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Salmonella enterica Serovar Gallinarum Requires the *Salmonella* Pathogenicity Island 2 Type III Secretion System but Not the *Salmonella* Pathogenicity Island 1 Type III Secretion System for Virulence in Chickens

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Salmonella enterica **serovar Gallinarum is a host-specific serotype that causes the severe systemic disease fowl typhoid in domestic poultry and a narrow range of other avian species but rarely causes disease in mammalian hosts. Specificity of the disease is primarily at the level of the reticuloendothelial system, but few virulence factors have been described other than the requirement for an 85-kb virulence plasmid. In this work, by making functional mutations in the type III secretion systems (TTSS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2, we investigated the role of these pathogenicity islands in interactions between *Salmonella* serovar Gallinarum and avian cells in vitro and the role of these pathogenicity islands in virulence in chickens. The SPI-1 mutant showed decreased invasiveness into avian cells in vitro but was unaffected in its ability to persist within chicken macrophages. In contrast the SPI-2 mutant was fully invasive in nonphagocytic cells but failed to persist in macrophages. In chicken infections the SPI-2 mutant was attenuated while the SPI-1 mutant showed full virulence. In oral infections the SPI-2 mutant was not observed in the spleen or liver, and following intravenous inoculation it was cleared rapidly from these sites. SPI-2 function is required by *Salmonella* serovar Gallinarum for virulence, primarily through promoting survival within macrophages allowing multiplication within the reticuloendothelial system, but this does not preclude the involvement of SPI-2 in uptake from the gut to the spleen and liver. SPI-1 appears to have little effect on virulence and survival of *Salmonella* serovar Gallinarum in the host.**

Salmonella enterica serovar Gallinarum causes the severe systemic disease fowl typhoid in domestic poultry and other avian species (28). Although fowl typhoid has been largely eradicated in Europe and North America through control measures, the disease still causes substantial economic losses in Latin America, Africa, and Asia, where the intensification of the poultry industry is in its infancy. Serovar Gallinarum is host specific, causing mortality rates of 50% or more in the domestic fowl (28, 34). In contrast serovar Gallinarum does not cause systemic disease in experimentally infected mice or other laboratory mammals and has only rarely been reported to cause enteritis in humans (5, 26, 28). Host specificity of *Salmonella* serovar Gallinarum is expressed primarily at the level of the reticuloendothelial system (5), though other factors, particularly interaction with the intestinal epithelia, may also play a role (21, 26).

The molecular and cellular mechanisms of fowl typhoid are relatively poorly understood. The 85-kb serovar Gallinarum plasmid is essential for virulence (2), and several plasmid virulence genes have been identified (31). Two pathogenicity islands, *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2, which play key roles in mediating disease by *Salmonella enterica* through their respective type III secretion systems (TTSS) (19), have been described. The TTSS mediate the translocation of various virulence-associated effector proteins from the bacteria into the host cells (12, 18, 40). Several of the SPI-1 TTSS-dependent effector proteins have been identified in serovar Dublin. *sopA*, *sopB*, *sopD*, *sopE*, and *avrA* appear to have homologues in serovar Gallinarum as detected by Southern blotting (1, 15, 20, 42, 43), though their functional expression has not been confirmed. In contrast, the cellular and molecular mechanisms of both *Salmonella*-mediated enteritis in mammals and typhoid-like disease in serovar Typhimurium-infected mice have been studied extensively, particularly the roles of the TTSS encoded by SPI-1 and SPI-2 (12, 18, 40).

In this study we aim to determine the role of the SPI-1 and SPI-2 TTSS on the virulence, uptake, distribution, and pathology of serovar Gallinarum infections in the chicken, through experimental infections with functional knockout mutations in either the SPI-1 TTSS (*spaS*) or the SPI-2 TTSS (*ssaU*). Here we report that a functional SPI-1 TTSS is not required for serovar Gallinarum to cause fowl typhoid. The SPI-2 TTSS is required for development of fowl typhoid, primarily at the level of macrophage interaction.

MATERIALS AND METHODS

Bacterial strains. A spontaneous nalidixic acid-resistant (Nal^r) mutant of serovar Gallinarum 9 (35) was used as the parent strain. This strain has been well characterized for virulence in poultry (2, 5). The mutation in *spaS* was made by conjugation of suicide plasmid pSS1 (20) into the serovar Gallinarum 9 Nal^r mutant. The integration of the plasmid pSS1 into the serovar Gallinarum 9 chromosome by a single recombination event resulted in an insertion mutant that we called the serovar Gallinarum *spaS* mutant. The *ssaU* mutant was made as

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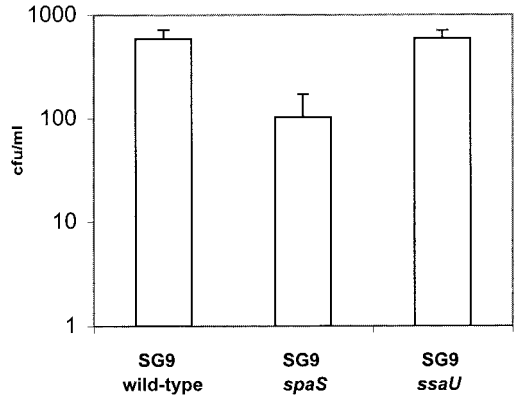

FIG. 1. Invasion of *Salmonella* serovar Gallinarum 9 (SG9) and SPI-1 (*spaS*) and SPI-2 (*ssaU*) mutant strains into primary CKC with 1 h of contact time at an MOI of 10. The values shown (mean plus standard error of the mean [error bar]) are based on three separate independent experiments.

follows. An internal DNA fragment of *ssaU* was amplified by PCR with primers SU1 (5′-ATA TTT ATC TAG TCG ACT GGT TTC CAT C-3′) and SU2 (5′-CGT CAA GCA GAG CTC TAT ACG CTA TTA C-3′), which were designed from the known sequence of the serovar Typhimurium *ssaU* (16). The amplified fragment was digested with *Sal*I and *Sst*I and cloned into the suicide vector pDM4. The resulting plasmid, pSS2, was conjugated into the serovar Gallinarum 9 Nal^r mutant. The integration of the suicide plasmid pSS2 into the chromosome by a single recombination event resulted in an insertion mutant that we called the serovar Gallinarum *ssaU* mutant.

All strains were grown from stocks maintained at −70°C in Luria-Bertani (LB) broth supplemented with 30% (vol/vol) glycerol. Bacteria were cultured for 18 h in LB broth at 37°C in an orbital shaking incubator at 150 rpm.

Host cell invasion. The invasiveness of the mutants into nonphagocytic host cells was determined using primary chick kidney cells (CKC) as previously described (3, 21). CKC were seeded to 10^6 cells/ml, and bacteria were added at a multiplicity of infection (MOI) of 10. Differences in invasiveness were assessed by variance analysis using the Minitab for Windows statistical package (Minitab Ltd., Coventry, West Midlands, United Kingdom).

Persistence in macrophages. The persistence of the strains in macrophages was determined by a gentamicin protection assay using the HD11 chicken macrophage-like cell line (7). Cells were seeded at 4×10^5/ml and grown at 41°C in 5% CO_2 and RPMI 1640 medium (Life Technologies, Paisley, United Kingdom) containing 20 mM L-glutamine (Life Technologies), 2.5% fetal bovine serum, 2.5% chicken serum, 10% tryptose phosphate broth, and penicillin and streptomycin (each at 10 U/ml). Cells were grown for 48 h and then adjusted in antibiotic-free medium to give a final concentration of 10^6/ml.

Serovar Gallinarum 9 and the serovar Gallinarum *spaS* and *ssaU* mutants were grown to late log phase in LB broth and added separately to the HD11 cells at an MOI of 10. The bacteria were incubated with cells for 1 h, and then the medium was replaced with RPMI 1640 containing gentamicin sulfate (100 μg/ml) and incubated for 1 h to kill extracellular bacteria. The cells were then washed three times with antibiotic-free medium. The initial count of bacterial invasion and/or uptake (1-h survival) was made by lysing cells with 0.1% Triton X-100 in phosphate-buffered saline and then plating the lysate onto LB agar. To determine the persistence of *Salmonella* bacteria, cells were maintained in medium containing gentamicin sulfate (20 μg/ml) to inhibit growth and reinvasion of any bacteria released by lysed macrophages. At 4 and 24 h postinvasion the monolayer was washed three times in phosphate buffer and the cells were lysed in 100 μl of a 0.1% Triton X-100 solution. Viable counts of the released bacteria were determined and are represented as CFU per milliliter (3). The viability of macrophages was determined at each time point by measuring the release of lactate dehydrogenase using the Cytox 96 nonradioactive cytotoxicity assay (Promega UK, Southampton, United Kingdom).

Experimental animals. Three-week-old specific-pathogen-free Rhode Island Red chickens were obtained from the Poultry Production Unit, Institute for Animal Health, Compton, United Kingdom. Birds were maintained in wire cages at an ambient temperature of 20°C. Birds were watered and fed ad libitum on a vegetable protein-based diet (Special Diet Services, Manea, Cambridgeshire, United Kingdom).

Determination of virulence. Three groups of 10 chickens were infected orally with 10^8 CFU of serovar Gallinarum 9, serovar Gallinarum *spaS* mutant, or serovar Gallinarum *ssaU* mutant in a volume of 0.1 ml of LB broth. Animals were observed regularly over 4 weeks following infection. Birds that became sick from typhoid were killed humanely, once they reached specific end points based on signs of anorexia, failure to drink, ruffled feathers, or lethargy.

Bacterial distribution and pathogenesis experiments. (i) Oral inoculation. Three groups of 20 chickens were infected as described above. Five birds from each group were killed for postmortem analysis at 1, 3, 7, and 14 days following infection. At postmortem, liver, spleen and ileal tissue, heart blood, and cecal contents were removed aseptically and bacteriological analysis was performed as previously described (4). Samples were plated onto brilliant green agar (Oxoid, Unipath, Basingstoke, Hampshire, United Kingdom) containing sodium nalidixate (20 μg/ml) and novobiocin (1 μg/ml) (both from Sigma Chemical Co., Poole, Dorset, United Kingdom) and then incubated at 37°C for 18 h. All bacteria were assessed for maintenance of their insertion mutations, and these were found to be stable. At postmortem, differences in gross pathology were noted, and spleen, liver, ileal, and cecal tonsil tissue were removed and placed in 10% formalin, embedded in paraffin wax, cut, and stained with hematoxylin and eosin for histological examination.

(ii) Intravenous inoculation. Three groups of 20 chickens were inoculated intravenously via the wing vein with 10^4 CFU of serovar Gallinarum 9, serovar Gallinarum *spaS* mutant, and serovar Gallinarum *ssaU* mutant in a volume of 0.1 ml of LB broth. Birds were taken for postmortem analysis at 4 h and at 1, 3, and 5 days postinfection. Spleen and liver tissue and cecal contents were taken for bacteriological analysis as described above, and any gross pathology seen at postmortem was noted.

RESULTS

Cell invasion. All strains were invasive into CKC (Fig. 1). Both the parent strain and *ssaU* mutant showed a similar degree of invasiveness. In contrast, the *spaS* mutant was significantly less invasive than the other strains ($P < 0.05$).

Persistence in macrophages. All three strains were internalized by macrophages. However, by 4 h postinfection the serovar Gallinarum *ssaU* mutant was eliminated while the parent strain and *spaS* mutant both persisted for 24 h following infection (Fig. 2). Similar levels of cell lysis occurred with the parent strain (52% lysis at 4 h) and the *ssaU* mutant strain (50% lysis at 4 h). The *spaS* mutant was slightly less lytic, with 40% lysis found after 4 h. This indicates that the differences in persis-

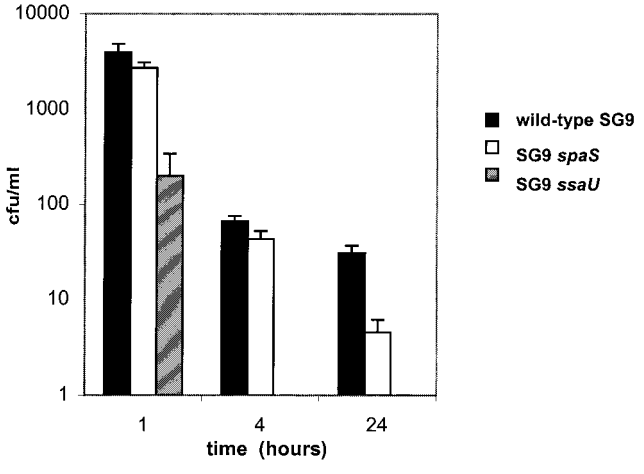

FIG. 2. Uptake and persistence of *Salmonella* serovar Gallinarum 9 (SG9) and SPI-1 (*spaS*) and SPI-2 (*ssaU*) mutant strains in the HD11 chicken macrophage-like cell line, determined by gentamicin protection assay. Bacteria were added at an MOI of 10. The results shown are mean (plus standard error of the mean [error bar]) values based on three independent experiments.

TABLE 1. Distribution in tissue of *Salmonella* serovar Gallinarum (SG9) and *spaS* and *ssaU* mutant strains following oral infection[a]

Tissue	SG9 strain	Mean $\log_{10}$ CFU/g of tissue at day postinfection (SEM)			
		1	3	7	14
Spleen	Parent	<1	4.05 (0.07)	4.5 (0.15)	4.23 (0.09)
	spaS mutant	<1	3.35 (0.13)	4.59 (0.29)	4.24 (0.29)
	ssaU mutant	<1	<1	<1	<1
Liver	Parent	<1	4.13 (0.11)	4.33 (0.09)	4.23 (0.09)
	spaS mutant	<1	3.36 (0.13)	4.59 (0.22)	4.38 (0.45)
	ssaU mutant	<1	<1	<1	<1
Cecal content	Parent	2.90 (0.11)	1.51 (0.94)	2.66 (0.67)	4.51 (0.37)
	spaS mutant	3.51 (0.28)	0.40 (0.40)	3.59 (0.52)	5.18 (0.40)
	ssaU mutant	1.55 (0.95)	<1	<1	<1
Ileum	Parent	<1	0.65 (0.40)	3.85 (0.14)	4.24 (0.04)
	spaS mutant	1.24 (0.77)	0.40 (0.40)	3.35 (0.97)	4.82 (0.37)
	ssaU mutant	<1	<1	<1	<1

[a] Oral infection with 10^8 CFU in 3-week-old Rhode Island chickens. Viable counts are mean values from five animals at each time point.

tence of the *ssaU* mutant strain are not due to increased ability to cause macrophage lysis but are related to the ability to survive within host macrophages.

Virulence. Six out of ten birds died or were killed because of severe fowl typhoid in both the serovar Gallinarum 9 and serovar Gallinarum *spaS* mutant orally infected groups, a final mortality of 60%. However, the death rates were different in the two groups, with mortalities of 50 and 30% of birds infected with the parent strain and *spaS* mutant, respectively, requiring culling by 13 days postinfection. At the termination of the experiment, 3 weeks postinfection, the remaining birds in these groups showed lesions typical of fowl typhoid on postmortem examination and *Salmonella* could be recovered from the livers of all birds. In contrast no mortality or morbidity occurred in the group infected with the serovar Gallinarum *ssaU* mutant. At the termination of the experiment none of the birds in this group showed signs of illness and no salmonellae were recovered from their livers.

Bacterial distribution and pathogenesis. (i) Experiment 1. From 8 days post-oral infection birds infected with serovar Gallinarum 9 and the serovar Gallinarum *spaS* mutant began to demonstrate signs of fowl typhoid. At postmortem examination at 1 and 3 days following infection, no indications of fowl typhoid were seen. At 7 days following infection pronounced splenomegaly was found at postmortem examination of birds infected with serovar Gallinarum 9 and the serovar Gallinarum *spaS* mutant. At postmortem examination at 14 days postinfection these groups displayed classical signs of gross fowl typhoid pathology, including hepatosplenomegaly, necrotic lesions, liver and spleen lesions, bronzed livers, and hemorrhaging in the ileum. Histological examination of the liver and spleen revealed lesions showing marked infiltration of heterophils (avian polymorphonuclear cells), lymphocytes, and macrophages typical of fowl typhoid (data not presented) (34). In contrast the serovar Gallinarum *ssaU* mutant showed no indications of causing disease throughout the experiment or at postmortem examination. No gross pathology or histopathological changes were found in this group.

Serovar Gallinarum 9 and the serovar Gallinarum *spaS* mutant could be detected in the spleen, liver, and ileum from 3 days postinfection onwards and in cecal contents from the first day postinfection (Table 1). However, viable counts of the *spaS* mutant in the liver and spleen were lower than those of the parent at day 3. In contrast the serovar Gallinarum *ssaU* mutant could not be recovered from any of the tissues, even following enrichment with selenite broth, though it was recovered from the cecal contents at 1 day postinfection. From 7 days postinfection serovar Gallinarum 9 and the serovar Gallinarum *spaS* mutant were also detected in heart blood, indicating systemic bacteremia. Interestingly, of the three strains studied the *spaS* mutant was isolated in the highest numbers from the ileal and cecal contents early in infection, suggesting that it was not taken up from the gut as rapidly.

(ii) Experiment 2. Birds infected intravenously with both serovar Gallinarum 9 and the serovar Gallinarum *spaS* mutant showed signs of systemic salmonellosis at 4 days following infection. At postmortem examination 3 days following infection, the serovar Gallinarum 9-infected birds showed splenomegaly. At postmortem examination 5 days following infection, pronounced hepatosplenomegaly, spleen and liver lesions, gut hemorrhaging, and watery gut contents were observed. No signs of disease were found in birds infected with the serovar Gallinarum *ssaU* mutant. Bacteria could be found in the spleens of birds from all three groups 4 h after intravenous inoculation (Table 2). However, by 24 h postinfection, the serovar Gallinarum *ssaU* mutant had been cleared. In contrast the numbers of both serovar Gallinarum 9 and the serovar Gallinarum *spaS* mutant in the spleen and liver of infected groups rose from 1 day postinfection, with bacteria subsequently found in the cecal contents and heart blood of these groups at 5 days postinfection (Table 2).

DISCUSSION

The data presented here indicate that the SPI-2-encoded TTSS is required for serovar Gallinarum to produce systemic fowl typhoid in chickens but that the SPI-1-encoded TTSS is not essential.

Murray and Lee (25) have previously shown that deletion of SPI-1 does not affect the ability of serovar Typhimurium to pass the epithelial barrier in a mouse model. However, the study used a mutant of *hilA*, which has previously been shown

TABLE 2. Distribution in tissue of *Salmonella* serovar Gallinarum (SG9) and *spaS* and *ssaU* mutant strains following intravenous infection[a]

Tissue	SG9 strain	Mean $\log_{10}$ CFU/g of tissue at day post infection (SEM)			
		1	3	7	14
Spleen	Parent	1.00 (0.63)	2.58 (0.19)	4.40 (0.23)	4.54 (0.10)
	spaS mutant	1.30 (0.81)	2.32 (0.20)	3.98 (0.19)	4.75 (0.26)
	ssaU mutant	1.30 (0.53)	<1	<1	<1
Liver	Parent	<1	1.81 (0.48)	4.27 (0.28)	4.68 (0.24)
	spaS mutant	<1	1.76 (0.45)	4.16 (0.28)	4.56 (0.18)
	ssaU mutant	<1	<1	<1	<1
Cecal content	Parent	<1	<1	<1	2.64 (0.0.24)
	spaS mutant	<1	<1	<1	1.74 (0.72)
	ssaU mutant	<1	<1	<1	<1

[a] Intravenous infection with 10^4 CFU in 3-week-old Rhode Island chickens. Viable counts are mean values from five animals at each time point.

to differentially regulate type III secreted proteins (13), and a complete deletion of SPI-1. In particular the complete deletion of SPI-1 means a distinction is not made between the function of the TTSS and other genes on the island. Although they do use a well-defined mutation in *invG*, it has been previously noted that mutations in *invG* could have different effects dependent on the mutation used (30). In this study we used specific mutations in genes encoding the structural components of the SPI-1 TTSS and the SPI-2 TTSS; *spaS* and *ssaU*, respectively. SpaS and SsaU are major structural components of the TTSS apparatus, and disruption of the genes coding for these components destroys the function of the respective TTSS to translocate effector proteins (20, 42). Although there may be pleiotropic effects associated with these mutations, they should be restricted to defects in the ability of *Salmonella* to translocate effector proteins. Our results confirm that the SPI-1 translocational machinery is required for maximal in vitro invasion of nonphagocytic CKC, but it is not required for serovar Gallinarum to cause systemic disease.

Infection with the serovar Gallinarum *ssaU* mutant caused no mortality in 3-week-old chickens; it was cleared rapidly from the spleen and liver following oral or intravenous infection. The SPI-2 mutant also failed to cause any pathological changes and showed poor survival in chicken macrophages. This is in contrast to the serovar Gallinarum *spaS* mutant, which was fully virulent when compared with the parental serovar Gallinarum 9, albeit with a slight delay in appearance of morbidity and mortality. Serovar Gallinarum *spaS* was recovered from organs at similar levels as the parent strain, produced the typical pathological changes associated with fowl typhoid and persisted well in chicken macrophages. However, in in vitro assays of invasion into nonphagocytic cells, the *spaS* mutant is significantly less invasive than either the parent strain or the *ssaU* mutant. This phenotype correlates to the previously described role of SPI-1-mediated entry of nonphagocytic cells (14). A functional SPI-1 system is therefore not required by serovar Gallinarum to produce fowl typhoid. This is in agreement with some of the observations of Murray and Lee (25) for the role of SPI-1 in serovar Typhimurium in murine typhoid-like disease. We have previously shown that serovar Gallinarum appears to preferentially invade the cecal tonsil and Peyer's patch during the early stages of fowl typhoid (6, 23). This apparent tropism for lymphoid tissue is also displayed by serovar Typhimurium in infection of mice (24). It appears therefore that serovar Gallinarum may gain entry primarily through gut-associated lymphoid tissue rather than nonphagocytic cells. Reports on experiments using ligated loops of mice have debated the role of M cells in the murine disease, indicating that the preparation of cultures prior to inoculation can have a marked effect on results (10, 11, 27); however, inoculation in this study has been carried out by the oral route, and hence the gene regulation might be thought of as equivalent to that of a natural infection. We intend to investigate the role of SPI-1 and SPI-2 in entry of lymphoid tissue. Although the SPI-1 TTSS of serovar Gallinarum is not essential to the production of systemic disease, it may play a role in pathogenesis. The data suggest that the kinetics of disease progression might be slower in the *spaS* strain, as the bacteria appeared in higher numbers in ileum and cecal contents earlier in infection than did the parent strain and viable counts recovered from the spleen at 3 days post-oral infection were also lower (Table 1). Further detailed experiments, particularly of the early stages of infection, would be needed to confirm this apparent effect. Interestingly, while mutations in *invH* in *Salmonella* serovar Typhimurium UK1 have little effect on systemic spread in chickens (29), *Salmonella* serovar Typhimurium *invH* mutants show a greatly reduced colonization of systemic sites during calf enteritis. These results suggest that there is a distinction within SPI-1 for mediation of enteritis and typhoid-like disease (41).

Loss of function of the SPI-2 TTSS in serovar Gallinarum led to attenuation in vivo. Similar loss of SPI-2 function in serovar Typhimurium leads to attenuation of typhoid-like disease in mice and gastroenteritis in cattle (8, 9, 17, 32, 36). As with serovar Typhimurium (9), experimental oral infection with serovar Gallinarum SPI-2 mutants did not lead to uptake to the spleen and liver, though colonization of the Peyer's patches by serovar Typhimurium was seen in mice (9). Mutants of *sseD*, a SPI-2 dependent effector protein in serovar Dublin were fully invasive but were attenuated for systemic disease in calves (8, 22). Uptake of the serovar Gallinarum *ssaU* mutant to the spleen could be demonstrated after infection of chickens by the intravenous route, as also occurs with serovar Typhimurium SPI-2 mutants following intraperitoneal administration to mice (33). This suggests that to cause systemic disease in their respective hosts, *Salmonella* serovars Gallinarum, Typhimurium, and Dublin require the SPI-2 TTSS during translocation of bacteria from the gut-associated lymphoid tissue to

the spleen, suggesting a role of macrophage-like cells in this translocation process. However, the main function of the SPI-2 is likely to be in the replication and survival of *Salmonella* within macrophages. The SPI-2 system is required by serovar Gallinarum to promote survival within the HD11 macrophage-like cell line in vitro. SPI-2 TTSS is required by serovar Typhimurium for survival and replication in RAW 267.4 murine macrophage-like cells (17). As yet we have been unable to demonstrate proliferation of any *Salmonella* serovar in avian macrophages in vitro (unpublished observations).

The mechanisms by which SPI-2 acts to promote survival within macrophages are beginning to be elucidated. SPI-2 interferes with intracellular trafficking and is involved in the inhibition of phagosolysosome maturation in macrophages containing *Salmonella* (37). SPI-2 function is also required by intracellular *Salmonella* to avoid killing by reactive oxygen intermediates (ROI) (38). This appears to be achieved through excluding NADPH phagocyte oxidase (Phox) from vacuoles containing *Salmonella*. Killing by ROI has been shown to be important in serovar Typhimurium host resistance in the mouse; mice deficient in gp91*phox* oxidase activity succumb rapidly to salmonellosis (39). Evidence for the importance of ROI-mediated killing of *Salmonella* in chickens has been found in inbred lines of chickens genetically resistant to systemic salmonellosis. Macrophages from *Salmonella*-resistant lines of birds that possess the *SAL1* resistant haplotype show increased killing of serovar Gallinarum and an increased ROI but not nitric oxide production in vitro, indicating that ROI-mediated killing may be important in host resistance to systemic infection (P. Wigley, S. D. Hulme, N. Bumstead, and P. A. Barrow, unpublished results).

In this work we have shown the importance of the SPI-2 TTSS in a naturally occurring form of systemic, typhoid-like salmonellosis that has considerable economic and veterinary importance worldwide. We also confirm that SPI-1 is not essential for cell invasion and the production of systemic fowl typhoid, though it may play a role in early stages of infection. In contrast SPI-2 TTSS function is absolutely necessary for serovar Gallinarum to cause disease by enabling the bacteria to survive within macrophages and multiply within the reticuloendothelial system. Additionally the SPI-2 system may be involved in translocation from the gut to the liver and spleen, suggesting that this mechanism involves survival and transport within phagocytes.

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REFERENCES

1. **Bakshi, C. S., V. P. Singh, M. W. Wood, P. W. Jones, T. S. Wallis, and E. E. Galyov.** 2000. Identification of SopE2, a *Salmonella* secreted protein which is highly homologous to SopE and involved in bacterial invasion of epithelial cells. J. Bacteriol. **182:**2341–2344.
2. **Barrow, P. A., J. M. Simpson, M. A. Lovell, and M. M. Binns.** 1987. Contribution of *Salmonella gallinarum* large plasmid towards virulence in fowl typhoid. Infect. Immun. **55:**388–392.
3. **Barrow, P. A., and M. A. Lovell.** 1989. Invasion of Vero cells by *Salmonella* species. J. Med. Microbiol. **28:**59–67.
4. **Barrow, P. A., and M. A. Lovell.** 1991. Experimental infection of egg-laying hens with *Salmonella enteritidis* phage type 4. Avian Pathol. **20:**335–348.
5. **Barrow, P. A., M. B. Huggins, and M. A. Lovell.** 1994. Host specificity of *Salmonella* infections in chickens and mice is expressed in vivo primarily at the level of the reticuloendothelial system. Infect. Immun. **62:**4602–4610.
6. **Barrow, P. A., M. A. Lovell, and B. A. D. Stocker.** 2000. Protection against fowl typhoid by parental administration of live SL5828, an *aroA-serC* (aromatic dependent) mutant of a wild-type *Salmonella* Gallinarum strain made lysogenic for P22 *sie*. Avian Pathol. **29:**423–431.
7. **Beug, H., A. vonKirchbach, G. Doderlein, J. F. Conscience, and T. Graf.** 1979. Chicken hemapoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation. Cell **18:**375–390.
8. **Bispham, J., B. N. Tripathi, P. R. Watson, and T. S. Wallis.** 2001. *Salmonella* pathogenicity island-2 influences both systemic salmonellosis and *Salmonella*-induced enteritis in calves. Infect. Immun. **69:**367–377.
9. **Cirillo, D. M., R. H. Valdivia, D. M. Monack, and S. Falkow.** 1998. Macrophage-dependent induction of the *Salmonella* pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. **30:**175–188.
10. **Clark, M. A., B. H. Hirst, and M. A. Jepson.** 1998. Inoculum composition and *Salmonella* pathogenicity island 1 regulate M-cell invasion and epithelial destruction by *Salmonella typhimurium*. Infect. Immun. **66:**724–731.
11. **Clark, M. A., K. A. Reed, J. Lodge, J. Stephen, B. H. Hirst, and M. A. Jepson.** 1996. Invasion of murine intestinal M cells by *Salmonella typhimurium inv* mutants severely deficient for invasion of cultured cells. Infect. Immun. **64:**4363–4368.
12. **Darwin, K. H., and V. L. Miller.** 1999. Molecular basis of the interaction of *Salmonella* with the intestinal mucosa. Clin. Microbiol. Rev. **12:**405–428.
13. **Eichelberg, K., and J. E. Galán.** 1999. Differential regulation of *Salmonella typhimurium* type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and HilA. Infect. Immun. **67:**4099–4105.
14. **Galán, J. E.** 1996. Molecular genetic bases of *Salmonella* entry into host cells. Mol. Microbiol. **20:**263–271.
15. **Hardt, W. D., and J. E. Galán.** 1997. A secreted *Salmonella* protein with homology to an avirulence determinant of plant pathogenic bacteria. Proc. Natl. Acad. Sci. USA **94:**9887–9892.
16. **Hensel, M., J. E. Shea, B. Raupach, D. Monack, S. Falkow, C. Gleeson, T. Kubo, and D. W. Holden.** 1997. Functional analysis of *ssaK/U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* pathogenicity island 2. Mol. Microbiol. **24:**155–167.
17. **Hensel, M., J. E. Shea, S. R. Waterman, R. Mundy, T. Nikolaus, G. Banks, A. Vazquez-Torres, C. Gleeson, F. C. Fang, and D. W. Holden.** 1998. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. **30:**163–174.
18. **Hensel, M.** 2000. *Salmonella* pathogenicity island 2. Mol. Microbiol. **36:**1015–1023.
19. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62:**379–433.
20. **Jones, M. A., M. W. Wood, P. B. Mullan, P. R. Watson, T. S. Wallis, and E. E. Galyov.** 1998. Secreted effector proteins of *Salmonella dublin* act in concert to induce enteritis. Infect. Immun. **66:**5799–5804.
21. **Kaiser, P., L. Rothwell, E. E. Galyov, P. A. Barrow, J. Burnside, and P. Wigley.** 2000. Differential cytokine expression in avian cells in response to invasion by *Salmonella enteritidis* and *Salmonella gallinarum*. Microbiology **146:**3217–3226.
22. **Klien, J. R., and B. R. Jones.** 2001. *Salmonella* pathogenicity island 2-encoded proteins SseC and SseD are essential for virulence and are substrates of the type III secretion system. Infect. Immun. **69:**737–743.
23. **Lowry, V. K., G. I. Tellez, D. J. Nisbet, G. Garcia, O. Urquiza, L. H. Stanker, and M. H. Kogut.** 1999. Efficacy of *Salmonella enteritidis*-immune lymphokines on horizontal transmission of *S. arizonae* in turkeys and *S. gallinarum* in chickens. Int. J. Food Microbiol. **48:**139–148.
24. **Mittrucker, H. W., and S. H. Kaufmann.** 2000. Immune response to infection with *Salmonella typhimurium* in mice. J. Leukoc. Biol. **67:**457–463.
25. **Murray, R. A., and L. A. Lee.** 2000. Invasion genes are not required for *Salmonella enterica* serovar Typhimurium to breach the intestinal epithelium: evidence that *Salmonella* pathogenicity island 1 has alternative functions during infection. Infect. Immun. **68:**5050–5055.
26. **Pascopella, L., B. Raupach, N. Ghori, D. Monack, S. Falkow, and P. L. Small.** 1995. Host restriction phenotypes of *Salmonella typhi* and *Salmonella gallinarum*. Infect. Immun. **63:**4329–4335.
27. **Penhieter, K. L., N. Mathur, D. Giles, T. Fahlen, and B. D. Jones.** 1997. Non-invasive *Salmonella typhimurium* mutants are avirulent because of an inability to enter and destroy M cells of ileal Peyer's patches. Mol. Microbiol. **24:**697–709.
28. **Pomeroy, B. S., and K. V. Nagaraja.** 1991. Fowl typhoid, p. 87–99. *In* B. W. Calnek et al. (ed.), Diseases of poultry, 9th ed. Iowa State University Press, Ames, Iowa.
29. **Porter, S. B., and R. Curtiss III.** 1997. Effect of *inv* mutations on virulence and colonisation in 1-day-old White leghorn chicks. Avian Dis. **41:**45–57.
30. **Reed, K. A., M. A. Clark, T. A. Booth, C. J. Hueck, S. I. Miller, B. H. Hirst,**

and M. A. Jepson. 1998. Cell-contact-stimulated formation of filamentous appendages by *Salmonella typhimurium* does not depend on the type III secretion system encoded by pathogenicity island 1. Infect. Immun. **66:**2007–2017.
31. **Rychlik, I., M. A. Lovell, and P. A. Barrow.** 1998. The presence of genes homologous to the K88 genes *faeH* and *faeI* on the virulence plasmid of *Salmonella gallinarum*. FEMS Microbiol. Lett. **159:**255–260.
32. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **93:**2593–2597.
33. **Shea, J. E., R. C. Beuzon, C. Gleeson, R. Mundy, and D. W. Holden.** 1999. Influence of the *Salmonella typhimurium* pathogenicity island 2 type III secretion system on bacterial growth in the mouse. Infect. Immun. **67:**213–219.
34. **Shivaprasad, H. L.** 2000. Fowl typhoid and pullorum disease. Rev. Sci. Technol. **19:**405–424.
35. **Smith, H. W.** 1956. Observations on experimental fowl typhoid. J. Comp. Pathol. **65:**37–54.
36. **Tsolis, R. M., L. G. Adams, T. A. Ficht, and A. J. Baumler.** 1999. Contribution of *Salmonella typhimurium* virulence factors to diarrheal disease in calves. Infect. Immun. **67:**4879–4885.
37. **Uchiya, K., M. A. Barbieri, K. Funato, A. H. Shah, P. D. Stahl, and E. A. Groisman.** 1999. A *Salmonella* virulence protein that inhibits cellular trafficking. EMBO J. **18:**3924–3933.
38. **Vazquez-Torres, A., Y. Xu, J. Jones-Carson, D. W. Holden, S. M. Lucia, M. C. Dinauer, P. Mastroeni, and F. C. Fang.** 2000. *Salmonella* pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science **287:**1655–1658.
39. **Vazquez-Torres, A., J. Jones-Carson, P. Mastroeni, H. Ischiropoulos, and F. C. Fang.** 2000. Antimicrobial actions of the NADPH phagocyte oxidase and inducible nitric oxide synthase in experimental salmonellosis. I. Effects on microbial killing by activated peritoneal macrophages in vitro. J. Exp. Med. **192:**227–236.
40. **Wallis, T. S., and E. E. Galyov.** 2000. Molecular basis of salmonella-induced enteritis. Mol. Microbiol. **36:**997–1005.
41. **Watson, P. R., E. E. Galyov, S. M. Paulin, P. W. Jones, and T. S. Wallis.** 1998. Mutation in *invH*, but not *stn*, reduces *Salmonella*-induced enteritis in cattle. Infect. Immun. **66:**1432–1438.
42. **Wood, M. W., M. A. Jones, P. W. Watson, A. M. Siber, B. A. McCormick, S. Hedges, R. Rosqvist, T. S. Wallis, and E. E. Galyov.** 2000. The secreted effector protein of *Salmonella dublin*, SopA, is translocated into eukaryotic cells and influences the induction of enteritis. Cell. Microbiol. **2:**293–303.
43. **Wood, M. W., M. A. Jones, P. W. Watson, S. Hedges, T. S. Wallis, and E. E. Galyov.** 1998. Identification of a pathogenicity island required for *Salmonella* enteropathogenicity. Mol. Microbiol. **29:**883–891.

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