



HAL
open science

Salmonella Typhimurium resides largely as an extracellular pathogen in porcine tonsils, independently of biofilm-associated genes csgA, csgD and adrA

Alexander van Parys, Filip Boyen, Jiri Volf, Elin Verbrugge, Bregje Leyman, Ivan Rychlik, Freddy Haesebrouck, Frank Pasmans

► **To cite this version:**

Alexander van Parys, Filip Boyen, Jiri Volf, Elin Verbrugge, Bregje Leyman, et al.. Salmonella Typhimurium resides largely as an extracellular pathogen in porcine tonsils, independently of biofilm-associated genes csgA, csgD and adrA. Veterinary Microbiology, 2009, 144 (1-2), pp.93. 10.1016/j.vetmic.2009.12.021 . hal-00514613

HAL Id: hal-00514613

<https://hal.science/hal-00514613>

Submitted on 3 Sep 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: *Salmonella* Typhimurium resides largely as an extracellular pathogen in porcine tonsils, independently of biofilm-associated genes *csgA*, *csgD* and *adrA*

Authors: Alexander Van Parys, Filip Boyen, Jiri Volf, Elin Verbrugghe, Bregje Leyman, Ivan Rychlik, Freddy Haesebrouck, Frank Pasmans



PII: S0378-1135(09)00612-9
DOI: doi:10.1016/j.vetmic.2009.12.021
Reference: VETMIC 4718

To appear in: *VETMIC*

Received date: 9-11-2009
Revised date: 11-12-2009
Accepted date: 16-12-2009

Please cite this article as: Van Parys, A., Boyen, F., Volf, J., Verbrugghe, E., Leyman, B., Rychlik, I., Haesebrouck, F., Pasmans, F., *Salmonella* Typhimurium resides largely as an extracellular pathogen in porcine tonsils, independently of biofilm-associated genes *csgA*, *csgD* and *adrA*, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.12.021

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25

TITLE

***Salmonella* Typhimurium resides largely as an extracellular pathogen
in porcine tonsils, independently of biofilm-associated genes *csgA*,
csgD and *adrA***

RUNNING TITLE

***Salmonella* Typhimurium: an extracellular pathogen in porcine tonsils**

Alexander Van Parys^{a,*}, Filip Boyen^a, Jiri Volf^b, Elin Verbrugghe^a, Bregje Leyman^a, Ivan
Rychlik^b, Freddy Haesebrouck^a and Frank Pasmans^a

^a University of Ghent, Faculty of Veterinary Medicine, Dept. of Pathology, Bacteriology and
Avian Diseases, Salisburylaan 133, 9820 Merelbeke, Belgium

^b Veterinary Research Institute, Hudcova 70, 621 00 Brno, Czech Republic

* Corresponding author: University of Ghent, Faculty of Veterinary Medicine, Dept. of
Pathology, Bacteriology and Avian Diseases, Salisburylaan 133,
9820 Merelbeke, Belgium

Tel: +32 9 264 73 76

Fax: +32 9 264 74 94

E-mail address: alexander.vanparys@ugent.be (A. Van Parys)

26 **Abstract**

27 Persistent *Salmonella* Typhimurium infections in pigs are a major concern for food
28 safety and human health. Tonsils play a key role in the persistence of *Salmonella*
29 Typhimurium in pigs. Previous studies indicated that *Salmonella* virulence genes involved in
30 invasion and intracellular survival are of little importance for the colonization of porcine
31 tonsils, suggesting a predominantly extracellular location of the *Salmonella* bacteria. Biofilm
32 formation might promote extracellular persistence of *Salmonella* Typhimurium. The aim of
33 this study was to determine whether the bacterium resides predominantly intra- or
34 extracellularly in tonsils of pigs and to examine the contribution of biofilm-associated genes
35 *csgA*, *csgD* and *adrA* in *Salmonella* persistence in porcine tonsils. Single cell suspensions
36 were prepared from tonsils of orally inoculated pigs (2×10^7 colony forming units (CFU) wild
37 type *Salmonella* Typhimurium) to determine the ratio of extracellular versus intracellular
38 bacteria. Both at 5 and 28 days post inoculation (pi), the majority of *Salmonella* bacteria was
39 found extracellularly in porcine tonsils. To determine the contribution of biofilm formation in
40 extracellular persistence, pigs were orally inoculated with a mixture of 2×10^7 CFU of the
41 *Salmonella* Typhimurium wild type strain and 2×10^7 CFU of one of the *Salmonella*
42 Typhimurium *csgA*, *csgD* or *adrA* mutants. At ten days pi, equal numbers of both wild type
43 and mutant *Salmonella* bacteria were found not only in tonsils, but also in ileum, ileum
44 contents, ileocecal lymph nodes and faeces.

45 In conclusion, we showed that *Salmonella* Typhimurium resides extracellularly in
46 porcine tonsils, using a biofilm independent mechanism.

47

48 **Keywords:** *Salmonella* Typhimurium; Pig; Extracellular; Biofilm; Tonsil

49

50

51 1. Introduction

52 In European countries, *Salmonella enterica* subspecies *enterica* serovar Typhimurium
53 (*Salmonella* Typhimurium) is the serovar most frequently isolated from slaughter pigs
54 (Anon., 2007 and 2008). Porcine carcass contamination with *Salmonella* Typhimurium can
55 largely be attributed to persistently infected pigs (Botteldoorn et al., 2003). In most cases the
56 bacterium will subclinically colonize the palatine tonsils, secondary lymphoid organs situated
57 at the oropharynx. They play a vital role in both innate and adaptive immune responses due to
58 the presence of antimicrobial peptides and a variety of immune cells (Perry and Whyte, 1998;
59 Horter et al., 2003). Even though tonsils are a predilection site for *Salmonella* persistence in
60 pigs (Wood et al., 1989), virulence mechanisms necessary for cell invasion and intracellular
61 survival do not contribute to tonsillar colonization (Boyen et al., 2006b and 2008b),
62 suggesting that *Salmonella* Typhimurium resides mainly extracellularly in porcine tonsils.

63 Biofilm formation is a mechanism used by several bacteria to survive in an
64 extracellular context or in hostile environments with for example low pH or limited oxygen
65 (Rosenberg et al., 2008; Boyen et al., 2009). Biofilms are a multicellular behaviour mode, in
66 which bacteria are embedded into a self-produced extracellular matrix. Regulation of biofilm
67 formation in *Salmonella* Typhimurium has been intensively studied in the past years and
68 several genes playing a role in *Salmonella* Typhimurium biofilm formation were identified
69 (Römling, 2005). For example, CsgD stimulates biofilm production indirectly by
70 transcriptional activation of the curli biosynthesis operon *csgDEFG-csgBAC* and *adrA*, a
71 gene crucial for cellulose biosynthesis (Römling, 2005). The role of biofilm formation in
72 *Salmonella* Typhimurium persistence in pigs is still unknown.

73 It was the aim of the present study to determine whether *Salmonella* Typhimurium
74 persists intracellularly or extracellularly in tonsils of pigs. Additionally, the role of biofilm
75 formation in persistence of *Salmonella* Typhimurium in porcine tonsils was determined.

76 2. Materials and Methods

77 2.1 Bacterial strains and growth conditions

78 *Salmonella* Typhimurium strain 112910a, phage type 120/ad, was isolated from a pig
79 stool sample and characterized previously (Boyen et al., 2008b). A spontaneous nalidixic acid
80 resistant derivative of the wild type strain (WT_{nal}) was used in the first *in vivo* experiment to
81 minimize irrelevant bacterial growth when plating tonsillar, intestinal and faecal samples. In
82 the second *in vivo* experiment, the WT_{nal} strain was used together with one of the 3 deletion
83 mutants *csgA*, *csgD* or *adrA*. These deletion mutants were constructed according to the one-
84 step inactivation method described by Datsenko and Wanner (2000) and slightly modified for
85 use in *Salmonella* Typhimurium as described before (Boyen et al., 2006a). Primers used to
86 create the gene-specific linear PCR fragments are summarized in Table 1.

87 For the oral inoculation of pigs, the bacteria were grown for 16 h at 37 °C in 5 ml
88 Luria-Bertani broth (LB; Sigma Aldrich Chemie GmbH, Steinheim, Germany) on a shaker.
89 The bacteria were washed twice in Hank's buffered salt solution (HBSS; Gibco Life
90 Technologies, Paisley, Scotland) and centrifuged at 2300 x g for 10 min at 4 °C and diluted in
91 HBSS to the appropriate concentration of 10⁷ colony forming units (CFU) per ml. The
92 number of viable *Salmonella* bacteria per ml inoculum was determined by plating 10-fold
93 dilutions on brilliant green agar (BGA; Lab M Limited, Lancashire, UK) supplemented with
94 20 µg/ml nalidixic acid (BGA^{NAL}; Sigma Aldrich Chemie GmbH, Steinheim, Germany) for
95 selective growth of the WT_{nal} strain and BGA supplemented with 25 µg/ml chloramphenicol
96 (BGA^{CA}; Sigma Aldrich Chemie GmbH, Steinheim, Germany) for selective growth of the
97 mutant strains.

98 The *in vitro* colony morphology exhibited by the wild type *Salmonella* Typhimurium
99 and the *csgA*, *csgD* and *adrA* mutants on agar at different incubation temperatures was
100 examined by plating the strains on LB without salt, supplemented with 20 µg/ml Coomassie

101 Brilliant Blue (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 40 µg/ml Congo Red
102 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) as described by Malcova et al. (2008).
103 The plates were incubated at 28 °C and 37 °C for 7 consecutive days and were then visually
104 examined.

105

106 2.2 Experimental animals

107 The animal experiments were approved by the ethical committee of the Faculty of
108 Veterinary Medicine, Ghent University (EC 2007/052 and EC 2008/074).

109 In both *in vivo* trials, 4-week-old piglets (commercial closed line based on Landrace)
110 from a serologically *Salmonella* negative breeding herd were used. The piglets tested negative
111 for *Salmonella* at faecal sampling. The piglets arrived at the facility 7 days before they were
112 inoculated and were housed in separate isolation units at 25°C under natural day-night rhythm
113 with ad libitum access to feed and water.

114

115 2.3 Localization of *Salmonella* Typhimurium in porcine tonsils

116 **Experimental inoculation of piglets**

117 One week after their arrival at the facility, 12 individually housed experimental
118 animals were orally inoculated with approximately 2×10^7 CFU of a stationary phase culture
119 of the WT_{nal} in 2 ml HBSS. Five and 28 days post oral inoculation (pi), 8 and 4 piglets
120 respectively were humanely euthanized. Samples of tonsils were collected to prepare 'single
121 cell suspensions' and cryosections.

122

123 **Localization of *Salmonella* Typhimurium in porcine tonsils using the single cell** 124 **suspension method**

125 Adhering muscle and fat tissue was removed from the tonsillar tissue and the tonsil
126 was rinsed once with HBSS. The tissue was subsequently treated with 10 mM dithiothreitol
127 (DTT; Bio-Rad Laboratories, Hercules, California, USA) for 10 min to remove mucus rests.
128 After tissue samples were cut to small pieces, they were treated twice with 25 ml collagenase
129 from *Clostridium histolyticum* Type V (100 u/ml; Sigma-Aldrich Chemie GmbH, Steinheim,
130 Germany) on a shaker for 45 min at 37 °C to obtain a single cell suspension. The single cell
131 suspension was then filtered through a 64 µm pore size filter and transferred to a 50 ml tube.
132 The number of cells per ml as well as their viability was determined using trypan blue
133 staining and a counting chamber (Bürker, Marienfeld, Germany).

134 The single cell suspension was further processed as follows. Firstly, 500 µl of the cell
135 suspension was treated with 500 µl Triton X-100 2% (Sigma Aldrich Chemie GmbH,
136 Steinheim, Germany) and incubated for 10 min at room temperature on a shaker, causing cell
137 lysis, and plated on BGA^{NAL} to assess the total number of intra- and extracellular *Salmonella*
138 bacteria in the tonsillar tissue. Secondly, the remaining cell suspension was centrifuged (2300
139 x g, 10 min, 4 °C) and the obtained cell pellet was resuspended in 5 ml RPMI (Gibco Life
140 Technologies, Paisley, Scotland) supplemented with 100 µg/ml gentamicin (Gibco Life
141 Technologies, Paisley, Scotland) and incubated for 1 h at 37 °C on a shaker, killing
142 extracellular salmonellae. The suspension was then centrifuged (2300 x g, 10 min, 4 °C) and
143 washed three times with HBSS. The cells were counted as described above. Five hundred µl
144 of this cell suspension was treated with 500 µl Triton X-100 2% and incubated for 10 min at
145 room temperature on a shaker, causing cell lysis, and plated on BGA^{NAL} to assess the number
146 of intracellular salmonellae. If negative at direct plating, the samples were pre-enriched for 16
147 h in BPW at 37 °C, enriched for 16 h at 37 °C in tetrathionate broth (Merck KGaA,
148 Darmstadt, Germany) and plated again on BGA^{NAL}. Samples that were negative after direct
149 plating but positive after enrichment were presumed to contain 50 CFU *Salmonella* per gram

150 tissue (detection limit for direct plating). Samples that remained negative after enrichment
151 were presumed to contain less than 50 CFU *Salmonella* per gram. After determining the
152 overall CFU of *Salmonella* bacteria and the intracellular CFU, the percentage of bacteria
153 residing extracellularly in porcine tonsils was calculated.

154

155 **Localization of *Salmonella* Typhimurium in porcine tonsils using immunohistochemical** 156 **staining of cryosections**

157 A small piece of the tonsil was embedded in a capsule with KP-CryoBlock (Klinipath,
158 Duiven, The Netherlands), frozen in liquid nitrogen and stored at -70 °C. The frozen tissue
159 parts were sectioned at 8 µm with a cryotome, dried for 1 h at 37°C and fixed in acetone for 5
160 min. The cryosections were washed with distilled water and incubated with HBSS + 0.2%
161 saponin (Sigma Aldrich Chemie GmbH, Steinheim, Germany) for 20 min at room
162 temperature. Image-iT FX signal enhancer (Invitrogen, Oregon, USA) was brought onto the
163 sections for 30 min at room temperature. Tissue sections were washed twice with HBSS +
164 0.2% saponin. The sections were pre-incubated with 100 µl 30% (v/v) goat serum for 30 min
165 at room temperature to reduce background staining and washed twice with HBSS + 0.2%
166 saponin. Sections were incubated for 45 min at room temperature with 100 µl of a primary
167 antibody mixture containing a polyclonal rabbit antibody targeting the *Salmonella* O4 somatic
168 antigen (Pro Lab Diagnostics, Neston, UK) and a mouse monoclonal antibody targeting
169 CD172α (AbD Serotec, Kidlington, UK), diluted respectively 1:100 and 1:50 in HBSS +
170 0.2% saponin. Thereafter, they were washed twice with HBSS + 0.2% saponin. CD172α is
171 defined as a specific myelomonocytic transmembrane receptor of 90-115 kDa molecular
172 weight (Piriou-Guzylack and Salmon, 2008). The sections were subsequently incubated for 30
173 min at room temperature with 100 µl of a secondary antibody mixture containing goat anti-
174 rabbit Alexa Fluor 488 (Molecular Probes, Oregon, USA) and goat anti-mouse Alexa Fluor

175 568 (Molecular Probes, Oregon, USA), both diluted 1:200 in HBSS + 0.2% saponin and
176 washed twice with HBSS + 0.2% saponin. The sections were dried in the dark at room
177 temperature and fixed with VectaShield (Vector Laboratories Inc., Burlingame, USA).
178 Sections were evaluated using a Leica DMRB fluorescence microscope (Leica Inc., Wetzlar,
179 Germany). Pictures were taken using a Moticam 2300 3MP camera (Moticam Instruments
180 Inc., Richmond, Canada) and Motic Images Advanced 3.2 (Motic China Group Co. Ltd.)
181 imaging software. Subsequently, 100 bacteria in each tonsil sample were visually counted and
182 the percentage of bacteria residing intracellularly was calculated.

183

184 2.4 The role of biofilm formation in *Salmonella* Typhimurium persistence in pigs

185 **Experimental inoculation of piglets**

186 Twenty-one experimental piglets were randomly divided in 3 groups of 6 animals,
187 housed in 3 pairs per group, and 1 negative control group of 3 animals. Five days later, the 3
188 negative control pigs were humanely euthanized and samples of tonsils, ileum, ileum
189 contents, ileocecal lymph nodes and faeces were collected for bacteriological analysis. One
190 week after their arrival at the facility, each group of 6 piglets was orally inoculated with a
191 mixture of approximately 2×10^7 CFU WT_{nal} and 2×10^7 CFU of one of the three
192 chloramphenicol-resistant deletion mutants *csgA*, *csgD* or *adrA* in 2 ml HBSS. Ten days after
193 oral inoculation, all 18 piglets were humanely euthanized and samples of tonsils, ileum, ileum
194 contents, ileocecal lymph nodes and faeces were collected for bacteriological analysis.

195

196 **Bacteriological analysis**

197 All tissue and contents samples were stored for maximum 1 week at -70 °C until
198 further processing. The samples were thawed, weighed and 10% (w/v) suspensions were
199 made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was

200 homogenized with a Colworth stomacher 400 (Seward and House, London, UK). The
201 homogenized samples were examined for the presence of the wild type *Salmonella*
202 Typhimurium and the mutants by plating 10-fold dilutions on BGA^{NAL} and BGA^{CA}
203 respectively. BGA plates were incubated for 16 h at 37 °C. The samples were pre-enriched for
204 16 h in BPW at 37 °C and, if negative at direct plating, enriched for 16 h at 37 °C in
205 tetrathionate broth and then again plated on BGA^{NAL} and BGA^{CA}. Samples that were negative
206 after direct plating but positive after enrichment were presumed to contain 50 CFU
207 *Salmonella* per gram tissue (detection limit for direct plating). Samples that remained
208 negative after enrichment were presumed to contain less than 50 CFU *Salmonella* per gram
209 tissue and were assigned value '1' prior to further calculation.

210 After determining the number of wild type and mutant CFU for all samples derived
211 from all piglets, these data were converted logarithmically prior to calculation of the average
212 differences between log values of WT and mutant per tissue sample per group and prior to
213 statistical analysis.

214

215 2.5 Statistical analysis

216 For analysis of the results from the mixed infection *in vivo* trial, an unpaired student's
217 *t*-test was performed to determine whether the log₁₀ value of the wild type/mutant ratio of the
218 samples was significantly different from the log₁₀ value of the wild type/mutant ratio in the
219 inoculum, using the SPSS Statistics 17.0 software (SPSS Inc., Chicago, USA). Differences
220 with a *P*-value ≤ 0.05 were considered significant.

221

222 **3. Results**

223 3.1 *Salmonella* Typhimurium predominantly resides extracellularly in porcine tonsils

224 Five and 28 days post oral inoculation (pi), 97.0 ± 2.0 % and 97.6 ± 3.6 % of the
225 *Salmonella* bacteria respectively was found to reside extracellularly in the tonsils using the
226 single cell suspension method.

227 Using immunohistochemistry on cryosections of tonsils, 97.4 ± 1.6 % and 86.0 ± 11.6
228 % of *Salmonella* bacteria resided extracellularly at 5 and 28 days pi respectively, confirming
229 the results obtained by single cell suspensions.

230

231 3.2 Biofilm formation does not contribute to extracellular persistence of *Salmonella* 232 *Typhimurium* in porcine tonsils

233 After 7 days incubation at 28 °C, the wild type *Salmonella* Typhimurium grew as a
234 biofilm-associated rough and dry (cf. RDAR) morphotype on LB without salt, supplemented
235 with Coomassie Brilliant Blue and Congo Red (Malcova et al., 2008). After incubation at 37
236 °C, the wild type strain no longer exhibited the RDAR morphotype but the smooth and white
237 (SAW) morphotype, indicating the absence of biofilm formation under these conditions
238 (Malcova et al, 2008). The biofilm-associated RDAR morphotype was absent in all of the
239 three *Salmonella* Typhimurium mutants at 28 °C as well as at 37 °C, giving the colonies a
240 white ($\Delta csgD$), purple/red ($\Delta csgA$) and purple/red ($\Delta adrA$) colour and a smooth appearance
241 (cf. SAW morphotype) (Fig. 1).

242 Following bacteriological analysis, the *csgA* mutant strain was found to be slightly
243 impaired in the ileum compared to the wild type strain. The opposite was true for the faeces,
244 in which the wild type *Salmonella* was impaired in colonization and persistence capacity
245 compared to the $\Delta csgD$ *Salmonella* Typhimurium. Nonetheless, these differences were not
246 significant due to high standard deviations with *P*-values of 0.24 and 0.29 respectively.
247 Furthermore, in none of the other samples of all three groups the output ratio wild type/mutant
248 was significantly different from the wild type/mutant ratio in the inoculum. This finding

249 showed that none of the *csgA*, *csgD* or *adrA* *Salmonella* mutants were significantly
250 impaired in colonization and persistence capacity compared to the wild type strain in
251 any of the organs examined ($P > 0.05$).

252 The average \log_{10} values of the ratio wild type/mutant for all samples per group of six
253 piglets are summarized in Fig. 2.

254

255 4. Discussion

256 *Salmonella* virulence genes are often clustered in several regions of the chromosome
257 termed '*Salmonella* pathogenicity islands' (SPI). Macrophages can be actively entered by
258 *Salmonella* Typhimurium in a SPI-1 dependent manner (Horter et al., 2003; Donné et al.,
259 2005; Takaya et al., 2005; Boyen et al., 2006a). Although these phagocytic cells are also
260 present in the tonsils, previous work using a SPI-1 deficient strain, showed no difference in
261 colonizing capacity in the tonsils between the mutant and the wild type strain (Boyen et al.,
262 2006b), while there was a significant colonization defect in the gut. The results from the
263 present study explain this finding since salmonellae were mostly found extracellularly in the
264 palatine tonsils. Using gentamicin-based single cell suspensions, we found that the major part
265 of bacteria (97.0 ± 2.0 %) resided extracellularly 5 days pi. One would expect that this
266 percentage would decrease over time, since intracellular survival of *Salmonella* Typhimurium
267 in macrophages is correlated with systemic spread and long-term persistence of the pathogen
268 in mice and pigs (Riber and Lind, 1999; Boyen et al., 2008a). Nevertheless, at 28 days pi still
269 most *Salmonella* bacteria (97.6 ± 3.6 %) were detected extracellularly. The data obtained by
270 immunohistochemistry confirmed this observation in the tonsils.

271 Localization and behaviour of *Salmonella* spp. in animal tissues is serovar and host
272 specific. Pullinger et al. (2007) found that systemic translocation of the host-adapted serovar
273 *Salmonella* Dublin predominantly occurs in a cell-free niche in efferent lymph in cattle. Our

274 results show that *Salmonella* Typhimurium predominantly survives in an extracellular niche
275 in the tonsils in pigs, indicating the host and serovar dependency of *Salmonella* pathogenesis.
276 This study confirmed the previous finding by Gray et al. (1995) that the host-adapted
277 *Salmonella* serovar Choleraesuis resided largely extracellularly in porcine tonsils. Although
278 we found that *Salmonella* Typhimurium resides largely as an extracellular pathogen in tonsils,
279 this does not rule out the importance of intracellular survival of *Salmonella* Typhimurium in
280 macrophages in dissemination of the bacterium through the porcine body, resulting in
281 persistent *Salmonella* infection.

282 A unique characteristic of the tonsils is the presence of tonsillar crypts, epithelial
283 diverticula that considerably increase the available surface for bacterial adherence (Perry and
284 White, 1998). Since it was stated earlier that these crypts play a major role in the persistence
285 of several microorganisms in pigs (Horter et al., 2003), we wanted to determine if *Salmonella*
286 Typhimurium resides extracellularly in porcine tonsils by formation of biofilms. However,
287 despite residing extracellularly, biofilm-associated genes *csgA*, *csgD* and *adrA* do not
288 contribute to persistent *Salmonella* Typhimurium colonization of tonsils in pigs. Since the
289 *Salmonella* Typhimurium genome possesses thirteen fimbrial loci (Clayton et al., 2008), it is
290 possible that the remaining fimbrial operons in our *csgA* and *csgD* mutants still provide the
291 function and enable *Salmonella* adhesion to the extracellular surface of tonsillar cells.

292 Another explanation could be the temperature dependency of *Salmonella* biofilm
293 formation mediated by *csgA*, *csgD* and *adrA* genes. We showed that the wild type strain
294 optimally produces the biofilm-associated RDAR morphotype at 28 °C and that expression of
295 this morphotype is inhibited at 37 °C, confirming earlier *in vitro* research in *Escherichia coli*
296 (Brombacher et al., 2005) and *Salmonella* Typhimurium (Römling et al., 1998). Basic body
297 temperature in pigs is in the range of 38-40 °C (Kaliszan et al., 2005). Despite the fact that
298 this is the temperature at which RDAR colony morphotype expression seems to be inhibited

299 *in vitro*, biofilm formation *in vivo* might behave differently. Downregulation of biofilm-
300 associated gene expression at temperatures above 32 °C *in vitro* might be counterbalanced *in*
301 *vivo* by a variety of environmental and physiological cues such as osmolarity, growth rate,
302 pH, O₂ level, the presence of important ions like iron and the availability of specific nutrients
303 e.g. glucose and phosphate (Römling et al., 1998; Gerstel and Römling, 2003; Brombacher et
304 al., 2005; Goller and Romeo, 2008). Nevertheless, there was no significant colonization
305 defect in any of the biofilm deficient mutant strains in any of the examined samples in the
306 current setup.

307

308 **5. Conclusion**

309 We have shown that *Salmonella* Typhimurium persists predominantly extracellularly
310 in the tonsils of pigs, independently of mutations in biofilm-associated genes *csgA*, *csgD* and
311 *adrA*. This finding is in sharp contrast with the general consensus that *Salmonella*
312 Typhimurium persists as an intracellular pathogen in the tonsils. Since most research in this
313 area was performed using murine, bovine, ovine and not porcine models (Alpuche-Aranda et
314 al., 1994; Ohl and Miller, 2001; Monack et al., 2004; Wick, 2007), this experiment
315 emphasizes the host-specificity of *Salmonella* Typhimurium pathogenesis.

316

317 **Acknowledgements**

318 The technical assistance of Gunter Massaer, Rosalie Devloo and Nathalie
319 Vanrysselberghe is greatly appreciated. This work was supported by the Institute for the
320 Promotion of Innovation by Science and Technology in Flanders (IWT Vlaanderen), Brussels,
321 Belgium (grant IWT Landbouw 040791); the Research Foundation-Flanders (FWO) and the
322 Special Research Fund (BOF), University of Ghent, Belgium.

323

324 **References**

325 Alpuche-Aranda, C.M., Racoosin, E.L., Swanson, J.A., Miller, S.I., 1994. *Salmonella*
326 stimulate macrophage macropinocytosis and persist within spacious phagosomes. *J. Exp.*
327 *Med.* 179, 601-608.

328

329 Anonymous, 2007. The Community Summary Report on Trends and Sources of Zoonoses,
330 Zoonotic Agents, Antimicrobial resistance and Foodborne outbreaks in the European Union in
331 2005. European Food Safety Authority.
332 http://www.efsa.europa.eu/EFSA/DocumentSet/Zoonoses_Report_EU_en_2005,3.pdf.

333

334 Anonymous, 2008. Report of the task force on zoonoses data collection on the analysis of the
335 baseline survey on the prevalence of *Salmonella* in slaughter pigs, in the EU, 2006-2007. Part
336 A: *Salmonella* prevalence estimates. *The EFSA Journal* 135, 1-111.

337

338 Botteldoorn, N., Heyndrickx, M., Rijpens, N., Grijspeerdt, K., Herman, L., 2003. *Salmonella*
339 on pig carcasses: positive pigs and cross contamination in the slaughterhouse. *J. Appl.*
340 *Microbiol.* 95, 891-903.

341

342 Boyen, F., Pasmans, F., Donné, E., Van Immerseel, F., Adriaensen, C., Hernalsteens, J.P.,
343 Ducatelle, R., Haesebrouck, F., 2006a. Role of SPI-1 in the interactions of *Salmonella*
344 Typhimurium with porcine macrophages. *Vet. Microbiol.* 113, 35-44.

345

346 Boyen, F., Pasmans, F., Van Immerseel, F., Morgan, E., Adriaensen, C., Hernalsteens, J.P.,
347 Decostere, A., Ducatelle, R., Haesebrouck, F., 2006b. *Salmonella* Typhimurium SPI-1 genes
348 promote intestinal but not tonsillar colonization in pigs. *Microbes Infect.* 8, 2899-2907.

349

350 Boyen, F., Haesebrouck, F., Maes, D., Van Immerseel, F., Ducatelle, R., Pasmans, F., 2008a.

351 Non-typhoidal *Salmonella* infections in pigs: A closer look at epidemiology, pathogenesis and

352 control. *Vet. Microbiol.* 130, 1-19.

353

354 Boyen, F., Pasmans, F., Van Immerseel, F., Morgan, E., Botteldoorn, N., Heyndrickx, M.,

355 Volf, J., Favoreel, H., Hemalsteens, J.P., Ducatelle, R., Haesebrouck, F., 2008b. A limited

356 role for *SsrA/B* in persistent *Salmonella* Typhimurium infections in pigs. *Vet. Microbiol.* 128,

357 364-373.

358

359 Boyen, F., Eeckhaut, V., Van Immerseel, F., Pasmans, F., Ducatelle, R., Haesebrouck, F.,

360 2009. Quorum sensing in veterinary pathogens: Mechanisms, clinical importance and future

361 perspectives. *Vet. Microbiol.* 135, 187-195.

362

363 Brombacher, E., Baratto, A., Dorel, C., Landini, P., 2006. Gene expression regulation by the

364 curli activator CsgD protein: Modulation of cellulose biosynthesis and control of negative

365 determinants for microbial adhesion. *J. Bacteriol.* 188, 2027-2037.

366

367 Clayton, D.J., Bowen, A.J., Hulme, S.D., Buckley, A.M., Deacon, V.L., Thomson, N.R.,

368 Barrow, P.A., Morgan, E., Jones, M.A., Watson, M., Stevens, M.P., 2008. Analysis of the role

369 of 13 major fimbrial subunits in colonisation of the chicken intestines by *Salmonella enterica*

370 serovar Enteritidis reveals a role for a novel locus. *BMC Microbiol.* 8, 228.

371

372 Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes of

373 *Escherichia coli* K-12 using PCR products. *P. Natl. Acad. Sci. USA* 97, 6640-6645.

374

375 Donné, E., Pasmans, F., Boyen, F., Van Immerseel, F., Adriaensen, C., Hernalsteens, J.P.,
376 Ducatelle, R., Haesebrouck, F., 2005. Survival of *Salmonella* serovar Typhimurium inside
377 porcine monocytes is associated with complement binding and suppression of the production
378 of reactive oxygen species. *Vet. Microbiol.* 107, 205-214.

379

380 Gerstel, U., Römling, U., 2003. The *csgD* promoter, a control unit for biofilm formation in
381 *Salmonella* Typhimurium. *Res. Microbiol.* 154, 659-667.

382

383 Goller, C.C., Romeo, T., 2008. Environmental influences on biofilm development.
384 In: *Current Topics in Microbiology and Immunology*, vol. 322, Springer-Verlag Berlin,
385 Berlin, pp. 37-66.

386

387 Gray, J.T., Fedorka-Cray, J.T., Stabel, T.J., Ackerman, M.R., 1995. Influence of inoculation
388 route on the carrier state of *Salmonella-Choleraesuis* in swine. *Vet. Microbiol.* 47, 43-59.

389

390 Horter, D.C., Yoon, K.J., Zimmerman, J.J., 2003. A review of porcine tonsils in immunity
391 and disease. *Anim. Health Res. Rev.* 4, 143-155.

392

393 Kaliszan, M., Hauser, R., Kaliszan, R., Wiczling, P., Buczynski, J., Penkowski, M., 2005.
394 Verification of the exponential model of body temperature decrease after death in pigs. *Exp.*
395 *Physiol.* 90, 727-738.

396

- 397 Malcova, M., Hradecka, H., Karpiskova, R., Rychlik, I., 2008: Biofilm formation in field
398 strains of *Salmonella enterica* serovar Typhimurium: Identification of a new colony
399 morphology type and the role of SGI1 in biofilm formation. *Vet. Microbiol.* 129, 360-366.
400
- 401 Monack, D.M., Bouley, D.M., Falkow, S., 2004. *Salmonella* Typhimurium persists within
402 macrophages in the mesenteric lymph nodes of chronically infected Nramp1^{+/+} mice and can
403 be reactivated by IFN γ neutralization. *J. Exp. Med.* 199, 231-241.
404
- 405 Nollet, N., Maes, D., Duchateau, L., Hautekiet, V., Houf, K., Van Hoof, J., De Zutter, L., De
406 Kruif, A., Geers, R., 2005. Discrepancies between the isolation of *Salmonella* from
407 mesenteric lymph nodes and the results of serological screening in slaughter pigs. *Vet. Res.*
408 36, 545-555.
409
- 410 Ohl, M.E., Miller, S.I., 2001. *Salmonella*: A model for bacterial pathogenesis. *Annu. Rev.*
411 *Med.* 52, 259-274.
412
- 413 Perry, M., Whyte, A., 1998. Immunology of the tonsils. *Rev. Immunol. Today* 19, 414-421.
414
- 415 Piriou-Guzylack, L., Salmon, H., 2008. Membrane markers of the immune cells in swine: an
416 update. *Vet. Res.* 39, 54.
417
- 418 Pullinger, G.D., Paulin, S.M., Charleston, B., Watson, P.R., Bowen, A.J., Dziva, F., Morgan,
419 E., Villarreal-Ramos, B., Wallis, T.S., Stevens, M.P., 2007. Systemic Translocation of
420 *Salmonella enterica* serovar Dublin in cattle occurs predominantly via efferent lymphatics in

421 a cell-free niche and requires type III secretion system 1 (T3SS-1) but not T3SS-2. Infect.
422 Immun. 75, 5191-5199.
423
424 Riber, U., Lind, P., 1999. Interaction between *Salmonella* Typhimurium and phagocytic cells
425 in pigs. Phagocytosis, oxidative burst and killing in polymorphonuclear leukocytes and
426 monocytes. Vet. Immunol. Immunop. 67, 259-270.
427
428 Römling, U., Sierralta, W.D., Eriksson, K., Normark, S., 1998. Multicellular and aggregative
429 behaviour of *Salmonella* Typhimurium strains is controlled by mutations in the *agfD*
430 promoter. Mol. Microbiol. 28, 249-264.
431
432 Römling, U., 2005. Characterization of the *rdar* morphotype, a multicellular behaviour in
433 enterobacteriaceae. Cell. Mol. Life Sci. 62, 1234-1246.
434
435 Rosenberg, L.E., Carbone, A.L., Römling, U., Uhrich, K.E., Chikindas, M.L., 2008. Salicylic
436 acid-based poly(anhydride esters) for control of biofilm formation in *Salmonella enterica*
437 serovar Typhimurium. Lett. Appl. Microbiol. 46, 593-599.
438
439 Salles, M.W.S., Middleton, D.M., 2000. Lymphocyte subsets in porcine tonsillar crypt
440 epithelium. Vet. Immunol. Immunop. 77, 133-144.
441
442 Takaya, A., Suzuki, A., Kikuchi, Y., Eguchi, M., Isogai, E., Tomoyasu, T., Yamamoto, T.,
443 2005. Derepression of *Salmonella* pathogenicity island 1 genes within macrophages leads to
444 rapid apoptosis via caspase-1- and caspase-3-dependent pathways. Cell. Microbiol. 7, 79-90.
445

446 Wick, M.J., 2007. Monocyte and dendritic cell recruitment and activation during oral
447 *Salmonella* infection. Immunol. Lett. 112, 68-74.

448

449 Wood, R.L., Pospischil, A., Rose, R., 1989. Distribution of persistent *Salmonella*
450 Typhimurium infection in internal organs of swine. Am. J. Vet. Res. 50, 1015-1021.

451

452

453

454

455

456

457

458

459

460

461

462

463

464

465

466

467

468

469

470

471 **Captions to Table and Figures**

472

473 **Table 1.** Primers used in this study to create the *csgA*, *csgD* and *adrA* deletion mutants.

474

475 **Fig. 1.** *In vitro* colony morphology of *Salmonella* Typhimurium strains after 7 days
476 incubation at 28°C on LB plates without salt, supplemented with 20 µg/ml Coomassie
477 Brilliant Blue and 40 µg/ml Congo Red. **A.** wild type strain, dry and rough morphotype; **B.**
478 Δ *adrA* strain, smooth and purple morphotype; **C.** Δ *csgA* strain, smooth and purple/red
479 morphotype; **D.** Δ *csgD* strain, smooth and purple/red morphotype.

480

481 **Fig. 2.** Recovery of bacteria from various organs of six piglets orally inoculated with a
482 mixture of the wild type *Salmonella* Typhimurium and *csgA*, *csgD* or *adrA* *Salmonella*
483 Typhimurium mutants. The log₁₀ value of the ratio of CFU per gram sample of the wild type
484 and the biofilm-associated gene mutants is given as the mean ± standard deviation.

Primers	Sequences
<i>csgA</i> Forward	5'-CAACGCTAATACCGTTACGACTTTTAAATCAATCCGATGGGG GTTTTACCTGTGTAGGCTGGAGCTGCTTC-3'
<i>csgA</i> Reverse	5'-GAAAAAAAAACAGGGCTTATGCCCTGTTTTTTTATTAGCGCAG ACGCTAAACATATGAATATCCTCCTTAG-3'
<i>csgD</i> Forward	5'-TTTATGGGGGCAGCTGTCAGATGTGCGATTAATAAAAGTGGA GTTTCATCTGTGTAGGCTGGAGCTGCTTC-3'
<i>csgD</i> Reverse	5'-CGAACAGTAACTCTGCTGCTACAATCCAGGTCAGATAGCGTT TCATGGCCCATATGAATATCCTCCTTAG-3'
<i>adrA</i> Forward	5'-ATACTTCCTCCATGCGCTCTGTTTCTATAATTTGGGAAAATTG TTTCTAATGTGTAGCTGGAGCTGCTTC-3'
<i>adrA</i> Reverse	5'-TGTATGGAAAATCAGAGGCGCTCAGTAAATCCTGAAgCCCG GCTGGACGCATATGAATATCCTCCTTAG-3'









