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Original article

Dam methylation regulates the expression of SPI-5-encoded *sopB* gene in *Salmonella enterica* serovar Typhimurium

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Abstract

DNA adenine methylation is an essential factor in *Salmonella* virulence. Here, we investigate the involvement of DNA adenine methylase (Dam) in the expression and translocation of a SPI-5-encoded effector of *S.* Typhimurium. SopB expression and secretion were determined using SopB–FLAG-tagged wild type and *dam* strains of *S.* Typhimurium. Western blot and quantitative reverse transcriptase PCR analysis showed that the *dam* mutant expresses lower levels of SopB protein and *sopB* mRNA than the wild type strain under SPI-1 and SPI-2 inducing conditions in vitro. SopB secretion was also considerably impaired in the absence of *dam*. In agreement with in vitro experiments, SopB synthesis in *dam* mutants recovered from infected epithelial cells and from murine mesenteric lymph nodes was reduced by 40% respect to the wild type strain ($p < 0.05$). SopB translocation was neither detected in the cytosol of epithelial cells nor in the cytosol of cells isolated from mesenteric lymph nodes infected with the *dam* mutant. Taken together, our results demonstrate that, in *S.* Typhimurium, Dam methylation modulates the expression and translocation of SPI-5-encoded SopB effector.

Keywords: *Salmonella enterica* serovar Typhimurium; DNA adenine methylase; SopB; SPI-5

1. Introduction

SopB is a type 3 secreted *Salmonella* effector protein with phosphoinositide phosphatase activity and a distinct GTPase binding domain [1,2]. The *sopB* gene is located in the SPI-5 pathogenicity island and is well conserved in all sequenced *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strains [3]. It is known that SopB plays a multifaceted role in early and late stages of infection. For instance, although SopB aids bacterial invasion by localizing to the plasma membrane early in infection [4], it later relocalizes to the SCV [5] where it alters the phosphoinositide lipids composition to reduce the recruitment of Rab8, Rab13, Rab23, and Rab35 and prevent phagolysosome formation [6,7]. SopB has a number of other roles in establishing the intracellular niche such as, the induction of iNOS long after invasion [8] and activation of serine protein kinase Akt [9], among others.

DNA adenine methylation (Dam) is an essential factor in *Salmonella* virulence [10,11]. It is generally accepted that Dam methylation regulates virulence genes that participate in the intestinal stage of infection [10,11]. In fact, Dam methylation is not a direct regulator of gene expression but a mechanism that controls DNA–protein interactions [12], and the levels of many transcriptional regulators vary depending on physiological and environmental conditions. In this work

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we present evidence that Dam methylation regulates the SPI-5-encoded *sopB* gene.

2. Materials and methods

2.1. Bacterial strains

This work was carried out using FLAG-tagged strains of *S.* Typhimurium derived from strain ATCC 14028. SSM2796 (*sopB*::3xFLAG) (kindly provided by Dr. Uzzau). ST1702 (*sseJ*::3xFLAG) strains of *S.* Typhimurium were obtained using the method described by Uzzau et al. [13]. The high-frequency generalized transducing bacteriophage P22HT was used for the transduction. The Δ*dam-230 zge-6313::Tn10d*Cm^R allele was transduced from *S.* Typhimurium SV4712 strain (kindly provided by Dr. Casadesús) into the tagged strains SSM2796 and ST1702 resulting in *dam* strains called STD2796 (*sopB*::3 X FLAG Δ*dam-230 zge-6313::Tn10d*Cm^R) and STD1702 (*sseJ*::3 X FLAG Δ*dam-230 zge-6313::Tn10d*Cm^R). For complementation experiments, pIZ833 carrying a *dam* gene was introduced into the mutant strains by electroporation [14]. Mutants *invG::aphT* (Km^R) *sopB*::3xFLAG, *invG::aphT* (Km^R) *sseJ*::3xFLAG, *ssaK::aphT* (Km^R) *sopB*::3xFLAG and *ssaK::aphT* (Km^R) *sseJ*::3xFLAG were constructed by phage P22-mediated transduction from *invG::aphT* (Km^R) (SB1171) [15] and *ssaK::aphT* (Km^R) (ST0303) strains of *S.* Typhimurium to SSM2796 (*sopB*::3xFLAG) and ST1702 (*sseJ*::3xFLAG) mutants. Gene deletion was verified by PCR.

2.2. Culture conditions

For in vitro studies, bacteria were grown to exponential phase under different culture conditions. To mimic the intestinal environment [16] bacteria were grown at 37 °C without aeration in a Luria–Bertani (LB) broth containing 0.3 M NaCl. An intracellular milieu was recreated by growing bacteria in MgM minimal medium containing 0.1% casaminoacids at 37 °C with aeration [16] at pH 6.

For in vivo studies, bacterial inocula used to infect cells or animals were prepared growing the tagged strains overnight under SPI-1 non-inducing conditions (LB at 28 °C) as previously described Giacomodonato et al. [17]. In this way, the residual expression of SopB from in vitro bacterial growth was minimized.

Cultures were centrifuged, diluted in sterile saline and inoculated to cultured cells or mice. Viable bacteria in the inoculum were quantified by dilution and plating onto LB agar plates with appropriate antibiotics.

2.3. Expression and secretion of SopB in vitro

For the isolation of cell-associated proteins, 1.5 ml of bacterial cultures were centrifuged and resuspended in 100 μl of H_2O and immediately mixed with 100 μl of Laemmli buffer. For the isolation of proteins released into the culture supernatants (secreted proteins), bacteria were pelleted by centrifugation and 2 ml of supernatant was collected from each sample. Supernatants were then filtered (0.45 μm pore size), and the proteins were precipitated with 25% trichloroacetic acid and sedimented by high-speed centrifugation (14,000 *g* for 30 min). The pellet was washed in cold acetone and suspended in phosphate-buffered saline (PBS) and Laemmli buffer. Four independent extractions for each sample were added together to minimize differences in protein recovery from sample to sample. The proteins (cell-associated and secreted proteins) were then boiled for 5–10 min, and an aliquot of each sample was separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) [18]. Finally, effector proteins were immunodetected as described below.

2.4. Expression and secretion of SopB in infected eukaryotic cells

Human laryngeal epithelial (HEp-2) cells (ATCC, CCL-23), were maintained in DMEM containing 10% fetal bovine serum. Infected monolayers (multiplicity of infection = 10:1) were incubated for 20 min at 37 °C in 5% CO_2, washed twice with PBS, and then incubated in fresh tissue culture medium containing 100 μg ml^{-1} of gentamicin for 1 h to remove extracellular bacteria and finally in fresh tissue culture medium containing 10 μg ml^{-1} gentamicin for the remainder of the experiment. At 24 h post-infection monolayers were washed twice with cold HBSS and lysed with 1.0 ml of HBSS containing 0.1% Triton X-100 and 1 mM PMSF as described Kubori et al. [19]. This procedure lyses the infected cells but does not affect the integrity of the bacterial membrane [20]. An aliquot of this suspension was used to determine the number of intracellular bacteria by plating serial dilutions onto LB agar plates. Cell lysates were collected in chilled microfuge tubes, and centrifuged at 17,000 *g* for 15 min at 4 °C to separate the soluble fraction, containing bacterial proteins that have been translocated into the host cell cytosol, from the insoluble fraction, which contains the internalized bacteria. The soluble fraction was filtered through a 0.45 μm pore-size filter and subjected to 10% trichloroacetic acid precipitation and sedimented by high-speed centrifugation (14,000 *g* for 30 min). The pellet was washed in cold acetone and resuspended in PBS and Laemmli buffer. The insoluble fraction was washed once with cold PBS and suspended in an appropriate volume of PBS and Laemmli buffer. The protein extracts were boiled for 5–10 min, and resolved on 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE). Finally, effector proteins were immunodetected as described below.

2.5. Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions, from bacteria culture (exponential phase) and from infected HEp-2 cells in six-well plates. Contaminating DNA was digested with RNase-free DNase I (Epicentre Biotechnologies), and the purity of all RNA preparations was confirmed by subjecting them to PCR analysis using primers specific for the gene encoding the 16S rRNA. After inactivation of DNase, RNA was used as a

template for reverse transcription-PCR. Complementary cDNA was synthesized using random hexamer primers (Invitrogen), deoxynucleoside triphosphates, and Moloney murine leukemia virus M-MLV reverse transcriptase (Invitrogen). Relative quantitative real-time PCR was performed with an appropriate primer set, cDNAs, and Mezcla Real (Biodynamics) that contained nucleotides, polymerase, reaction buffer, and Green dye, using a Rotor-Gene 6000 real-time PCR machine (Corbett Research). The primer sequence were 16S rRNA forward 5′ GCCGCAAGGTTAAAACTCAA 3′ and reverse 5′ AAGGCACCAATCCATCTCTG 3′, *sopB* forward 5′ GGAATTGTAAAAGCGGCAAA 3′, and reverse 5′ TTTTCTGTCCACCGCTATCC 3′, *sseJ* forward 5′ GCCGATGCATTTAAGGTGAT 3′, and reverse 5′ TTTTCTGTCCACCGCTATCC 3′. The amplification program consisted of an initial incubation for 3 min at 95 °C, followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C 20 s. A no-template control was included for each primer set. Melting curve analysis verified that each reaction contained a single PCR product. For the relative gene expression analysis, a comparative cycle threshold method (ΔΔCT) was used [21]. The number of copies of each sample transcript was determined with the aid of the Rotor-Gene 6000 Series Software Version 1.7. Briefly, the amplification efficiencies of the genes of interest and the 16S rRNA gene used for normalization were tested. Then each sample was first normalized for the amount of template added by comparison to the 16S rRNA gene (endogenous control). These values were further normalized using the wild type sample (calibrator treatment). Hence, the results were expressed relative to the value for the calibrator sample, which was 1.

2.6. Mice

Six to 8-week old BALB/c mice were obtained from our vivarium, maintained under standard conditions and provided with food and water *ad libitum*. At the end of the experiment, mice were killed with carbon dioxide. All experimental protocols were approved by the Animal Ethics Committee, University of Buenos Aires, Argentina.

2.7. Expression and translocation of SopB in vivo

Mice were inoculated intraperitoneally with 10^7 CFU/mouse of the *Salmonella*-tagged strains and were euthanized 24 h post inoculation. Mesenteric lymph nodes (MLN) were removed and incubated for 20 min in 3 ml of HBSS containing 100 μg ml^{-1} of gentamicin, following by 3 washes in 10 ml of HBSS without antibiotic, before single cell suspensions were prepared using an iron mesh sieve. Then, the isolated cells were processed as described above to analyze the expression and translocation of SopB in HEp-2 cells.

2.8. Western blot analysis

The gels were blotted onto a Hybond-P membrane (GE Health-care, Madrid, Spain). 3xFLAG fusion proteins were immunodetected using mouse anti-FLAG M2-peroxidase (HRP) mAbs (Sigma, St Louis MO). Some blots were reprobed with polyclonal antibodies to actin (Sigma) and to GroEL (Sigma) as eukaryotic and bacterial cytosolic protein markers, respectively. Goat anti-rabbit HRP-conjugated antibodies (Sigma) were used as secondary antibodies. The reacting bands were detected by enhanced chemiluminescence (ECL) (Luminol, Santa Cruz Biotechnology, Santa Cruz, CA) in an Image Quant 300 cabinet (GE Healthcare) following the manufacturer instructions. Blots were photographed, and the intensity of the signals expressed in arbitrary units was determined by densitometry analysis using the public domain NIH Image J software (http://rsb.info.nih.gov/nih-image/).

2.9. Statistical analysis

The data represented as mean ± standard deviation (SD) were from triplicates and were analyzed for statistical significance using a nonparametric Mann–Whitney test using the GraphPad Prism software version 5.

3. Results

3.1. Dam mutants of S. Typhimurium synthesize and secrete low levels of SopB

To investigate the capacity of *Salmonella dam* strain to synthesize and secrete SopB, bacteria were grown under culture conditions resembling the intestinal (SPI-1 conditions) or the intracellular (SPI-2 conditions) environment as described in materials and methods. The relative amount of the effector protein present in the whole bacterial extract and in the supernatant was quantified (Fig. 1A and B).

Under SPI-1 conditions, SopB expression and secretion were reduced in the *dam* mutant compared to wild type bacteria, as revealed by Western blot densitometry analysis (Fig. 1A and B). Similarly, under SPI-2 conditions SopB expression in the *dam* mutant was reduced respect to the wild type strain, whereas no secreted SopB was detected in the culture supernatant (Fig. 1A and B). The reduced expression of SopB in *dam* mutants was corroborated by qRT-PCR. As shown in Table 1, the transcript level of *sopB* relative to 16S rRNA gene expression was 55% and 32% lower under intestinal and intracellular conditions, respectively. It is important to note that pIZ833 plasmid did not complement the effect of the lack of *dam* on *sopB* expression and SopB expression/secretion. Failure in restoring certain virulence traits after complementation of *dam* mutants has been reported earlier [10,11,31] and could be explained by the fact that overproduction of Dam methylase reproduces certain phenotypes of mutant strains lacking the Dam protein [22,23]. To investigate whether the lack of complementation is a consequence of Dam overproduction we constructed wt/pIZ833 strain. We found that the expression and secretion of SopB protein and *sopB* mRNA in both wt/pIZ833 and *dam* strains are comparable (Table 1; Fig. 1A and B). These results confirm that pIZ833 is functional, and strongly suggest that

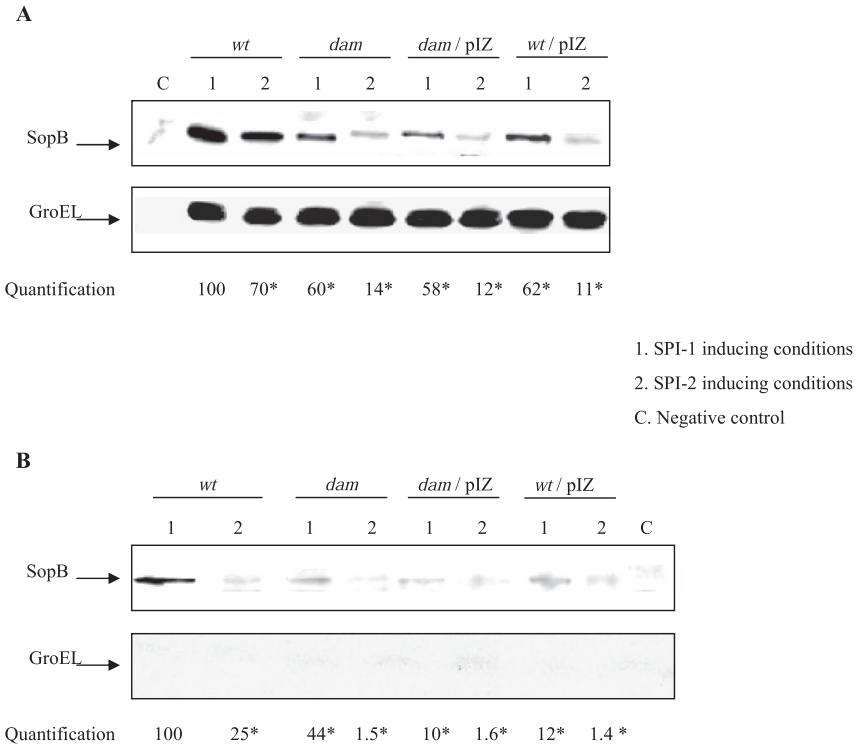

Fig. 1. Analysis of SopB expression (A) and secretion (B) in vitro by Western blot. Wild type, *dam*, *dam*/pIZ833 and wt/pIZ833 SopB-tagged strains of *S.* Typhimurium were grown under SPI-1 (lanes #1) and SPI-2 culture conditions (lanes #2); lane C = negative control. Expression (A) and secretion (B) were investigated in whole bacterial extracts and supernatants, respectively. Samples were subjected to SDS-PAGE and tagged proteins were detected by anti-FLAG antibodies. Each lane was loaded with material from approximately 10^6 CFU. For quantification of effector levels present in whole bacterial extracts (A), GroEL was used as loading control, and the ratio tagged protein/GroEL was normalized to 100 in the wild type background. SopB secretion (B) was normalized to 10^6 CFU. Results were expressed relative to the value for the wild type background, which was 100. GroEL was used as a control of lack of bacterial cell lysis. Data are representative from three independent experiments. *$p < 0.05$ with respect to the wild type strain (Mann–Whitney test).

complementation experiments failed because of Dam overexpression. Therefore, *dam*/pIZ833 strain was not included as a control in the experiments that follow.

3.2. SopB synthesis and translocation is impaired in intracellular dam mutants of S. Typhimurium

We investigated the synthesis and secretion of SopB in *dam* mutants infecting HEp-2 cells. Confluent HEp-2 cells were inoculated with SopB–FLAG tagged *dam Salmonella* mutant. Twenty-four hour post-infection cells were mechanically disrupted and centrifuged at low speed to separate cell lysates into soluble fraction (containing host cell cytoplasm and membranes) and insoluble fraction (containing intact bacteria, host cell nuclei, unbroken host cells and host cells cytoskeleton). Western blot analysis of insoluble fraction revealed that *dam* mutant expressed lower levels of SopB compared to the wild type strain ($p < 0.05$) (Fig. 2A). Immunoblotting analysis of the soluble fraction showed that SopB is translocated only by wild type strain (Fig. 2B). Despite the fact that SopB was expressed in intracellular *dam* mutants, this effector could not be detected in the soluble fraction (Fig. 2B). Lack of SopB translocation in intracellular *dam* mutants could be explained by the detection limit of our

Table 1
Analysis of *sopB* expression under different culture conditions by qRT-PCR.

	Gene	*dam*		*dam*/pIZ		wt/pIZ	
		Relat. Express ± SD	Relat. Express (%)	Relat. Express ± SD	Relat. Express (%)	Relat. Express ± SD	Relat. Express (%)
SPI-1 culture conditions	*sopB*	0.55* ± 0.02	55	0.39* ± 0.02	39	0.42* ± 0.05	42
	sseJ	0.00 ± 0.00	0	0.00 ± 0.00	0	0.00 ± 0.00	0
SPI-2 culture conditions	*sopB*	0.32* ± 0.03	32	0.31* ± 0.05	31	0.29* ± 0.03	29
	sseJ	0.71* ± 0.05	71	2.24* ± 0.15	224	1.95* ± 0.09	195

Relative expression of *sopB* and *sseJ* mRNA determined by qRT-PCR. Total mRNA was harvested from cultures of wt, *dam*, *dam*/pIZ833 and wt/pIZ833 strains. The relative mRNA amount was determined by reverse transcription real-time quantitative PCR and related to mRNA levels in wt strain, set as 1. Values are means ± SD of three independent mRNA extractions performed in triplicates. * Significant difference $p < 0.05$ with respect to wt strain.

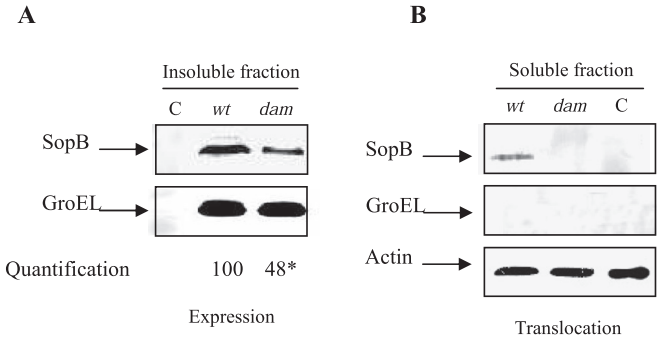

Fig. 2. Analysis of SopB expression (A) and translocation (B) in HEp-2 cells by Western blot. Epithelial cells were infected with wild type and *dam* SopB-tagged strains of *S.* Typhimurium for 24 h. Post-infection cells were processed as indicated in materials and methods to obtain an insoluble fraction containing intact bacteria and a soluble fraction containing translocated effectors. Both fractions were analyzed by immunoblotting using anti-FLAG antibodies. Lane C = control uninfected cell cultures. As a control for the host cell cytosolic fraction some blots were reprobed with polyclonal antibodies to actin. GroEL was used as a control for the lack of bacterial cell lysis. Each lane was loaded with material from approximately 10^6 CFU. For quantification of effector levels present in the insoluble fraction (A), GroEL was used as loading control, and the ratio tagged protein/GroEL was normalized to 100 in the wild type background. Data are representative from three independent experiments. *$p < 0.05$ with respect to the wild type strain (Mann–Whitney test).

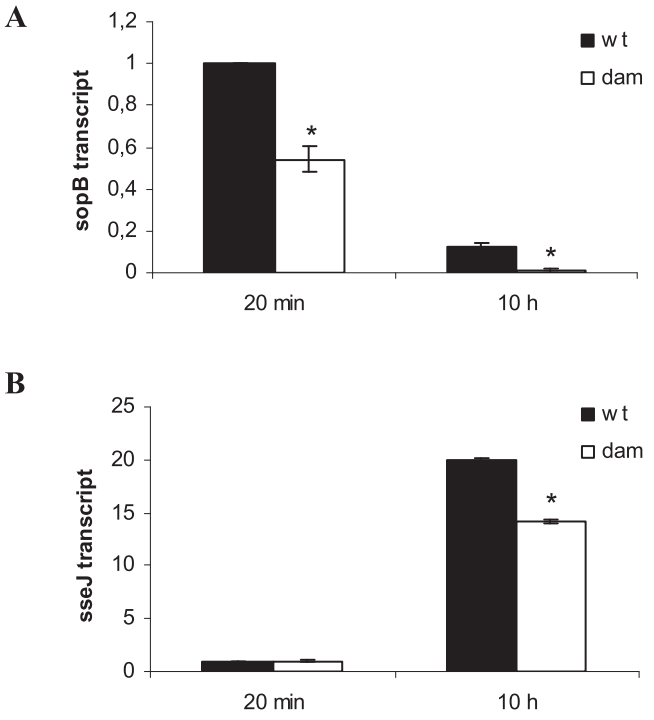

Fig. 3. Analysis of *sopB* expression in HEp-2 cells by qRT-PCR. Epithelial cells were infected with wild type or *dam* strains of *S.* Typhimurium for 20 min or 10 h. Post-infection cells were processed as indicated in materials and methods to obtain total mRNA. *sopB* (A) and *sseJ* mRNA levels (B) from bacteria colonizing HEp-2 cells were measured by qRT-PCR at the indicated times. The mRNA amount was related to mRNA levels in wild type strain at 20 min post-infection, set as 1. Values are means ± SD of three independent mRNA extractions performed in triplicates. * Significant difference $p < 0.05$ with respect to wild type strain (Mann–Whitney test).

method. Moreover, it is important to consider that SPI-1 dependent secretion in general could be decreased since Dam is a positive regulator of SPI-1 [24].

To expand upon these results we used qRT-PCR to directly measure *sopB* mRNA levels in intracellular bacteria, as described in materials and methods. As predicted from previous studies [8] we found that *sopB* mRNA levels were detectable for at least 10 h post-infection overlapping with the maximal induction of *sseJ* (Fig. 3). Again, we observed that the transcript level of *sopB* mRNA was lower in the *dam* mutant respect to the wild type strain under early (20 min) and late (10 h) stages of infection (Fig. 3).

3.3. SopB synthesis and translocation is impaired in intracellular dam mutants of S. Typhimurium in the murine model

To investigate the synthesis and translocation of SopB in *dam* mutants during murine infection, mice were infected intraperitoneally with tagged strains of *Salmonella*. Animals received a high dose of *Salmonella* that, in turn, yielded a sufficient number of infecting bacteria (to be recovered to investigate SopB expression), and also provided an adequate amount of infected cells (to be isolated to determine SopB translocation). In a previous report, we demonstrated that 24 h after inoculation, SopB continues to be expressed by wild type infecting bacteria recovered from MLN [25]. Once again, intracellular *Salmonella dam* mutant expressed lower levels of SopB respect to the wild type strain ($p < 0.05$) (Fig. 4A and C). Then, we investigated the in vivo translocation of SopB in the cytosol of infected cells isolated from MLN during murine *Salmonella* infection. Results showed that SopB translocation in infected cells recovered from MLN was evident only for the wild type strain (Fig. 4B).

In order to identify whether SopB translocation under intracellular conditions was via the SPI-1 or SPI-2 type three secretion system (T3SS), we analyzed SopB secretion in two genetic backgrounds *invG::aphT* (Km^R) and *ssaK::aphT* (Km^R).

Both InvG and SsaK are structural components of the SPI-1 and SPI-2 T3SS respectively. These mutations render the bacteria defective for SPI-1 T3SS- or SPI-2 T3SS-dependent secretion, respectively [26–28]. These mutations were transduced into SopB–FLAG and SseJ–FLAG strains. Interestingly, analysis in vitro by Western blot confirmed that SopB was secreted by both the SPI-1 and SPI-2 T3SSs under SPI-1 and SPI-2 inducing conditions respectively, whereas SseJ was secreted only via the SPI-2 T3SS (Fig. 5A). Then, we investigated the SopB translocation in infecting HEp-2 cells. Confluent HEp-2 cells were inoculated with wild type and Δ*ssaK* SopB–FLAG tagged *Salmonella* strains as indicated in materials and methods. Western blot analysis of insoluble and soluble fraction revealed that Δ*ssaK* (SPI-2 T3SS) mutant was capable of expressing SopB in the insoluble fraction containing intact bacteria at the similar level compared with wild type strain. However, delivery of SopB into the cytosol of HEp-2 cells was not observed following infection with the

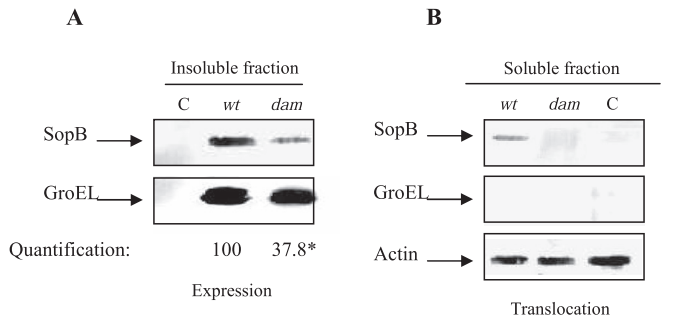

Fig. 4. Analysis of SopB expression (A) and translocation (B) in MLN by Western blot. Groups of mice were inoculated intraperitoneally with 10^7 CFU of the wild type and *dam* SopB-tagged strains of *S.* Typhimurium and euthanized at 24 h post-infection. MLN were removed and processed as indicated in materials and methods to obtain an insoluble fraction containing intact bacteria and a soluble fraction containing translocated effectors. Both fractions were analyzed by immunoblotting using anti-FLAG antibodies. Lane C = sample from control uninfected mice. As a control for the host cell cytosolic fraction some blots were reprobed with polyclonal antibodies to actin. GroEL was used as a control for the lack of bacterial cell lysis. Each lane was loaded with material from approximately 10^6 CFU. For quantification of effector levels present in the insoluble fraction (A), GroEL was used as loading control, and the ratio tagged protein/GroEL was normalized to 100 in the wild type background. Data are representative from three independent experiments. $*p < 0.05$ with respect to the wild type strain (Mann–Whitney test).

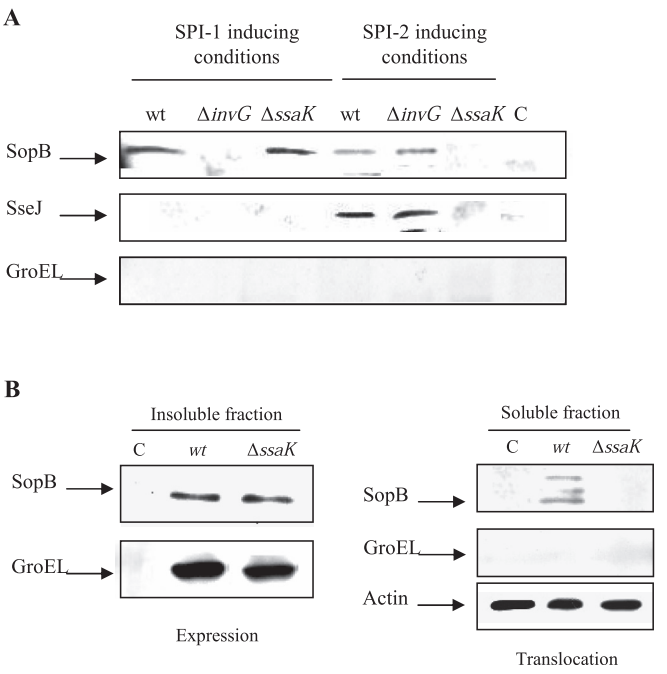

Fig. 5. Analysis of SPI-1 T3SS- and SPI-2 T3SS-dependent secretion of SopB in vitro (A) and in HEp-2 culture cells (B) by Western blot. (A) Wild type, Δ*invG* (SPI-1 T3SS defective) and Δ*ssaK* (SPI-2 T3SS defective) SopB-tagged and SseJ-tagged strains of *S.* Typhimurium were grown under SPI-1 and SPI-2 culture conditions as indicated in materials and methods. Effector's secretion was investigated in bacterial supernatants. SseJ was used as control for SPI-2 T3SS-dependent secretion. Lane C = negative control. Samples were subjected to SDS-PAGE and tagged proteins were detected by anti-FLAG antibodies. (B) Epithelial cells were infected with wild type and Δ*ssaK* (SPI-2 T3SS defective) SopB-tagged strains of *S.* Typhimurium for 24 h. Post-infection cells were processed as indicated in materials and methods to obtain an insoluble fraction containing intact bacteria and a soluble fraction containing translocated effectors. Both fractions were analyzed by immunoblotting using anti-FLAG antibodies. Lane C = control uninfected cell cultures. As a control for the host cell cytosolic fraction some blots were reprobed with polyclonal antibodies to actin. GroEL was used as loading control and as a control for the lack of bacterial cell lysis. Each lane was loaded with material from approximately 10^6 CFU. Data are representative from three independent experiments.

Δ*ssaK* mutant (Fig. 5B). These findings demonstrate that SopB is translocated into host cells at late times post-infection in a SPI-2 dependent manner.

4. Discussion

Dam is a well-characterized enzyme with a role in various basic cellular processes ranging from DNA replication, mismatch repair to expression of virulence genes in an increasing number of human and animal pathogens including *S.* Typhimurium [12,23,29]. Most studies about gene regulation by Dam methylation are carried out under SPI-1 inducing conditions because the majority of virulence defects so far described in *Salmonella dam* mutants affect the intestinal stage of infection [10,11]. In fact, we have shown previously that Dam methylation modulates SPI-1-dependent effectors of *Salmonella* such as SipA, SopA, SopD, and SopE2 in vitro under conditions that mimic the extracellular environment typical of the intestinal lumen [17]. Here we show that *dam* mutants of *S.* Typhimurium synthesize and secrete lower levels of SopB compared to wild type strain. Interestingly, we also found that SopB is expressed and secreted under SPI-1 and SPI-2 conditions.

The reduced secretion/translocation of SopB in *dam* mutants could be caused by multiple factors. The first and simplest explanation could be the diminished expression of the *sopB* gene. In this regard, the facts that the *dam* mutant exhibits reduced levels of *sopB* mRNA and that the amount of SopB detected by Western blot is diminished, indicate that the expression of *sopB* gene is controlled (directly or indirectly) by Dam methylation. In theory, the expression of *sopB* could be regulated directly by Dam methylation since five putative methylated-GATC sequences are present downstream of the +1 codon. However, it is important to mention that *sopB*, is cooperatively regulated by InvF and HilA [30] and lowered levels of all SPI-1-encoded transcriptional regulators (HilA, HilC, HilD, and InvF) were found in *Salmonella dam* mutants, hereby confirming that the entire SPI-1 is under Dam-dependent control [24]. Given that these master regulators of SPI-1 are affected by Dam methylation, it is possible that the effect of Dam methylation/*sopB* was indirect. Moreover, it has been previously shown that *sigE* (*pipC*) (class IA chaperone that binds SopB) expression is down-regulated in a *dam* mutant [31]. SigE is essential for SopB stability, secretion and translocation [32]. Besides, Dam methylation is required to activate the expression of certain SPI-1 syringe-encoding genes [31]. The regulation of *sopB* in *S.* Typhimurium is highly complex and not completely understood; therefore, further experiments are necessary to investigate whether the expression of *sopB* is regulated directly or indirectly by Dam methylation.

In vitro and in vivo experiments showed that Dam methylation also modulates SopB expression under intracellular conditions. We have shown earlier that *Salmonella* effectors involved in invasion processes continue to be expressed in vivo during systemic infection [25,34]. For instance, SopB protein was found in both phases of *Salmonella* infection [6,25]. However, the amount of SopB detected at day 5 was lower than that observed in bacteria recovered at 12 h post-intraperitoneally inoculation [34,35]. Knodler et al. demonstrated by RT-PCR and Northern analysis that *sopB* expression was markedly increased in SPI-1 inducing media compared with SPI-2 inducing media [33]. In this way, our experiments performed in cultured cells strongly suggest that persistence of SopB during *Salmonella* infection is due -in part- to *de novo* synthesis. These results are consistent with those obtained by Patel et al. [6]. They observed that a proportion of SopB detected late in infection represented newly synthesized protein. Moreover, we show here that SopB expression is impaired in *dam* mutants at early and late stages of *Salmonella* infection.

Balbontín et al. confirmed that the inefficient invasion of epithelial cell cultures found in *Salmonella dam* mutants is correlated with altered secretion patterns of invasion proteins encoded inside SPI-1 [31]. Earlier Raffatellu et al. demonstrated the participation of SipA, SopA,B,D,E2 during the invasion process [18]. More recently, Tahoun et al. showed that SopB is able to transform follicle associated intestinal epithelial cells into M cells in order to enhance bacterial translocation across the intestinal mucosa [36]. They proved that SopB is necessary and sufficient to induce this cellular transformation. Therefore, the diminished amount of SopB in *dam* mutants could contribute to their defective mucosal invasion.

It is known that interaction between SopB and Cdc42 present in host cells is involved in bacterial intracellular replication [37]; therefore, SopB contributes to establish the *Salmonella* intracellular replicative niche in the mammalian host. It is important to note that SopD is also involved in systemic disease in mice and is required for optimal replication in mouse macrophages [38]. The need for Dam methylation to up regulate the expression of *sopB* and *sopD* (data not shown) could explain -in part- the deficient intracellular replication registered in the *dam* mutant. We observed a significant defect in proliferation of the *dam* strain within HEp-2 cells [17]. Moreover, we found an impaired colonization of spleen, Peyer's patches and MLN by the *dam* mutant (data not shown). Low persistence of *Salmonella dam* mutant in these organs could be, to some extent, the consequence of the increased sensitivity toward H_2O_2 compared with the wild type strain of *S.* Typhimurium, as described recently [39].

Knodler et al. demonstrated that SPI-5 encodes effectors that can be induced by distinct regulatory cues and that are targeted to different T3SS [33]. In this regard, SPI-5 encodes the SPI-1 T3SS translocated effector SopB. In contrast, the adjacently encoded PipB effector is part of the SPI-2 regulon and it is translocated by the SPI-2 T3SS to the *Salmonella*-containing vacuole. Thus, it is clear that a functional and regulatory cross-talk exists between three chromosomal PAIs, SPI-1, SPI-2 and SPI-5. Effectors encoded within a single PAI can be induced by dissimilar environmental signals, translocated by distinct T3SS and up or down regulated by Dam methylation. Balbontin et al. showed that *pipB*, *pipC* are up regulated by Dam methylation, but *pipA*, *orfX* and *pipD* are down regulated under SPI-1 inducing conditions [31]. We reveal here that the expression of *sopB* is up regulated by Dam methylation.

Interestingly, we demonstrated in vitro and in infected epithelial cells that SopB is secreted by the SPI-1 and SPI-2 T3SSs. The fact that SopB is translocated by both syringes could explain why or how it participates in intestinal and systemic phases of *Salmonella* infection. Our findings are consistent with observed by Geddes et al. who identified effectors that were secreted by both T3SSs like SptP, SlrP, AvrA, SteA, and SteB [27]. Similarly, Brumell et al. demonstrated that SopD, originally identified as an SPI-1 effector, is also a dual effector [40]. Another example is PipB2, a T3SS2 effector, that is also a substrate of T3SS1 [41]. These findings confirm the functional overlap between these virulence-related secretion systems.

In summary, through in vitro and in vivo experiments we showed that Dam methylation modulates SopB expression and translocation. This effect was evident at both, early and late stages of *S.* Typhimurium infection. We extended our initial analyses of Dam methylation's influence on virulence functions in *Salmonella* and suggested a role of DNA methylation in the regulation of the expression of a SPI-5-encoded *Salmonella sopB* gene.

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