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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INTRODUCTION

*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.

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., intragastrically; iNOS, inducible nitric oxide synthase; i.p., intraperitoneally; 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 pathogenesis 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 5′ ends of these oligonucleotides were complementary to the first 20 nt of the pSUB11 3 × FLAG coding region (GACTAAAGACCATGACGG, forward primers) and to the 20 nt of the pSUB11 priming site 2 (CATATGAAATATCCTCCTTAG, 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 (14000 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. LD₅₀ was calculated by the method of Reed & Muench (1938).

**Organ colonization.** Groups of 10 mice were inoculated i.p. with 10⁵ or 10⁷ 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 10⁵ or 10⁶ 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 (10⁵ and 10⁶ c.f.u. per mouse) of tagged serovar Typhimurium strains. A different group of animals were inoculated i.g. with 10⁶ 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 10⁵ c.f.u. per mouse were euthanized at 12–18 h post-infection. Mice receiving 10⁵ c.f.u. i.p. were euthanized at day 5 post-inoculation. On the other hand, mice inoculated i.g. with 10⁶ 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 MgCl₂, 20 mM Tris/HCl, pH 7.5, 1 % Triton-X100) supplemented with protease inhibitors (complete EDTA-freet 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.
SipA, SopA, SopB, SopD and SopE2 were synthesized and secreted in vivo 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 (10² c.f.u. per mouse). In this

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>LD₅₀*</th>
<th>Colonization (i.p.)</th>
<th>Colonization (i.g.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.p.</td>
<td>log c.f.u. per spleen</td>
<td>log c.f.u. per MLN</td>
</tr>
<tr>
<td>ATCC 14028</td>
<td>&lt; 10</td>
<td>8.10 ± 0.31 †</td>
<td>7.50 ± 0.22 †</td>
</tr>
<tr>
<td>SSM 3213</td>
<td>&lt; 10</td>
<td>8.35 ± 0.37 †</td>
<td>7.87 ± 0.81 †</td>
</tr>
<tr>
<td>SSM 3214</td>
<td>&lt; 10</td>
<td>8.08 ± 0.12 ‡</td>
<td>6.77 ± 0.50 §</td>
</tr>
<tr>
<td>SSM 3215</td>
<td>&lt; 10</td>
<td>7.91 ± 0.12 ‡</td>
<td>7.23 ± 0.21 †</td>
</tr>
</tbody>
</table>

*LD₅₀ was calculated by the method of Reed & Muench (1938). No differences were observed between the LD₅₀ 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 10⁷ c.f.u. per mouse.
‡Organ colonization at day 5 after i.p. inoculation with 10⁶ c.f.u. per mouse.
§Organ colonization at day 5 after i.p. inoculation with 10⁵ 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 the route of inoculation.
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, SopA, 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 StpP 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
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<sup>−/−</sup> mice. Therefore, there appears to be considerable
functional overlap between SPI-1 and SPI-2 during patho-
genesis. Most studies focus on the role played by SPI-1
effectors during the intestinal phase of salmonellosis, over-
looking 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. 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<sup>7</sup> c.f.u. or (b) 1 × 10<sup>2</sup> 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, respec-
tively, 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 pro-
tein extracts from two mice were pooled.
Each lane was loaded with material from
approximately 1 × 10<sup>7</sup> 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).
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 \textit{Salmonella} spp. resides (Patel & Galan, 2005). Absence of \textit{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 \textit{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 \textit{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 \textit{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 \textit{Salmonella} infection. Interest, 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 \textit{Salmonella} systemic infection in mice.

In contrast to other \textit{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 \textit{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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