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A limited role for SsrA/B in persistent *Salmonella* Typhimurium infections in pigs

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ABSTRACT

Virulence genes regulated by the SsrA/B system are indispensable for systemic disease in BALB/c mice. The role of this regulating system in the pathogenesis of *Salmonella* Typhimurium infections in pigs is not documented. In the present study, the interactions of *Salmonella* Typhimurium and an *ssrA/B* mutant were compared *in vitro* and *in vivo*. The *ssrA/B* mutant strain displayed decreased *Salmonella* Pathogenicity Island 2 (SPI-2) expression levels, showed a replication defect in mouse macrophages and was attenuated in a mouse model after oral inoculation. Using real time qRT-PCR and a porcine ileal loop model, it was shown that the *ssrA/B* mutant strain was not significantly attenuated in overall virulence and SPI-1 expression in specific. Flowcytometric analysis demonstrated that the *ssrA/B* mutant strain was defective in intracellular replication in porcine macrophages. After oral inoculation of piglets with the wild type strain or the *ssrA/B* mutant strain, the animals of both groups excreted *Salmonella* and were colonized by *Salmonella* to the same extent. In an intravenous mixed infection model, the *ssrA/B* mutant strain was defective in the colonization of several internal organs. These results suggest that the *ssrA/B* gene of *Salmonella* Typhimurium plays a limited role in the persistent intestinal colonization of pigs.

Key words

Salmonella Typhimurium – pig – macrophage – *ssrA/B*

1. Introduction

Salmonellosis is one of the leading zoonoses in the world. Human infections with non-typhoidal *Salmonella* are generally foodborne and are a major public health concern. Over the last two decades, the emergence of *Salmonella* strains carrying multiple antibiotic resistance genes has led to an increased risk for hospitalization, invasive illness, and death. This is particularly the case for *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium; Velge et al., 2005).

Second to *Salmonella* Enteritidis, *Salmonella* Typhimurium is the most common serotype associated with human salmonellosis in Europe (Fisher, 2004). Due to a drop in *Salmonella* Enteritidis infections in poultry in 2005 and 2006 (Collard et al., 2007), the relative importance of *Salmonella* Typhimurium infections in pigs might increase the next few years. *Salmonella* Typhimurium is the most frequently isolated serotype from pigs and pork (Anonymous, 2004). Infected pigs can shed *Salmonella* for at least 28 weeks (Wood et al., 1989). These carrier pigs are a vast reservoir of *Salmonella* Typhimurium and pose an important threat to animal and human health (Berends et al., 1997). The mechanism underlying this carrier state of infection is unknown.

The virulence genes located on *Salmonella* Pathogenicity Island 2 (SPI-2), which encodes a type III secretion system (T3SS), may play a role in the persistence of *Salmonella* in food-producing animals since these genes are indispensable for the induction of disease and systemic persistence in mice (Cirillo et al., 1998; Hensel et al., 1998). The two-component regulatory system, *ssrA/B*, responds to environmental signals (Löber et al., 2006) and controls the expression of the type III secretion system and the

secreted effector proteins. Through interaction with the intracellular traffic in macrophages and dendritic cells, the injected SPI-2 effector proteins manage to create a safe niche for the salmonellae inside the phagocyte.

It becomes increasingly clear that the pathogenesis of *Salmonella* infections varies depending on the host - strain combination (Pasmans et al., 2003; Morgan et al., 2004). Therefore, pathogenesis studies that use both the host species of interest and relevant *Salmonella* strains are of crucial importance.

The aim of the present study was to determine the contribution of SsrA/B to the intestinal and systemic colonization and the intestinal persistence of pigs by a *Salmonella* Typhimurium field strain.

2. Materials and Methods

2.1. Bacterial strains and plasmids

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) strain 112910a was isolated from a pig stool sample on a pig farm with a persistent *Salmonella* problem and was used as the wild type strain (WT) in which all mutant strains were constructed. The construction and characterization of the non-polar deletion mutant in the major SPI-1 regulatory protein HilA, has been described before (Boyen et al., 2006a; Boyen et al., 2006c). A mutant strain in which the *ssrA* gene was deleted (designated SsrA/B) was constructed accordingly. This region also contains a promoter region of the *ssrB* gene, thus resulting in the functional destruction of the

complete *ssrA/B* system. Briefly, *ssrA* was first substituted by a PCR-modified kanamycin resistance cassette with the aid of a helper plasmid encoding the λ Red system. Primers used to create the PCR- modified resistance cassettes for the *ssrA/B* gene were

5'- TAGTGATCAAGTGCCAAAGATTTTGCAACAGGCAACTGGAGGGAAGCATTTG
 TGTAGGCTGGAGCTGCTTC-3' and 5'-
 AAGATCTTATATTCTTTTCATTTTGCTGCCCTCGCGAAAATTAAGATAATACAT
 ATGAATATCCTCCTTAG-3'. The mutant allele was subsequently transduced, using bacteriophage P22HT_{int}, in a fresh wild type *Salmonella* background to discount effects on virulence due to unlinked mutations. In the last step, the antibiotic resistance cassette was eliminated using the helperplasmid pCP20. The targeted gene was completely deleted from the start codon till the stop codon. This was confirmed by sequencing.

For all *in vitro* experiments and the loop assays, the WT and SsrA/B strains were used. For the *in vivo* assays, an invasive, spontaneous nalidixic acid resistant derivative (WT_{nal}) was used. The *ssrA/B::kan* mutation was moved by P22 transduction into the WT_{nal} strain, resulting in the SsrA/B_{kan/nal} strain. This strain was used in the mixed *in vivo* assay. For the oral infection model, the kanamycin resistance cassette was eliminated as described above, resulting in the SsrA/B_{nal} strain. At different stages of the construction, bacteriophage P22 sensitivity was tested to confirm the smooth phenotype.

For flowcytometric analysis, fluorescence microscopy and confocal imaging, the pFPV25.1 plasmid expressing green fluorescent protein under the constitutive promoter of *rpsM* was used (Valdivia and Falkow, 1996).

2.2. Cell cultures and growing conditions

The mouse macrophage cell line RAW 264.7 or porcine pulmonary alveolar macrophages (PAM) were used for all experiments. PAM were isolated and kept in culture as previously described (Boyen et al., 2006a; Dom et al., 2004).

For intracellular survival assays, stationary phase cultures were obtained following growth overnight with aeration in Luria-Bertani (LB) broth and opsonized with serum obtained from *Salmonella* negative pigs. For the mouse infection model, bacterial strains were grown to stationary phase, washed in phosphate buffered saline (PBS) and resuspended at a concentration of $\sim 2.5 \times 10^9$ CFU/ml in PBS. The inocula for the oral infection models and the intestinal loop model were prepared as described previously (Boyen et al., 2006b). For the intravenous infection assay, overnight cultures of both strains were assembled, washed three times in PBS and resuspended to obtain $\sim 3 \times 10^8$ CFU/ml in PBS.

2.3. SPI-1 and SPI-2 expression studies

The relative expression levels of the *hilA* (encoding a major SPI-1 regulator), *sipA* (encoding a SPI-1 effector), *ssrA/B* (encoding a major SPI-2 regulator) and *sifB* (encoding a T3SS-2 secreted effector) genes of the *Salmonella* Typhimurium wild type strain and the *ssrA/B* mutant strain were compared in LB broth. A *hilA* mutant strain was used as an internal control.

Total RNA was extracted from 1 ml of the bacterial culture using the RNeasy Mini Kit and the RNAprotect Bacteria reagent (Qiagen, Valencia, CA, USA) followed by a DNase treatment. To quantify the expression of the genes of interest, the quantitative real time reverse transcriptase PCR method (qRT-PCR) according to (Botteldoorn et al., 2006) was used. Primers used are shown in the Supplementary Data 1. As control house keeping genes 16S rRNA, *gmk* and *rpoD* were used and the normalisation factor (NF) was calculated by using the GeNorm software (Vandesompele et al., 2002).

2.4. Intestinal loop model

To check the behaviour of the *ssrA/B* mutant strain during the intestinal phase of infection, an intestinal loop model was performed on 6-week-old piglets. This model has been described in detail elsewhere (Watson et al., 1995; Boyen et al., 2006c). In short, intestinal loops of 6-7 cm in length were ligated and inoculated with $1-2 \times 10^9$ CFU *Salmonella* Typhimurium 112910a or its isogenic deletion mutants in *hilA* and *ssrA/B*. The *hilA* mutant strain was used as an internal control. Approximately 10 ml of blood was removed from the piglets to isolate the polymorphonuclear leucocytes (PMNs). The isolated PMNs were labeled with 111 Indium and reinjected intravenously. The influx of PMNs in the intestinal wall and in the lumen of the gut, as assessed by the counts per minute (cpm) emitted from 111 Indium-labelled PMNs within each loop, was recorded 12 h after injection of the loops.

2.5. Flow cytometric analysis of intracellular replication

To assess the intramacrophagal replication deficit of the *ssrA/B* mutant strain, RAW 264.7 cells or PAM were seeded in 25 cm² culture flasks at a density of approximately 5 x 10⁶ cells per flask and were allowed to attach overnight. The cells were inoculated with the wild type strain or the *ssrA/B* mutant strain carrying the pFPV25.1 plasmid, at a multiplicity of infection (moi) of 1:1. To synchronize the infection, the flasks were centrifuged at 365 x g for 5 min. After 25 min incubation at 37°C under 5% CO₂, the cells were washed and fresh medium supplemented with 100 µg/ml gentamicin was added. After additional 60 min incubation at 37°C under 5% CO₂, the cells were washed. To assess the initial bacterial load, cells were released using trypsin and maintained on ice, protected from light until use. To assess intracellular growth, fresh medium supplemented with 15 µg/ml gentamicin was added and cells were released and handled as described 6 hours after inoculation.

Flow cytometric measurements were made using a FACScantoTM cytometer (Becton-Dickinson, Erembodegem, Belgium). Macrophages were discriminated from bacteria and debris based on forward (FSC) and side (SSC) light scatter. GFP fluorescence was recorded using the FL1 channel (emission wavelength: 515-545 nm). Data were expressed in arbitrary units and both the average fluorescence and the median fluorescent value of infected macrophages were calculated from the fluorescence histograms using the FACSDiva software (Becton-Dickinson, Erembodegem, Belgium).

2.6. Experimental infection of BALB/c mice

Seven-week-old female BALB/c mice were randomly divided in 2 groups, anesthetized with isoflurane and inoculated orally with ca. 1×10^8 CFU of *Salmonella* Typhimurium 112910a or *Salmonella* Typhimurium 112910a Δ ssrA/B. On day 1 and day 4 after inoculation, a subset of animals of both groups were humanely killed. For each animal, cecum, spleen and liver were removed, homogenized and the number of CFU/g tissue was determined on brilliant green agar (BGA) plates.

2.7. Experimental infections of piglets

In the experimental infection studies, 5-week-old piglets, obtained from a serologically negative breeding herd, that were negative for *Salmonella* at fecal sampling were used. Oral experimental infections were performed in piglets as described before (Boyen et al., 2006b). In short, animals of groups 1 and 2 were orally inoculated with ca. 1×10^7 CFU of *Salmonella* Typhimurium 112910a or *Salmonella* Typhimurium 112910a Δ ssrA/B respectively in 2 ml PBS. Group 3, the negative control group, was sham-inoculated with 2 ml PBS. The rectal temperature and the clinical condition were monitored and fresh fecal samples were collected on several days for bacteriological analysis. On days 5 and 28 pi, 5 piglets of each group were euthanized. Samples of several organs were taken for bacteriological analysis. All samples were examined for the presence of the *Salmonella* strains by plating tenfold dilutions on BGA supplemented with 20 μ g/ml nalidixic acid. If negative at direct plating, the samples were pre-enriched in buffered peptone water, enriched in tetrathionate broth and plated on BGA supplemented with 20 μ g/ml nalidixic acid. Samples that were negative after direct

plating but positive after enrichment were presumed to contain 83 CFU/g. Samples that remained negative were presumed to contain 0 CFU/g.

For the mixed infection assay, 11 experimental animals were intravenously inoculated with approximately 1.5×10^8 CFU of each strain of a 1:1 mixture of the WT_{nal} and the SsrA/B_{kan/nal} strains in 0.5 ml PBS. The negative control group consisted of 3 piglets which were sham-inoculated with PBS. The *Salmonella*-inoculated piglets were euthanized on days 1 (5 piglets) and 3 (6 piglets) after inoculation. Sham-inoculated piglets were euthanized 3 days after inoculation. Samples of tonsils, lung, liver, spleen, kidney, bronchial, mesenteric and ileocecal lymph nodes, ileum and cecum were taken for bacteriological analysis. The ileum and cecum tissue samples were separated from their contents and were rinsed in PBS. Both the contents and the rinsed intestinal samples were bacteriologically examined. All samples were processed as described before (Boyen et al., 2006c). The ratio WT_{nal} / SsrA/B_{kan/nal} was calculated for all samples derived from each piglet. These data were converted logarithmically prior to statistical analysis. The appropriate detection limits were used to estimate the minimum ratios when samples were not positive after direct plating.

2.8. Ethical considerations

The animal work presented here was approved by the Ethical committee of the Faculty of Veterinary Medicine of the Ghent University (EC 2004/103 and EC 2006/104) and in the UK were conducted according to the requirements of the Animal (Scientific Procedures) Act 1986.

2.9. Statistical analysis

All *in vitro* experiments were carried out in triplicate with three repeats per experiment, unless otherwise stated. The data from the intestinal loops were analyzed by one-way analysis of variance methods, using the SPSS 12.0 software for windows. A Student's *t*-test was used to determine whether the flow cytometric fluorescence values of both strains differed and to determine whether the log value of the WT_{nal} / SipB_{kan/nal} ratio of the samples was significantly different from the log value of the WT_{nal} / SipB_{kan/nal} ratio of the inoculum. In the oral infection assay, statistical analysis was performed using a non-parametric Kruskal-Wallis test. Differences with a *P* value ≤ 0.05 were considered significant.

3. Results

3.1. The *ssrA/B* mutant strain is attenuated in a BALB/c infection model

SPI-2 deficient *Salmonella* Typhimurium strains are severely attenuated in virulence in a BALB/c infection model. Deletion of the *ssrA/B* gene resulted in a systemic colonization defect in this model, as shown in the Supplementary Data 2. Although the *ssrA/B* mutant strain and the wild type strain were found in similar numbers in the murine ceca 1 day after inoculation, the *ssrA/B* mutant strain was found in lower numbers in the ceca 4 days after inoculation, though not statistically significant (*P* >

0.05). The *ssrA/B* mutant strain was found in significantly ($P < 0.05$) lower numbers in liver and spleen 4 days after inoculation.

3.2. The *ssrA/B* mutant strain is defective in SPI-2 expression

To check the deletion of *ssrA/B* and the expression of downstream T3SS-2 - secreted effector genes, the expression levels of *ssrA/B* and *sifB* (Miao and Miller, 2000) were measured using qRT-PCR. The *ssrA/B* gene was expressed in the wild type strain and the *hilA* mutant strain, but not in the *ssrA/B* mutant strain. The expression of *sifB* was considerably decreased in the *ssrA/B* mutant strain. Both the expression of *hilA* (a major SPI-1 regulator) and *sipA* (coding for a SPI-1 effector protein) were dramatically diminished in the *hilA* mutant strain, but not in the *ssrA/B* mutant strain (Supplementary Data 3).

3.3. The *ssrA/B* mutant strain elicits an intestinal inflammatory response

In the porcine intestinal loop assays, the virulence of the wild type strain and the *ssrA/B* mutant strain in the intestinal phase of the infection were compared. Twelve hours post inoculation, both the wild type strain and the *ssrA/B* mutant strain, but not the *hilA* mutant strain, induced intestinal inflammation. There was no statistically significant difference in neutrophil influx between the wild type strain and the *ssrA/B* mutant strain ($P > 0.05$). These results are shown in the Supplementary Data 4.

3.4. The *ssrA/B* deletion mutant shows impaired intracellular replication in murine and porcine macrophages

At time point 0 h, both the mean fluorescence and the median fluorescent value of the infected RAW 264.7 cells and PAM were similar for the wild type strain and the *ssrA/B* mutant strain. At 6 h after inoculation, however, both the mean fluorescence and the median fluorescent value of the infected macrophages was significantly higher in the cells infected with the wild type strain, compared to the cells infected with the *ssrA/B* mutant strain. These results are shown in Table 1.

3.5. The *ssrA/B* deletion mutant is not impaired in porcine colonization after oral inoculation

Sham-inoculated control piglets did not develop disease signs and *Salmonella* was not isolated from any of the samples taken from these animals throughout the experiment.

The animals from both *Salmonella*-inoculated groups had a slight increase in temperature during the first few days after inoculation and some presented with mild diarrhea. The animals of both groups showed a similar fecal excretion pattern (data not shown). Two weeks after inoculation, the piglets intermittently shed *Salmonella* at enrichment level. Although during the last few days of the experiment, the *ssrA/B* mutant strain was shed by fewer animals, this difference was not significant due to the low level of shedding reached at this stage of the infection.

At days 5 and 28 pi, the animals of both groups were infected to the same extent in the gut, the contents of the gut and the gut-associated lymphoid tissue (Table 2). No significant differences ($P > 0.05$) were seen. In addition, no significant differences were seen in the colonization of the internal organs, both at days 5 and 28 pi ($P > 0.05$). However, the low numbers of bacteria in these organs might interfere with a meaningful statistical analysis.

3.6. The *ssrA/B* deletion mutant is attenuated after intravenous inoculation of piglets

In order to get higher numbers of bacteria in the internal organs, we used the intravenous mixed infection model. One day after inoculation, both strains were recovered from all pigs inoculated intravenously with a 1:1 mixture of the WT_{nal} and SsrA/B_{kan/nal} strains. The numbers of bacteria in the blood, the heart, the tonsils and the gut samples were very low, impairing a meaningful quantitative comparison between both strains in these organs. Bacteria were found in relatively higher numbers, although still low, in the liver, spleen, kidney, lungs and bronchial lymph nodes. In these organs, except for the liver, the output ratio WT_{nal} / SsrA/B_{kan/nal} was not significantly different ($P > 0.05$) from the ratio in the inoculum.

Three days pi, neither strain could be recovered from the blood samples. The number of WT_{nal} bacteria found in most of the organs was comparable with the number of WT_{nal} bacteria found 1 day after inoculation. The number of SsrA/B_{kan/nal} bacteria, however, showed an overall decrease. The number of positive lymphoid tissues (tonsils,

gut associated lymphoid tissue) was markedly lower for the *ssrA/B* mutant strain. For the kidney, heart, ileal wall and the bronchial and mesenteric lymph nodes, this resulted in an output ratio $WT_{nal} / SsrA/B_{kan/nal}$ that was significantly higher ($P < 0.05$) than in the inoculum. The numbers of bacteria in the tonsils, lungs and contents of ileum and cecum were very low, impairing a meaningful quantitative comparison between both strains. The average log values of the ratio $WT_{nal} / SsrA/B_{kan/nal}$ for all samples are summarized in Figure 1.

4. Discussion

In this report we characterized an *ssrA/B* deletion mutant in a porcine field strain of *Salmonella* Typhimurium. The deletion was confirmed, both at the transcriptional level and the phenotypic level, using qRT-PCR, a RAW replication model and a BALB/c mouse model. Recently it has been found that the expression of SPI-2 encoded genes is regulated exclusively through the induction of the SsrA/B regulatory system (Löber et al., 2006). This means that disabling the SsrA/B system undeniably results in the loss of the SPI-2 T3SS. Nevertheless, it can not be ruled out that the SsrA/B system also regulates genes which are not associated to SPI-2. Using qRT-PCR, it was confirmed that the expression of the SPI-2 secreted effector gene *sifB* was abolished in the *ssrA/B* mutant strain. The overall virulence and, specifically, the expression of SPI-1 were checked in different assays, confirming that the attenuation in mice is linked to the deletion of *ssrA/B*. Even though we are aware that these phenomena were described before, we strongly believe that these experiments were necessary as a positive control for our

341 *ssrA/B* mutant strain, since it was made in a porcine field strain that has not been used
342 before in pathogenesis studies in mice.

343 Using GFP-expressing bacteria, it was shown that the *ssrA/B* mutant strain was
344 slightly attenuated in intracellular replication in PAM. This defect was more subtle
345 compared to the defect that was observed in mouse macrophages. However, it can not be
346 ruled out that the difference observed in this assay, may also be attributed to the nature of
347 the cells used (primary cells vs. cell line).

348 In the first part of this pathogenesis study, we chose to perform a single *in vivo*
349 assay that resembled the natural infection, which is often established with low or
350 moderate numbers of *Salmonella* (Loynachan and Harris, 2005), to be able to investigate
351 the biological relevance of SsrA/B in the course of infection in the field. The fact that
352 very few differences were seen in the long-term intestinal colonization of pigs orally
353 inoculated with an *ssrA/B* mutant strain and its isogenic parental wild type strain may
354 come as a surprise. However, a closer look at pathogenesis studies performed in various
355 host species may shed a clearer light on this matter. In laboratory mice, SsrA/B-regulated
356 genes have an important impact on the pathogenesis of *Salmonella* Typhimurium
357 infections, particularly on the systemic phase of the infection (Cirillo et al., 1998; Hensel
358 et al., 1998), but also on the enteric phase (Hapfelmeier et al., 2005). Data obtained in
359 food-producing animals, however, are scarce. The role of SsrA/B-regulated genes in host-
360 restricted/adapted serotypes seems consistent in the literature: they are important
361 virulence factors for systemic disease and as a consequence for colonization of the host.
362 For example, for *Salmonella* Gallinarum and *Salmonella* Pullorum, which cause severe
363 systemic disease in fowl, SPI-2 is a prerequisite for virulence and colonization in

chickens (Jones et al., 2001; Wigley et al., 2002). In mice and calves, SPI-2 genes are required for virulence in *Salmonella* Choleraesuis (Dunyak et al., 1997) and *Salmonella* Dublin (Bispham et al., 2001) infections respectively. In broad host range serotypes, however, like *Salmonella* Enteritidis and *Salmonella* Typhimurium (except in NRAMP^{-/-} mice), the importance of SPI-2 is less described. In calves, a *Salmonella* Typhimurium SPI-2 mutant strain was attenuated in colonization after oral inoculation in a signature-tagged mutagenesis assay (Coombes et al., 2005), but was still able to cause lethal infections (Tsolis et al., 1999). SPI-2 was found to play a role in the colonization of *Salmonella* Typhimurium in calves, but not in chickens (Morgan et al., 2004). In addition, a screening of 7,680 *Salmonella* Enteritidis mutants for attenuation in a chicken macrophage infection model resulted in the detection of mutations in several flagellar, LPS and SPI-1 associated genes, but not in SPI-2 genes (Zhao et al., 1999). Only recently, it has been shown that an *ssrA* mutant strain promotes reproductive tract colonization, but is not essential for intestinal colonization of chickens with *Salmonella* Enteritidis (Bohez et al., 2007). In a rabbit ileal loop model and in calves, *Salmonella* Typhimurium SPI-2 mutant strains induced inflammation and fluid accumulation to the same extent as the wild type strain (Tsolis et al., 1999; Everest et al., 1999). Considering these reports, the results shown here are not as surprising as expected at first sight. During the preparations of this manuscript, Brumme et al. (2007) found that a SPI-2 deletion mutant was not able to induce clinical symptoms of porcine salmonellosis, whereas the SPI-2 mutant infected group showed similar quantities of salmonellas in the organs as the wild-type infected pigs. Almost simultaneously, Carnell et al. (2007) identified SPI-2 related genes in a signature-tagged mutagenesis assay in pigs as

important for the colonization of the porcine gut. Even though the results of both of these assays are highly interesting and relevant in the used models, very high inoculation doses were used (10^{10} cfu – 10^{11} cfu per piglet), which do not necessarily reflect the natural situation of the subclinical carriage of *Salmonella* Typhimurium in the field.

It is generally accepted that mixed inoculum assays are more capable of discriminating differences in the ability of strains to colonize the host. Using a mixed inoculum assay and a high inoculation dose, we could indeed show that an *ssrA/B* mutant strain is attenuated for the systemic colonization of internal organs of pigs after intravenous injection. These findings are consistent with the intramacrophagal replication defect of the *ssrA/B* mutant strain we observed *in vitro*. The mixed intravenous infection protocol, however, does not fit closely to the natural route of infection and bacteria may reach their host cells in a more artificial manner. Recently, it has been shown that the magnitude of the intracellular SPI-2 gene expression is dependent on the mechanism of internalization by macrophages (Drektrah et al., 2007). This phenomenon may additionally explain the differences that were seen in both *in vivo* experiments.

In conclusion, we have shown that an *ssrA/B* mutant of a porcine field strain of *Salmonella* Typhimurium is fully capable of colonizing the intestines of pigs and to establish a long term intestinal persistent infection after oral inoculation. Using an intravenous mixed infection model, however, the *ssrA/B* mutant strain was defective in the systemic colonization of several internal organs. This work contributes to the recent insights in the serotype- and host-dependent pathogenesis of salmonellosis in food producing animals.

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Figure 1: Recovery of bacteria from various organs of piglets at days 1 and 3 after intravenous inoculation with an equal mixture of WT_{nal} and SsrA/B_{kan/nal}. The log value of the ratio of the number of CFU/g sample of WT_{nal} and SsrA/B_{kan/nal} is given as the mean $\pm$ standard deviation. An asterisk indicates that the output ratio was significantly different ($P < 0.05$) from that present in the inoculum.

Table 1: Mean and median fluorescent values of infected RAW 264.7 cells and porcine macrophages (PAM) at 0 h and 6 h after inoculation. The average values of 3 independent experiments $\pm$ sd are shown. Both the mean and median fluorescent values of the cells infected with the *ssrA/B* mutant strain at 6 h pi were statistically significant lower ($P < 0.05$) than the values of the cells infected with the wild type strain and are indicated with a “*”.

	Mean fluorescence $\pm$ sd		Median fluorescent value $\pm$ sd	
RAW 264.7	0 h	6 h	0 h	6 h
WT	1165 $\pm$ 110	3236 $\pm$ 488	831 $\pm$ 26	1862 $\pm$ 189
ΔssrA/B	1073 $\pm$ 124	1594 $\pm$ 298*	801 $\pm$ 57	1042 $\pm$ 166*
PAM	0 h	6 h	0 h	6 h
WT	744 $\pm$ 84	1028 $\pm$ 202	594 $\pm$ 42	710 $\pm$ 126
ΔssrA/B	749 $\pm$ 59	674 $\pm$ 122*	578 $\pm$ 38	531 $\pm$ 74*

Table 2: Post mortem bacteriological findings at days 5 and 28 after oral inoculation of piglets with 1×10^7 CFU of the wild type *Salmonella* Typhimurium strain or the *ssrA/B* mutant strain. The number of positive tissues in relation to the total number of tissues (frequency) and the average number of CFU ($\log_{10}$) $\pm$ sd per gram tissue are shown. Samples only positive after enrichment were given a value of 83 CFU/g.

	Tissue	Wild type strain		<i>ssrA/B</i> mutant strain	
		Frequency	$\log_{10}$ CFU/g $\pm$ sd	Frequency	$\log_{10}$ CFU/g $\pm$ sd
Day 5 pi	Mand. ln.	4/5	1.56 ± 1.11	3/5	0.98 ± 1.17
	Tonsil	3/5	1.33 ± 1.57	4/5	3.14 ± 1.77
	Lung	1/5	0.2 ± 0.44	2/5	0.4 ± 0.55
	Heart	2/5	0.4 ± 0.55	1/5	0.2 ± 0.45
	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
	Kidney	2/5	0.78 ± 1.27	2/5	0.6 ± 0.55
	Ileocecal ln.	5/5	3.69 ± 0.62	5/5	4.04 ± 0.79
	Jejunum	5/5	2.97 ± 1.97	5/5	3.30 ± 1.48
	Ileum	5/5	4.92 ± 0.52	5/5	5.17 ± 1.39
	Cecum	5/5	3.85 ± 0.68	5/5	3.40 ± 2.29
	Content jejunum	5/5	2.64 ± 1.70	4/5	1.18 ± 1.04
	Content ileum	5/5	4.40 ± 0.73	5/5	3.92 ± 2.74
	Content cecum	4/5	2.74 ± 1.56	4/5	2.53 ± 2.70
Day 28 pi	Mand. ln.	0/5	0 ± 0	2/5	0.74 ± 1.18
	Tonsil	2/5	0.76 ± 1.23	4/5	1.88 ± 1.74
	Lung	0/5	0 ± 0	1/5	0.2 ± 0.45
	Heart	0/5	0 ± 0	1/5	0.2 ± 0.45
	Liver	0/5	0 ± 0	1/5	0.2 ± 0.45
	Spleen	0/5	0 ± 0	2/5	0.4 ± 0.55
	Kidney	0/5	0 ± 0	0/5	0 ± 0
	Ileocecal ln.	4/5	1.32 ± 1.34	4/5	0.98 ± 0.68
	Jejunum	2/5	0.4 ± 0.55	2/5	0.4 ± 0.55
	Ileum	5/5	1.72 ± 1.61	5/5	1.18 ± 0.41
	Cecum	5/5	1.45 ± 1.01	2/5	1.04 ± 0.79
	Content jejunum	0/5	0 ± 0	1/5	0.2 ± 0.45
	Content ileum	5/5	1.58 ± 1.29	4/5	0.8 ± 0.45
	Content cecum	5/5	1 ± 0	2/5	0.4 ± 0.55