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

22

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

36

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

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## 45 1. Introduction

46

47 *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) is a facultative  
48 intracellular bacterium with a broad host range. It can cause foodborne illness in humans,  
49 following the consumption of chicken products (Guard-Petter, 2001). Poultry products become  
50 contaminated as asymptomatic carrier birds enter the slaughterhouse and asymptomatic carrier  
51 hens lay contaminated eggs. Infection of one day-old chickens with high doses of *Salmonella*  
52 Enteritidis can cause severe clinical salmonellosis with a high rate of mortality, while this serovar  
53 produces a chronic asymptomatic carrier state in low dosed infected young chickens and infected  
54 adult birds (Gast and Benson, 1995; Desmidt *et al.*, 1997; Van Immerseel *et al.*, 2004). These  
55 carrier birds may excrete the bacteria intermittently until slaughter age (6 weeks) in broilers until  
56 the end of the reproduction cycle in layer type chickens and thus constitute an important source  
57 of *Salmonella* infection (Gast and Benson, 1995; Desmidt *et al.*, 1997; Humphrey *et al.*, 1991).  
58 *Salmonella* Enteritidis specifically interacts with the reproductive organs of hens leading to egg  
59 contamination (De Buck *et al.*, 2004). Following oral ingestion, *Salmonella* colonizes the  
60 chicken gut, especially the caeca, where it penetrates the mucosal epithelium (Desmidt *et al.*,  
61 1997). Interaction between *Salmonella* and the epithelium triggers infiltration of phagocytic cells  
62 to the infected site, where these cells take up the bacteria. *Salmonella* is capable to survive and  
63 replicate within bacterially modified macrophage spacious phagosomes, which can be attributed  
64 to the type III secretion system (TTSS) encoded by *Salmonella* pathogenicity island 2 (SPI-2)  
65 (Knodler and Steele-Mortimer, 2003). The infected phagocytes disseminate to the internal  
66 organs, such as liver, spleen and the reproductive tissues and thus spread the bacteria within the  
67 host (Turnbull and Snoeyenbos, 1974; Barrow *et al.*, 1999).

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

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

80

## 81 **2. Materials and methods**

82

### 83 *2.1. Bacterial strain*

84

85 *Salmonella* Enteritidis phage type 4, strain 76Sa88 Nal<sup>r</sup> was used in the experiments. This  
86 strain was isolated from an outbreak of salmonellosis on a poultry farm. The nalidixic acid  
87 resistant strain and its 76Sa88 wild type progenitor strain are well-characterized (Bohez *et al.*,  
88 2006).

89

### 90 *2.2. Construction of defined mutant*

91

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

96

### 97 2.3. *In vitro* experiments

98

#### 99 2.3.1. *Invasion and survival in T84 and HD11 cell lines*

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

108

#### 109 2.3.2. *Cytotoxicity assay*

110

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

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

118

119 
$$\text{Cytotoxicity} = (\text{experimental value} - \text{low control}) / (\text{positive control} - \text{low control}) \times 100$$

120

#### 121 *2.4. Experimental inoculation of chickens*

122

123 The experimental protocols were approved by the ethical committee of the Faculty of  
124 Veterinary Medicine, Ghent University. Bacteria used for inoculation were grown for 20 h at  
125 37°C in Luria-Bertani Broth (LB) on a shaker platform. The number of CFU/ml was determined  
126 by plating 6 x 20 µl of a tenfold dilution series of the suspensions on BGA. Thereafter the plates  
127 were incubated aerobically for 20 h at 37°C. The suspensions were kept at 4°C until they were  
128 used in the assays. The bacterial cultures were diluted in phosphate buffered saline (PBS) to the  
129 respective inoculation density.

130

##### 131 *2.4.1. Oral inoculation of one day-old SPF chicks*

132

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

139 group at the beginning of the experiment and cultured for *Salmonella* as described below. The  
140 chickens had *ad libitum* access to drinking water and feed. One day-old chicks were randomly  
141 divided in two groups of 61 and 62 chickens and orally inoculated in the crop using a plastic tube  
142 with  $10^8$  colony forming units (CFU) of *Salmonella* Enteritidis 76Sa88 parent strain or its  
143 *Salmonella* Enteritidis  $\Delta$ *ssrA* mutant in a volume of 0.2 ml of PBS. At day 2, 4, 7, 10, 14, 21, 28,  
144 36 and 42 post-hatch cloacal swabs were taken for the detection of *Salmonella*. For  
145 bacteriological analysis of caeca, liver and spleen, four chickens per group were euthanized at  
146 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  
147 6 and 9 chickens were euthanized in the groups infected with *Salmonella* Enteritidis 76Sa88  
148 parent strain and its  $\Delta$ *ssrA* mutant, respectively.

149

#### 150 2.4.2. Intra-peritoneal inoculation of five day- old SPF chicks

151

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

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

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

167

#### 168 2.4.3. Intravenous inoculation of adult SPF laying hens

169

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

181

#### 182 2.4.4. Bacteriological analysis of cloacal swabs and organ samples

183

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

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

195 Samples that were negative after titration but positive after *Salmonella* enrichment were  
196 presumed to contain 10<sup>1</sup> CFU/g organs. Samples that were negative after enrichment were  
197 presumed to have 0 CFU/g. The mean CFU/g tissue was calculated for each group.

198

#### 199 2.4.5. Bacteriological examination of eggs

200

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

207

208 2.4.6. *Statistical analysis*

209

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

214 In the intra-peritoneal and intravenous infection experiment, statistical analysis of organ  
215 colonisation was performed by general analysis of variance and the independent T-test.,  
216 respectively. Statistical differences in egg production were determined by the chi-square analysis  
217 and differences in positive laid eggs by the Fisher's exact T-test. Differences with P-values below  
218 0.05 were significant. All statistical analyses were performed using SPSS 12.0.

219

220 **3. Results**

221

222 *3.1. Invasion in T84 human epithelial cells and uptake and proliferation in chicken macrophage*  
223 *HD11 cells*

224

225 No statistically significant differences were observed in invasion of both *Salmonella*  
226 Enteritidis wild type strain and its  $\Delta ssrA$  mutant strain in T84 cells and HD11 cells. The *ssrA*  
227 mutant was significantly ( $p=0.006$ ) reduced in its intramacrophage survival compared to the wild  
228 type strain. The relative intramacrophage survival percentage (6 h / 1 h) of the  $\Delta ssrA$  mutant was  
229 62 % of that of the wild type strain.

230

231 3.2. LDH assay

232  
233 The percentage of cytotoxicity at 6 h post-infection of HD11 cells with the *Salmonella*  
234 Enteritidis *ssrA* mutant strain ( $20\% \pm 2.2$ ) was similar to that of *Salmonella* Enteritidis 76Sa88  
235 wild type strain ( $21\% \pm 2.4$ ). No statistically significant differences could be observed. The  
236 results are calculated relative to triton-exposed cells (100 %).

237

238 3.3. In vivo trials

239  
240 The pathogenic responses may vary between different serotypes, even strains, and  
241 different host infection models.

242

243 3.3.1. Oral inoculation of one day- old SPF chicks

244

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

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

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

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

270

### 271 3.3.2. Intra-peritoneal infection of five day-old SPF chicks

272

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

276 post-infection. In about 50% of the animals *Salmonella* could not be detected at direct plating. At  
277 24 h post-infection, the *ssrA* mutant was statistically significantly reduced in liver and spleen  
278 colonization compared to its isogenic wild type strain. At direct plating, the *ssrA* mutant strain  
279 could not be isolated from any liver sample, whereas 5 of the 6 animals were positive for the wild  
280 type strain. Of the spleen samples, only 1 of 6 samples was positive at direct plating for the *ssrA*  
281 mutant, while all samples were positive for the wild type strain. Compared to its isogenic wild  
282 type strain, the numbers of *ssrA* mutant bacteria colonizing liver and spleen was about 100 and  
283 1000 fold, respectively, reduced (statistical significant with  $p < 0.05$ ). At 48 h post-infection, no  
284 statistically significant differences could be detected anymore, although a decrease in liver and  
285 spleen colonization by the *ssrA* mutant strain was still present.

286

### 287 3.3.3. Intravenous infection of adult SPF white LSL hens

288

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

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

300 lesions of oviducts and ovaries. The oviducts were reduced in size and the ovaries were shrunked  
301 and had discolored follicles. These pathological changes in the reproductive tract were absent in  
302 the birds infected with the *ssrA* mutant. Animals infected with the *ssrA* mutant showed a  
303 significantly ( $p < 0.01$ ) lower colonization in ovary and oviduct in comparison to animals infected  
304 with the wild type strain, i.e. a decrease of about log 2.4 and log 2.9, respectively. In addition, the  
305 oviduct samples of only 4 of 9 chickens were positive for the *ssrA* mutant strain at direct plating,  
306 while all oviduct samples were positive in birds infected with the wild type strain. Egg  
307 production was reduced after infection with both *Salmonella* Enteritidis parent strain and its *ssrA*  
308 mutant derivative (Table 2). From day 7 to day 13 post-infection, laying hens in the *ssrA* mutant  
309 inoculated group showed a significant higher egg production. At day 1 and 3 post-infection, 2  
310 and 1 egg(s) of a total number of 18 laid eggs, respectively, were found positive for *Salmonella*  
311 Enteritidis wild type strain (Table 2). The *ssrA* mutant strain could be isolated from the content of  
312 1 egg of a total number of 33 laid eggs at day 3 post-infection.

313

#### 314 4. Discussion

315

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

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

324 systemic spread and internal organ colonization. The reduced *ssrA* colony counts in the oviducts  
325 and ovaries are indicative for the requirement of SPI-2 by *Salmonella* Enteritidis to spread to the  
326 reproductive tract tissues. This is reflected in the fact that less reproductive tract lesions were  
327 observed in the group of animals inoculated with the *ssrA* mutant and the faster recovery of egg  
328 production after intravenous inoculation with the *ssrA* mutant. *In vitro* studies show that the *ssrA*  
329 mutant of *Salmonella* Enteritidis was as invasive as the parent strain in T84 intestinal epithelial  
330 cells but severely attenuated in intramacrophage survival in chicken macrophages. The ability of  
331 *Salmonella* Typhimurium to induce systemic infection in mice has been shown to be dependent  
332 on its survival within macrophages (Cirillo *et al.*, 1998; Shea *et al.*, 1999). Susceptible mice  
333 develop a severe systemic disease often resulting in death (Santos *et al.*, 2001; Mastroeni *et al.*,  
334 2004), as is the case with other host-specific serotypes in other animal species (Pardon *et al.*,  
335 1988; Uzzau *et al.*, 2000), including *Salmonella* Gallinarum biovar Pullorum in poultry (Pomeroy  
336 *et al.*, 1991; Snoeyenbos *et al.*, 1991). The *Salmonella* Pathogenicity Island 2 type three secretion  
337 system is an essential virulence locus for systemic disease in host-specific *Salmonella* serotypes  
338 (Jones *et al.*, 2001; Wigley *et al.*, 2002; Bispham *et al.*, 2001). Whereas an *ssrA* mutant in the  
339 host non-specific serotype Enteritidis did not show colonization defects in one day-old chicks, the  
340 *ssrA* mutant in the host-specific serotype Gallinarum was found to be fully attenuated for its  
341 virulence in one day-old chicks following oral infection (Shah *et al.*, 2005). In addition, a SPI-2  
342 mutant of *Salmonella* Gallinarum carrying a mutation in the *ssaU* gene was neither detected in  
343 the internal organs of orally infected 1 week-old chickens (biovar Pullorum, Wigley *et al.*, 2002)  
344 nor in those of 3 week-old chickens (biovar Gallinarum, Jones *et al.*, 2001). The involvement of  
345 SPI-2 in systemic colonization by host-specific serotypes in chickens was attributed to  
346 intramacrophage survival (Wigley *et al.*, 2001). In this study, we have shown that in the host non-

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

349         The SPI-2 regulator *ssrA* is not an important factor for the enteric phase of the infection  
350 with this host non-specific serotype Enteritidis after oral inoculation of chickens: caecal  
351 colonization was not affected by the mutation, while spleen and liver colonization showed  
352 reduced colonization levels. Also Morgan *et al.* (2004) could not detect a role of SPI-2 in caecal  
353 colonization of chickens, although these authors only studied colonization early post-infection.  
354 Moreover, in rabbit ileal loops SPI-2 seems not required for the induction of enteropathogenic  
355 responses to serotype Typhimurium (Everest *et al.*, 1999). SPI-2 is also not required for induction  
356 of enteritis in calves by *Salmonella* Typhimurium (Tsolis *et al.*, 1999a). In contrast, Morgan *et al.*  
357 (2004) and Tsolis *et al.* (1999b) identified SPI-2 as an essential factor for efficient gut  
358 colonization of cattle.

359 In conclusion, the SPI-2 regulator *ssrA* plays an important role in reproductive tract colonization  
360 of *Salmonella* Enteritidis in laying hens. *SsrA* however does not play a major role in colonization  
361 of the chicken gut after oral infections with the host non-specific serotype Enteritidis. The SPI-2  
362 knock-out mutant thus appears to be safe for laying hens, a characteristic which could hold  
363 promise for the future development of live vaccines. To provide complete elimination of  
364 prolonged shedding and subsequently egg contamination via egg shell penetration, extra  
365 attenuation of a live vaccine, for example by mutation of the gene candidate *hilA* (Bohez *et al.*,  
366 2006), is needed.

367

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369

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373

#### 374 **References**

375

376 Barrow, P.A., 1999. Virulence of *Salmonella enterica* serovar Enteritidis. In : Saeed, A.M. (ed.).  
377 *Salmonella enterica* serovar Enteritidis in humans and animals, Iowa State University Press,  
378 Ames, USA, p. 173-181.

379

380 Bispham, J., Tripathi, B.N., Watson, P.R., Wallis, T.S., 2001. *Salmonella* pathogenicity island 2  
381 influences both systemic salmonellosis and *Salmonella*-induced enteritis in calves. Infect.  
382 Immun. 69, 367-377.

383

384 Bohez, L., Ducatelle, R., Pasmans, F., Botteldoorn, N., Haesebrouck, F., Van Immerseel, F.,  
385 2006. *Salmonella enterica* serovar Enteritidis colonization of the chicken caecum requires the  
386 HilA regulatory protein. Vet Microbiol. 116, 202-210.

387

388 Cirillo, D.M., Valdivia, R.H., Monack, D.M., Falkow, S., 1998. Macrophage-dependent  
389 induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in  
390 intracellular survival. Mol Microbiol. 30, 175-188.

391

392 Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *Escherichia*  
393 coli K-12 using PCR products. Proc. Natl. Acad. Sci. USA. 97, 6640-6645.

394  
395 De Buck, J., Van Immerseel, F. , Haesebrouck, F. , Ducatelle R., 2004. Colonization of the  
396 chicken reproductive tract and egg contamination by *Salmonella*. J. Appl. Microbiol. 97, 233–  
397 245.  
398  
399 Desmidt, M., Ducatelle, R., Haesebrouck, F., De Groot, P.A., Verlinden, M., Wijffels, R.,  
400 Hinton, M., Bale, J.A., Allen, V.M., 1996. Detection of antibodies to *Salmonella* Enteritidis in  
401 sera and yolks from experimentally and naturally infected chickens. Vet. Rec. 1338, 223-226.  
402  
403 Desmidt, M., Ducatelle, R., Haesebrouck, F., 1997. Pathogenesis of *Salmonella* Enteritidis phage  
404 type four after experimental infection of young chickens. Vet. Microbiol. 56, 99-109.  
405  
406 Everest, P., Ketley, J., Hardy, S., Douce, G., Khan, S., Shea, J., Holden, D., Maskell, D., Dougan.  
407 G., 1999. Evaluation of *Salmonella* typhimurium mutants in a model of experimental  
408 gastroenteritis. Infect. Immun. 67, 2815-2821.  
409  
410 Gast, R.K., Benson, S.T., 1995. The comparative virulence for chicks of *Salmonella* Enteritidis  
411 phage type 4 isolates and isolates of phage types commonly found in poultry in the United States.  
412 Avian Dis. 39, 567-574.  
413  
414 Guard-Petter, J., 2001. The chicken, the egg and *Salmonella* Enteritidis. Environ. Microbiol. 3,  
415 421-430.  
416

- 417 Humphrey, T.J., Chart, H., Baskerville, A. and Rowe B. 1991. The influence of age on the  
418 response of SPF hens to infection with *Salmonella* Enteritidis PT4. *Epidemiol Infect.* 106, 33–43.  
419
- 420 Jones, M.A., Wigley, P., Page, K.L. Hulme, S.D., Barrow, P.A., 2001. *Salmonella* enterica  
421 serovar Gallinarum requires the *Salmonella* pathogenicity island 2 type III secretion system but  
422 not the *Salmonella* pathogenicity island 1 type III secretion system for virulence in chickens.  
423 *Infect. Immun.* 69, 5471-5476.  
424
- 425 Knodler, L.A., Steele-Mortimer, O., 2003. Taking Possession: Biogenesis of the *Salmonella*-  
426 Containing Vacuole. *Traffic.* 4, 587-599.  
427
- 428 Löber, S., Jäckel, D., Kaiser, N., Hensel, M., 2006 Regulation of *Salmonella* pathogenicity island  
429 2 genes by independent environmental signals. *International Journal of Medical Microbiology*  
430 296, 435-447.  
431
- 432 Mastroeni, P., Sheppard, M., 2004. *Salmonella* infections in the mouse model: host resistance  
433 factors and in vivo dynamics of bacterial spread and distribution in the tissues. *Microbes Infect.*  
434 6, 398-405.  
435
- 436 Morgan, E., Campbell, J.D., Rowe, S.C., Bispham, J., Stevens, M.P., Bowen, A., Barrow, P.A.,  
437 Maskell, D.J., Wallis, T.S., 2004. Identification of host-specific colonization factors of  
438 *Salmonella enterica* serovar Typhimurium. *Mol. Microbiol.* 54, 994-1010.  
439

440 Pardon, P., Sanchis, R., Marly, J., Lantier, F., Pepin, M., Popoff, M., 1988. [Ovine salmonellosis  
441 caused by *Salmonella abortus ovis*] *Ann Rech Vet.* 19, 221-235. Review. French.

442  
443 Pomeroy, B. S., Nagaraja, K. V, 1991. Fowl typhoid. In B. W. Calnek, H. J. Barnes, C. W. Beard,  
444 W. M. Reid, and H. W. Yoder (ed.), *Diseases of poultry*. Iowa State University Press, Ames. p.  
445 87-99.

446  
447 Santos, R.L., Zhang, S., Tsohis, R.M., Kingsley, R.A., Adams, L.G., Baumler, A.J., 2001. Animal  
448 models of *Salmonella* infections: enteritis versus typhoid fever. *Microbes Infect.* 3, 1335-44.

449  
450 Shah, D.H., Lee, M.J., Park, J.H., Lee, J.H., Eo, S.K., Kwon, J.T., Chae, J.S., 2005. Identification  
451 of *Salmonella* gallinarum virulence genes in a chicken infection model using PCR-based  
452 signature-tagged mutagenesis. *Microbiology.* 151, 3957-3968.

453  
454 Shea, J.E., Beuzon, C.R., Gleeson, C., Mundy, R., Holden, D.W., 1999. Influence of the  
455 *Salmonella* typhimurium pathogenicity island 2 type III secretion system on bacterial growth in  
456 the mouse. *Infect. Immun.* 67:213-219.

457  
458 Snoeyenbos, G. H., 1991. Pullorum disease. In B. W. Calnek, H. J. Barnes, C. W. Beard, W. M.  
459 Reid, and H. W. Yoder (ed.), *Diseases of poultry*. Iowa State University Press, Ames. p. 73-86.

460  
461 Tsohis, R.M., Adams, L.G., Ficht, T.A., Baumler, A.J., 1999a. Contribution of *Salmonella*  
462 typhimurium virulence factors to diarrheal disease in calves. *Infect. Immun.* 67, 4879-4885.

463

- 464 Tsolis, R.M., Townsend, S.M., Miao, E.A., Miller, S.I., Ficht, T.A., Adams, L., Baumler, A.J.,  
465 1999b. Identification of a putative *Salmonella enterica* serotype typhimurium host range factor  
466 with homology to IpaH and YopM by signature-tagged mutagenesis. *Infect. Immun.* 67, 6385-  
467 6393.
- 468
- 469 Turnbull, P.C., Snoeyenbos, G.H., 1974. Experimental salmonellosis in the chicken. 1. Fate and  
470 host response in alimentary canal, liver, and spleen. *Avian Dis.* 18, 153-77.
- 471
- 472 Uzzau, S., Brown, D.J., Wallis, T.S., Rubino, S., Leori, G., Bernard, S., Casadesus, J., Platt, D.J.,  
473 Olsen, J.E., 2000. Host adapted serotypes of *Salmonella enterica*. *Epidemiol. Infect.* 125, 229-  
474 255.
- 475
- 476 Van Immerseel, F., De Buck, J., Pasmans, F., Bohez, L., Boyen, F., Haesebrouck, F. and  
477 Ducatelle, R. 2004. Intermittent long-term shedding and induction of carrier birds after infection  
478 of chickens early posthatch with a low or high dose of *Salmonella* Enteritidis. *Poult Sci.* 83,  
479 1911-1916.
- 480
- 481 Wigley, P., Berchieri, A., Jr, Page, K.L., Smith, A.L., Barrow, P.A., 2001. *Salmonella enterica*  
482 serovar Pullorum persists in splenic macrophages and in the reproductive tract during persistent,  
483 disease-free carriage in chickens. *Infect. Immun.* 69, 7873-7879.
- 484
- 485 Wigley, P., Jones, M.A., Barrow, P.A., 2002. *Salmonella enterica* serovar Pullorum requires the  
486 *Salmonella* pathogenicity island 2 type III secretion system for virulence and carriage in the  
487 chicken. *Avian Pathol.* 31, 501-506.

**Figure 1.** Log<sub>10</sub>CFU of *Salmonella* Enteritidis 76Sa88 wild type strain (●) and *Salmonella* Enteritidis  $\Delta$ *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  $\Delta$ *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.** Log<sub>10</sub>CFU of *Salmonella* Enteritidis 76Sa88 wild type strain (●) and *Salmonella* Enteritidis  $\Delta$ *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.

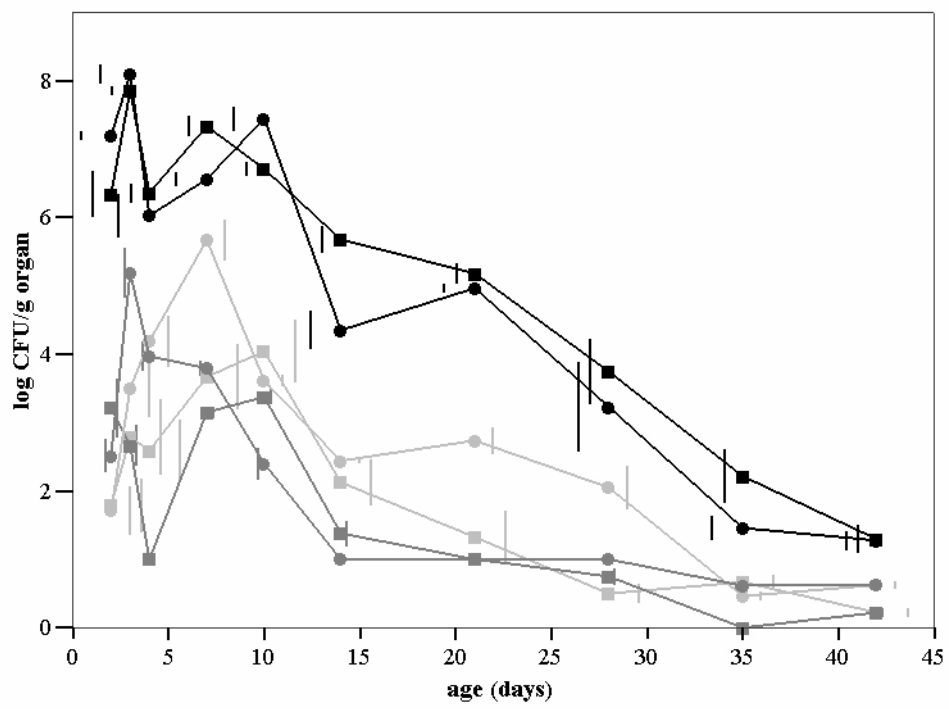


Figure 1

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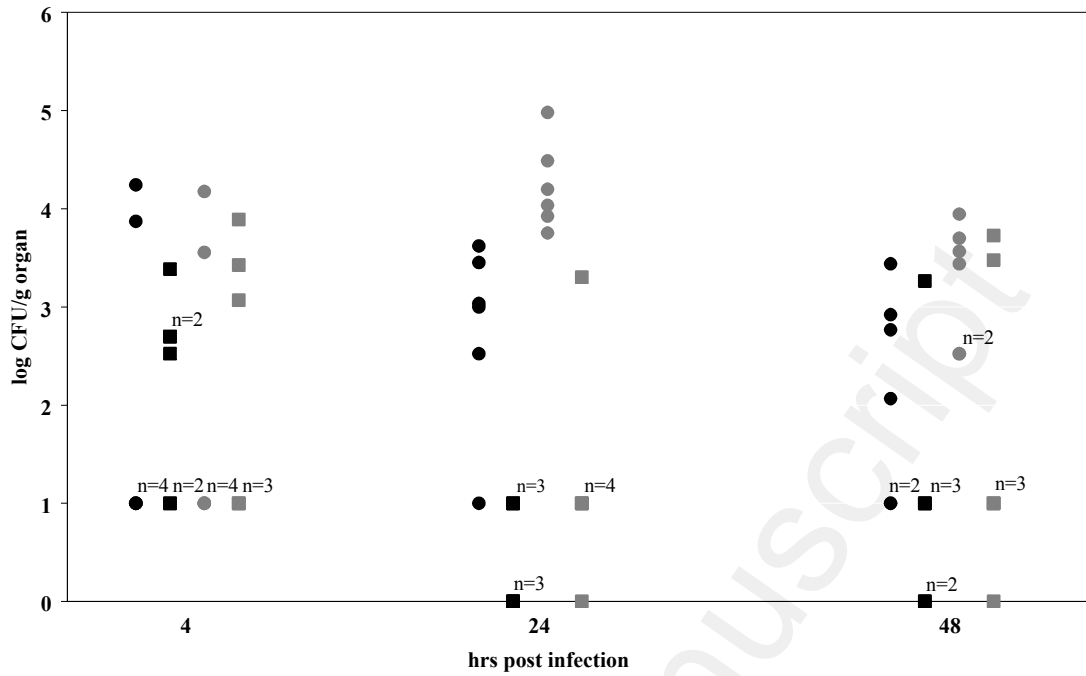


Figure 2

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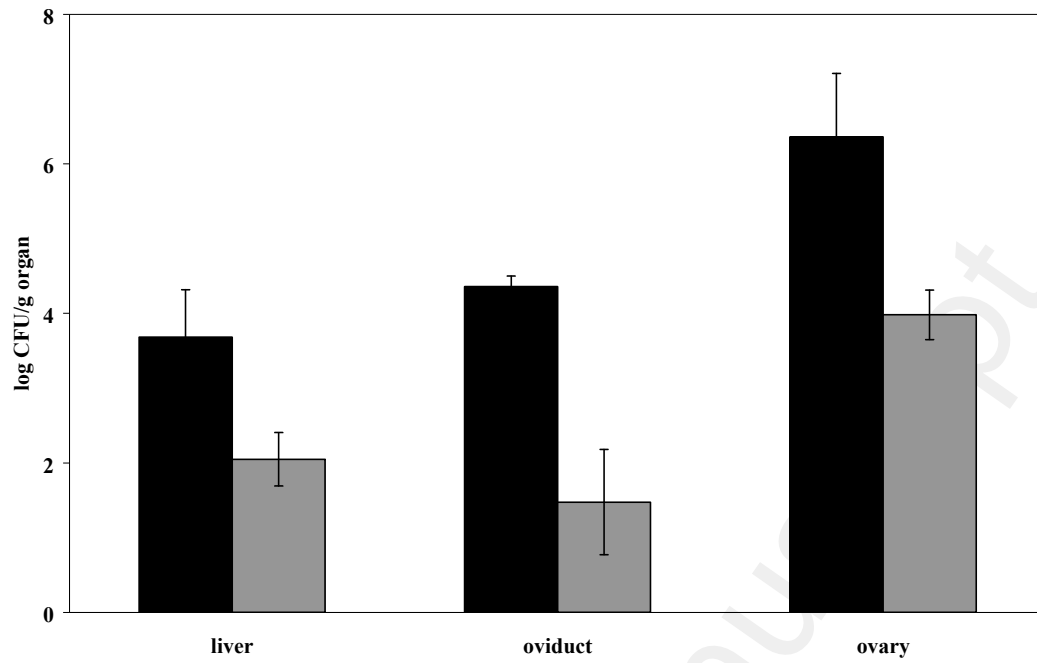


Figure 3

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**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  $\Delta$ *ssrA* mutant strain (SsrA). Samples were taken at 2, 4, 7, 10, 14, 21, 28, 36 and 42 days of age.

<sup>a</sup> Numbers of positive samples after enrichment / total number of samples.

<sup>b</sup> 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  $\Delta$ *ssrA* mutant strain (SsrA).

<sup>a</sup> Numbers of eggs laid/ total number of laying hens.

<sup>b</sup> Number of positive egg contents.

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Table 1

	days of age								
	2	4	7	10	14	21	28	36	42
WT	61 <sup>a</sup> /61(61 <sup>b</sup> )	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 <sup>a</sup> /6(2 <sup>b</sup> )	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)

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