

## Subspecies IIIa and IIIb Salmonellae Are Defective for Colonization of Murine Models of Salmonellosis Compared to *Salmonella enterica* subsp. I Serovar Typhimurium<sup>∇</sup>

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**Non-subspecies I salmonellae are commensals of cold-blooded vertebrates and cause sporadic disease in mammals. The reasons why non-subspecies I salmonellae do not circulate in populations of warm-blooded vertebrates, but instead only cause occasional disease in this niche, are unknown. We examined the ability of *Salmonella enterica* subsp. IIIa (subsp. *arizonae*) and subsp. IIIb (subsp. *diarizonae*) isolates to grow competitively with subspecies I (serovar Typhimurium) ATCC 14028 in vitro, to colonize *Salmonella*-sensitive BALB/c mice, and to persist in the intestine of *Salmonella*-resistant CBA/J mice in competitive infections. Subspecies IIIa had severely reduced intestinal colonization, intestinal persistence, and systemic spread in mice. Subspecies IIIa is nonmotile on swarming agar and thus may also have reduced motility under viscous conditions in vivo. Surprisingly, subspecies IIIb colonizes the intestinal tract of BALB/c mice normally yet does not spread systemically. Subspecies IIIb colonization of the intestine of CBA/J mice is reduced late in infection. In order to understand why these isolates do not colonize systemic sites, we determined that subspecies IIIa and IIIb are not internalized well and do not replicate in J774-A.1 murine macrophages, despite normal adherence to these cells. We further show that selected effectors of both type III secretion systems 1 and 2 are secreted by subspecies IIIa and IIIb in vitro but that each of these isolates secretes a different combination of effectors. We outline the phenotypic differences between these subspecies and subspecies I and provide a possible explanation for the inability of these strains to spread systemically in murine models.**

The species *Salmonella enterica* contains six subspecies (10). Subspecies I serovar Enterica is responsible for the overwhelming majority of salmonellosis in mammals and birds, while non-subspecies I isolates cause only sporadic disease in mammalian (including humans) and avian species and are primarily described as nonpathogenic commensals of cold-blooded vertebrates (56, 58). The reasons for the overwhelming epidemiologic dominance of subspecies I salmonellae in mammalian and avian salmonellosis are unknown but may be related to an increased ability of this subspecies to be transmitted and persist within the warm-blooded vertebrate population.

An estimated 93,000 cases of human salmonellosis are attributed to amphibian and reptile contact each year; this constitutes ~7% of the total annual cases of salmonellosis in the United States (14). Subspecies IIIa and IIIb are considered reptile associated, and they colonize the reptilian intestinal tract asymptotically and are excreted in feces (6, 11, 49, 58). Both subspecies IIIa and IIIb can cause disease in various warm-blooded vertebrates, including humans, domestic poultry, sheep, wild birds, and cats (1, 2, 18, 50). Subspecies IIIa and IIIb are observed as the cause of disease in humans,

especially in the young and in immunocompromised individuals. Many human cases of subspecies IIIa and IIIb salmonellosis can be linked to contact with a reptile, although other sources are possible (49).

Subspecies IIIa and IIIb can colonize the human intestine and can be isolated upon fecal culture from infected individuals (26, 49). Furthermore, subspecies IIIa and IIIb infections are invasive in young children and immunocompromised individuals, resulting in serious systemic disease, including sepsis and meningitis (14). Despite the fact that subspecies IIIa and IIIb can colonize the intestinal tract of warm-blooded vertebrates and cause disease in these hosts, the reasons why these subspecies do not circulate more widely in populations of warm-blooded vertebrates are unknown.

We hypothesized that subspecies IIIa and IIIb are not as epidemiologically successful as subspecies I in warm-blooded vertebrates because they may be unable to colonize and persist in the intestinal tracts of these hosts. In this study we determined the ability of subspecies IIIa and IIIb to colonize the mammalian intestine, persist there for prolonged periods, and spread systemically by using competitive infections with subspecies I (serovar Typhimurium) in murine models commonly employed to study salmonellosis.

### MATERIALS AND METHODS

**Bacterial strains and media.** The bacterial strains used in this study are described in Table 1. The subspecies I isolates used in these experiments are derivatives of *S. enterica* serovar Typhimurium ATCC 14028. Mutants for the type III secretion system 1 (TTSS-1) effector SipB (STM2885), TTSS-1 machinery ( $\Delta$ imvA; STM2896), and TTSS-2 effectors SseG (STM1404) and SifA

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TABLE 1. Strains used in this study

Strain name	Genotype	Reference or source
HA348	ATCC 14028 NaI <sup>r</sup>	This work
HA350	SGSC4693 NaI <sup>r</sup> (subsp. IIIa)	This work
HA378	SGSC4692 NaI <sup>r</sup> (subsp. IIIb)	This work
HA458	HA348 $\Delta invA::Kan$	This work
HA816	ATCC 14028 $\Delta sseG::Kan$	This work
HA817	ATCC 14028 $\Delta sifA::Cm$	This work
HA818	ATCC 14028 $\Delta sipB::Kan$	This work
AJB715	ATCC 14028 <i>phoN</i> <sup>r</sup>	36
SGSC4693	IIIa (62:z4,z23:-) (SARC5 RSK2980, CDC346-86, and ATCC BAA-731)	7
SGSC4692	IIIb [61:1,v:1,5,(7)] (CDC 01-0005 and ATCC BAA-639)	ATCC

(STM1224) in the subspecies I ATCC 14028 background were generated by Lambda Red swap essentially as previously described (20). The *S. enterica* subsp. IIIa (subsp. *arizonae*) isolate used in this study was SGSC4693 (62:z4,z23:-; also known as SARC5 RSK2980, CDC346-86, or ATCC BAA-731), and the complete genome sequence is publicly available for this isolate (GenBank Accession number CP000880.1). The *S. enterica* subsp. IIIb (*diarizonae*) isolate used in this study was SGSC4692 [61:1,v:1,5,(7), also known as CDC 01-0005], and this isolate is currently being sequenced (54, 55). Spontaneous nalidixic acid-resistant isolates of these strains were generated in our laboratory by selecting resistant colonies on Luria-Bertani (LB) plates containing 50  $\mu$ g/ml nalidixic acid, and these strains are listed in Table 1. All strains were routinely grown in LB broth or on LB plates containing 50  $\mu$ g/ml nalidixic acid or 50  $\mu$ g/ml kanamycin when appropriate.

For determination of different subspecies CFU counts from competitive infections we used a simple colorimetric assay for PhoN expression. Mutations in *phoN* do not affect either virulence or intestinal persistence in subspecies I (36). IIIa (2980) lacks *phoN* activity and forms white colonies on medium containing 5 bromo-4-chloro-3-indolyl- $\beta$ -D-phosphate (XP; 20 mg/liter), while IIIb used in these experiments is *phoN* positive and forms blue colonies on XP-containing medium. IIIa and IIIb can thus be differentiated from subsp. I isolates with either an intact (in the case of IIIa) or deleted *phoN* locus.

Swimming and swarming motilities of both subspecies IIIa and IIIb were tested and compared to the motility of subsp. I serovar Typhimurium ATCC 14028. Swimming motility was assayed on LB plates containing 0.3% Bacto agar (Difco), while swarming motility was assayed on plates containing 0.6% Bacto agar and 0.5% glucose as previously described (51). Three  $\mu$ l of stationary-phase culture in LB broth was spotted onto motility plates and incubated for 5 h at 37°C, and photographs were taken to capture the movement of all isolates spotted on the same plate. The diameter of each spotted sample was measured at this point and compared to that of subsp. I serovar Typhimurium and was also evaluated after 24 h of incubation on individual swarming motility plates. All assays were performed in triplicate and each experiment was repeated on three separate occasions.

**Organ colonization in *Salmonella*-susceptible BALB/c mice.** We examined the competitive growth of subsp. IIIa (SGSC4693) and IIIb (SGSC4692) isolates with a commonly studied subsp. I isolate, serovar Typhimurium ATCC 14028 (HA348 ATCC 14028 NaI<sup>r</sup> and AJB715 ATCC 14028 NaI<sup>r</sup> *phoN*) in 8- to 10-week-old female *Salmonella*-sensitive BALB/c mice (Jackson Laboratories) in mixed infections using the following protocol: HA348 (wild type) versus HA350 (IIIa) and AJB715(wild type) versus HA378 (IIIb). *Salmonella* strains used as inocula were grown to stationary phase at 37°C with aeration and mixed in a 1:1 ratio of subsp. I to non-subsp. I. Inocula were serially diluted and titers were determined for bacterial CFU to determine the exact ratios for these strains.

Groups of six mice were inoculated intragastrically by gavage with approximately  $2 \times 10^7$  bacteria in 200  $\mu$ l of LB. Infected mice were observed daily for signs of illness and were euthanized after the development of signs, at 4 to 5 days postinfection (inactivity/reluctance to move, ruffled fur, or crouching together). Immediately after euthanasia Peyer's patches, ceca, mesenteric lymph nodes, livers, and spleens of infected mice were excised and homogenized in 5 ml ice-cold phosphate-buffered saline (PBS). Organ homogenates were serially diluted and plated to determine the ratio of subsp. I to non-subsp. I from the tissues of infected animals. Data are expressed as the ratio of subsp. I CFU versus non-subsp. I CFU, were normalized to the input ratio, and were converted

logarithmically. Statistical significance was determined using Student's *t* test and a *P* value of <0.05.

**Persistence in *Salmonella*-resistant CBA/J mice.** Subspecies IIIa and subsp. IIIb were tested for the ability to persist in the intestine of *Salmonella*-resistant CBA/J mice (Jackson Laboratories) in competitive infections with virulent subsp. I ATCC 14028 derivatives. Strains were grown to stationary phase at 37°C with aeration and were mixed 1:1 prior to inoculation. Groups of four to six 8- to 10-week-old CBA/J mice were infected intragastrically by gavage with an equal mixture of subsp. I and non-subsp. I isolates, approximately  $2 \times 10^9$  total CFU in 100  $\mu$ l LB (3). Approximately 100 mg of feces was collected at various time intervals (1, 3, 6, 9, 12, 15, 21, 30, and 40 days postinfection), serially diluted, and plated for enumeration of CFU of subsp. I strain versus non-subsp. I strain. Data are expressed as the ratio of subsp. I versus the non-subsp. I CFU, were normalized to the input ratio, and were converted logarithmically. Statistical significance was determined using Student's *t* test and a *P* value of <0.05.

**Cell association, invasion, and intracellular replication.** The ability of subsp. IIIa and IIIb to associate with, be internalized by, and replicate inside J774-A.1 macrophages was tested. J774-A.1 cells were propagated in Dulbecco's modified Eagle's medium (Cellgro) supplemented with 10% fetal bovine serum (PAA Laboratories), and plated at a density of  $3.5 \times 10^5$  cells per well in 24-well dishes for all infections. Bacteria used for infecting J774-A.1 macrophages were grown to stationary phase without aeration in LB broth supplemented with 0.3 M NaCl, conditions that induce TTSS-1 expression (4, 25). J774-A.1 cells were infected with *Salmonella* at a multiplicity of infection of 50:1 (*Salmonella*:J774) and incubated for 1 h at 37°C with 5% CO<sub>2</sub> in a humidified tissue culture incubator. The actual titer of the inoculum in each experiment was determined by serial dilution and plating on appropriate bacteriologic media. J774-A.1 monolayers were washed three times with 1 ml of sterile PBS prior to lysis, to enumerate cell-associated bacteria, or treated with 100  $\mu$ g/ml gentamicin sulfate for 1.5 h at 37°C with 5% CO<sub>2</sub> in a humidified tissue culture incubator. For enumeration of intracellular bacteria within J774-A.1 cells, gentamicin was removed and monolayers were washed with sterile PBS three times. Infected monolayers were lysed in 1% Triton X-100, and intracellular CFU were enumerated by serial dilution and plating. For assessment of intracellular growth, infected, gentamicin-treated monolayers were washed with sterile PBS and fresh Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10  $\mu$ g/ml gentamicin was added. Infected monolayers were incubated for 24 h, washed three times with sterile PBS, and lysed, and intracellular CFU were enumerated. At each stage when infected cells were lysed, the number of viable J774-A.1 cells in duplicate monolayers infected with each strain was assessed by 0.4% trypan blue (Cellgro) exclusion and counting viable cells. Each experiment was performed on three separate occasions and by evaluating samples in triplicate.

**Analysis of SPI-1 and SPI-2 effector expression and secretion.** In order to determine whether TTSS-1 and TTSS-2 are functional in subsp. IIIa and IIIb, we grew these strains under previously published conditions for TTSS-1 and TTSS-2 expression and determined whether several effectors of this system were being produced and secreted (17, 47). Cultures were normalized by optical density at 600 nm, and bacterial pellets were collected by centrifugation and resuspended in sodium dodecyl sulfate (SDS) sample buffer to generate whole-cell lysate. Proteins in the culture supernatants were precipitated with trichloroacetic acid (TCA) precipitated (6% TCA final concentration) overnight at 4°C. Both proteins from whole-cell lysates and TCA-precipitated proteins from bacteria grown under TTSS-1- and TTSS-2-inducing conditions were separated by SDS-polyacrylamide gel electrophoresis. These proteins were analyzed by Western blotting using specific antisera against the effector proteins SipB (1:10,000 dilution), SipC (1:10,000 dilution), SopB (1:2,000 dilution), SseG (1:2,000 dilution), and SifA (1:2,000 dilution) (antisera were kind gifts of Daoguo Zhou and John Brumell). For detection of primary antibodies we used the WesternDot 625 Western blot kit according to the manufacturer's instructions (Invitrogen).

## RESULTS

**Subspecies I colonizes murine models better than non-subsp. I salmonellae.** We compared the abilities of subsp. I ATCC 14028 and subsp. IIIa or subsp. IIIb to colonize the organs of orally infected *Salmonella*-susceptible BALB/c mice 5 days postinfection. The subsp. IIIa isolate we tested, SGSC4693 (HA350), colonized the intestinal tract very poorly compared to subsp. I (Fig. 1A). In the ceca and Peyer's patches of infected mice, subsp. IIIa colonized 100- to 1,000-fold more

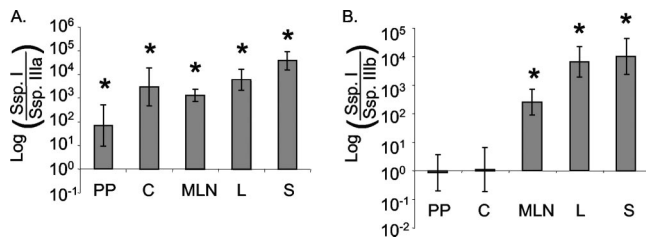


FIG. 1. Non-subspecies I salmonellae colonize BALB/c mice differently than subsp. I ATCC 14028. Mice were infected with an equal mixture of subsp. I (Ssp. I) and non-subsp. I strains and were euthanized 4 days postinfection. Peyer's patches (PP), mesenteric lymph nodes (MLN), spleen (S), liver (L), and cecum (C) were collected, and bacteria were recovered and enumerated. A. Colonization of tissues in BALB/c mice by HA348 (subsp. I) versus HA350 (subsp. IIIa). B. AJB715 (subsp. I) versus HA378 (subsp. IIIb). Data were plotted as logarithmically converted normalized means of output ratios  $\pm$  standard errors. Statistical significance was determined using a Student's two-tailed *t* test, and asterisks indicate that normalized output ratios were significantly statistically different from the equivalent ratio in the inoculum with a *P* value of  $<0.05$ .

poorly at this early time after infection than the subsp. I isolate that was concurrently inoculated. Subspecies IIIa also failed to spread systemically, colonizing the liver and spleen in far lower numbers than the subsp. I isolate (concurrent infection). In contrast, subsp. IIIb (SGSC4692) was equally capable of intestinal colonization in *Salmonella*-susceptible BALB/c mice at 5 days postinfection as subsp. I was (Fig. 1B). Subspecies IIIb colonized the Peyer's patches and cecum in BALB/c mice in numbers equivalent to subsp. I. Despite good colonization of the intestinal tract in BALB/c mice, however, the subsp. IIIb isolate tested here was nearly completely unable to spread systemically and colonized systemic organs very poorly.

We assessed intestinal colonization and long-term fecal shedding in *Salmonella*-resistant mice using intragastric inoculation of groups of six 8-week-old CBA/J mice. We monitored fecal shedding on day 1 and at 3-day intervals for 30 days postinfection. Our results are shown in Fig. 2. Consistent with our experiments in *Salmonella*-susceptible BALB/c mice (Fig. 1), subsp. IIIa also had a defect in initial intestinal colonization of *Salmonella*-resistant CBA/J mice compared to subsp. I (Fig. 2A). This colonization defect was followed by severely reduced fecal shedding of subsp. IIIa from *Salmonella*-resistant mice for 40 days of infection compared to subsp. I ATCC 14028. In contrast, subsp. IIIb colonized the intestine of *Salmonella*-resistant CBA/J indistinguishably from subsp. I and was shed in the feces at levels similar to subsp. I for over 20 days postinfection (Fig. 2B). After 27 days of infection, subsp. IIIb exhibited a statistically significant defect in fecal shedding compared to ATCC 14028.

**Cell association, invasion, and intracellular growth in macrophages.** The ability to adhere to, be internalized by, and replicate in eukaryotic cells, notably macrophages, is intimately associated with the pathogenesis of subsp. I (24) and is important for virulence and systemic colonization by this organism (12, 23). Because subsp. IIIa and IIIb were unable to spread systemically in BALB/c mice and colonize systemic organs, we postulated that defects in cell association, internalization, and intracellular growth in macrophages may be responsible for these defects in intestinal colonization and systemic spread.

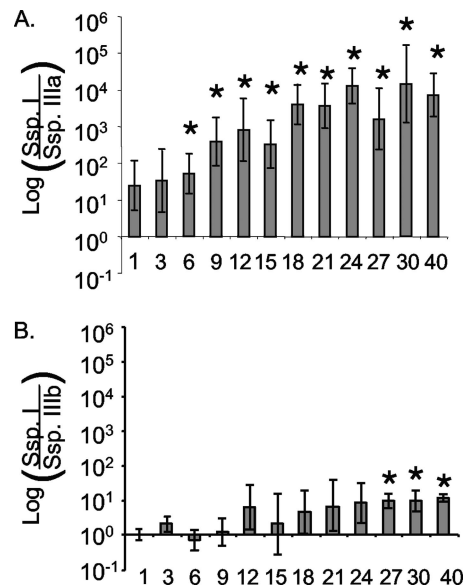


FIG. 2. Non-subsp. I salmonellae differ from subsp. I ATCC 14028 in their ability to persist in the intestine of *Salmonella*-resistant CBA/J mice. (A) *S. enterica* subsp. IIIa (HA350) does not persist in the intestine relative to *S. enterica* subsp. I over 40 days of infection. (B) *S. enterica* subsp. IIIb (HA378) has an intestinal persistence defect relative to subsp. I. Data are represented as the log (mean of the subsp. I CFU/non-subsp. I CFU ratio) recovered from feces at the indicated time points postinfection. Data were converted logarithmically and displayed graphically, and statistical significance was determined using a Student's two-tailed *t* test and a *P* value of  $<0.05$ . Error bars denote standard errors, and a \* denotes a result statistically significantly different from inoculum.

Thus, we tested the ability of subsp. IIIa and subsp. IIIb isolates to associate with, become intracellular in, and replicate in cultured murine macrophages of the J774A.1 lineage. Both subsp. IIIa and IIIb associated with cultured J774 macrophages in higher numbers than subsp. I in our assays (Fig. 3A). Despite this finding, both subsp. IIIa and subsp. IIIb were internalized poorly and at levels similar to our noninvasive serovar Typhimurium  $\Delta invA$  mutant (Fig. 3A). In addition, both the subsp. IIIa and IIIb isolates that we studied replicated poorly inside macrophages in our assays, while both the subsp. I isolate used here and the  $\Delta invA$  mutant replicated intracellularly (Fig. 3B). Additional experiments showed that the growth of subsp. IIIa and subsp. IIIb during competitive growth in vitro in rich media is indistinguishable from subsp. I ATCC 14028 (data not shown).

**Analysis of SPI-1 and SPI-2 secreted effectors.** We used published conditions for the expression of the subsp. I TTSS-1 and TTSS-2 and the secretion of effectors from these systems to determine whether our subspecies IIIa and IIIb isolates were secreting effectors of these systems (Table 2) (45). Bacteria were grown under SPI-1- or SPI-2-expressing conditions (17, 47) and collected by centrifugation, and the secreted proteins in the supernatant were precipitated with TCA. Whole-cell lysates and TCA precipitates were examined by SDS-polyacrylamide gel electrophoresis and Western analysis with antibodies specific to particular effectors of these TTSS.

We determined that the TTSS-1 effectors SopB and SipC (see Table 2 for *S. enterica* subsp. *arizonae* homologs

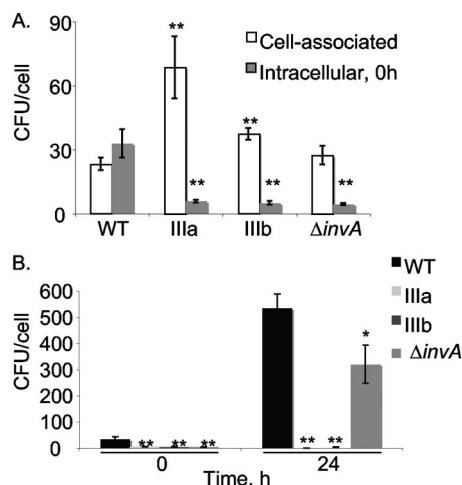


FIG. 3. Subspecies IIIa and IIIb isolates have reduced invasiveness and do not replicate intracellularly in J774 murine macrophages. (A) Attachment and invasion of J774.A1 murine macrophages of virulent wild-type HA348 (WT; subsp. I ATCC 14028 derivative), HA350 (IIIa), HA378 (IIIb), and HA458 (HA348  $\Delta invA::Kan$ ) at a multiplicity of infection of 50. (B) Replication of bacteria inside J774.A1 murine macrophages. Intracellular growth was quantified after a standard gentamicin protection assay as described in Materials and Methods. Data are shown as the means of three experiments, with each assay performed in triplicate, and standard errors. Statistical significance: \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ .

SARDI01910 and SARDI00089) are present in the whole-cell lysates of subsp. I, IIIa, and IIIb grown under TTSS-1-inducing conditions (Fig. 4A). Of these effectors, we found that SopB and its homologs (SARDI01910 in IIIa; IIIb was not sequenced) are secreted into the medium from subsp. I, IIIa, and IIIb under TTSS-1-inducing in vitro conditions. Thus, the TTSS-1 secretion system in all of the subspecies isolates examined here is likely functional and the effector SopB is produced and secreted. In contrast, proteins recognized by anti-SipC antiserum were produced by all the subspecies we examined but they were only secreted by subsp. I. Therefore, although the TTSS-1 appears to be present and functional as shown by the secretion of SopB in all subspecies examined here, a different complement of effectors is being secreted from subsp. I than from the IIIa and IIIb isolates that we studied.

Both IIIa and IIIb encode a homolog of the *sipB* gene (Table

TABLE 2. TTSS-1 and TTSS-2 effector homologs in *S. enterica* subsp. IIIa examined in this study

TTSS and effector(s)	SARI homolog	Length (aa) <sup>a</sup> in subspecies:		% Identity	% Similarity
		I (LT2)	IIIa		
<b>TTSS-1</b>					
SipB, SspB	SARI00088	593	594	92	94
SipC, SspC	SARI00089	409	409	91	94
SopB, SigD	SARI01910	561	561	90	94
<b>TTSS-2</b>					
SseG	SARI01576	228	196	83	91
SifA	SARI01766	336	336	66	79

<sup>a</sup> aa, amino acids.

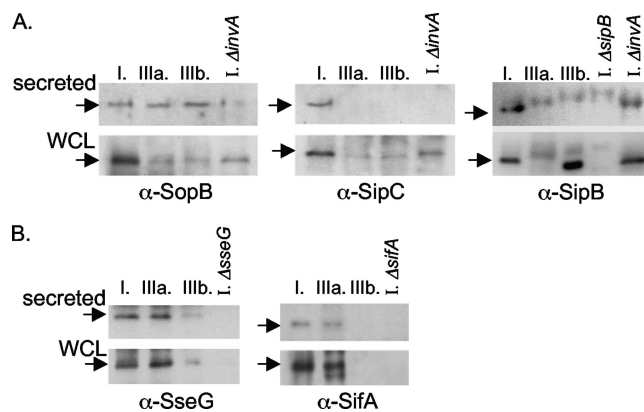


FIG. 4. TTSS-1 and TTSS-2 effector production and secretion from non-subsp. I isolates. We studied the production and secretion of effectors of TTSS-1 and TTSS-2 by using published conditions for the expression of these systems combined with Western analysis with specific antisera. (A) TTSS-1 effector production and secretion were examined in whole-cell lysates (WCL) and from TCA precipitates of secreted proteins using specific antisera against SopB, SipC, and SipB. (B) TTSS-2 effector protein production and secretion were examined in whole-cell lysates and TCA precipitates of secreted proteins using specific antisera against SseG and SifA.

2) (41). In our experiments, however, only subsp. I and subsp. IIIb produced a protein that was strongly reactive with anti-SipB antiserum in the whole-cell lysate under TTSS-1-inducing conditions (Fig. 4A). However, this strongly anti-SipB-reactive protein was not secreted from subsp. IIIb into the medium in our experiments. Although subsp. IIIa encodes a homolog with 92% identity and 94% similarity at the protein level to SipB (SARDI000880 (Table 2), subsp. IIIa did not produce a protein that reacted strongly with anti-SipB antiserum in our experiments. Further mutational analysis will determine whether the weakly reactive anti-SipB bands of similar size produced by subsp. IIIa are SipB homologs. To summarize our findings for the TTSS-1 effectors we examined, there was a variable pattern of production and secretion of these effectors from subsp. IIIa and IIIb relative to subsp. I under the in vitro conditions used here.

We also examined the production and secretion of effectors of TTSS-2 in subsp. I, IIIa, and IIIb, using published conditions for TTSS-2 expression and effector secretion. Subspecies IIIa and IIIb encode homologs of the *sseG* gene, while only subsp. I and subsp. IIIa encode the TTSS-2 effector SifA (45). In our experiments, proteins that reacted with anti-SseG antiserum were produced and secreted from subsp. I, IIIa, and IIIb (Fig. 4B). These data suggest that TTSS-2 in subsp. IIIa and IIIb is functional and that the effector SseG is secreted. Subspecies IIIa produces and secretes a protein that is recognized by anti-SifA antiserum, while subsp. IIIb does not (Fig. 4B). These data are in agreement with the fact that subsp. IIIb does not encode a *sifA* homolog (45). To summarize, the TTSS-2 of subsp. IIIa and IIIb is likely functional and the effector SseG is secreted by both of these non-subspecies I serovars, while the effector SifA is produced and secreted only from subsp. IIIa.

**Motility.** We tested our subsp. IIIa and IIIb isolates for both swimming and swarming motilities by using previously established assay methods. Subspecies IIIb is diphasic and was mo-

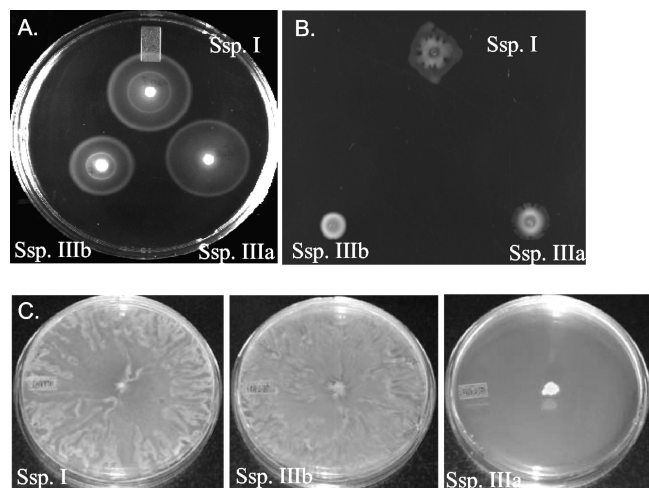


FIG. 5. Subspecies (Ssp.) IIIa and IIIb have altered swimming and swarming motility compared to subsp. I. Three microliters of bacterial culture was spotted onto swimming assay plates (0.3% Bacto Agar) (A) or swarming assay plates (0.6% Bacto agar with 0.5% glucose) (B) and incubated for 5 h at 37°C. (C) Three  $\mu$ l of each isolate was spotted on individual swarming plates and incubated for 24 h at 37°C. The diameter of each area of spotted bacteria was measured at both 5 h and 24 h postinoculation.

tile in both our swimming (Fig. 5A) and swarming (Fig. 5B) assays and swarmed to the same diameter at 24 h compared to subsp. I (Fig. 5C). Subspecies IIIa, in contrast, is known to be monophasic and was comparable to subsp. I in swimming motility (Fig. 5A) but was completely unable to swarm (Fig. 5B and C). After 24 h of incubation on motility plates, subsp. I isolates had a mean swarm diameter of 85 mm (standard deviation, 0), while subsp. IIIa had a mean swarm diameter of 6 mm (standard deviation, 0.81 mm) (Student's two-tailed *t* test,  $P < 1.5 \times 10^{-9}$ ) (Fig. 5C). Using video microscopy we noted that this subsp. IIIa isolate is completely nonmotile on swarming agar (data not shown). Subspecies IIIb, in contrast, is diphasic and was motile in both our swimming and swarming assays and swarmed to the same diameter at 24 h as subsp. I (Fig. 5C).

## DISCUSSION

Subspecies I salmonellae cause the vast majority of salmonellosis in mammals and birds, although non-subspecies I serovars are capable of causing sporadic disease. The factors that allow subspecies I salmonellae to be so epidemiologically dominant in mammals and birds are unknown. In this study we determined that the non-subspecies I isolates that we examined, IIIa SGSC4693 and IIIb SGSC4692, poorly colonize murine models that are commonly used to study the pathogenesis of subsp. I salmonellae compared to subsp. I ATCC 14028.

Although the genome sequence of the subsp. IIIa isolate used in these studies has recently become publicly available, the remaining isolates used here are currently being sequenced, making a direct comparison of their genomic content and comprehensive analysis of our observations possible in the near future. Both small-scale gene-by-gene studies and more comprehensive genomic comparisons between subsp. I salmo-

nellae and non-subspecies I salmonellae have been undertaken (8, 15, 19, 27, 34, 36, 40, 41, 45, 46, 52, 53). These studies identified several areas of genomic variability, including fimbrial operons and other adhesins (36, 37, 45), flagellins, and TTSS apparatus and effectors that, along with other loci, may be relevant to our observations.

We have shown that subsp. IIIa SGSC4693 is highly defective in intestinal colonization and persistence compared to subsp. I. One major area of genetic difference between subsp. I and subsp. IIIa is in the complement of fimbriae and other adhesins that are encoded by these subspecies (15, 45, 52). In subsp. I salmonellae, fimbriae are important factors for colonization of mucosal surfaces in the intestine of mammalian hosts (57). Subspecies I LT2 possesses 12 fimbrial operons of the chaperone-usher assembly class, while subspecies IIIa possesses only two of these, *fim* and *bcf*, but may have additional still-undefined fimbrial operons (45). Subspecies I mutants with the *fim* operon deleted have a reduced ability to attach to HeLa cell monolayers (5), but these mutants colonize and persist in the intestine of *Salmonella*-resistant CBA/J mice to levels very similar to the isogenic wild type (57). Similarly, in subsp. I the *bcf* operon is unnecessary for colonization of the murine intestine until 20 days postinfection (57), but other fimbrial operons such as *std* and *stb* are critical both for colonization and persistence. These studies are consistent with our data that subsp. IIIa is heavily disadvantaged both in initial colonization of the murine intestine and in persistence in this niche, and the two fimbrial operons IIIa is known to possess (*fim* and *bcf*) are unimportant for these processes during infection of murine models with subsp. I.

We have also shown that subsp. IIIa is motile individually in liquid media but is completely unable to swarm across the surface of more solid media. Subspecies IIIa is the most divergent subspecies among *S. enterica* and is monophasic (45). The ability of subsp. IIIa to colonize the intestinal tract may also be limited by its lack of motility in environments of high viscosity. Perhaps this isolate is unable to penetrate viscous environment of the mucous layer covering the intestinal epithelium, although this remains to be shown.

In contrast to subsp. IIIa, subsp. IIIb SGSC4692 colonizes the intestine well in the murine models used in our study but has a persistence defect in this niche. Subspecies IIIb is known to possess four fimbrial operons, *fim*, *bcf*, *std*, and *stb*, but may also have additional, still-unidentified fimbrial operons (45). Clinical isolates of subsp. IIIb have also been reported to have *agfA* (43). In subsp. I deletions of the *std* or *stb* fimbrial operons result in strains that are defective for initial colonization of the intestine and for persistence in this niche (57). Our data, and those of others showing that subsp. IIIb possesses the *std* and *stb* operons and colonizes the murine intestinal tract well, are consistent with the hypothesis that fimbria encoded by *stb* and *std* are very important for intestinal colonization. Subspecies IIIb also lacks the *lpf* operon, which is important for intestinal persistence after 20 days of infection in murine models and for biofilm formation on Hep-2 cells and explanted chicken intestinal tissue (39, 57). The apparent lack of *lpf* in subsp. IIIb may contribute to the mild intestinal persistence defect of this isolate.

Other adhesins known to be important for intestinal persistence in subsp. I include proteins encoded at the CS54 island

by *shdA* (STM2513), the neighboring gene *ratB* (STM2514), and a third protein chromosomally encoded elsewhere by *misL* (STM3757). ShdA and MisL are autotransporter proteins that bind extracellular matrix components in vitro (21, 35). Mutants for *misL* and *shdA* in subsp. I show reduced colonization of the cecum and/or Peyer's patches in BALB/c mice at 5 days postinfection (21, 36). These mutants also have very late defects (21 to 25 days postinfection) in intestinal persistence in *Salmonella*-resistant CBA/J mice (21, 36). *ratB* mutants in subsp. I colonize the intestine well but are highly defective for intestinal persistence beginning at 5 days postinfection (36). *shdA*, *ratB*, and *misL* are absent in the subsp. IIIa and subsp. IIIb isolates that have been examined, including the subsp. IIIa isolate used in our studies (36, 37, 45). These data are consistent with our observations that subsp. IIIa is defective for colonization and persistence in the intestinal tract of mice. However, despite the apparent lack of these factors in subsp. IIIb, the subsp. IIIb isolate tested here was not defective for colonization of the Peyer's patches and cecum in BALB/c mice (21, 36, 37) and had a milder persistence defect in identical animal models than either *misL*, *shdA*, or *ratB* mutants of subsp. I. Our subsp. IIIb isolate may possess alternate adhesins that partially complement the functions of MisL, ShdA, and RatB, and we may be able to determine this when the complete genome sequence for this isolate is completed.

The ability to replicate in macrophages is important for subsp. I to colonize systemic sites in the mouse (12, 23). Both the subsp. IIIa and subsp. IIIb isolates we tested failed to spread beyond the intestine and colonize systemic organs in murine models, and we hypothesized that subsp. IIIa and IIIb may be unable to replicate in macrophages. We showed that these isolates adhere to J774-A.1 murine macrophages in culture, yet they are not internalized and do not replicate once intracellular. The SPI-1-encoded TTSS-1 and the SPI-2-encoded TTSS-2 are essential for cellular invasion and for intracellular replication, abilities that are important for salmonellae to invade host cells and spread systemically. Both subsp. IIIa and subsp. IIIb possess SPI-1 and SPI-2, but the genetic islands encoding these TTSS, the fidelity of these secretion systems, and the distribution, production, and secretion of the effectors of these systems, and the *spv* genes, have not been extensively studied outside of subsp. I salmonellae (15, 24, 29, 30, 45).

Recent reports have indicated that subsp. IIIa either lacks or has several divergent components of TTSS-1 encoded in SPI-1, including *invA* and *invH* (15). *avrA*, an effector of the TTSS-1 that inhibits interleukin-8 production by inhibiting NF- $\kappa$ B signaling as well as preventing the ubiquitination of  $\beta$ -catenin, is also absent from subsp. IIIa (16, 22, 33, 41). We showed that the TTSS-1 effector SopB, an inositol phosphatase that activates CDC42, Rac1, RhoG, AktA, and chloride secretion and disrupts tight junctions (9, 38, 42, 44, 59), is produced and secreted from subsp. IIIa in vitro, indicating that the TTSS-1 is likely functional in subsp. IIIa in our experiments. Despite its presence in whole-cell lysate, we could not detect the secretion of SipC, a second TTSS-1 effector that nucleates and bundles actin (13, 28, 48) under in vitro conditions that allow the secretion of this protein from subsp. I. Finally, the TTSS-1 effector SipB, which binds to and activates caspase-1 to induce autophagy and apoptosis in macrophages (31, 32), does not appear to be produced or secreted from subsp. IIIa in our

experiments. Thus, the TTSS-1 of subsp. IIIa is functional but secretes a different complement of effectors than the TTSS-1 of subsp. I under similar conditions. A more complete analysis of the presence or absence of TTSS-1 effector homologs and their production and secretion in vivo from subsp. IIIa may provide additional insight into the inability of subsp. IIIa to colonize the murine intestine.

Genetic variability in SPI-2, encoding the TTSS-2, in subsp. IIIa has also been reported recently (15). We showed that the TTSS-2 effector SseG, a participant in *Salmonella*-induced filament (Sif) formation and microtubule bundling during the intracellular growth of subsp. I, is produced and secreted from subsp. IIIa. A second effector of the TTSS-2, SifA, involved in inducing Sif formation and maintenance of the *Salmonella*-containing vacuole, is also produced and secreted from subsp. IIIa. These data strongly suggest that the TTSS-2 machinery is functional in our in vitro experiments in subsp. IIIa. A more complete comparison of the presence and primary sequence of the 20 or so known effector protein homologs of the TTSS-2 in subsp. IIIa may yield additional clues as to why subsp. IIIa is unable to replicate intracellularly in macrophages and spread systemically in murine models of infection.

SPI-1 and SPI-2 regions encoding the TTSS-1 and TTSS-2 secretion systems themselves have not been extensively studied in subsp. IIIb beyond microarray analysis for genomic content (45). Subspecies IIIb is known to encode homologs for the following TTSS-1 effectors: SipA, SipB, SipC, AvrA, SopB, and SopD (41), but the presence of the remaining known effectors of TTSS-1 has been studied only by microarray analysis. The gene for the TTSS-1 effector SopA appears to be missing or divergent in subsp. IIIb in these microarray studies (45). We showed that subsp. IIIb produces and secretes SopB, suggesting that the TTSS-1 is functional in our assays. However, although subsp. IIIb produced two additional effector proteins, SipB and SipC, these proteins were not detectable in the secreted fraction. This finding indicates that despite a functional TTSS-1, subsp. IIIb secretes a different complement of effectors than subsp. I. Further work will be necessary to determine whether a unique complement of TTSS-1 effectors is secreted from subsp. IIIb during infection and how this may affect intestinal colonization and persistence.

Finally, subsp. IIIb produced and secreted the TTSS-2 effector SseG, indicating that the TTSS-2 apparatus is also functional in our subsp. IIIb isolate. An additional effector called SifA does not have a homolog in subsp. IIIb (45). In agreement with this published finding, SifA was not detected in whole-cell lysates in our experiments. Thus, the TTSS-2 in subsp. IIIb also secretes a different complement of effectors, lacking at least one effector known to be important for intracellular growth of subsp. I. If variability in effector production and secretion also occurs in vivo, it may contribute to the inability of IIIb to grow intracellularly and to its failure to colonize systemic sites in *Salmonella*-susceptible BALB/c mice. Completion of the genome sequence and subsequent complete analysis of the TTSS-2 effector homologs present in subsp. IIIb should provide additional insight into the differences between subsp. I and subsp. IIIb TTSS-2 effector complements present in both organisms.

The non-subsp. I *Salmonella* isolates we studied in this work colonize the intestine differently than subsp. I, and they are

defective for fecal shedding and intestinal persistence. This altered colonization of the murine intestinal tract is likely the result of multiple factors, including different complement of fimbrial operons and adhesins and potentially differential production and secretion of effectors of the TTSS in non-subsp. I isolates relative to subsp. I. The inability of subsp. IIIa and IIIb to colonize and/or persist in the intestinal tract are likely to be partially responsible for the failure of non-subsp. I isolates to circulate in populations of warm-blooded vertebrates relative to subsp. I salmonellae, as these factors are critical to transmission of salmonellae from host to host. Furthermore, we showed that subsp. IIIa and IIIb are strongly defective for systemic colonization. The inability of the subsp. IIIa and IIIb isolates tested here to access systemic sites may be partially the result of different complements of effectors of the type three secretion systems in these isolates relative to subsp. I and may reduce the ability of these isolates to persist long term in an infected host, thus reducing transmission of these subspecies in warm-blooded vertebrates.

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