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Original article

The temperature-sensing protein TlpA is repressed by PhoP and dispensable for virulence of *Salmonella enterica* serovar Typhimurium in mice

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Abstract

TlpA is a temperature-sensing, coiled-coil protein, encoded on the pSLT virulence plasmid of *Salmonella enterica* serovar Typhimurium. TlpA was previously presumed to play a role in the pathogenicity of *Salmonella*. Herein we show that TlpA is tightly regulated, differentially expressed in response to environmental and physiological signals, and can be secreted in vitro. Expression of *tlpA* was found to be repressed in modified minimal medium containing limiting concentrations of Mg^{2+} and in the stationary phase of growth, but induced in rich LB broth and in response to elevated temperatures. The response regulator PhoP was found to play a key role in the repression of *tlpA* in conjunction with two other regulators, RpoS and TlpA itself. In addition, we demonstrate that TlpA is dispensable for intracellular proliferation of *S*. Typhimurium within host cells and for virulence in mice. Based on presented homology of TlpA to the IncP plasmid encoded protein, KfrA, and to SMC family members, a potential function for TlpA is discussed. Cumulatively, our data do not support the previous hypothesis that TlpA plays a role in the pathogenicity of *Salmonella* per se, but may suggest an alternative function for TlpA unrelated to host infection.

Keywords: *Salmonella enterica* serovar Typhimurium; pSLT; Virulence plasmid; Coiled-coil protein; Pathogenicity; Temperature sensing; PhoP; KfrA; SMC

1. Introduction

Salmonella enterica is a Gram-negative facultative intracellular pathogen that infects animal and human hosts. The nature and the severity of the disease are dependent upon the bacterial serovar and the host species [1]. *S*. Typhimurium causes gastroenteritis in humans and calves, whereas in mice it leads to a typhoid-like systemic infection. While a significant number of virulence factors are clustered on the virulence plasmid or within large regions of the chromosome called *Salmonella* pathogenicity islands (SPI), they are also found scattered throughout different loci in the genome [2]. The pathogenic potential of *S. enterica* as well as many other Gram-negative bacteria is indicated by the possession of a specialized type III secretion system (TTSS) that is used to deliver virulence effector proteins directly into the cellular environment of the eukaryotic host.

TlpA is a 371-amino-acid, cytoplasmic protein, encoded on the 96-kb pSLT virulence plasmid of *S. enterica* serovar Typhimurium [3], and characterized by a remarkably long α-helical coiled-coil motif [4,5]. The N-terminus of TlpA is a sequence specific DNA-binding domain that can act as an autoregulatory repressor. It has been shown that TlpA can be found in a temperature-dependent two-state equilibrium, between unfolded monomers and highly α-helical coiled-coil oligomers. At physiological temperatures transcription of *tlpA* is kept in check by the repressing activity of TlpA, which in its dimeric and folded coiled-coil conformation is able to bind to the *tlpA* promoter and repress transcription. Elevated temperature leads to a shift in the equilibrium that favors the non-functional unfolded monomeric form, which leads to increased transcription of *tlpA* [6–8].

Entry from the ‘‘cold’’ environment into the ‘‘warm’’ host is believed to be one of the central cues triggering virulence

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factors in pathogenic bacteria [9]. TlpA was the first documented case of a temperature-sensing gene regulator and was presumed to be an ideal sensor of environmental signals. Based on that, TlpA was suggested to play a role in the pathogenicity of *S*. Typhimurium [6–8]; however, a documented function in virulence has not been reported.

2. Materials and methods

2.1. Bacterial strains and in vitro growth conditions

Bacterial strains and plasmids used in this study are listed in the supplementary data, appendix no. 1. *S*. Typhimurium SL1344 was used as the wild-type strain, and all mutants used in this study were isogenic derivatives of SL1344. Bacterial cultures were routinely maintained in Luria–Bertani (LB) medium or in modified magnesium minimal medium (MgM) containing 80 mM MES (pH 5.8), 5 mM KCl, 7.5 mM $(NH_4)SO_4$, 0.5 mM K_2SO_4, 337 μM K_2HPO_4/KH_2PO_4 (pH 7.4), 20 mM $MgCl_2$, 38 mM glycerol, and 0.1% casamino acids.

2.2. Construction of S. Typhimurium SL1344 mutant strains

Refer to supplementary data, appendix no. 2.

2.3. Construction of TlpA::HA, CyaA, and LacZ fusion proteins

Refer to supplementary data, appendix no. 3.

2.4. Tissue culture conditions and bacterial infection

The human epithelial HeLa and the murine macrophages J774-A.1 and RAW264.7 cell lines were purchased from the American Type Culture Collection (ATCC). All cell lines were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FCS) at 37 °C in a humidified atmosphere with 5% CO_2. For additional details regarding the bacterial infection of tissue cultures refer to supplementary data, appendix no. 4.

2.5. cAMP assays

HeLa cells were seeded in 24-well plates and infected for 2, 4, or 8 h with *S*. Typhimurium SL1344 strains expressing TlpA-CyaA or SseK1-Cya. The cyclic AMP (cAMP) enzyme immunoassay (EIA) was performed according to the manufacturer's instructions for determination of intracellular cAMP with the nonacetylation EIA procedure.

2.6. Sub-cellular fractionation of infected HeLa cells

Infection of HeLa cells was performed as described in the supplementary data, appendix no. 4. At 8 h postinfection, the cells were washed, scraped into PBS, and centrifuged for 5 min at 1000 × *g* and 4 °C. The resultant pellet was resuspended in HB buffer (250 mM sucrose, 3 mM imidazole, 0.5 mM EDTA; pH 8) containing protease inhibitors, and mechanically disrupted by repeated passage through a 22-gauge needle. Ultracentrifugation fractionation was performed as previously described [10].

2.7. β-Galactosidase assays

β-Galactosidase assays were performed as described elsewhere [11]. *Salmonella* strains were grown in LB for 3 h (logarithmic phase), 16 h (stationary phase) or for 16 h in MgM medium. The substrate for β-galactosidase hydrolysis was *o*-nitrophenyl-β-D-galactopyranoside.

2.8. Bacterial infection of mice

Female BALB/c mice were purchased from Jackson Laboratories, housed at the University of British Columbia Animal Facility in sterilized filter-topped cages and given food and water ad libitum. For detailed infection procedures, refer to supplementary data, appendix no. 5.

2.9. Statistical analysis

The statistical significance between different values of the β-galactosidase assays was calculated using the Student's *t*-test. $P < 0.05$ was considered to be statistically significant. The statistical analysis that was used to evaluate the Competitive Index experiment results was the Wilcoxon rank sum test against a hypothetical value of 1.00.

3. Results

3.1. Phylogenetic distribution of TlpA among Salmonella serovars

The bacterial genus *Salmonella* is divided into two species, *S. bongori* and *S. enterica*. *S. enterica* itself is comprised of six subspecies: *enterica*, *salamae, arizona, diarizonae, indica, houtenae,* or I, II, IIIa, IIIb, IV, and VI, respectively [12]. In order to map the phylogenetic distribution of *tlpA* amongst *Salmonella* serovars, we performed a BLAST search against the currently available genome sequences of *Salmonella* strains. This analysis enabled us to identify several proteins homologous to *S*. Typhimurium SL1344 TlpA, as presented in Table 1. TlpA homologs were found in five different serovars, all of which are members of *S. enterica* subsp. *enterica* (group I) including: Typhimurium, Enteritidis, Dublin, Choleraesuis, and Gallinarum. Interestingly, no TlpA homologs were found in other *Salmonella* serovars outside of *S. enterica* subspecies I.

A common feature of these serovars is the presence of serovar-specific virulence plasmids, typically 50–100 kb in size, which share considerable homologies [13]. Correspondingly to the location of *tlpA* on pSLT, the locus of *tlpA* homologs

Table 1
Phylogenetic distribution of TlpA among *Salmonella* serovars

Serovar	Strain	Subspecies	Presence of TlpA homolog	Locus	Contig[a] or Accession number[b]	Homology to *tlpA* (% identity)
Typhimurium	SL1344	I	+	pSLT	salt10-417e06.q1k[a]	–
	LT2 (SGSC1412)	I	+	pSLT	NP_490538[b]	100
	DT104	I	+	DT104 plasmid	DT104_plasmid[a]	100
	DT2	I	+		DT2_31g01.q1k[a]	99
	D23580	I	+		D23580-151g11.p1k[a]	100
Enteritidis	PT4	I	+	PT4 plasmid	PT4_plasmid[a]	99
Dublin	OU7025	I	+	pOU1113	YP_271807[b]	99
	OU7432	I	+	pOU1115	DQ115388[b]	100
Choleraesuis	B67	I	+	pSCV50	AAS58919[b]	99
	RF-1	I	+	pKDSC50	NP_073266[b]	100
Gallinarum	287/91	I	+	Gallinarum plasmid	Gallinarum plasmid[a]	99
Infantis		I	–			
Typhi	CT18	I	–			
	Ty2	I	–			
Paratyphi A	ATCC 9150	I	–			
Arizona		IIIa	–			
Bongori	12419	V	–			

[a] Contig number is given for the homologs that were identified using the *Salmonella* spp. comparative sequencing BLAST server at the Welcome Trust Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/salmonella).
[b] An accession number is given for the homologs that were identified using BLAST against *Salmonella* genomes at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi).

in the other serovars was identified in their serovar-specific virulence plasmids. As shown in Table 1, a remarkable identity that ranged between 99%–100% was found between *tlpA* and its homologs. It is noteworthy that the *tlpA* polymorphism distributed evenly intra-serovar and inter-serovars. For comparison, the *spv* locus, which is also encoded on pSLT was found in different isolates of subspecies I, II, IIIa IV and VII, encoded either on the plasmid or on the chromosome [14].

3.2. TlpA shows sequence similarity to the DNA-binding protein KfrA, the translocated effector LepB from Legionella pneumophila and to members of the SMC family

Bioinformatics analysis allowed us to identify several proteins that show sequence homology to TlpA, including the IncP plasmid encoded protein, KfrA. The degree of the homology varied from 27% identity/47% similarity (E-value 3e-22) to KfrA from *Achromobacter xylosoxidans* (CAI47877) up to 41% identity/58% similarity (E-value 9e-66) to a protein annotated as KfrA from an uncultured bacterium that was isolated from an activated sludge (CAG27823). Fig. 1 shows the homology of TlpA to these proteins using a ClustalW analysis. KfrA is encoded within the central control region (*ctl*) in several plasmids of the IncP group, and has been proposed to be involved in plasmid partition [15,16].

In addition, an intriguing sequence similarity was observed between TlpA and the C-terminus of LepB, a translocated effector protein from *Legionella pneumophila*, which showed 22% identity and 47% similarity over 316 amino acids (E-value 8e-11). LepB has recently been shown to be transported via the Icm/Dot type IV secretion system into host cells and to be involved in non-lytic exit of *Legionella* from its protozoan host [17]. Furthermore, some similarity (E-value 1e-06—0.003) was also found between the C-terminus of TlpA and several members of the SMC family, which are known to be involved in cell division and chromosome partitioning [18].

3.3. The expression profile of TlpA in response to different environmental signals

In order to gain insights into the nature of TlpA, we were interested in identifying specific environmental signals affecting its expression and specifically, growth conditions that mimic the intracellular environment. To do so, a tandem hemagglutinin (2HA) epitope-tagged TlpA was cloned under the control of its native promoter and was used to examine the expression profile of TlpA, under the following conditions: elevated temperatures, nutrient limitation, acidic pH, reactive oxygen and nitrogen intermediates, and limitation of divalent cations. As shown in Fig. 2, the expression of TlpA-HA was found to be clearly temperature-dependent. Higher expression levels were detected when cultures were grown at 37 °C in comparison to 26 °C and a significant induction was observed following temperature shifting from 26 °C or 37 °C to 43 °C. Higher expression of TlpA-HA was also evident following shifting from 26 °C to 37 °C. These results are in agreement with previous observations [7] and indicate that the construct used is indeed reliable for monitoring the expression pattern of TlpA. Interestingly, the expression of TlpA-HA in MgM medium was found to be dramatically lower than in LB, indicating that the expression of TlpA is being repressed under these conditions. Minimal acidic medium is considered to reflect the intracellular conditions and was previously shown to induce the expression of SPI-2 virulence genes, which are required for intracellular survival of *Salmonella* [19]. Other growth conditions tested, including exposure to hydrogen-peroxide, paraquat, which

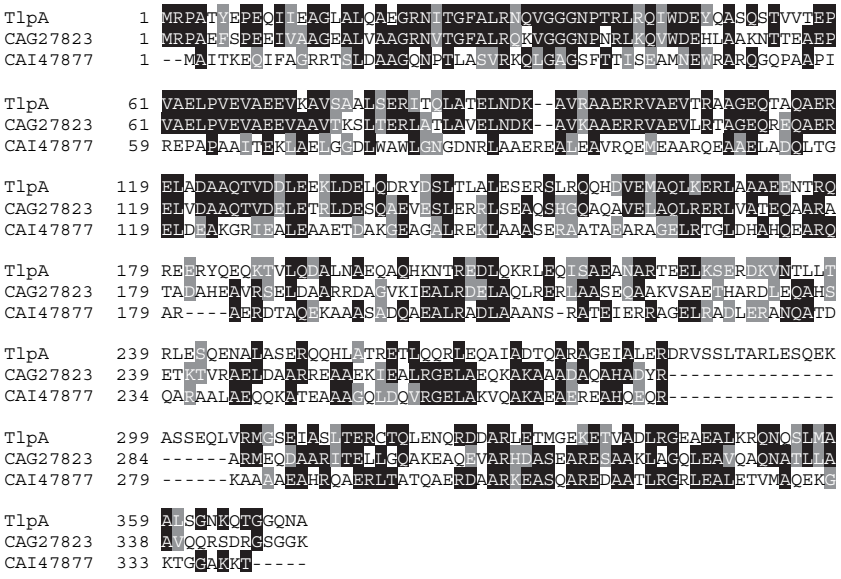

Fig. 1. TlpA shows sequence similarity to KfrA. Amino acid sequence alignment of KfrA from *Achromobacter xylosoxidans* (CAI47877); KfrA from an uncultured bacterium (CAG27823), and TlpA from *Salmonella* Typhimurium (Q56080, TlpA) is presented. Sequence alignments were performed by ClustalW and the output was reformatted by BoxShade 3.21. Amino acid identity is shown in black and similar amino acids are shown in gray.

generates elevated levels of superoxide ($O_2^{\cdot-}$) within the bacterial cytosol [20]; nitric oxide, acidic LB medium (pH 5), and limitation of divalent cations were not found to have a detectable effect on the expression of TlpA-HA.

3.4. The response regulator PhoP represses the expression of tlpA

The distinct expression profile of TlpA in different growth conditions suggested that TlpA is being specifically regulated. To shed some light on the regulation of TlpA, the expression of a *tlpA::lacZ* translational fusion was tested in different genetic backgrounds and under different growth conditions.

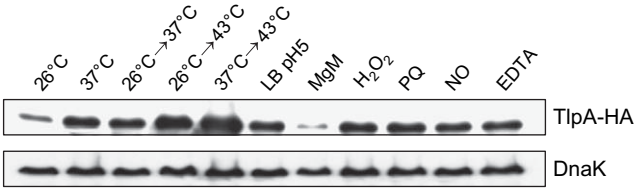

Fig. 2. The expression of TlpA-HA in response to different environmental signals. Wild-type *S*. Typhimurium expressing TlpA-HA from its native promoter was grown under the following conditions (from left to right): in LB medium (pH 7.3) at 26 °C; at 37 °C; at 26 °C for 3.5 h and then shifted to 37 °C for 2 h; at 26 °C for 3.5 h and then shifted to 43 °C for 2 h; at 37 °C for 3.5 h and then shifted to 43 °C for 2 h; in acidic (pH 5) LB medium at 37 °C (LB pH5); in acidic (pH 5.8) MgM medium at 37 °C (MgM); in LB medium (pH 7.3) at 37 °C for 3.5 h and then in the presence of 100 μM H_2O_2 for 2 h (H_2O_2); in LB medium (pH 7.3) at 37 °C for 3.5 h and then in the presence of 100 μM paraquat for 2 h (PQ); in LB medium (pH 5) at 37 °C for 3.5 h and then in the presence of 1 mM $NaNO_2$ for 2 h (NO); in LB medium (pH 7.3) at 37 °C for 3 h and then in the presence of 100 μM EDTA for 2 h (EDTA). Western blot analysis was performed with an anti-HA monoclonal antibody (top panel). To show equal amounts of proteins loaded, the same blot was re-probed for DnaK (bottom panel).

The mutant strains that were initially tested included *tlpA*, three global regulators (*phoP*, *sirA* and *ompR*), SPI-1 and SPI-2 specific regulators, *hilA* and *ssrB*, respectively, and the stationary phase alternative sigma factor *rpoS*. The expression of *tlpA::lacZ* was evaluated during growth in LB (at logarithmic and stationary phase) and in MgM medium. As shown in Fig. 3, notably lower levels of *tlpA::lacZ* were found in the wild-type background, when the bacteria were cultured in MgM medium (Fig. 3C) vs. LB (Fig. 3A, $P < 0.0001$). This result is in agreement with the lower TlpA-HA levels that were detected using a Western blot analysis (Fig. 2), and provides further support to the finding that TlpA is being repressed under growth in MgM medium conditions.

The expression of *tlpA::lacZ* in the wild-type strain was found to be more than 3 fold higher during logarithmic growth (Fig. 3A) than in stationary phase (Fig. 3B, $P < 0.0001$), implying that TlpA is down-regulated at the stationary stage of growth. In agreement with that, during stationary phase, but not at logarithmic phase, higher expression of *tlpA::lacZ* was found in the *rpo*S background vs. the wild-type (Fig. 3B, $P < 0.0001$), suggesting a possible role for RpoS in the regulation of *tlpA*.

Nonetheless, the most consistent difference observed in all of the tested conditions was the higher expression level of *tlpA::lacZ* in the *tlpA* and *phoP* mutants. These differences were found to be more pronounced in MgM medium, in which the expression in *tlpA* and *phoP* mutants was high by more than 2- and 3-fold, respectively, than in the wild-type ($P < 0.0001$). This observation suggests that *tlpA::lacZ* is being suppressed by TlpA and is in agreement with a previous study showing that TlpA has a self-repression activity [7].

Likewise, the higher expression of *tlpA::lacZ* in the *phoP* mutant indicated that PhoP is involved in the regulation of *tlpA*. The PhoPQ two-component system acts as

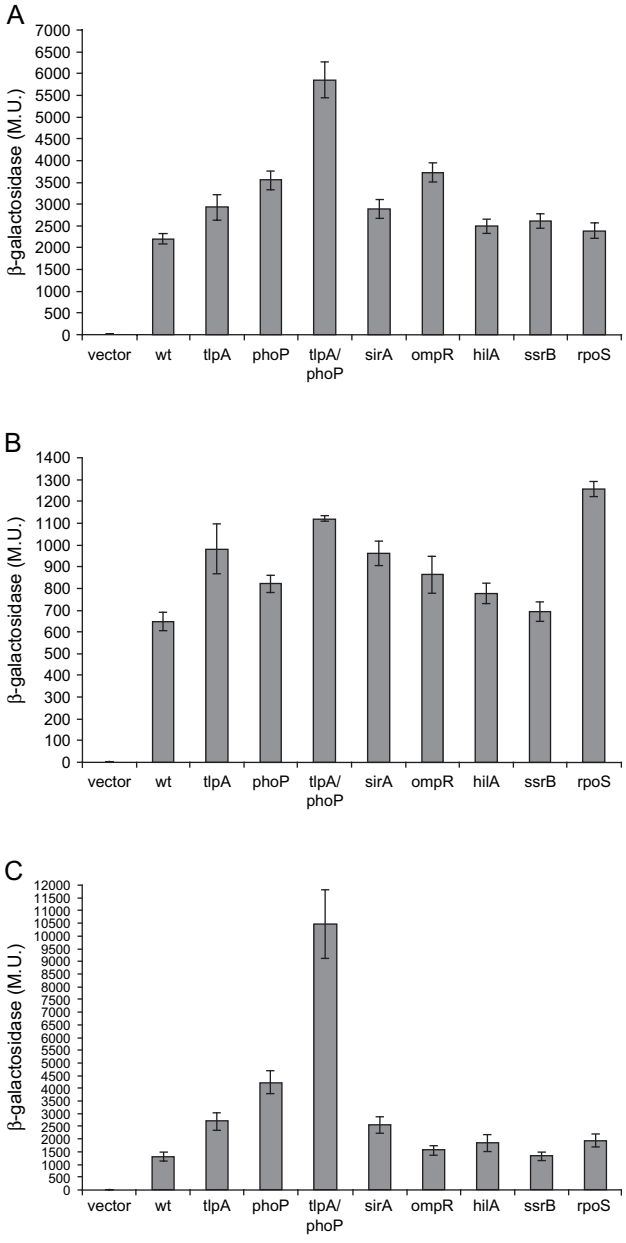

Fig. 3. The expression of *tlpA::lacZ* in different *S*. Typhimurium regulatory mutants and under different growth conditions. Wild-type *S*. Typhimurium carrying the pMC1403 vector (vector), or the *tlpA::lacZ* fusion (wt), and *S*. Typhimurium mutant strains (*tlpA, phoP, tlpA*/*phoP, sirA, ompR, hilA, ssrB* and *rpoS*) harboring *tlpA::lacZ* were grown in LB to a late-logarithmic phase (OD_{600} ~1) (A); stationary phase (OD_{600} ~3.5) (B); or in MgM medium (OD_{600} ~1) (C). The β-galactosidase specific activity values are presented in Miller units (M.U.) and are an average of at least four independent cultures, with the standard deviation indicated by the error bars.

a transcriptional regulator that responds to Mg^{2+} starvation [21]. To further investigate the possibility that PhoP regulates *tlpA*, we constructed a double mutant strain lacking both *tlpA* and *phoP* genes (OG2008). As can be clearly seen in Fig. 3, the expression level of *tlpA::lacZ* was found to be significantly higher in this strain, in all of the examined conditions. The most dramatic effect was seen in MgM medium, in which the expression of *tlpA::lacZ* was 8 fold higher in the *tlpA*/*phoP* double mutant vs. the wild-type (Fig. 3C). These results indicate that PhoP plays a pivotal role as a repressor in the regulation of *tlpA*. Consistent with that, a putative PhoP binding motif was identified in the upstream region of *tlpA*, suggesting that PhoP may directly repress *tlpA* expression (see Section 4).

3.5. TlpA is secreted in vitro in a TTSS-independent manner and is not translocated into host cells

The similarity that was found between TlpA and the *Legionella* translocated effector LepB led us to investigate whether TlpA can also be secreted and/or delivered into host cells during infection. Whole bacterial cell lysates and secreted protein fractions were tested for the presence of TlpA-HA during growth in LB (SPI-1 inducing conditions) and in acidic MgM medium (SPI-2 inducing conditions). Interestingly, secretion assays showed that TlpA-HA can be secreted into the medium while growing in LB (Fig. 4A) and to a lesser extent during growth in minimal medium (data not shown). The lack of a predicted N-terminal signal peptide suggested that the secretion of TlpA-HA might involve a Sec-independent mechanism, such as TTSS. To test this possibility, we examined the observed secretion in SPI-1 and SPI-2 TTSS mutants (*invA* and *ssaR*, respectively). As seen in Fig. 4A, expression and secretion of TlpA-HA were found to be similar in the wild-type and these mutant backgrounds. Similar results were observed in an *invA*/*ssaR* double mutant (data not shown). Given these results, we concluded that the secretion of TlpA-HA in vitro is not TTSS-dependent.

Having said that, it has been recently shown that another *Salmonella* virulence plasmid-encoded protein, the SpvB toxin, is introduced into the host in a SPI-1 and SPI-2 TTSS-independent mechanism [22]. Therefore, we were interested in examining possible transport of TlpA into host cells by a TTSS-independent manner. To address that, we employed a *cyaA* gene fusion approach, which was also used to show the translocation of LepB-CyaA from *Legionella* into host cells [17]. A protein fusion between TlpA and the catalytic domain of adenylate cyclase of *Bordetella pertussis* (*tlpA-cyaA*) was constructed and *Salmonella* strains carrying either *tlpA-cyaA* or *sseK1-cyaA*, as a positive control [23], were used to infect HeLa cells. Following infection, we assayed the intracellular levels of cAMP as a measure of translocation of the CyaA fusion proteins. As can be seen in Fig. 4B, we were able to detect high levels of SseK1-CyaA that was translocated into host cells at 8 h postinfection, but failed to detect translocation of TlpA-CyaA.

To further examine the possibility that TlpA might be translocated into host cells, we used another methodology. HeLa cells were infected with wild-type SL1344 harboring TlpA-HA or SseK1-HA and analyzed by subcellular fractionation [10]. At 8 h postinfection, cells were disrupted into three fractions: the pellet (fraction P), which contained whole cells, insoluble host components, and intact bacteria; the host membranes (fraction M); and the host cytosol components (fraction C). Western analysis (Fig.4C) detected SseK1-HA

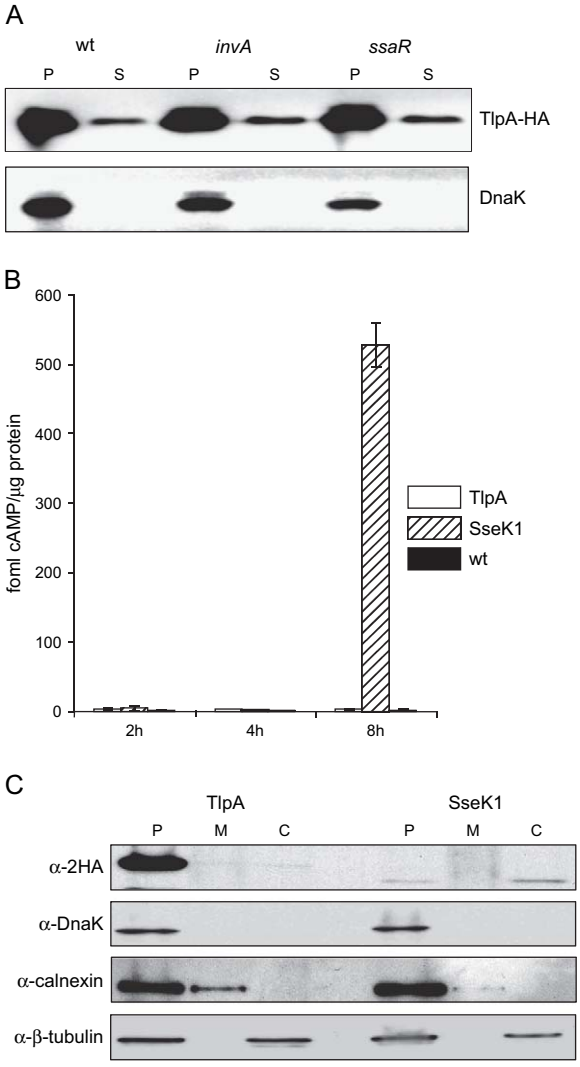

Fig. 4. TlpA is secreted in vitro but not translocated into host cells. (A) Wild-type *S*. Typhimurium (wt) and two mutant strains *invA* or *ssaR* expressing TlpA-HA were grown in LB. Whole bacterial lysate pellets (P) and culture media supernatant (S) were analyzed by Western blot using an anti-HA antibody (top panel). To show that the TlpA-HA detected in the supernatant is not a result of bacterial cell lysis, the blot was reprobed for the bacterial cytoplasmic protein, DnaK (bottom panel). (B) HeLa cells were infected for 2, 4, and 8 h with *S*. Typhimurium (wt), *S*. Typhimurium expressing *tlpA-cyaA* (TlpA), or *sseK1-cyaA* (SseK1). Translocation of TlpA-CyaA and SseK1-CyaA was assayed by determining the intracellular cAMP levels in infected cell extracts. Values represent the average of three independent samples analyzed in duplicates, with the standard deviation indicated by the error bars. (C) HeLa cells were infected with the indicated *Salmonella* strains expressing TlpA-HA or SseK1-HA for 8 h, following a sub-cellular fractionation. The resultant pellet (P), membrane (M), and cytosol (C) fractions were subjected to Western analysis. DnaK was included as a marker for intact bacteria; calnexin was included as a marker for host membranes; and β-tubulin was included as a marker for the host cytosol.

in the pellet, and also in fraction C, confirmed its translocation into the host cytosol. In contrast, TlpA-HA was detected only in the whole cells pellet providing further support to the finding that TlpA is not translocated into host cells. Collectively, these results suggest that TlpA is not a translocated effector protein.

3.6. TlpA is dispensable for intracellular growth and virulence in mice

In order to directly evaluate the possible contribution of TlpA to the virulence of *Salmonella*, we constructed a *S*. Typhimurium derived strain containing an in-frame deletion of *tlpA* and measured intracellular growth to examine the ability of *tlpA* strain to survive and replicate within murine macrophages (J774-A.1 and RAW264.7) and human epithelial (HeLa) cell lines. Our results show that the *tlpA* mutant was able to survive and proliferate to a similar extent as the isogenic wild-type strain in the three cell lines examined. The SPI-2 TTSS structural gene mutant, *ssaR*, which was used as a negative control, was found to be dramatically impaired for intracellular replication (Fig. 5A). These results suggest that TlpA is required neither for invasion nor for intracellular proliferation within host cells.

To examine whether TlpA is involved in the virulence of *Salmonella* in vivo, we used the susceptible BALB/c murine infection model. Wild-type, *tlpA*, and *ssaR* strains were used to infect mice by intraperitoneal injection. As illustrated in Fig. 5B, the virulence of the wild-type and the *tlpA* mutant strains was found to be indistinguishable ($P = 0.9156$), as was measured by the survival time of the infected mice; while the *ssaR* strain showed complete attenuation in virulence ($P = 0.0025$ for *ssaR* vs. wild-type).

In some cases, comparing survival-time is not sensitive enough to reveal moderate virulence attenuation, while the Competitive Index (C.I.) approach is considered to be more precise [24]. Fig. 5C summarize the C.I. results of two independent experiments, in which mice were infected orally with 1:1 mixed inoculum of wild-type and *tlpA* strains. The C.I. geometrical mean that was calculated for the spleen and the liver was 1.05 ($P = 0.85$) and 1.06 ($P = 0.78$), respectively. A different experiment was preformed by intraperitoneal injection of these strains and as can be seen in Fig. 5D, similar results were obtained showing geometrical C.I. mean of 1.03 ($P = 0.92$) and 1.08 ($P = 0.46$) in the spleen and in the liver, respectively. These results are in agreement with the in vitro study and together suggest that the loss of *tlpA* is insufficient to cause detectable attenuation in a susceptible murine host, and in the tissue culture model.

3.7. Assessing the role of TlpA in pSLT stability

Given the results suggesting that TlpA does not play a role in the pathogenicity of *Salmonella*, we were interested in testing alternative function for TlpA. As mentioned, significant sequence similarity of TlpA was found to KfrA. Since KfrA has been proposed to be involved in plasmid partition [15,16], and taking into consideration the homology of TlpA to SMC proteins, we were interested in examining a related role for TlpA. To do so, marked strains were constructed in the wild-type and in a *tlpA* background, by introducing a Cm resistance cassette into pSLT (OG-2009 and OG-2010, respectively). To evaluate the contribution of TlpA to the stability of pSLT, both strains were grown in LB without

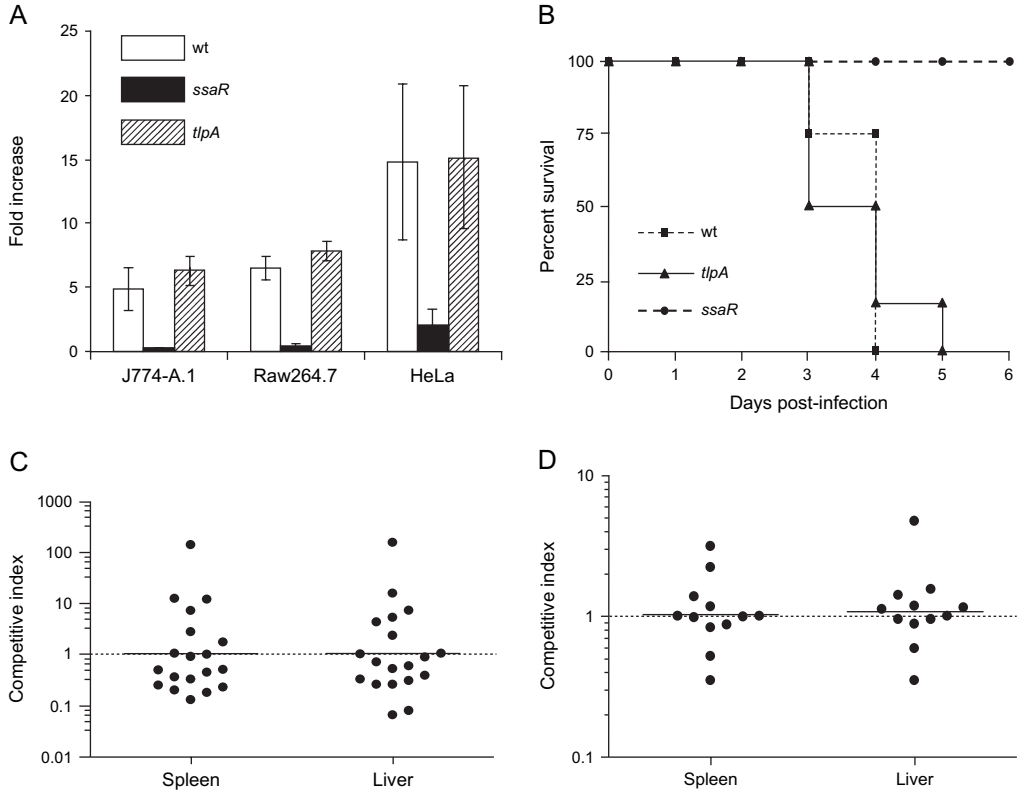

Fig. 5. TlpA is dispensable for intracellular proliferation in host cells and for virulence in mice. (A) J774-A.1, RAW264.7, and HeLa cell lines were infected with *S.* Typhimurium wild-type (wt), a *tlpA* mutant (*tlpA*), or SPI-2 mutant strain (*ssaR*). Intracellular replication is expressed as the increase in intracellular CFU from 2 to 24 h after infection. Each value represents the average of three independent samples within the same experiment, with the standard deviation indicated by the error bars. (B) Female BALB/c mice were infected with 5×10^4 CFU of wild-type *S.* Typhimurium (squares), *ssaR* (circles) or *tlpA* (triangles) mutant strains. The survival of infected mice over time is shown. (C) Competitive index analysis of an oral infection. Two groups of female BALB/c mice were infected orally with 1×10^6 CFU of 1:1 mixture of *tlpA* and a marked wild-type strain. Mice were sacrificed 4 days postinfection and bacteria were recovered from the liver and the spleen. The CFU number of each strain was determined and a C.I. value was calculated as described in Section 2. Each dot represents a single C.I. value from an individual mouse; a geometrical mean of all the C.I. values in each organ is shown. A competitive index of 1 indicates that the two strains are proliferating equally in vivo. (D) C.I. analysis of an intraperitoneal infection. Female BALB/c mice were infected by i.p. injection with 8×10^4 CFU of *tlpA* and a marked wild-type strain. Mice were euthanized 2 days postinfection and the bacteria were recovered from the liver and the spleen. Analysis was done as described above.

antibiotic at 37 °C and 40 °C. Every 24 h for 7 days, the cultures were diluted 1:150 into fresh LB. After 7 days, serial dilutions of the different cultures were plated for colony numeration on LB + Sm (to select for *S.* Typhimurium) and on LB + Cm (to select for pSLT +). The obtained results showed no significant difference in the number of CFU recovered from OG2009 vs. OG2010, at 37 °C or 40 °C (data not shown). These results indicate that the absence of TlpA does not affect plasmid loss rate, under the preformed conditions.

4. Discussion

TlpA has been previously characterized as a unique temperature-sensing, coiled-coil, autoregulator [6–8]. The ability of TlpA to sense temperature changes on one hand, and control gene expression on the other hand, has led to the hypothesis that TlpA plays a role in the virulence of *Salmonella* [6–8].

To gain insights into the nature of TlpA, we examined various physiological and environmental growth conditions to identify signals affecting *tlpA* expression. We showed that TlpA is induced in LB and by elevated temperatures but not in response to other stress conditions, assumed to mimic the intracellular milieu. Additionally, we demonstrated that TlpA is being repressed in MgM medium, by using two independent approaches (Figs. 2 and 3).

Differential expression of TlpA was also found to be growth-phase dependent. Higher expression of *tlpA::lacZ* was observed during logarithmic growth in comparison to the stationary phase (Fig. 3A and B). This observation suggests that *tlpA* is down-regulated during the stationary phase. In agreement with this, higher expression levels of *tlpA::lacZ* were found in stationary phased culture, in the absence of the alternative stationary phase sigma factor RpoS, indicating that RpoS plays a role in the regulation of *tlpA*. In addition to RpoS, our data suggest that at least two other regulators are involved in controlling *tlpA* expression. Elevated expression of *tlpA::lacZ* was found in the *tlpA* mutant background, supporting the notion that TlpA represses its own promoter [7].

Another major regulator that was found in our analysis is the response regulator PhoP. A double mutant lacking both *tlpA* and *phoP* resulted in extensively higher expression of *tlpA::lacZ,* indicating an additive repression effect of TlpA and PhoP on the expression of *tlpA* (Fig. 3). In *Salmonella*, PhoP is the response regulator of the PhoP/PhoQ two-component

system, which responds to environmental Mg^{2+} [21]. It is believed that by monitoring the availability of extracellular Mg^{2+}, PhoPQ allows *Salmonella* to sense the transition from an extracellular environment to a subcellular location and to activate a set of virulence factors essential for intracellular survival [25,26]. Therefore, the regulation of *tlpA* by PhoP may explain and provide a molecular mechanism to the suppressed expression of *tlpA* under MgM medium, containing low Mg^{2+} concentration.

It has been shown that a PhoP recognition sequence consists of a direct repeat of the hexanucleotide (T/G)GTTTA separated by a spacer of five nucleotides. Nonetheless, a conserved thymine in the first half site (at position 3) and two conserved thymines together with one conserved adenine in the second half site (at positions 3, 4, and 6, respectively) were sufficient for DNA binding of PhoP in a DNA footprinting analysis [27,28]. Examination of the upstream region of *tlpA* revealed a potential PhoP box (TG**T**CTGTCTCTGG**TT**A**A**) 241 bp upstream from the first methionine. The presence of a PhoP binding sequence in the promoter region would indicate that the repression of *tlpA* by PhoP is direct; however, further studies are needed in order to confirm this.

A bioinformatics analysis revealed that TlpA shares sequence similarity with KfrA and with the *L. pneumophila* effector LepB. Based on that and considering the previous hypothesis that TlpA plays a role in virulence, we were interested in studying possible function of TlpA as an effector and/or in *Salmonella* pathogenesis. Our results show that TlpA can be secreted into the culture media (Fig. 4A); however, TlpA secretion was found to be TTSS-independent. Furthermore, two different approaches (Fig. 4B and C) suggested that TlpA is not transported into host cells. Together, these results indicate that TlpA does not act as a translocated effector protein and direct to a different function from LepB.

To evaluate directly the contribution of TlpA to the pathogenicity of *Salmonella* in vivo, we studied the ability of a *tlpA* mutant strain to cause systemic infection in susceptible BALB/c mice. The results presented here (Fig. 5) from both survival-time and C.I. experiments indicate that TlpA is dispensable for virulence in mice. These results are also in agreement with the in vitro study, showing that TlpA is not required for intracellular invasion or multiplication inside host cells. Collectively, our data do not support the previous hypothesis that TlpA plays a role in the pathogenicity of *Salmonella per se*, but imply an alternative function which is not directly involved in the virulence of *Salmonella*.

Unraveling TlpA function may be facilitated by the homology found with KfrA, and the SMC proteins. Several common characters can be attributed to TlpA and KfrA, which may suggest a related function: (1) significant degree of sequence similarity and a common coiled-coil secondary structure; (2) comparable episomal localization; (3) both have an N-terminal DNA-binding domain; and (4) both act as an autoregulatory repressor [6,16].

The function of the *kfrA* gene product is as yet unknown; however, it has been proposed to be involved in plasmid partition [15,16], although a deletion of *kfrA* did not seem to affect the plasmid loss rate [29,30]. The somewhat homology of TlpA to the SMC family, known to be involved in chromosome partitioning [18], might also suggest a putative function, related to DNA-segregation. Similar to the effect of the *kfrA* deletion, our results showed that the absence of TlpA did not affect the loss rate of pSLT; and therefore, suggest that either TlpA has only an auxiliary role in pSLT stability, or that TlpA has a different function, conceivably, in other aspects of plasmid biology.

The absence of TlpA homologs from *E. coli* or other related Enterobacteria genomes; the fact that TlpA was found exclusively in several serovars of a single subspecies of *S. enterica*; and their extremely low degree of sequence polymorphism, all suggest that *tlpA* is "evolutionary new" in the *Salmonella* genome and was acquired by horizontal gene transfer only recently. Possibly, this event happened after the speciation of *S. enterica* into the subspecies *enterica*, but before the divergence of Typhimurium, Enteritidis, Dublin, Choleraesuis, and Gallinarum to different serovars. Indeed, *tlpA* and the two open reading frames located immediately downstream (PSLT49 and PSLT50) have a higher G + C content (59%) in comparison to the rest of the chromosome and the virulence plasmid (53% in both cases) [31], which is often indicative of lateral gene transfer events. We would like to propose that the homologous protein, KfrA (or its molecular ancestor) may have been the origin of such acquisition and they both may fulfill a similar function in the biology of their host plasmid.

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Appendix A. Supplementary information

Supplementary information for this manuscript can be downloaded at 10.1016/j.micinf.2006.04.015.

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