller, 1993; Pegues *et al.*, 1995), InvF (Darwin & Miller, 1999, 2000; Eichelberg & Galán, 1999), Dam (López-Garrido & Casadesús, 2010), RcsC/RcsD/RcsB phosphorelay system (Arricau *et al.*, 1998; Lin *et al.*, 2008; Winter *et al.*, 2009), AcrA, AcrB and TolC (Webber *et al.*, 2009), Lon (Boddicker & Jones, 2004), CsrA (Altier *et al.*, 2000; Lawhon *et al.*, 2003) and SirA/SirC (Mizusaki *et al.*, 2008; Rakeman *et al.*, 1999) (reviewed by Golubeva *et al.*, 2012). The best-characterized direct regulators of the TTSS of SPI-1 are HilA (Bajaj *et al.*, 1995, 1996; Boddicker *et al.*, 2003; Thijs *et al.*, 2007) and InvF (Darwin & Miller, 1999, 2000; Eichelberg & Galán, 1999), while other protein factors directly or indirectly exert their activities through these factors. We have shown that in addition to protein factors, HilE is regulated by the small RNA (sRNA) isrM encoded in SPI-1 (Gong *et al.*, 2011). It is not clear though how *Salmonella* senses diverse environmental signals and induces the complex network of regulatory factors for the TTSS in response. We propose that the physiological and energetic state of *Salmonella* serves as an important signal to activate the TTSS including that of SPI-1. Disparate TTSS-inducing signals which include contact with host cells, salts, acids, oxygen and organic acids are all capable of altering the membrane potential of *Salmonella*, and it is possible that altered membrane potential is the direct signal that activates the TTSS instead of an individual stimulus. This would explain how *Salmonella* activates the TTSS of SPI-1 in response to seemingly disparate signals. We recognize that our methods of measuring intrabacterial pH and ATP levels most likely measured the mean pH and ATP levels inside bacteria and we cannot rule out the possibility that a redistribution of protons and/or ATP in the K^+ transporter mutants and cation transport modulator-treated *Salmonella* was the reason for the inhibition of the TTSS of SPI-1. Currently there is no method available to measure local distribution of protons and ATP because of the small size of bacteria.

Type III secretion systems are widely used by a variety of pathogenic bacteria to secrete virulence factors to facilitate the infection and colonization of their hosts. Many pathogenic bacteria infect multiple hosts ranging from cold-blooded animals, such as flies and turtles, to warm-blooded animals such as birds, rodents and humans. The combination of individual pathogens, their diverse hosts and the conditions the pathogens encounter is so large that it is difficult to speculate how pathogens manage to regulate their TTSS in response to each combination. Our hypothesis that pathogenic bacteria regulate the TTSS through their physiological state helps to explain how these bacteria activate the TTSS in response to external environments. Since K^+ and K^+ transporters play important roles in the physiological state of bacteria, they are likely involved in the pathogenesis of other pathogenic bacteria in addition to *Salmonella*. Indeed, K^+ transporters and channels have been shown to be important for stress resistance and pathogenesis of a number of bacterial species such as *Pseudomonas aeruginosa* (Ueda & Wood, 2008), *Vibrio vulnificus* (Chen *et al.*, 2004), *Mycobacterium tuberculosis* (Cholo *et al.*, 2006) and *Staphylococcus aureus* (Xue *et al.*, 2011). Further analysis using genetic and physiological tools will elucidate the interaction of physiological state of pathogenic bacteria and their virulence characteristics including the secretion of virulence factors through the TTSS.

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