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The *Salmonella* Pathogenicity Island 2 regulator *ssrA* promotes reproductive tract but not intestinal colonization in chickens

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Abstract

Using a deletion mutant in the regulator of SPI-2, *ssrA*, we investigated the role of SPI-2 in invasion, intestinal colonization and reproductive tract infection of chickens by *Salmonella* Enteritidis. The *ssrA* mutant was fully invasive in phagocytic and nonphagocytic cells but failed to persist within chicken macrophages. The ability of *Salmonella* Enteritidis to cause disease in orally infected 1 day-old chicks was not altered when *ssrA* was deleted. Furthermore, caecal colonization was not affected, while spleen and liver showed reduced colonization. Following intraperitoneal and intravenous infection of one day-old chicks, internal organ colonization was strongly reduced. After intravenous inoculation in adult laying hens bacterial numbers of the *ssrA* mutant were significantly lower in oviducts and ovaries as compared to the wild type strain. The chickens showed less reproductive tract lesions and the recovery of egg production was faster compared to the wild type strain infected chickens. These findings indicate that the SPI-2 regulator *ssrA* promotes reproductive tract colonization, but is not essential for intestinal colonization of chickens with the host non-specific serotype Enteritidis.

Keywords: *Salmonella* Enteritidis; *Salmonella* Pathogenicity Island 2 regulator *ssrA*; colonization; poultry

1. Introduction

Salmonella enterica serovar Enteritidis (*Salmonella* Enteritidis) is a facultative intracellular bacterium with a broad host range. It can cause foodborne illness in humans, following the consumption of chicken products (Guard-Petter, 2001). Poultry products become contaminated as asymptomatic carrier birds enter the slaughterhouse and asymptomatic carrier hens lay contaminated eggs. Infection of one day-old chickens with high doses of *Salmonella* Enteritidis can cause severe clinical salmonellosis with a high rate of mortality, while this serovar produces a chronic asymptomatic carrier state in low dosed infected young chickens and infected adult birds (Gast and Benson, 1995; Desmidt *et al.*, 1997; Van Immerseel *et al.*, 2004). These carrier birds may excrete the bacteria intermittently until slaughter age (6 weeks) in broilers until the end of the reproduction cycle in layer type chickens and thus constitute an important source of *Salmonella* infection (Gast and Benson, 1995; Desmidt *et al.*, 1997; Humphrey *et al.*, 1991). *Salmonella* Enteritidis specifically interacts with the reproductive organs of hens leading to egg contamination (De Buck *et al.*, 2004). Following oral ingestion, *Salmonella* colonizes the chicken gut, especially the caeca, where it penetrates the mucosal epithelium (Desmidt *et al.*, 1997). Interaction between *Salmonella* and the epithelium triggers infiltration of phagocytic cells to the infected site, where these cells take up the bacteria. *Salmonella* is capable to survive and replicate within bacterially modified macrophage spacious phagosomes, which can be attributed to the type III secretion system (TTSS) encoded by *Salmonella* pathogenicity island 2 (SPI-2) (Knodler and Steele-Mortimer, 2003). The infected phagocytes disseminate to the internal organs, such as liver, spleen and the reproductive tissues and thus spread the bacteria within the host (Turnbull and Snoeyenbos, 1974; Barrow *et al.*, 1999).

SPI-2 is an essential virulence locus for systemic disease in host-specific *Salmonella* serotypes including *Salmonella* Pullorum in poultry, and SPI-2 mutants fail to induce systemic disease and clinical signs in the infected animals (Jones *et al.*, 2001; Wigley *et al.*, 2002; Bispham *et al.*, 2001).

The exact role of SPI-2 in the colonization and systemic spread of host non-specific serotypes and in the subsequent development of asymptomatic carriers in chickens however is currently not clear. Therefore, in this study, the role of *ssrA* in shedding, gut and internal organ colonization was investigated after oral inoculation of one day-old chickens with *Salmonella* Enteritidis. Organ colonization was evaluated after intraperitoneal inoculation of 5 day-old chickens and intravenous inoculation of adult laying hens to assess the role of *ssrA* in organ colonization, including oviduct and ovaria colonization and egg contamination, after passing the mucosal barrier of the gut.

2. Materials and methods

2.1. Bacterial strain

Salmonella Enteritidis phage type 4, strain 76Sa88 Nal^r was used in the experiments. This strain was isolated from an outbreak of salmonellosis on a poultry farm. The nalidixic acid resistant strain and its 76Sa88 wild type progenitor strain are well-characterized (Bohez *et al.*, 2006).

2.2. Construction of defined mutant

A deletion mutant in the *Salmonella* Pathogenicity Island 2 (SPI-2) encoded regulator SsrA was constructed according to the one-step inactivation method, first described by Datsenko and Wanner (2000). The targeted gene, *ssrAI*, was completely deleted from the start codon till the stop codon, as confirmed by sequencing.

2.3. *In vitro* experiments

2.3.1. *Invasion and survival in T84 and HD11 cell lines*

Invasion in T84 human colon carcinoma cell line was performed according to the setup of Bohez *et al.* (2006.) Invasion and proliferation in HD11 chicken macrophage cells were determined by seeding the HD11-cells in a 24-well cell culture plates (Greiner, Frickenhausen, Germany) at a density of $5 \cdot 10^5$ cells/well in culture medium. The bacteria were grown to stationary phase (20 h) at 37°C with shaking and added to the cells at a multiplicity of infection of 10 (CFU/cell). Invasion and proliferation were determined after 1 h and 6 h of incubation, respectively. Experiments were performed in triplicate with 3 repeats in each experiment. Statistical analysis was performed by analysis of variance methods using SPSS 12.0 software.

2.3.2. *Cytotoxicity assay*

Cytotoxicity was assessed by measuring the lactate dehydrogenase (LDH) release (Cytotoxicity detection kit, Roche Diagnostic Corporation, Indianapolis, USA) of HD11 chicken macrophage cells. This assay is performed according to setup of Bohez *et al.* (2006). The bacteria were grown to stationary phase (20 h) at 37°C with shaking and added to the cells at a multiplicity of infection of 10 (CFU/cell). Cytotoxicity was determined after 6 h of incubation.

The percentage of cytotoxicity was established using the following formula, instructed by the Cytotoxicity detection kit (Roche):

$$\text{Cytotoxicity} = (\text{experimental value} - \text{low control}) / (\text{positive control} - \text{low control}) \times 100$$

2.4. Experimental inoculation of chickens

The experimental protocols were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University. Bacteria used for inoculation were grown for 20 h at 37°C in Luria-Bertani Broth (LB) on a shaker platform. The number of CFU/ml was determined by plating 6 x 20 µl of a tenfold dilution series of the suspensions on BGA. Thereafter the plates were incubated aerobically for 20 h at 37°C. The suspensions were kept at 4°C until they were used in the assays. The bacterial cultures were diluted in phosphate buffered saline (PBS) to the respective inoculation density.

2.4.1. Oral inoculation of one day-old SPF chicks

Specific pathogen free (SPF) White Leghorn chickens (Charles River Laboratories, Brussels, Belgium) were hatched in isolation and housed in HEPA-filtered rooms. Twenty-five chicks were euthanized immediately after hatching and serum samples were taken for the detection of maternal antibodies against *Salmonella* Enteritidis by means of a previously described anti *Salmonella* Enteritidis ELISA, based on lipopolysaccharides of *Salmonella* Enteritidis (Desmidt *et al.*, 1996). Furthermore, cloacal swabs were taken from 5 chickens per

group at the beginning of the experiment and cultured for *Salmonella* as described below. The chickens had *ad libitum* access to drinking water and feed. One day-old chicks were randomly divided in two groups of 61 and 62 chickens and orally inoculated in the crop using a plastic tube with 10^8 colony forming units (CFU) of *Salmonella* Enteritidis 76Sa88 parent strain or its *Salmonella* Enteritidis Δ ssrA mutant in a volume of 0.2 ml of PBS. At day 2, 4, 7, 10, 14, 21, 28, 36 and 42 post-hatch cloacal swabs were taken for the detection of *Salmonella*. For bacteriological analysis of caeca, liver and spleen, four chickens per group were euthanized at day 2, 3, 4, 7, 10, 14, 21 and 28 post-hatch. On day 36 and day 42 of life 13 and 16 animals, and 6 and 9 chickens were euthanized in the groups infected with *Salmonella* Enteritidis 76Sa88 parent strain and its Δ ssrA mutant, respectively.

2.4.2. Intra-peritoneal inoculation of five day- old SPF chicks

An intra-peritoneal infection is very difficult in one day-old chickens because of the presence of the yolk sac. Therefore five day-old chickens were used in which the yolk sac was already reduced.

Specific pathogen free (SPF) White Leghorn eggs (Charles River Laboratories) were hatched and housed in isolation. Immediately after hatching, serum samples were taken from 10 chickens to detect maternal antibodies against *Salmonella* Enteritidis by means of an indirect ELISA (Desmidt *et al.*, 1996). Before the start of the experiment cloacal swabs were taken from all chickens and cultured for *Salmonella* as described below. Feed and drinking water were provided *ad libitum*. Five day-old chicks were randomly divided in two groups of 24 chickens each and housed in separate HEPA-filtered rooms. The birds were intra-peritoneally inoculated

with 10^5 colony forming units (CFU) of *Salmonella* Enteritidis 76Sa88 parent strain or its isogenic Δ ssrA mutant in a volume of 0.2 ml of PBS with 10 mg/ml methylene blue solution (UCB, Leuven, Belgium). Blue staining beneath the abdominal skin indicated a well performed intra-peritoneal infection. At 4, 24 and 48 h post-infection six chickens per group were euthanized and samples of caeca, liver and spleen were collected for the detection of *Salmonella*,

2.4.3. Intravenous inoculation of adult SPF laying hens

Fifteen SPF Lohmann white LSL hens (Lohmann Tierzucht GMBH, Cuxhaven, Germany) were housed in two separate HEPA-filtered rooms with both 10 animals. The birds received 16 h of light per day and had free access to food and water. At the age of 18 weeks, serum samples were taken from three hens of each group to test for anti-*Salmonella* Enteritidis antibodies in an LPS-ELISA (Desmidt *et al.*, 1996). On week 21, all the hens were in lay. At 24 weeks of age, all the animals were intravenously inoculated in the wing vein with 250 μ l containing 2×10^8 cfu of the *Salmonella* Enteritidis 76Sa88 wild type strain or its Δ ssrA mutant strain. This protocol was already used to produce high levels of internal egg contamination (De Buck *et al.*, 2004). Eggs were collected daily during 13 days. Two weeks after inoculation, all the animals were euthanized and liver, oviduct and ovary were aseptically removed for bacteriological analysis for *Salmonella*.

2.4.4. Bacteriological analysis of cloacal swabs and organ samples

Cloacal swabs were directly inoculated on BGA plates with 20 µg/ml nalidixic acid, which were incubated for 20 h at 37°C. When negative after direct inoculation, samples were pre-enriched in buffered peptone water (BPW, Oxoid, Basingstoke, England) overnight at 37°C, after which samples were enriched by addition of 1 ml of this suspension to 9 ml tetrathionate - brilliant green broth. After incubation overnight at 37°C, a loopful of this suspension was plated on BGA.

Samples of caeca, liver, spleen, oviduct and ovary were homogenised and 10-fold dilutions were made in buffered peptone water. For each dilution 6 x 20 µl were inoculated on BGA plates with 20 µg/ml nalidixic acid. After incubation overnight (37°C) the number of CFU/g tissue was determined by counting the bacterial colonies. For samples which were negative after titration, pre-enrichment and enrichment was performed, as described above.

Samples that were negative after titration but positive after *Salmonella* enrichment were presumed to contain 10¹ CFU/g organs. Samples that were negative after enrichment were presumed to have 0 CFU/g. The mean CFU/g tissue was calculated for each group.

2.4.5. Bacteriological examination of eggs

Upon collection, faeces on the surface of the eggs were removed and the eggs were decontaminated by dipping in ethanol (95%) for 1 min. The eggs were broken aseptically and homogenized. A volume of 40 ml of BPW per egg was added and incubated 48 h at 37 °C. Enrichment was done in tetrathionate brilliant green broth at 37°C for 24 h, where after a loopful of enrichment broth culture was streaked onto BGA plates supplemented with 20 µg/ml nalidixic acid.

2.4.6. Statistical analysis

For each time point in the oral infection experiment, logistic regression (binary outcome variables) and general analysis of variance (continuous outcome variables) methods were used to determine significant differences between treated groups for the cloacal swab analysis and for analysis of the samples of caeca, liver and spleen, respectively.

In the intra-peritoneal and intravenous infection experiment, statistical analysis of organ colonisation was performed by general analysis of variance and the independent T-test., respectively. Statistical differences in egg production were determined by the chi-square analysis and differences in positive laid eggs by the Fisher's exact T-test. Differences with P-values below 0.05 were significant. All statistical analyses were performed using SPSS 12.0.

3. Results

3.1. Invasion in T84 human epithelial cells and uptake and proliferation in chicken macrophage HD11 cells

No statistically significant differences were observed in invasion of both *Salmonella* Enteritidis wild type strain and its $\Delta ssrA$ mutant strain in T84 cells and HD11 cells. The *ssrA* mutant was significantly ($p=0.006$) reduced in its intramacrophage survival compared to the wild type strain. The relative intramacrophage survival percentage (6 h / 1 h) of the $\Delta ssrA$ mutant was 62 % of that of the wild type strain.

3.2. LDH assay

The percentage of cytotoxicity at 6 h post-infection of HD11 cells with the *Salmonella* Enteritidis *ssrA* mutant strain ($20\% \pm 2.2$) was similar to that of *Salmonella* Enteritidis 76Sa88 wild type strain ($21\% \pm 2.4$). No statistically significant differences could be observed. The results are calculated relative to triton-exposed cells (100 %).

3.3. In vivo trials

The pathogenic responses may vary between different serotypes, even strains, and different host infection models.

3.3.1. Oral inoculation of one day- old SPF chicks

No differences in clinical signs were observed between animals inoculated with either the parent strain or the $\Delta ssrA$ mutant. Both groups showed dullness, pasted vents and reduced appetite in the first days after inoculation. In the group inoculated with the *Salmonella* Enteritidis $\Delta ssrA$ mutant mortality was 27% compared with 15% in the group inoculated with *Salmonella* Enteritidis 76Sa88 wild type strain.

The chickens of both groups showed a similar faecal excretion pattern during the whole experiment (Table 1). Almost all cloacal samples were positive for *Salmonella* after enrichment in the 6 weeks of study. At direct plating in both groups a decrease in shedding was observed

from day 21 of age and at the end of the experiment about 20% of the chickens yielded positive swabs in both groups.

The ability of *Salmonella* Enteritidis 76Sa88 wild type strain and its $\Delta ssrA$ mutant strain to colonize caeca, liver and spleen is presented in Fig.1. Analysis of the number of *Salmonella* ($\log_{10}$ CFU/gr organ) recovered from the caeca showed no statistically significant differences between both groups. At 2 days post-infection a maximum level of colonization (ca. 10^8 CFU/g) was observed for both strains. Afterwards, the numbers of colonizing bacteria in the caeca gradually decreased over time. At 42 days the caecal samples of all animals remained positive after enrichment. One day after infection the counts of *Salmonella* Enteritidis 76Sa88 wild type strain and its *ssrA* mutant strain were similar both in spleen (ca. 10^2 cfu/g) and liver (ca. 10^3 cfu/g). The wild type strain peaked in liver and spleen at days 3 and 7 of age, respectively, reaching levels of about log 5.2 and log 5.7, while at these time points the *ssrA* mutant only reached levels of log 2.7 and log 3.7 for liver and spleen colonization. When comparing the numbers of wild type strain and the *ssrA* mutant recovered from the spleen and liver over the full length of the experiment a significant ($p < 0.05$) difference was found for both organs. At the end of the experiment 63% of the animals were positive for the wild type strain after enrichment, while only 22% of the birds were positive for the *ssrA* mutant for both internal organs.

3.3.2. Intra-peritoneal infection of five day-old SPF chicks

The number of CFU per g liver and spleen of all inoculated animals at 4, 24 and 48 h after intra-peritoneal infection of five day-old chickens is presented in Fig.2. Similar levels of spleen and liver colonization were observed for the wild type strain and its *ssrA* mutant derivative at 4 h

post-infection. In about 50% of the animals *Salmonella* could not be detected at direct plating. At 24 h post-infection, the *ssrA* mutant was statistically significantly reduced in liver and spleen colonization compared to its isogenic wild type strain. At direct plating, the *ssrA* mutant strain could not be isolated from any liver sample, whereas 5 of the 6 animals were positive for the wild type strain. Of the spleen samples, only 1 of 6 samples was positive at direct plating for the *ssrA* mutant, while all samples were positive for the wild type strain. Compared to its isogenic wild type strain, the numbers of *ssrA* mutant bacteria colonizing liver and spleen was about 100 and 1000 fold, respectively, reduced (statistical significant with $p<0.05$). At 48 h post-infection, no statistically significant differences could be detected anymore, although a decrease in liver and spleen colonization by the *ssrA* mutant strain was still present.

3.3.3. Intravenous infection of adult SPF white LSL hens

In the group inoculated with the wild type strain, 2, 1 and 1 chicken(s) died on day 2, 3 and 5 after infection, compared to death of one chicken on day 6 after inoculation in the *ssrA* mutant inoculated group. At time of organ collection, day 14 after infection, 6 and 9 chickens were left in the wild type and *ssrA* mutant strain inoculated group, respectively.

Results of isolation of *Salmonella* Enteritidis wild type strain and *Salmonella* Enteritidis *ssrA* mutant strain from different organs at 2 weeks after intravenous infection of adult SPF laying hens are presented in Fig.3. Bacteriological analysis of the liver showed that the concentration of colonizing *ssrA* bacteria was significantly lower ($p=0.02$) in comparison to the wild type strain. At direct plating, 22% of the liver samples of chickens inoculated with the *ssrA* mutant were negative, while all liver samples were positive at direct plating for the wild type strain bacteria. Intravenous inoculation with *Salmonella* Enteritidis wild type strain resulted in

lesions of oviducts and ovaries. The oviducts were reduced in size and the ovaries were shrunked and had discolored follicles. These pathological changes in the reproductive tract were absent in the birds infected with the *ssrA* mutant. Animals infected with the *ssrA* mutant showed a significantly ($p<0.01$) lower colonization in ovary and oviduct in comparison to animals infected with the wild type strain, i.e. a decrease of about log 2.4 and log 2.9, respectively. In addition, the oviduct samples of only 4 of 9 chickens were positive for the *ssrA* mutant strain at direct plating, while all oviduct samples were positive in birds infected with the wild type strain. Egg production was reduced after infection with both *Salmonella* Enteritidis parent strain and its *ssrA* mutant derivative (Table 2). From day 7 to day 13 post-infection, laying hens in the *ssrA* mutant inoculated group showed a significant higher egg production. At day 1 and 3 post-infection, 2 and 1 egg(s) of a total number of 18 laid eggs, respectively, were found positive for *Salmonella* Enteritidis wild type strain (Table 2). The *ssrA* mutant strain could be isolated from the content of 1 egg of a total number of 33 laid eggs at day 3 post-infection.

4. Discussion

Various studies have, among others by means of an *ssrA* mutant, demonstrated that the function of the two-component regulatory system SsrA-B is crucial for the expression of all virulence genes within SPI-2 and various loci outside of SPI-2 encoding effector proteins of the SPI-2-TTSS (Cirillo *et al.*, 1998; Lober *et al.*, 2006).

The data presented here indicate that the SPI-2 regulator *ssrA* promotes reproductive tract infection of *Salmonella* Enteritidis in laying hens whereas it has no effect on intestinal colonization. The marked reduction in internal organ colonization observed after intraperitoneal and intravenous inoculation of the *ssrA* mutant confirms the role of the SPI-2 virulence genes in

systemic spread and internal organ colonization. The reduced *ssrA* colony counts in the oviducts and ovaries are indicative for the requirement of SPI-2 by *Salmonella* Enteritidis to spread to the reproductive tract tissues. This is reflected in the fact that less reproductive tract lesions were observed in the group of animals inoculated with the *ssrA* mutant and the faster recovery of egg production after intravenous inoculation with the *ssrA* mutant. *In vitro* studies show that the *ssrA* mutant of *Salmonella* Enteritidis was as invasive as the parent strain in T84 intestinal epithelial cells but severely attenuated in intramacrophage survival in chicken macrophages. The ability of *Salmonella* Typhimurium to induce systemic infection in mice has been shown to be dependent on its survival within macrophages (Cirillo *et al.*, 1998; Shea *et al.*, 1999). Susceptible mice develop a severe systemic disease often resulting in death (Santos *et al.*, 2001; Mastroeni *et al.*, 2004), as is the case with other host-specific serotypes in other animal species (Pardon *et al.*, 1988; Uzzau *et al.*, 2000), including *Salmonella* Gallinarum biovar Pullorum in poultry (Pomeroy *et al.*, 1991; Snoeyenbos *et al.*, 1991). The *Salmonella* Pathogenicity Island 2 type three secretion system is an essential virulence locus for systemic disease in host-specific *Salmonella* serotypes (Jones *et al.*, 2001; Wigley *et al.*, 2002; Bisham *et al.*, 2001). Whereas an *ssrA* mutant in the host non-specific serotype Enteritidis did not show colonization defects in one day-old chicks, the *ssrA* mutant in the host-specific serotype Gallinarum was found to be fully attenuated for its virulence in one day-old chicks following oral infection (Shah *et al.*, 2005). In addition, a SPI-2 mutant of *Salmonella* Gallinarum carrying a mutation in the *ssaU* gene was neither detected in the internal organs of orally infected 1 week-old chickens (biovar Pullorum, Wigley *et al.*, 2002) nor in those of 3 week-old chickens (biovar Gallinarum, Jones *et al.*, 2001). The involvement of SPI-2 in systemic colonization by host-specific serotypes in chickens was attributed to intramacrophage survival (Wigley *et al.*, 2001). In this study, we have shown that in the host non-

specific serotype Enteritidis, SPI-2 promotes the systemic spread to internal organs and reproductive tract tissue leading to persistent colonization of these organs.

The SPI-2 regulator *ssrA* is not an important factor for the enteric phase of the infection with this host non-specific serotype Enteritidis after oral inoculation of chickens: caecal colonization was not affected by the mutation, while spleen and liver colonization showed reduced colonization levels. Also Morgan *et al.* (2004) could not detect a role of SPI-2 in caecal colonization of chickens, although these authors only studied colonization early post-infection. Moreover, in rabbit ileal loops SPI-2 seems not required for the induction of enteropathogenic responses to serotype Typhimurium (Everest *et al.*, 1999). SPI-2 is also not required for induction of enteritis in calves by *Salmonella* Typhimurium (Tsolis *et al.*, 1999a). In contrast, Morgan *et al.* (2004) and Tsolis *et al.* (1999b) identified SPI-2 as an essential factor for efficient gut colonization of cattle.

In conclusion, the SPI-2 regulator *ssrA* plays an important role in reproductive tract colonization of *Salmonella* Enteritidis in laying hens. *SsrA* however does not play a major role in colonization of the chicken gut after oral infections with the host non-specific serotype Enteritidis. The SPI-2 knock-out mutant thus appears to be safe for laying hens, a characteristic which could hold promise for the future development of live vaccines. To provide complete elimination of prolonged shedding and subsequently egg contamination via egg shell penetration, extra attenuation of a live vaccine, for example by mutation of the gene candidate *hilA* (Bohez *et al.*, 2006), is needed.

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Figure 1. Log₁₀CFU of *Salmonella* Enteritidis 76Sa88 wild type strain (●) and *Salmonella* Enteritidis Δ ssrA mutant (■) in the caeca (black lines), liver (grey lines) and spleen (light grey lines) of chicks dosed orally at one day-old and then euthanized at day 2, 3, 4, 7, 10, 14, 21, 28, 36 and 42 of age. Each time point represents four chicks, with the exception of day 36 and day 42. In the groups infected with *Salmonella* Enteritidis 76Sa88 wild type strain 13 and 16 animals were euthanized on day 36 and day 42, respectively. In the groups infected with *Salmonella* Enteritidis Δ ssrA mutant 6 and 9 chickens were euthanized on day 36 and day 42, respectively. The error bars present the standard error of the means (SEM).

Figure 2. Log₁₀CFU of *Salmonella* Enteritidis 76Sa88 wild type strain (●) and *Salmonella* Enteritidis Δ ssrA mutant (■) in the liver (black dots) and spleen (grey dots) of chicks infected intra-peritoneally at five days-old. At 4, 24 and 48 hrs post-infection six chickens per group were euthanized. Each dot represents one chick, if more; the number of birds is indicated by n=.

Figure 3. Liver and reproductive tract colonization by *Salmonella* Enteritidis 76Sa88 wild type strain (black bars) and *Salmonella* Enteritidis 76Sa88 ssrA mutant strain (grey bars) 14 days after intravenous infection of adult SPF laying hens.

Figure 1

Figure 2

Figure 3

Table 1. Numbers of chickens positive at cloacal sampling after oral inoculation at day of hatch with 10^8 cfu *Salmonella* Enteritidis 76Sa88 wild type strain (WT) or its Δ *ssrA* mutant strain (SsrA). Samples were taken at 2, 4, 7, 10, 14, 21, 28, 36 and 42 days of age.

^a Numbers of positive samples after enrichment / total number of samples.

^b Number of positive samples after direct plating.

Table 2. Egg production and number of *Salmonella* positive eggs 1 to 13 days following intra-venous infection of adult SPF laying hens with *Salmonella* Enteritidis 76Sa88 wild type strain (WT) or its Δ *ssrA* mutant strain (SsrA).

^a Numbers of eggs laid/ total number of laying hens.

^b Number of positive egg contents.

Table 1

	days of age								
	2	4	7	10	14	21	28	36	42
WT	61 ^a /61(61 ^b)	53/53(52)	49/49(48)	45/45(45)	41/41(40)	37/37(22)	28/33(14)	25/29(10)	14/16(3)
SsrA	62/62(62)	49/49(48)	41/41(38)	35/35(34)	27/27(25)	23/23(13)	12/19(7)	11/15(8)	4/9(2)

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Table 2

	days post-infection												
	1	2	3	4	5	6	7	8	9	10	11	12	13
WT	5 ^a /6(2 ^b)	1/6(0)	2/6(1)	1/6(0)	1/6(0)	1/6(0)	1/6(0)	2/6(0)	1/6(0)	1/6(0)	0/6(0)	1/6(0)	1/6(0)
SsrA	2/9 (0)	1/9(0)	2/9(1)	1/9(0)	1/9(0)	2/9(0)	6/9(0)	2/9(0)	2/9(0)	5/9(0)	2/9(0)	2/9(0)	5/9(0)