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SipA, SopA, SopB, SopD and SopE2 effector proteins of *Salmonella enterica* serovar Typhimurium are synthesized at late stages of infection in mice

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Salmonella pathogenicity island (SPI)-1 is essential for invasion of non-phagocytic cells, whereas SPI-2 is required for intracellular survival and proliferation in phagocytes. Some SPI-1 effectors, however, are induced upon invasion of both phagocytic and non-phagocytic cells, suggesting that they may also be required post-invasion. In the present work, the presence was analysed of SipA, SopA, SopB, SopD and SopE2 effector proteins of *Salmonella enterica* serovar Typhimurium *in vitro* and *in vivo* during murine salmonellosis. Tagged (3 × FLAG) strains of *S. enterica* serovar Typhimurium were inoculated intraperitoneally or intragastrically to BALB/c mice and recovered from the spleen and mesenteric lymph nodes of moribund mice. Tagged proteins were detected by SDS-PAGE and immunoblotting with anti-FLAG antibodies. *In vitro* experiments showed that SPI-1 effector proteins SipA, SopA, SopB, SopD and SopE2 were secreted under SPI-1 conditions. Interestingly, it was found that *S. enterica* serovar Typhimurium continued to synthesize SipA, SopB, SopD and SopE2 in colonized organs for several days, regardless of the route of inoculation. Together, these results indicate that SPI-1 effector proteins may participate in the late stages of *Salmonella* infection in mice.

INTRODUCTION

Salmonella enterica has evolved refined mechanisms to invade, and survive and replicate within, host cells. The outcome of these interactions is determined by both the host species and the *Salmonella* serotype. For instance, *S. enterica* serovar Typhi causes a systemic disease in humans known as typhoid fever, whereas patients infected with *S. enterica* serovar Typhimurium (serovar Typhimurium) develop a localized gastroenteritis and lymphadenitis resulting in diarrhoea. Similar to humans, in calves, infection with serovar Typhimurium remains localized to the intestine. In contrast, serovar Typhimurium infection in mice results in a systemic typhoid-fever-like disease (Santos & Bäumler, 2004; Zhang *et al.*, 2003).

Two major virulence determinants involved in *Salmonella* pathogenesis are encoded in large chromosomal pathogenicity islands called *Salmonella* pathogenicity island (SPI)-1 and SPI-2 (Galan, 2001). Both SPI-1 and SPI-2 encode separate type III secretion systems (TTSSs) that introduce virulence proteins into the host environment, either by translocation directly into host cells or, possibly, by secretion into the vicinity of host cells (Galan, 2001; Waterman & Holden, 2003). Upon ingestion, *Salmonella* serotypes exhibit, in mammals, a tropism for intestinal lymphoid tissue (Reis *et al.*, 2003; Santos & Baümler, 2004; Tsolis *et al.*, 1999). In mice, serovar Typhimurium preferentially invades the M cells of the follicle-associated epithelium of Peyer's patches (Clark *et al.*, 1994; Jones *et al.*, 1994). Invasion of epithelial cells is governed by the *Salmonella* SPI-1-encoded TTSS-1 (Galan, 2001). Serovar Typhimurium senses environmental factors such as oxygen concentration, osmolarity and pH, which act as regulators for expression of TTSS-1 (Bajaj *et al.*, 1996). Alternatively, serovar Typhimurium can rapidly enter the bloodstream from the intestinal lumen by a TTSS-1-independent route. This pathway involves bacterial transport by CD-18-expressing phagocytes (macrophages and/or dendritic cells) to systemic sites of infection (Vazquez-Torres *et al.*, 1999). It is generally accepted that SPI-1 and SPI-2 TTSSs play a dichotomous role during the intestinal and systemic

Abbreviations: i.g., intragastric(ally); iNOS, inducible nitric oxide synthase; i.p., intraperitoneal(ly); MLN, mesenteric lymph node; serovar Typhimurium, *Salmonella enterica* serovar Typhimurium; SPI, *Salmonella* pathogenicity island; TTSS, type III secretion system.

phases of salmonellosis. Whereas TTSS-1 plays an essential function in colonization of the bovine intestine and in bovine enteropathogenesis (Zhang *et al.*, 2003), this virulence trait has been reported to have little or no role in systemic infection (Galan, 2001). Conversely, the SPI-2-encoded TTSS (TTSS-2) is more strongly related to systemic virulence and its associated pathology than to intestinal disease (Galan, 2001). It is also well documented that SPI-1 is essential for invasion of non-phagocytic cells, whereas SPI-2 is required for intracellular survival and proliferation in phagocytes (Marcus *et al.*, 2000).

In contrast to the current model of SPI-mediated pathogenesis, it has been shown that some SPI-1 effectors are induced upon invasion of both phagocytic and non-phagocytic cells, suggesting that they may also be required post-invasion (Pfeifer *et al.*, 1999). In this regard, elegant studies performed by Steele-Mortimer *et al.* (2002) have demonstrated that SPI-1 is essential for intracellular replication. On the other hand, Brown *et al.* (2005) have recently demonstrated that SPI-2 expression precedes penetration of the intestinal epithelium. Therefore, it is important to carefully consider the dichotomous roles of SPI-1 and SPI-2 in the intestinal and/or systemic paradigm of serovar Typhimurium infection (Coburn *et al.*, 2005; Schlumberger & Hardt, 2006). To analyse whether SPI-1 effector proteins participate in the late stages of murine salmonellosis, we investigated the presence of SipA, SopA, SopB, SopD and SopE2 during *Salmonella* infection of mice.

METHODS

Bacterial strains. This work was carried out using strains of serovar Typhimurium derived from strain ATCC 14028 and tagged with the 8 aa FLAG epitope tag peptide. Strains SSM 3213 (*sopA*::3 × FLAG *sopE2*::3 × FLAG *cat*::FLAG), SSM 3214 (*sopD*::3 × FLAG *sipA*::3 × FLAG *cat*::FLAG) and SSM 3215 (*sopB*::3 × FLAG *avrA*::3 × FLAG *cat*::FLAG) of serovar Typhimurium were obtained using the method described by Uzzau *et al.* (2001). 3 × FLAG epitope tails were added to the ends of the *sipA*, *sopA*, *sopB*, *sopD* and *sopE2* genes. The 3 × FLAG epitope is a sequence of three tandem FLAG epitopes (22 aa). For each tagged mutant, a pair of primers was designed to amplify a 3 × FLAG- and kanR-coding sequence by using plasmid pSUB11 (Uzzau *et al.*, 2001). The 3′ ends of these oligonucleotides were complementary to the first 20 nt of the pSUB11 3 × FLAG coding region (GACTACAAAGACCATGACGG, forward primers) and to the 20 nt of the pSUB11 priming site 2 (CATATGAATATCCTCCTTAG, reverse primers). The 5′ ends of the oligonucleotides were designed to be homologous to the last 40 nt of each tagged gene, not including the stop codon (forward primers), and to the 40 nt immediately downstream of the gene stop codon (reverse primers).

Preparation of secreted proteins. Bacterial strains were grown under conditions to induce SPI-1 gene expression, as described by Miki *et al.* (2004). Bacterial culture supernatants and pellets were obtained to investigate secreted proteins and cell-associated proteins, respectively (Pucciarelli *et al.*, 2002). Bacteria were grown in LB broth containing 0.3 M NaCl overnight at 37 °C without aeration (SPI-1-inducing conditions). For the isolation of proteins released into the culture supernatants (secreted proteins), cells were pelleted by centrifugation and 2 ml supernatant was collected from each sample. The supernatants were then filtered (0.45 μm pore size), and the proteins were precipitated with 25 % TCA 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. Four independent extractions for each sample were added together to minimize differences in protein recovery from sample to sample. The proteins were then boiled for 5–10 min, and an aliquot of each sample was separated by SDS-PAGE (10 % gel) (Raffatellu *et al.*, 2005). Finally, effector proteins were immunodetected using mouse anti-FLAG M2-peroxidase (HRP) mAbs (Sigma).

Animals. Six- to eight-week-old BALB/c mice were purchased from the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and kept in our animal house throughout the experiments. All experiments were performed in accordance with the guidelines of the School of Medicine Animal Care and Use Committee.

Virulence assays. Serial dilutions of bacterial suspensions were used to inoculate groups of six mice intragastrically (i.g.) (500 μl) or intraperitoneally (i.p.) (100 μl). Survival of infected mice was recorded for a minimum of 4 weeks. LD_{50} was calculated by the method of Reed & Muench (1938).

Organ colonization. Groups of 10 mice were inoculated i.p. with 10^2 or 10^7 c.f.u. per animal of the SSM strains and were euthanized at 5 days or 12–18 h after inoculation, respectively. Another group of 10 mice were inoculated i.g. with 10^6 or 10^8 c.f.u. per animal of the SSM strains and were euthanized at 8 or 5 days after inoculation, respectively. Spleens and mesenteric lymph nodes (MLNs) were removed and homogenized in 1 ml sterile saline. Appropriate dilutions were plated on tripticase soy agar (TSA) for determination of colony counts.

Murine salmonellosis. Groups of 10 mice were inoculated i.p. with two different lethal doses (10^2 and 10^7 c.f.u. per mouse) of tagged serovar Typhimurium strains. A different group of animals were inoculated i.g. with 10^6 c.f.u. per mouse of tagged serovar Typhimurium strains. To prepare the inocula, bacteria were grown overnight in LB at 37 °C. Cultures were diluted in physiological saline for i.p. and i.g. inoculation. Viable bacteria in inocula were quantified by dilution and plating onto LB agar plates containing appropriate antibiotics.

Preparation of bacterial extracts from spleens and MLNs. Bacterial extracts from spleens and MLNs of mice were prepared as described by Dominguez-Bernal *et al.* (2004), with modifications. Mice were euthanized when moribund. Animals infected i.p. with 10^7 c.f.u. per mouse were euthanized at 12–18 h post-infection. Mice receiving 10^2 c.f.u. i.p. were euthanized at day 5 post-inoculation. On the other hand, mice inoculated i.g. with 10^6 c.f.u. were euthanized at day 8 post-infection. Spleens and MLNs were aseptically recovered and homogenized in 1.5 ml cold double-distilled water. To determine bacterial counts, 100 μl of this homogenate was serially diluted in PBS and plated on TSA. The rest of the homogenate was centrifuged (9000 ***g***, 10 min, 4 °C) and resuspended in 500 μl freshly prepared lysis buffer (120 mM NaCl, 4 mM $MgCl_2$, 20 mM Tris/HCl, pH 7.5, 1 % Triton-X100) supplemented with protease inhibitors (complete EDTA-free cocktail, Roche). After 1 h incubation at 4 °C, samples were clarified by centrifugation at 1000 ***g*** for 2 min at 4 °C. Supernatants were further centrifuged (18 000 ***g***, 10 min, 4 °C) and the bacteria-containing pellets were washed once with cold PBS and resuspended in an appropriate volume of PBS and Laemmli buffer. Protein extracts were then boiled for 5–10 min, and an aliquot of each sample was resolved by 10 % SDS-PAGE for detection of 3 × FLAG-tagged proteins by Western blotting.

Immunodetection analysis. FLAG and 3 × FLAG fusion proteins were immunodetected using mouse anti-FLAG M2-peroxidase

(HRP) mAbs (Sigma). Detection was performed by chemiluminescence (Luminol, Santa Cruz Biotechnology). Blots were scanned, and the intensity of the signals was determined using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/).

RESULTS

Epitope tagging does not affect the invasive ability of serovar Typhimurium

In order to assess whether tagging of SPI-1 genes modifies the wild-type phenotype of serovar Typhimurium strains *in vivo*, we inoculated mice i.p. and i.g., as described above. The survival rate and the colonization of MLNs and spleens were determined at different time points. The results are summarized in Table 1. No differences were observed between the LD_{50} of the wild-type serovar Typhimurium (ATCC 14028) and any of the tagged *Salmonella* strains, regardless of the route of inoculation. Similarly, colonization of internal organs was not significantly different in any of the experimental groups studied. These results demonstrate that the tagging technique does not impair the invasiveness of the strains.

SipA, SopA, SopB, SopD and SopE2 are synthesized and secreted *in vitro* by tagged mutants of serovar Typhimurium

To investigate the capacity of tagged mutants to synthesize and secrete SPI-1 effector proteins, bacterial strains were grown under SPI-1 culture conditions (as described in Methods). SipA, SopA, SopB, SopD and SopE2 were synthesized and secreted by bacteria grown under SPI-1 conditions (Fig. 1). These results indicate that the SPI-1 secretion system is conserved and functioning in the tagged strains. We were unable to detect AvrA protein under any of the growth conditions tested. The whole *avrA* gene plus 300 nt downstream were sequenced in strain SSM 3215, confirming the correct fusion of the ORF with the 3 × FLAG coding sequence. It is worth pointing out here that the *avrA* gene is present in approximately 80 % of *S. enterica* serovars, although few of them synthesize the effector (Streckel *et al.*, 2004).

SipA, SopA, SopB, SopD and SopE2 are synthesized during murine salmonellosis

We investigated the synthesis of these SPI-1 effectors (associated with the initial stages of *Salmonella* infection) during acute lethal infection. Mice were inoculated i.p., a route of infection that does not require invasion of the intestinal epithelium (Galan & Curtiss, 1989), with high doses of tagged strains. In this way, we ensured that sufficient infecting bacteria could be recovered from the MLNs and spleen. We found that serovar Typhimurium recovered from internal organs 12–18 h after infection synthesized all the effector proteins studied (Fig. 2a). SopA was the effector detected in the lowest amount. Similar levels of the effector proteins, quantified as band intensity, were detected in bacteria recovered from MLNs and spleens at this early time point (Fig. 2c, black bars).

To rule out residual expression of the effector proteins from *in vitro* bacterial growth, we investigated longer periods after infection. For this purpose, animals were inoculated i.p. with low doses of bacteria (10^2 c.f.u. per mouse). In this

Table 1. Virulence of the different serovar Typhimurium strains used in this study

BALB/c mice were inoculated i.p. or i.g. with wild-type (ATCC 14028) and tagged (SSM 3213, SSM 3214 and SSM 3215) strains of serovar Typhimurium at the doses indicated in Methods. ND, Not determined.

Strain	LD_{50}*		Colonization (i.p.)		Colonization (i.g.)	
	i.p.	i.g.	log c.f.u. per spleen	log c.f.u. per MLN	log c.f.u. per spleen	log c.f.u. per MLN
ATCC 14028	<10	6×10^4	8.10 ± 0.31†	7.50 ± 0.22†	6.27 ± 0.51‡	ND
SSM 3213	<10	2.5×10^4	8.35 ± 0.37†	7.87 ± 0.81†	5.59 ± 0.46‡	ND
			8.08 ± 0.12§	6.77 ± 0.50§	7.51 ± 0.70‖	6.69 ± 0.54‖
SSM 3214	<10	2×10^4	7.91 ± 0.12†	7.23 ± 0.21†	6.41 ± 0.68‡	ND
			7.10 ± 0.80§	6.85 ± 0.71§	7.07 ± 0.50‖	6.70 ± 0.41‖
SSM 3215	<10	3×10^4	8.27 ± 0.30†	7.58 ± 0.36†	5.93 ± 0.32‡	ND
			7.54 ± 0.30§	6.74 ± 0.51§	7.14 ± 0.34‖	6.72 ± 0.26‖

*LD_{50} was calculated by the method of Reed & Muench (1938). No differences were observed between the LD_{50} of the wild-type and those of any of the tagged serovar Typhimurium strains, regardless the route of inoculation.

†Organ colonization at 12–18 h after i.p. inoculation with 10^7 c.f.u. per mouse.

‡Organ colonization at day 5 after i.g. inoculation with 10^8 c.f.u. per mouse.

§Organ colonization at day 5 after i.p. inoculation with 10^2 c.f.u. per mouse.

‖Organ colonization at day 8 after i.g. inoculation with 10^6 c.f.u. per mouse. Data are presented as mean ± SEM for 10 mice. No differences were observed in the amount of colonizing bacteria recovered from animals inoculated with the wild-type or with any of the tagged strains of serovar Typhimurium, regardless of the route of inoculation.

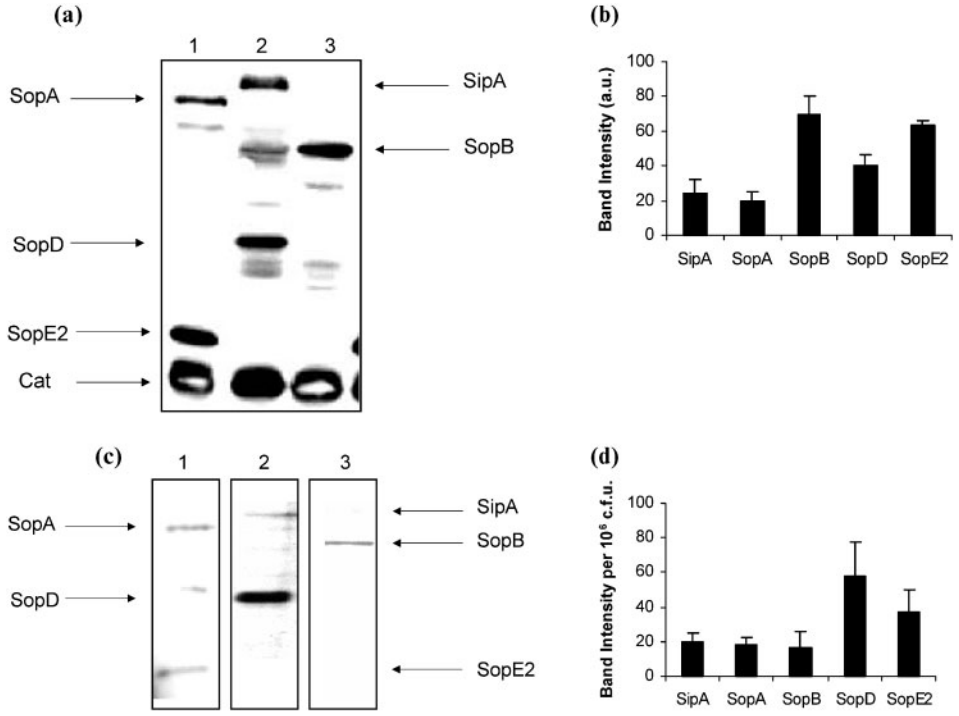

Fig. 1. Analysis of SipA, SopA, SopB, SopD and SopE2 (a) synthesis and (c) secretion *in vitro* under SPI-1 conditions. Bacterial pellets and bacterial supernatants were used to investigate cell-associated proteins and secreted proteins, respectively. Samples were subjected to SDS-PAGE and tagged proteins were detected by anti-FLAG antibodies. Each lane was loaded with material from approximately 10^7 c.f.u. Lanes: 1, SSM 3213; 2, SSM 3214; 3, SSM 3215. (b) Densitometric analysis of effector levels present in the whole bacterial extract. Effector levels were normalized to Cat expression and presented in arbitrary units (a.u.). The Cat protein was used as an internal marker because it is very stable. A constitutively expressed epitope-tagged gene such as *cat* can be used as a positive control or internal reference (Uzzau *et al*., 2001). (d) Densitometric analysis of effector levels present in bacterial supernatants. Protein loading was normalized to 10^6 c.f.u. Data are means ± SD from three independent experiments.

manner, we could recover infecting bacteria from MLNs and spleens several days after i.p. inoculation. We found that *Salmonella* strains isolated from mice after 5 days of infection continued to synthesize SipA, SopB, SopD and SopE2 (Fig. 2b). The amount of SipA, SopB and SopD detected at day 5 was significantly lower than that observed in bacteria recovered 12–18 h post-i.p. inoculation (Fig. 2c, white bars). SopA, the effector protein detected in the lowest amount at 12–18 h (Fig. 2a), was not detected at day 5 (Fig. 2b, c). Conversely, SopE2 levels at day 5 after i.p. infection were significantly higher than those detected at earlier time points. This was observed in bacteria isolated from both MLNs and spleens (Fig. 2c).

We next investigated whether these SPI-1 effector proteins were synthesized during the late stages of *Salmonella* infection acquired by the natural route. For that purpose, animals were inoculated i.g. with 10^6 c.f.u. per mouse of the tagged *Salmonella* strains. In these experiments, mice became moribund by day 8 post-inoculation. As shown in Fig. 3, serovar Typhimurium recovered from spleens and MLNs 8 days post-i.g. inoculation continued to synthesize SipA, SopB, SopD and SopE2. Once again, SopA was not detected.

DISCUSSION

Tagged strains of serovar Typhimurium were used to study *in vitro* synthesis and secretion of SPI-1 effector proteins SipA, SopA, SopB, SopD and SopE2. We also demonstrated *in vivo* that tagged strains are as virulent as the wild-type strain of serovar Typhimurium. Consequently, tagged strains were used to induce murine salmonellosis and to study effector protein synthesis at different stages of infection. Our data show that SPI-1 effectors, SipA, SopB, SopD and SopE2, are synthesized by the bacteria during the final phase of murine salmonellosis. Earlier work performed *in vitro* has demonstrated that some SPI-1 effectors, including SptP and SopB, persist within host cells for several hours after invasion, suggesting that there is continued secretion of these effectors post-invasion (Drecktrah *et al*., 2005; Kubori & Galan, 2003).

In vitro studies can provide attractive models for *in vivo* gene regulation; however, caution must be exercised when attempting to extrapolate relevant *in vivo* signals from environmental cues that regulate virulence genes *in vitro*. There is little direct evidence to identify the conditions that

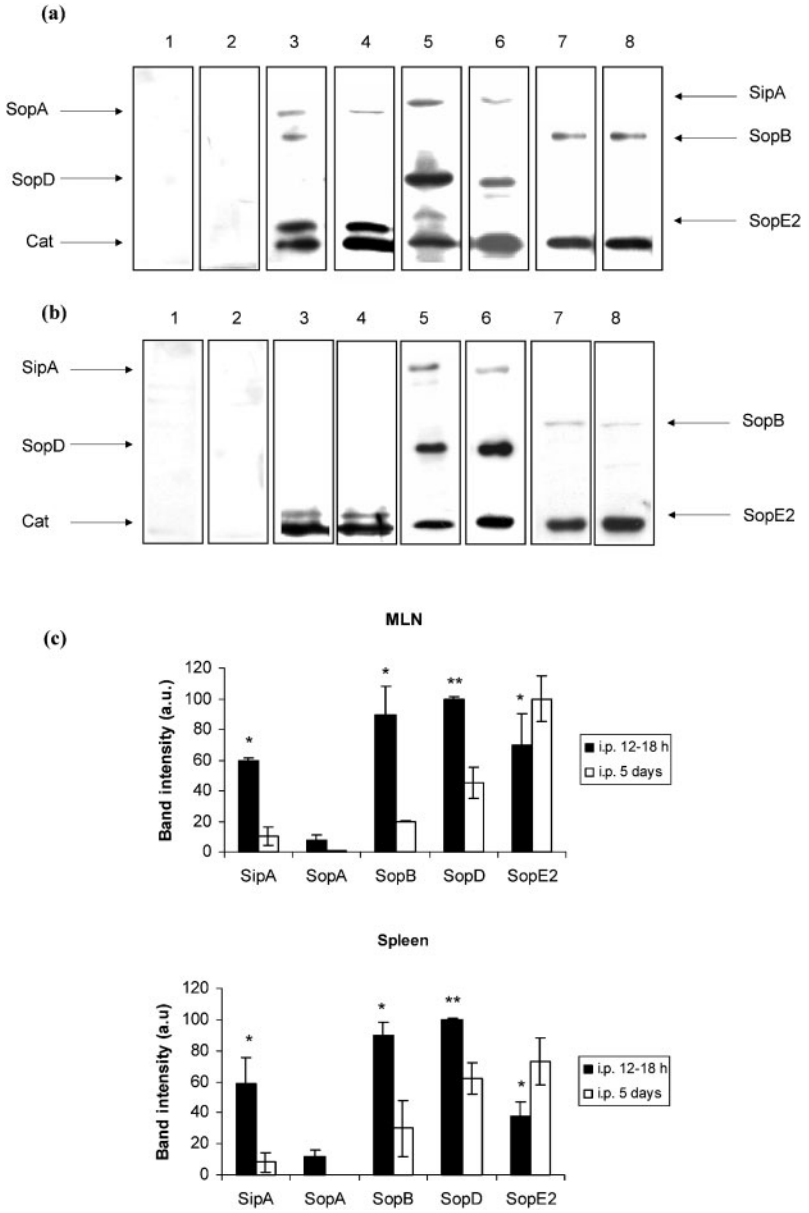

Fig. 2. Detection of SipA, SopA, SopB, SopD and SopE2 in internalized bacteria recovered from spleens and MLNs after i.p. inoculation. Mice were inoculated i.p. with (a) 1×10^7 c.f.u. or (b) 1×10^2 c.f.u. of tagged strains of serovar Typhimurium. Internalized bacteria were recovered from spleens and MLNs at (a) 12–18 h post-inoculation or (b) 5 days after inoculation. Proteins from internalized bacteria were extracted as described in Methods. Lanes: 1 and 2, MLN and spleen extracts, respectively, from control uninfected mice; 3 and 4, MLN and spleen extracts, respectively, from mice inoculated with SSM 3213; 5 and 6, MLN and spleen extracts, respectively, from mice inoculated with SSM 3214; 7 and 8, MLN and spleen extracts, respectively, from mice inoculated with SSM 3215. MLN protein extracts from two mice were pooled. Each lane was loaded with material from approximately 1×10^7 c.f.u. (c) Densitometric analysis of effector levels present in the whole bacterial extract. Effector levels were normalized to Cat expression and presented in arbitrary units (a.u.). Data are means ± SD from three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ (ANOVA).

bacteria encounter at different sites during infection. Signals that regulate virulence genes *in vitro* may not be the same as those modulating these genes *in vivo*. In some cases, *in vitro* cues may operate by an artificial process that bypasses the *in vivo* signalling mechanism (Lucas & Lee, 2000). To our knowledge, this is the first time that the synthesis of SPI-1 effector proteins has been documented in bacteria recovered from infected mice. SPI-1 effector proteins were detected several days after inoculation with low doses of the tagged strains, residual expression from the bacterial inoculum was therefore unlikely. Moreover, results from animals infected i.p. indicate that residual expression from the intestinal invasion stage could also be ruled out.

Most recently, Lawley *et al.* (2006) have shown by a microarray-based negative-selection screen that some SPI-1 genes contribute to long-term systemic infection in *Nramp1*^{r} mice. Therefore, there appears to be considerable functional overlap between SPI-1 and SPI-2 during pathogenesis. Most studies focus on the role played by SPI-1 effectors during the intestinal phase of salmonellosis, overlooking additional functions of SPI-1. The delayed synthesis of SipA, SopA, SopB, SopD and SopE2 demonstrated during murine infection suggests that SPI-1 effectors have potential actions in the post-invasion stages of the disease.

The effector protein genes *sopB*, *sopD* and *sopE2* are located in different regions of the *Salmonella* chromosome, and are present in a wide variety of *Salmonella* lineages, suggesting that these effector proteins may serve central virulence functions (Mirold *et al.*, 2001). Although SopB, SopD and SopE2 are clearly involved in host cell invasion (Raffatellu

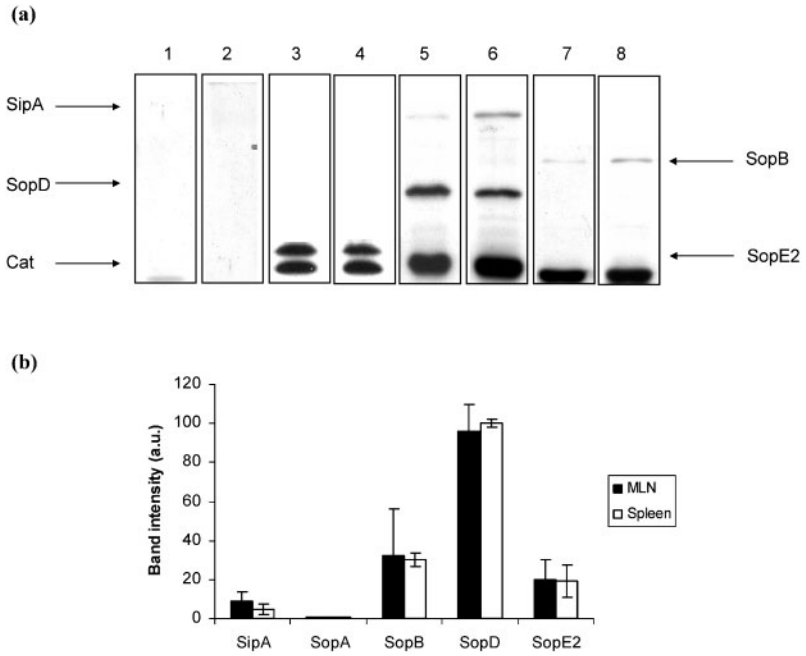

Fig. 3. (a) Detection of SipA, SopA, SopB, SopD and SopE2 in bacteria recovered from spleens and MLNs after infection through the natural route. Mice were inoculated i.g. with 1×10^6 c.f.u. and were euthanized 8 days after inoculation. Internalized bacteria were recovered from spleens and MLNs. Proteins from internalized bacteria were extracted as described in Methods. Lanes: 1 and 2, MLN and spleen extracts, respectively, from control uninfected mice; 3 and 4, MLN and spleen extracts, respectively, from mice inoculated with SSM 3213; 5 and 6, MLN and spleen extracts, respectively, from mice inoculated with SSM 3214; 7 and 8, MLN and spleen extracts, respectively, from mice inoculated with SSM 3215. Pooled MLN protein extracts from two mice were used in the experiments. Each lane was loaded with material from approximately 1×10^7 c.f.u. (b) Densitometric analysis of effector levels present in the whole bacterial extract. Effector levels were normalized to Cat expression and are presented in arbitrary units (a.u.). Data are means ± SD from three independent experiments.

et al., 2005), additional functions of these effectors should not be ruled out.

The role of SopB in the inflammatory response and in fluid secretion in the infected ileum has been discussed earlier (Zhang *et al.*, 2002). Furthermore, SopB could also participate in the development of murine salmonellosis after invasion and during the late stages of the disease. In this regard, it has been reported that SopB specifically stimulates inducible nitric oxide synthase (iNOS) production long after invasion (Drecktrah *et al.*, 2005). Moreover, it has been suggested that SopB participates in the creation of a spacious phagosome in which *Salmonella* spp. resides (Patel & Galan, 2005). Absence of *sopD* leads to a reduction of both fluid secretion and inflammatory responses during infection (Jones *et al.*, 1998; Zhang *et al.*, 2002). *In vitro* experiments using HeLa cells have shown that the expression of *sopD* is maintained at later stages of infection, suggesting that this effector may also play a role in systemic infection of the host (Brumell *et al.*, 2003). Here, we demonstrate that SopD is still present in bacteria infecting MLNs and spleens during late stages of murine salmonellosis. These results are in complete agreement with those reported earlier that show that *sopD* mutants of serovar Typhimurium are significantly reduced in their ability to replicate in the mouse spleen (Jiang *et al.*, 2004).

SopE2, a protein expressed by all strains of *Salmonella*, is introduced into host cells via the SPI-1 TTSS. Like its homologue SopE, SopE2 contributes to the bacterial invasion of epithelial cells (Buchwald *et al.*, 2002; Wallis & Galyov, 2000), and has also been implicated in the pathogenesis of diarrhoea and enteritis in calves (Zhang *et al.*, 2002). It is not clear whether this effect of SopE2 is related to its role in bacterial invasion or to some other function. It is well documented that SopE2 regulates epithelial interleukin (IL)-8 production (Huang *et al.*, 2004), and it is also involved in the upregulation of macrophage iNOS independently of effects on invasion (Cherayil *et al.*, 2000). We detected SopE2 in serovar Typhimurium recovered from MLNs and spleens 8 days after ingestion. This is believed to be the first time that SopE2 has been associated to late stages of *Salmonella* infection. Interesting, although not yet fully understood, is the fact that SopE2 synthesis increases significantly in infected organs by day 5 post-i.p. inoculation. Further studies are required to shed light on the possible role of this effector protein during *Salmonella* systemic infection in mice.

In contrast to other *S. enterica* effector proteins, such as SopB, SopD and SopE2, relatively little is known about SopA. Earlier work has demonstrated a role for SopA in the *Salmonella*-induced movement of polymorphonuclear leukocytes across the intestinal epithelium (Wood *et al.*, 2000) and shown that SopA acts in concert with other TTSS-1-secreted effector proteins (Zhang *et al.*, 2002). More recently, Layton *et al.* (2005) have reported that SopA localizes to mitochondria; the correlation of this fact with

the role of SopA in virulence remains unknown. We detected SopA in serovar Typhimurium infecting MLNs and spleens, although in very small amounts.

AvrA protein from serovar Typhimurium inhibits activation of the key proinflammatory NF-κB transcription factor and augments apoptosis in human epithelial cells (Collier-Hyams *et al.*, 2002). Interestingly, the *avrA* gene is prevalent in the majority of *S. enterica* serovars; however, only a small number of them usually produce the protein (Streckel *et al.*, 2004). Ben-Barak *et al.* (2006) have demonstrated that *avrA* expression is dependent on a specific regulatory function which appears to be differently modulated in the distinct *Salmonella* serovars. In our *in vitro* experiments, the lack of AvrA detection is remarkable, and might be due to non-permissive expression conditions in our standard culture procedure. Indeed, Streckel *et al.* (2004) have shown that some of the non-producer strains begin to produce AvrA in low-pH culture. On the other hand, the failure in the detection of AvrA *in vivo* is in agreement with the report of Lawley *et al.* (2006), who show that the *avrA* gene product lacks of an obvious role during long-term systemic infection; AvrA must be regarded as an effector protein involved in the enteritis pathway.

In summary, we detected *in vivo* the presence of SipA, SopB, SopD and SopE2 in serovar Typhimurium colonizing the MLNs and spleen for several days after inoculation. Further studies are needed to identify SPI-1-dependent functions at late stages of murine salmonellosis and to elucidate the mechanisms that facilitate the successful parasitic lifestyle of serovar Typhimurium.

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