

A *Salmonella* Typhimurium *ssrA* deletion mutant is not impaired in the colonization of pigs.

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The virulence genes located on the *Salmonella* Pathogenicity Island 2 (SPI-2) are indispensable for the induction of systemic disease and persistence in BALB/c mice after both oral and intraperitoneal inoculation. The role of this pathogenicity island in the pathogenesis of *Salmonella* Typhimurium infections in pigs is not documented. In the present study, the interactions of a porcine field strain of *Salmonella* Typhimurium and a non-polar isogenic SPI-2 (Δ *ssrA*) deletion mutant were compared in both *in vitro* and *in vivo* models. The *ssrA* mutant strain displayed decreased SPI-2 expression levels *in vitro* and was attenuated in a mouse model after oral inoculation. In contrast, 3 different tests could not detect significant differences in SPI-1 expression between the *ssrA* mutant strain and the wild type strain. As a control, significant differences were seen between the wild type strain and a SPI-1 (Δ *hilA*) mutant strain. In a single infection *in vivo* model, 2 groups of 10 piglets were inoculated with the wild type strain and the *ssrA* mutant strain, respectively. The course of infection was very similar in both groups. At day 5 and day 28 post inoculation, the animals of both groups were infected to the same extent in the gut and gut-associated lymphoid tissue, as well as in the internal organs. These results suggest that SPI-2 of *Salmonella* Typhimurium may not contribute to the colonization of pigs to the same extent as it contributes to the colonization of BALB/c mice.

INTRODUCTION

Salmonella Typhimurium is the most frequently isolated serotype from pigs and pork (Botteldoorn et al., 2003). Infected pigs can shed *Salmonella* for at least 28 weeks (Wood et al., 1989). These carrier pigs are a major reservoir of *Salmonella* Typhimurium and pose an important threat to animal and human health (Poppe et al., 1998). The mechanism of this carrier state is not yet known. The virulence genes located on the *Salmonella* Pathogenicity Island 1 (SPI-1) are of great importance in the invasion of intestinal cells in various animal species (Zhou and Galan, 2001). The virulence genes located on the *Salmonella* Pathogenicity Island 2 (SPI-2) are indispensable for the induction of systemic disease and persistence in mice after both oral and intraperitoneal inoculation (Shea

et al., 1999). The role of this pathogenicity island in the pathogenesis of *Salmonella* Typhimurium infections in pigs is not documented. It was the aim of the present study to determine the importance of SPI-2 in the colonization and persistence in pigs.

METHODS AND MATERIALS

Salmonella Typhimurium strain MB2486, isolated from a pig in Belgium, was used as the wild type strain (WT) to construct the *hilA* and *ssrA* deletion mutants, according to the one step inactivation method of Datsenko and Wanner (2000) with modifications for use in *Salmonella* Typhimurium (Boyen et al., 2006). The intracellular expression of the *ssrA* gene and of the SPI-2 effector gene *sifB* was quantified by real-time reverse transcription PCR, using SYBR Green, as described before

(Botteldoorn et al., 2006). The SPI-1 expression level (*hilA*, coding for the major regulating protein of SPI-1; *sipA*, coding for a SPI-1 effector protein) was measured in a late logarithmic culture in Luria-Bertani broth, also by real-time reverse transcription PCR, using SYBR Green. *In vitro* invasion and cytotoxicity assays were conducted on porcine pulmonary alveolar macrophages (PAM), as described before (Boyen et al., 2006). BALB/c mice (6 weeks old, 2 groups of 8 animals) and conventional *Salmonella* negative piglets (5 weeks old, 2 groups of 10 animals) were inoculated orally with approximately 10^8 and 10^7 colony forming units respectively of the wild type strain or the *ssrA* deletion mutant. As a control group, 4 animals were sham-inoculated with PBS. Four mice of each *Salmonella* inoculated group were humanely euthanized at day 1 and day 4 post inoculation. Five piglets of each *Salmonella* inoculated group were humanely euthanized at day 5 and day 28 post inoculation. Control animals were euthanized together with the last group of *Salmonella* inoculated animals. Internal organs of mice and piglets as well as faecal samples collected daily from the piglets were examined for the presence of *Salmonella* by means of plating ten-fold dilutions on Brilliant Green Agar.

RESULTS

The complete coding sequence of the *ssrA* gene was deleted, which was confirmed with PCR and sequencing of the surrounding area. The *ssrA* mutant strain displayed no intracellular *ssrA* expression and a decreased *sifB* expression level *in vitro*. The expression levels of 2 SPI-1 encoded proteins were not altered (Table 1).

Table 1: Relative expression levels of SPI-1 and SPI-2 related genes in the wild type, the ssrA mutant and the hilA mutant strain

Relative expression	Wild type	<i>ssrA</i> mutant	<i>hilA</i> mutant
Intracellular SPI-2 expression	<i>ssrA</i> : 1.0 <i>sifB</i> : 10.4	<i>ssrA</i> : 0.0 <i>sifB</i> : 2.6	Not Determined
SPI-1 expression in a logarithmic culture	<i>hilA</i> : 1.00 <i>sipA</i> : 0.10	<i>hilA</i> : 1.06 <i>sipA</i> : 0.07	<i>hilA</i> : 0.00 <i>sipA</i> : 0.01

In accordance with these results, 2 *in vitro* cell culture assays (invasion in PAM and induction of early cytotoxicity) could not show a difference in SPI-1 expression between the wild type strain and the *ssrA* mutant strain. As a control, the *hilA* mutant strain was significantly less invasive in PAM compared to the wild type strain and induced significantly less cell death 6 hours after incubation in PAM (Table 2).

Table 2: The invasion rate and cytotoxic effects of the wild type, the ssrA mutant and the hilA mutant strain in PAM

Phenotypical test	Wild type	<i>ssrA</i> mutant	<i>hilA</i> mutant
Invasion rate in PAM (\log_{10} , gentamicin protected bacteria +/- sem)	5.16 (+/- 0.10)	5.05 (+/- 0.13)	3.75 (+/- 0.10)
Early cytotoxicity in PAM (% viable cells, relative to control PAM +/- sem)	67.7 (+/- 10.2)	68.6 (+/- 4.8)	102.6 (+/- 8.3)

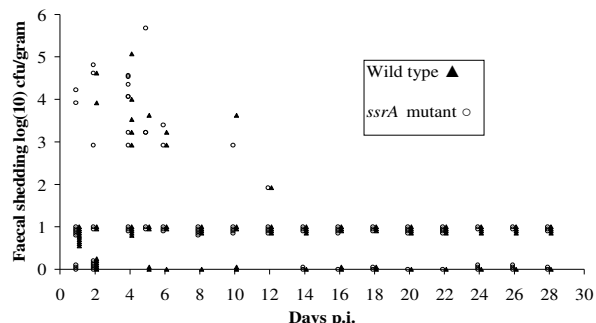
In the murine *in vivo* model, the caeca of both groups of mice were colonized to the same extent 1 day post inoculation. Four days after inoculation, however, the internal organs of the mice inoculated with the *ssrA* mutant strain were colonized with a 100-fold reduction compared to the wild type strain (Table 3).

Table 3: The colonization of different organs of BALB/c mice with the Salmonella Typhimurium wild type or ssrA mutant strain (\log_{10} cfu/gram tissue +/- sd)

Organ	Wild type	<i>ssrA</i> mutant
Caecum day 1	5.88 (+/- 1.13)	6.39 (+/- 1.20)
Caecum day 4	4.91 (+/- 1.43)	3.96 (+/- 0.39)
Liver day 4	3.02 (+/- 1.27)	1.03 (+/- 1.20)
Spleen day 4	3.79 (0.98)	1.37 (+/- 1.64)

The infection of the piglets inoculated with the *ssrA* mutant strain followed a similar course compared to the piglets infected with the wild type strain. The daily faecal excretion levels of both strains were not significantly different ($p>0.05$; non-parametric Kruskal-Wallis test; Figure 1). At days 5 and 28 post inoculation, the animals of both groups were infected to the

Figure 1: Faecal excretion of piglets inoculated with the wild type *Salmonella* strain or the *ssrA* deletion mutant. A value of 0 means negative for *Salmonella*, a value of 1 means positive for *Salmonella* only after pre-enrichment and enrichment.



same extent in the gut and gut-associated lymphoid tissue, as well as in the internal organs (Table 4). All sham inoculated animals, mice as well as piglets, remained negative for *Salmonella* throughout the experiment.

Table 4: The colonization of different organs of piglets with the *Salmonella* Typhimurium wild type and *ssrA* mutant strain

Tissue	Wild type strain		<i>ssrA</i> mutant strain		
	Frequency*	Log ₁₀ cfu g ⁻¹ ± sd	Frequency*	Log ₁₀ cfu g ⁻¹ ± sd	
Day 5 pi	Tonsil	3/5	1.33 ± 1.57	4/5	3.14 ± 1.77
	Liver	4/5	0.8 ± 0.45	2/5	0.4 ± 0.55
	Spleen	2/5	0.58 ± 0.86	1/5	0.2 ± 0.45
	Ileocecal ln.	5/5	3.69 ± 0.62	5/5	4.04 ± 0.79
	Ileum	5/5	4.92 ± 0.52	5/5	5.17 ± 1.39
Day 28 pi	Tonsil	2/5	0.76 ± 1.23	4/5	1.88 ± 1.74
	Liver	0/5	0 ± 0	1/5	0.2 ± 0.45
	Spleen	0/5	0 ± 0	2/5	0.4 ± 0.55
	Ileocecal ln.	4/5	1.32 ± 1.34	4/5	0.98 ± 0.68
	Ileum	5/5	1.72 ± 1.61	5/5	1.18 ± 0.41

*number of pigs positive/number of pigs inoculated

DISCUSSION

In NRAMP^{-/-} laboratory mice, SPI-2 has an important and highly documented impact on the pathogenesis of *Salmonella* Typhimurium infections, particularly on the systemic phase of the infection (Hensel et al., 1998; Shea et al., 1999), but also on the enteric phase (Coburn et al., 2005). Data describing the importance of SPI-2 in the systemic phase of infection obtained in food producing animals are less extensive. For host-restricted or host

adapted serotypes (ex. Gallinarum, Pullorum, Dublin, Choleraesuis) SPI-2 is a prerequisite for virulence and colonization in their respective hosts (Dunyak et al., 1997; Bispham et al., 2001; Jones et al., 2001; Wigley et al., 2002). However, for broad range serotypes, such as Enteritidis and Typhimurium, the role of SPI-2 in the pathogenesis of *Salmonella* infections in food producing animals is less described and less straightforward (Tsolis et al., 1999; Zhao et al., 2002; Morgan et al., 2004). In accordance, our results suggest that SPI-2 of *Salmonella* Typhimurium may not contribute to persistence in pigs to the same extent as it does in laboratory mice.

In conclusion, we have shown that a *ssrA* deletion mutant of a porcine field strain of *Salmonella* Typhimurium, that is attenuated *in vitro* and in an *in vivo* mouse model, is still capable of colonizing pigs and establishing a long term persistent infection. This work contributes to the recent insights in the serotype- and host-dependent pathogenesis of *Salmonella* infections in food producing animals.

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