AvrA effector protein of *Salmonella enterica* serovar Enteritidis is expressed and translocated in mesenteric lymph nodes at late stages of infection in mice

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Salmonellosis is a major health problem worldwide. *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) has been a primary cause of *Salmonella* outbreaks in many countries. AvrA is an SPI-1 effector protein involved in the enteritis pathway, with critical roles in inhibiting inflammation and apoptosis. In this work, we constructed an AvrA-FLAG-tagged strain of *S.* Enteritidis to analyse the expression profile of AvrA *in vitro*, in cell culture and *in vivo*. AvrA expression and secretion were observed *in vitro* under culture conditions that mimicked intestinal and intracellular environments. In agreement, bacteria isolated from infected cell monolayers expressed and translocated AvrA for at least 24 h post-inoculation. For *in vivo* experiments, BALB/c mice were inoculated by the natural route of infection with the AvrA-FLAG strain. Infecting bacteria and infected cells were recovered from mesenteric lymph nodes (MLN). Our results showed that AvrA continues to be synthesized *in vivo* up to day 8 post-inoculation. Moreover, AvrA translocation was detected in the cytosol of cells isolated from MLN 8 days after infection. Interestingly, we observed that AvrA is secreted by both type three secretion system (T3SS)-1 and T3SS-2. In summary, these findings indicate that AvrA expression is not constrained to the initial host–bacteria encounter in the intestinal environment as defined previously. The AvrA effector may participate also in systemic *S.* Enteritidis infection.

INTRODUCTION

Salmonellosis is considered one of the most widespread foodborne diseases in the world (Bollaerts *et al.*, 2008), and *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) is the main serotype responsible for human infections (Oliveira *et al.*, 2006; Moore *et al.*, 2007; Pang *et al.*, 2007). In Argentina, for instance, the proportion of salmonellosis cases attributed to this pathogen showed a 275-fold increase in the last decades (Hogue *et al.*, 1997; Rodrigue *et al.*, 1990; Morales & McDowell, 1999). Infection with *S. enterica* occurs mainly through the consumption of contaminated food; in particular, chicken eggs are considered a major source of infection (Perales & Audicana, 1988; Bäumler *et al.*, 2000; Hald *et al.*, 2004). The estimated number of human infections is greater than 93.8 million cases, with 155 000 deaths per year worldwide (Boyle *et al.*, 2007; Majowicz *et al.*, 2010; Hendriksen *et al.*, 2011). *S.* Enteritidis infection is generally confined to the intestinal mucosa and is usually self-limited, though systemic dissemination may occur in immunocompromised persons. Moreover, it is important to note that in developed countries up to 5 % of non-typhoidal *Salmonella* cases may be an invasive, extra-intestinal disease leading to bacteraemia and focal systemic infections.

Salmonella use a molecular 'needle', referred to as a type three secretion system (T3SS), to inject bacterial effector proteins into host cells. Stimulation of inflammation by effectors is crucial for *Salmonella* growth within the intestine (Stecher *et al.*, 2007). Effectors, such as SipA, SopE and SopB, are known to activate inflammation in host cells (Galyov *et al.*, 1997; Ehrbar *et al.*, 2003; Hapfelmeier *et al.*, 2004; Bruno *et al.*, 2009; Broberg & Orth, 2010). Uncontrolled inflammation is harmful to the host and eventually damages the niche occupied by *Salmonella* during infection. However, *Salmonella* secreted SspH 1, SptP and AvrA reverse the activation of signalling pathways induced by inflammatory *Salmonella* effectors (Ye *et al.*, 2007; McGhie *et al.*, 2009; Sun, 2009; Wu *et al.*, 2010).

Abbreviations: MLN, mesenteric lymph nodes; qRT-PCR, quantitative reverse transcriptase PCR; T3SS, type three secretion system.

AvrA is a multiple-function protein that plays a critical role in inhibiting inflammation, regulating epithelial apoptosis and enhancing proliferation during bacterial infections in cell culture models and acutely infected mice (Ye *et al.*, 2007; Jones *et al.*, 2008; Liao *et al.*, 2008; Du & Galán, 2009; Wu *et al.*, 2012). In this regard, it has been suggested that AvrA is primarily involved in the enteritis pathway (Lawley *et al.*, 2006).

Most *S. enterica* strains (approximately 80 %) contain the *avrA* gene; only some serovars such as *S. enterica* serovar Typhi (*S.* Typhi) and *S. enterica* serovar Paratyphi (*S.* Paratyphi) do not possess it. Some authors linked the absence of AvrA to the ability of these serovars to evade epithelial defences resulting in severe systemic disease (within macrophages) (Hardt & Galán, 1997; Prager *et al.*, 2000, 2003).

The modulation of gene expression in SPI-1 is remarkably complex and needs further characterization (Waterman & Holden, 2003; Ellermeier & Slauch, 2007). For example, in contrast to the current model of SPI-mediated pathogenesis, SPI-1 proteins SipA, SopA, SopB, SopD and SopE2 were found to be expressed by *Salmonella* in infected animals at the late stages of infection (Giacomodonato *et al.*, 2007). These results suggest that in addition to its generally recognized function in invasion, the SPI-1 factors may play an important role during systemic infection. Extensive studies have been carried out to investigate the expression of SPI-1 under different conditions *in vitro* (Löber *et al.*, 2006; Ellermeier & Slauch, 2007); however, little is known about the expression of *Salmonella* effectors *in vivo*, especially during the established phase of infection. In this study, we used an *S.* Enteritidis strain to study the expression profile of AvrA during late stages of murine salmonellosis. We demonstrated that AvrA effector can be expressed and translocated during early and late stages of systemic infection.

METHODS

Bacterial strains. This work was carried out using strains of *S.* Enteritidis derived from strain SS218 (an isolate from poultry collected from an Argentine farm) and tagged with the 8 aa FLAG epitope tag peptide. *S.* Enteritidis strains SE1702 (*avrA*::3×FLAG *cat*::FLAG), SE1703 (*sipA*::3×FLAG *cat*::FLAG) and SE1704 (*sseJ*::3×FLAG *cat*::FLAG) were obtained using the method described by Uzzau *et al.* (2001). 3×FLAG epitope tails were added to the ends of the *avrA*, *sipA* and *sseJ* genes. The 3×FLAG epitope is a sequence of three tandem FLAG epitopes (22 aa). A pair of primers was designed to amplify a 3×FLAG and Km^R coding sequence 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 (CATATG-AATATCCTCCTTAG, 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). Mutants *invG*::*aphT* (Km^R) *avrA*::3×FLAG, *invG*:: *aphT* (Km^R) *sseJ*::3×FLAG, *invG*::*aphT* (Km^R) *sipA*::3×FLAG,

ssaK::*aphT* (Km^R) *avrA*::3×FLAG, *ssaK*::*aphT* (Km^R) *sseJ*::3× FLAG and *ssaK*::*aphT* (Km^R) *sipA*::3×FLAG were constructed by phage P22-mediated transduction from *invG*::*aphT* (Km^R) and *ssaK*::*aphT* (Km^R) [previously obtained in our laboratory as described by Datsenko & Wanner (2000)] strains of *S.* Enteritidis to strains SE1702 (*avrA*::3×FLAG *cat*::FLAG), SE1703 (*sipA*::3×FLAG *cat*::FLAG) and SE1704 (*sseJ*::3×FLAG *cat*::FLAG) mutants. Gene deletion was verified by PCR.

Culture conditions. For *in vitro* studies, bacteria were grown to exponential phase under different culture conditions. To mimic the intestinal environment (Miki *et al.*, 2004) 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 % casamino acids at 37 °C with aeration at pH 6 (Miki *et al.*, 2004).

For *in vivo* studies, bacterial inocula used to infect cells or animals were prepared by growing the tagged strains overnight under SPI-1 non-inducing conditions (LB at 28 °C) as previously described Giacomodonato *et al.* (2009). In this way, the residual expression of AvrA from *in vitro* bacterial growth was ruled out.

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.

Expression and secretion of AvrA *in vitro*. For the isolation of cell-associated proteins, 1.5 ml bacterial culture was centrifuged and suspended in 100 μl of H_2O and immediately mixed with 100 μl of Laemmli buffer. To isolate the 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 PBS and Laemmli buffer. Four independent extractions for each sample were added together to minimize differences in protein recovery from sample to sample. Proteins (cell-associated and secreted proteins) were then boiled for 5–10 min, and an aliquot of each sample was separated by 10 % SDS-PAGE (Raffatellu *et al.*, 2005). Finally, effector proteins were immunodetected as described below.

Expression and secretion of AvrA in infected eukaryotic cells. Human laryngeal epithelial (HEp-2) cells (ATCC CCL-23), were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % FBS. Infected monolayers (m.o.i. 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 gentamicin ml^{-1} for 1 h to remove extracellular bacteria, and finally in fresh tissue culture medium containing 10 μg gentamicin ml^{-1} for the remainder of the experiment. At 20 min and 24 h post-infection monolayers were washed twice with cold HBSS and lysed with 1.0 ml HBSS containing 0.1 % Triton X-100 and 1 mM PMSF as described by Kubori & Galán (2003). This procedure lyses the infected cells, but does not affect the integrity of the bacterial membrane (Collazo & Galán, 1997). 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 suspended in PBS and Laemmli buffer. The insoluble fraction was washed once

with cold PBS and resuspended in an appropriate volume of PBS and Laemmli buffer. The protein extracts were boiled for 5–10 min, and resolved by 10 % SDS-PAGE. Finally, effector proteins were immunodetected as described below.

Subcellular fractionation of HEp-2 cells. HEp-2 cells were infected, washed, and harvested at 20 min and 24 h post-infection as described above. Homogenization buffer [20 mM HEPES (pH 7.2), 200 mM sucrose, 0.5 mM EGTA, 1 mM PMSF, 1× protease inhibitor cocktail (Roche)] was used. Cells were disrupted by mechanical lysis with a dounce homogenizer, and lysates were centrifuged twice at 20 000 *g* at 4 °C for 30 min to remove bacteria and debris. The resulting supernatant was centrifuged at 100 000 *g* at 4 °C to separate the membrane (pellet) from the cytoplasmic (supernatant) fractions. The pellet fraction was resuspended in an appropriate volume of PBS and Laemmli buffer. The soluble fraction was filtered through a 0.45 µm pore-size filter, 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 protein extracts were boiled for 5–10 min and resolved by 10 % SDS-PAGE. Finally, effector proteins were immunodetected as described below.

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 qRT-PCR. Complementary cDNA was synthesized using random hexamer primers (Invitrogen), deoxynucleoside triphosphates and Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Invitrogen). Relative quantitative real-time PCR was performed with an appropriate primer set, cDNAs and Mezcla Real (Biodynamics), which contained nucleotides, polymerase, reaction buffer and Green dye, using a Rotor-Gene 6000 real-time PCR machine (Corbett Research). The primer sequences were 16S rRNA forward 5′-GCCGCAAGGTTAAAACTCAA-3′ and reverse 5′-AAGGCACCAA-TCCATCTCTG-3′, *avrA* forward 5′-TGTTGAGCGTCTGGAAAGT-G-3′ and reverse 5′-CAGATTCAACGCCTTCCATT-3′, *sseJ* forward 5′-GCCGATGCATTTAAGGTGAT-3′ and reverse 5′-TTTTCTGTC-CACCGCTATCC-3′, and *sipA* forward 5′-CGTGACCACCTTTCC-ATCTT-3′ and reverse 5′-CCATTCGACTAACAGCAGCA-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. All samples were analysed in the same run for 16S expression for normalization. The number of copies of each sample transcript was determined with the aid of the Rotor-Gene 6000 Series Software Version 1.7. Quantification of gene expression was calculated using the comparative threshold cycle (C_t) method, normalized to the 16S control and efficiency of the reverse transcriptase reaction (relative quantity $2^{-\Delta\Delta C_t}$). The replicates were then averaged and fold induction was determined (Livak & Schmittgen, 2001).

Mice. Six to eight-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, Buenos Aires, Argentina.

Expression and translocation of AvrA *in vivo*. Mice were inoculated intragastrically with 10^6 c.f.u. per mouse of the tagged

Salmonella strains and were euthanized 8 days post-inoculation. Mesenteric lymph nodes (MLN) were aseptically removed and incubated for 20 min in 3 ml HBSS containing 100 mg gentamicin ml^{-1}, followed by three washes in 10 ml HBSS without antibiotic, before single cell suspensions were prepared using an iron mesh sieve. Then, the isolated cells were processed as described above to analyse the expression and translocation of AvrA in HEp-2 cells.

Western blot analysis. The gels were blotted onto a Hybond-P membrane (GE Health-care). The 3×FLAG fusion proteins were immunodetected using mouse anti-FLAG M2-horseradish peroxidase mAbs (Sigma). The reacting bands were detected by enhanced chemiluminescence (Luminol; Santa Cruz Biotechnology) in an Image Quant 300 cabinet (GE Healthcare) following the manufacturer's instructions.

Statistical analysis. Data are represented as the mean±SD from triplicates. One-way ANOVA was employed to assess the significance of the differences between the mean values of experimental groups using GraphPad Prism software version 5.

RESULTS

AvrA is synthesized and secreted *in vitro* under different culture conditions

Upon ingestion, *Salmonella* encounters different environments such as high osmolarity, basic pH and hypoxia in the intestine, and low nutrient levels and acidity inside the eukaryotic cell. To investigate the capacity of a tagged *Salmonella* strain to synthesize and secrete AvrA, bacteria were grown under different conditions resembling early and late stages of *Salmonella* infection as described in Methods. Results are shown in Fig. 1(a). We observed that AvrA was synthesized and secreted at the same level by bacteria grown under both culture conditions, whereas SipA expression and secretion were evident only under conditions that mimicked the intestinal milieu (Fig. 1a). This result was corroborated by qRT-PCR. As shown in Fig. 1(b), no differences were observed in the transcript levels of *avrA* of bacteria cultured under intestinal or intracellular conditions. Taken together these results suggest that AvrA can be synthesized and secreted not only by *Salmonella* located in the intestinal environment but also by intracellular bacteria.

Synthesis and secretion of AvrA by intracellular bacteria

We investigated the synthesis and secretion of AvrA in bacteria infecting HEp-2 cells. Confluent HEp-2 cells were inoculated with an AvrA-FLAG-tagged *Salmonella* strain. At 20 min and 24 h post-infection cells were mechanically disrupted and centrifuged at low speed to separate cell lysates into a soluble fraction (containing bacterial proteins that have been translocated into the host-cell cytosol) and an insoluble fraction (containing internalized bacteria). Western blot analysis of the insoluble fraction revealed that *S.* Enteritidis expressed similar levels of AvrA at both time points analysed (Fig. 2a). In the same way, immunoblotting
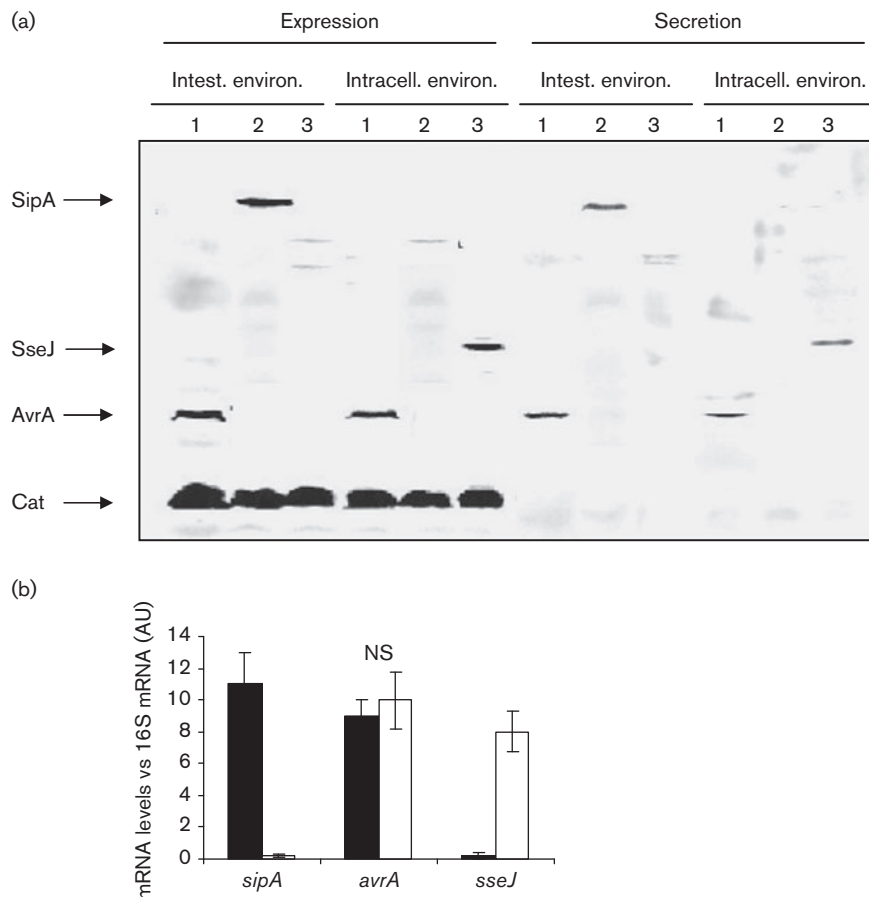
(a)

(b)

Fig. 1. (a) Analysis of AvrA expression and secretion *in vitro* by Western blot. AvrA-tagged, SipA-tagged and SseJ-tagged strains of *S.* Enteritidis were grown under different culture conditions that mimicked the intestinal environment and the intracellular environment. Expression and secretion 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 c.f.u. bacteria. Lanes: 1, SE1702 (*avrA*::3×FLAG *cat*::FLAG); 2, SE1703 (*sipA*::3×FLAG *cat*::FLAG); 3, SE1704 (*sseJ*::3×FLAG *cat*::FLAG). Data are representative from three independent experiments. (b) Analysis of *avrA* expression under different culture conditions by qRT-PCR. The relative mRNA amount was determined by real-time qRT-PCR and related to 16S mRNA levels. Values are means ± SD of three independent mRNA extractions performed in triplicate. AU, Arbitrary unit; NS, no significant difference between the two culture conditions (ANOVA). Black bars, intestinal environment; white bars, intracellular environment.

analysis of the soluble fraction showed that AvrA is translocated at early and late stages of *Salmonella* infection (Fig. 2a). SipA was expressed at 20 min post-infection and its translocation was observed only at early stages of infection. To analyse AvrA subcellular localization, we mechanically fractionated HEp-2 cells at 20 min and 24 h post-infection to separate the pellet containing plasma membranes from the cytoplasm. No AvrA was observed in the plasma membranes, but it was clearly present in the soluble fraction containing host cytoplasm at early and late stages of *Salmonella* infection Fig. 2(b). These results show the persistence of AvrA in the host cytosol after initial translocation. To expand upon these results we used qRT-PCR to directly measure *avrA* mRNA levels in intracellular bacteria, as described in Methods. As predicted from other

studies (Kerrinnes *et al.*, 2009), we found that *avrA* mRNA levels were detectable for at least 10 h post-infection overlapping with the maximal induction of *sseJ* (Fig. 3). Again, we observed that transcript levels of *avrA* mRNA were similar at early (20 min) and late (10 h) stages of infection (Fig. 3).

AvrA is synthesized and translocated during murine salmonellosis

Next we determined the length of time that AvrA is synthesized in infecting bacteria and for how long it is translocated into the cytosol of infected cells. To this purpose mice were infected by the natural route with the tagged strain of *Salmonella*. Animals received a high dose of
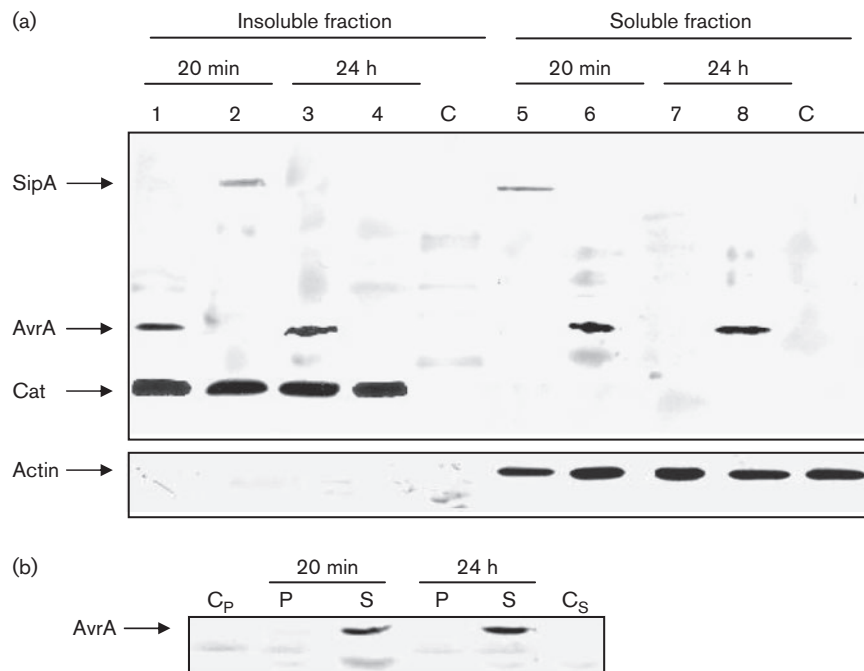
(a)

(b)

Fig. 2. Analysis of AvrA expression, translocation (a) and localization (b) in HEp-2 cells by Western blot. Epithelial cells were infected with SipA-tagged and AvrA-tagged strains of *S.* Enteritidis for 20 min and 24 h. (a) Post-infection cells were processed as indicated in Methods to obtain an insoluble fraction containing intact bacteria and a soluble fraction containing translocated effectors. Both fractions were analysed by immunoblotting using anti-FLAG antibodies. Lanes: 1, 3, 6 and 8, SE1702 (*avrA*::3×FLAG *cat*::FLAG); 2, 4, 5 and 7, SE1703 (*sipA*::3×FLAG *cat*::FLAG); C, control uninfected cell cultures. As a control for the host-cell cytosolic fraction some blots were reprobed with polyclonal antibodies to actin. (b) Subcellular localization of AvrA. Epithelial cells were infected with an AvrA-tagged strain of *S.* Enteritidis for 20 min and 24 h, and were fractionated into membrane insoluble (P) or soluble fractions (S) as described in Methods. Both fractions were analysed by immunoblotting using anti-FLAG antibodies. Cp and Cs, negative controls (pellet and soluble fraction from uninfected cell cultures, respectively). Each lane was loaded with material from approximately 10^6 c.f.u. bacteria. Data are representative from three independent experiments.

Salmonella that, in turn, yielded a sufficient number of infecting bacteria (to be recovered for AvrA expression analysis), and also provided an adequate amount of infected cells (to be isolated to determine AvrA translocation). Previously, we demonstrated that 24 h after inoculation, SopB continues to be expressed by wild-type infecting bacteria recovered from MLN (Giacomodonato *et al.*, 2011). As shown in Fig. 4, the expression of AvrA was still evident on day 8 following intragastric inoculation. However, at day 8 after infection SipA was weakly expressed (Fig. 4, lane 1, insoluble fraction), but was not translocated since it was not detected in the soluble fraction. In contrast, as shown in Fig. 4 (lane 2, insoluble and soluble fractions) translocation of AvrA during murine salmonellosis was evident for at least 8 days, coincident with the AvrA expression (Fig. 4).

To find out whether – under intracellular conditions – AvrA is translocated via T3SS-1, T3SS-2 or independently of both T3SSs, we analysed its secretion in three genetic backgrounds, *invG, ssaK and invG ssaK*. These mutations render the bacteria defective in secretion through T3SS-1, T3SS-2 or both T3SS-1/2, respectively (Kubori *et al.*, 2000;

Geddes *et al.*, 2005; Niemann *et al.*, 2011). *In vitro* analysis by Western blot confirmed that AvrA is secreted through T3SS-1 under intestinal conditions and through T3SS-2 under intracellular conditions; in the absence of both T3SSs the effector is not translocated (Fig. 5). This fact demonstrates that translocation of AvrA under intracellular conditions requires a functional T3SS-2.

DISCUSSION

The virulence-associated effector protein AvrA of *S. enterica*, which interferes with the first line of immune response of the target organism (Collier-Hyams *et al.*, 2002), is an important partner in the virulence phenotype of this pathogen (Streckel *et al.*, 2004; Ben-Barak *et al.*, 2006). Our study reveals that *S.* Enteritidis express AvrA at early and late stages of infection in the murine model.

Lawley *et al.* (2006) suggested that the *avrA* gene product lacks an obvious role during long-term systemic infection, and that AvrA must be regarded as an effector protein involved in the enteritis pathway. It has been demonstrated
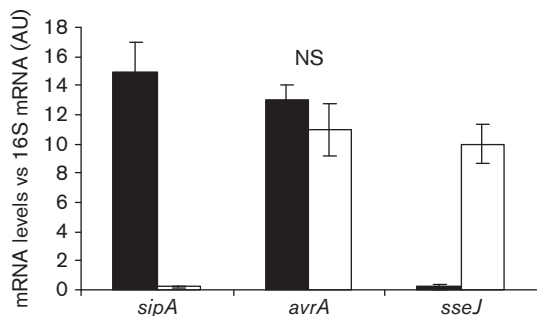
Fig. 3. Analysis of *avrA* expression in HEp-2 cells by qRT-PCR. Epithelial cells were infected with a wt strain of *S.* Enteritidis for 20 min (black bars) or 10 h (white bars). Post-infection cells were processed as indicated in Methods to obtain total mRNA. *sipA*, *avrA* and *sseJ* mRNA levels from bacteria colonizing HEp-2 cells were measured by qRT-PCR at the indicated times. *sipA* and *sseJ* were used as controls for SPI-1 and SPI-2 expression, respectively. The mRNA amount was related to 16S mRNA levels. Values are means ± SD of three independent mRNA extractions performed in triplicate. AU, Arbitrary unit; NS, no significant difference between the two times post-infection (ANOVA).
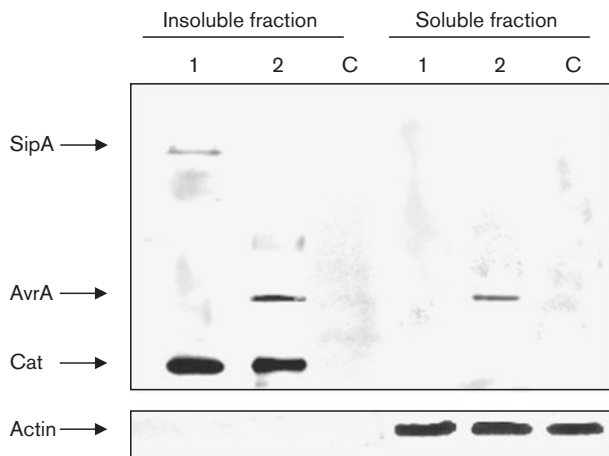

Fig. 4. Analysis of AvrA expression (insoluble fraction) and translocation (soluble fraction) in MLN by Western blot. Groups of mice were inoculated intragastrically with 10^6 c.f.u. of the AvrA- and SipA-tagged strains of *S.* Enteritidis, and euthanized at day 8 post-infection. MLN were removed and processed as indicated in Methods to obtain an insoluble fraction containing intact bacteria and a soluble fraction containing translocated effectors. Both fractions were analysed by immunoblotting using anti-FLAG antibodies. Lanes: 1, MLN protein extracts from mice inoculated with SE1703 (*sipA*::3×FLAG *cat*::FLAG); 2, MLN protein extracts from mice inoculated with SE1702 (*avrA*::3×FLAG *cat*::FLAG); C, MLN protein extracts from control uninfected mice. Pooled MLN protein extracts from two mice were used in the experiments. Each lane was loaded with material from approximately 10^6 c.f.u. bacteria. As a control for the host-cell cytosolic fraction some blots were reprobed with polyclonal antibodies to actin. Data are representative from three independent experiments.

that some SPI-1 effectors persist in host cells for several hours – or even days – after initial translocation, and play roles during systemic infection (Marcus *et al.*, 2002; Kubori & Galán, 2003; Drecktrah *et al.*, 2005; Giacomodonato *et al.*, 2007, 2009, 2011; Patel *et al.*, 2009; Gong *et al.*, 2010). Likewise, our results provide direct evidence that AvrA is expressed in both early and late stages of *Salmonella* infection.

Furthermore, this study demonstrates that SipA and AvrA are differentially expressed in *Salmonella* colonizing the epithelial cells and MLN. These results further suggest that different SPI-1 proteins are expressed by *S.* Enteritidis in specific tissues. Differential expression of these effector proteins may be important for bacterial pathogenesis in certain organs, such as gastroenteritis in the intestinal epithelium and typhoid fever during systemic infection in the MLN. In concurrence with our findings, Lu *et al.* (2012) demonstrated that AvrA has long-term effects in the *Salmonella*-infected intestine emphasizing the importance of AvrA in chronic bacterial infection.

Culture cell experiments allowed us to analyse the expression and translocation of *Salmonella* AvrA effector protein *in vivo*, at early and late time points. We found that AvrA is expressed and translocated within 20 min and 24 h following cell invasion. We observed that upon translocation from the bacteria and 24 h post-infection, AvrA localizes to the cytoplasm of infected cells. Shortly after its translocation AvrA is rapidly phosphorylated by the ERK pathway and in this way remains within the cell for an extended period of time (Du & Galán, 2009). Experiments performed *in vitro* and in cultured cells strongly suggest that persistence of AvrA during infection is due – in part – to *de novo* synthesis. These results are consistent with those obtained by Kerrinnes *et al.* (2009). They found that *avrA* transcription takes place constitutively in all *S. enterica* strains, but that *avrA* translation is regulated positively in a post-transcriptional manner by CsrA/CsrB of the Csr regulatory system.

Interestingly, we demonstrated that AvrA secretion in *S.* Enteritidis is dependent on both T3SS-1 and T3SS-2 systems. The fact that AvrA is translocated by both syringes supports the speculation that this effector protein participates in both intestinal and systemic phases of *Salmonella* infection. The involvement of AvrA in late phases of murine infection has been recently investigated using *Salmonella* mutants (Wu *et al.*, 2012). The authors observed that the loss of AvrA increases apoptosis in epithelial cells and macrophages during *Salmonella* infection. Moreover, the lack of AvrA results in a simultaneous loss of intracellular *Salmonella* carriage and an increase in microbial parenchymal burden in systemic lymphoid tissues, increasing mortality during late stages of infection. This finding that *S.* Enteritidis AvrA is secreted by both T3SSs is consistent with studies performed earlier in *S. enterica* serovar Typhimurium (*S.* Typhimurium) (Brumell *et al.*, 2003; Geddes *et al.*, 2005). In this regard, *S.* Typhimurium AvrA,
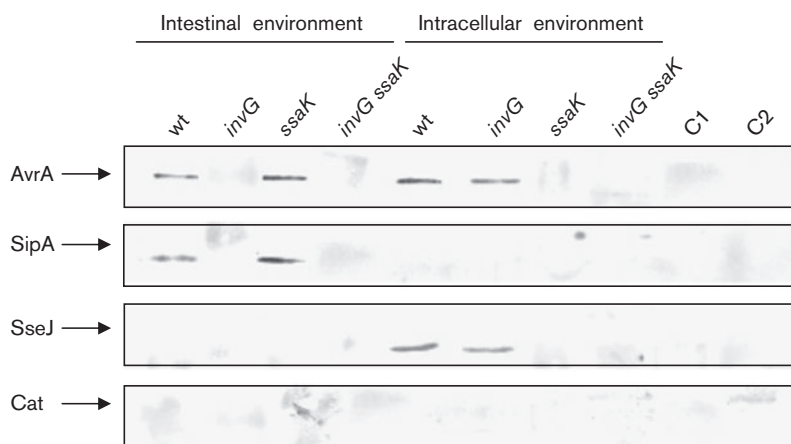
Fig. 5. Analysis of T3SS-1- and T3SS-2-dependent secretion of AvrA *in vitro* by Western blot. wt, *invG* (T3SS-1 defective), *ssaK* (TTSS-2 defective) and *invG ssaK* (lacking both T3SSs) strains of *S.* Enteritidis containing AvrA, SipA and SseJ tagged with FLAG were grown under culture conditions that mimicked the intestinal niche and the intracellular environment as indicated in Methods. The secretion of the effectors was investigated in bacterial supernatants. SipA and SseJ were used as controls for T3SS-1- and TTSS-2-dependent secretion, respectively. C1 and C2, negative controls (culture medium used for SPI-1 and SPI-2 inducing conditions, 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 c.f.u. bacteria. Data are representative from three independent experiments.

SptP, SlrP, SteA, SteB and SopD, originally identified as SPI-1-secreted proteins, are now considered as dual effectors.

We demonstrated as well that AvrA is expressed in *S.* Enteritidis recovered from MLN 8 days after infection. This result is in complete concurrence with those revealed by *in vitro* and cell culture experiments. Furthermore, we were able to demonstrate that AvrA is translocated for at least 8 days following intragastric infection. It is important to note that similar to AvrA, SopB has an anti-apoptotic effect through its phosphatase activity (Knodler *et al.*, 2005). We have previously shown that in *S.* Typhimurium SopB is also a dual effector. In a murine model of infection, the expression of SopB was detected for at least 5 days after a maximum expression on day 1 post-inoculation (Giacomodonato *et al.*, 2011). Here we show that, unlike SopB, the expression of AvrA during *Salmonella* infection is sustained for at least 8 days. Altogether, our results clearly show that these two dual effectors are differentially expressed *in vivo* by infecting *S.* Enteritidis. It is interesting to note that despite the anti-inflammatory effect of AvrA, its presence during late stages of *S.* Enteritidis infection prevented neither the systemic infection nor the death of infected mice.

In conclusion, our results indicate that the expression of AvrA and its function during murine infection are not constrained to the intestinal environment as previously defined. The *avrA* gene is prevalent in the majority of *S. enterica* serovars (80 %); however, only a small number of them usually express the protein. Significantly, *S.* Typhi and *S.* Paratyphi are strains that evade epithelial defences and result in severe systemic disease. These strains invariably lack an *avrA* allele (Prager *et al.*, 2000). However, the expression and translocation of AvrA in *S.* Enteritidis did not impede the development of a systemic

infection in the murine model. Our findings do not rule out a role for AvrA in avirulence (or virulence) as detection of this putative function will require the identification of a host in which this protein may be able to exert such an effect. Our work stresses the significance of analysing protein expression and translocation *in vivo* in the context of infection. Further investigation on kinetics, lifespan and function of SPI-1 effectors *in vivo* would provide a significant approach for analysing the function of these proteins in *Salmonella* pathogenesis, and would confirm the complementary behaviour of SPI-1 and SPI-2 effectors functions.

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