Net Replication of *Salmonella enterica* Serovars Typhimurium and Choleraesuis in Porcine Intestinal Mucosa and Nodes Is Associated with Their Differential Virulence[▽]

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Salmonella enterica **is a facultative intracellular pathogen of worldwide importance and causes a spectrum of diseases depending on serovar- and host-specific factors. Oral infection of pigs with** *S. enterica* **serovar Typhimurium strain 4/74 produces acute enteritis but is rarely fatal, whereas serovar Choleraesuis strain A50 causes systemic disease with a high mortality rate. With a porcine ligated ileal loop model, we observed that systemic virulence of serovar Choleraesuis A50 is not associated with enhanced intestinal invasion, secretory responses, or neutrophil recruitment compared to serovar Typhimurium 4/74. The net growth in vivo of serovar Choleraesuis A50 and serovar Typhimurium 4/74 was monitored following oral inoculation of pigs with strains harboring pHSG422, which exhibits temperature-sensitive replication. Analysis of plasmid partitioning revealed that the enteric virulence of serovar Typhimurium 4/74 relative to that of serovar Choleraesuis A50 is associated with rapid replication in the intestinal wall, whereas systemic virulence of serovar Choleraesuis A50 is associated with enhanced persistence in intestinal mesenteric lymph nodes. Faster replication of serovar Typhimurium, compared to that of serovar Choleraesuis, in the intestinal mucosa was associated with greater induction of the proinflammatory cytokines tumor necrosis factor alpha, interleukin-8 (IL-8), and IL-18 as detected by reverse transcriptase PCR analysis of transcripts from infected mucosa. During replication in batch culture and porcine alveolar macrophages, transcription of genes encoding components of type III secretion systems 1 (*sipC*) and 2 (*sseC*) was observed to be significantly higher in serovar Typhimurium 4/74 than in serovar Choleraesuis A50, and this may contribute to the differences in epithelial invasion and intracellular proliferation. The rapid induction of proinflammatory responses by strain 4/74 may explain why pigs confine serovar Typhimurium infection to the intestines, whereas slow replication of serovar Choleraesuis may enable it to evade host innate immunity and thus disseminate by stealth.**

Salmonella enterica subspecies *enterica* can be divided into more than 2,400 antigenically distinct serovars, some of which are important pathogens of humans and food-producing animals (50). The serovars can be divided into three broad classes (46). Ubiquitous serovars (e.g., Typhimurium) produce acute but self-limiting enteritis in a broad range of hosts, whereas host-specific serovars (e.g., Typhi) are associated with severe systemic disease in healthy outbred adults of a single species which may not involve diarrhea. Host-restricted serovars (e.g., Choleraesuis) are primarily associated with systemic disease in one host but may cause disease in a limited number of other species.

Clinical salmonellosis in pigs is mostly caused by serovars Choleraesuis and Typhimurium (36). Pigs infected with serovar Choleraesuis are lethargic and pyrexic and often have respiratory symptoms including pneumonia and coughing. Di-

arrhea may or may not be present, and cyanosis of the extremities is common. Gross lesions often include swollen mesenteric lymph nodes, enlargement of the spleen and liver, and congestion of the lungs. Mortality is high, particularly in intensively reared weaned pigs. In contrast, serovar Typhimurium typically causes watery diarrhea, anorexia, and pyrexia, but with a low mortality rate and little or no systemic involvement. Serovar Typhimurium infections may become persistent, involving asymptomatic excretion of the bacteria in the feces for several months postexposure. In a year-long randomized national abattoir survey in the United Kingdom, serovar Typhimurium was detected in the ceca of 11.1% of pigs presented for slaughter and on 2.1% of carcass swabs (15); thus, persistently infected pigs represent a significant reservoir of contamination of the food chain and environment. Serovar Choleraesuis was once the most frequently isolated serovar in pigs in the United Kingdom (41). While it is now rarely isolated in the United Kingdom, it remains a serious threat in the United States (36) and is also associated with sporadic cases of severe salmonellosis in humans (49; reviewed in reference 11).

The molecular mechanisms underlying the ability of *S. enterica* serovars to colonize the intestines of food-producing animals, and in some cases translocate to systemic sites, are poorly understood. Genome-wide mutagenesis has identified portfolios of serovar Typhimurium genes required for intesti-

nal colonization of cattle (32, 44), chickens (32), pigs (8), and mice (10, 24, 28, 38, 44) and revealed that *Salmonella* uses both conserved and host-specific colonization factors. Variation in the repertoire, sequence, and expression of such factors may explain the differential virulence of *S. enterica* serovars. Type III secretion systems (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2 play pivotal roles in the colonization of porcine intestines by serovar Typhimurium (5, 8), and mutation of a key SPI-1 regulator (*hilA*) is highly attenuating for serovar Choleraesuis following oral infection of pigs (29). Analysis of the complete genome sequence of serovar Choleraesuis strain SC-B67 recently revealed that it contains a higher proportion of deletions and pseudogenes than serovar Typhimurium LT2, particularly among genes involved in chemotaxis, signal transduction, and metabolism (12). The impact of such polymorphisms on the outcome of infections caused by serovars Typhimurium and Choleraesuis has received little attention.

The basis of systemic virulence of host-specific and -restricted serovars has so far not been definitively correlated with events during infection. No association could be detected between systemic virulence and the magnitude of intestinal invasion or induction of inflammatory or secretory responses by strains of host-restricted serovar Dublin in calves (34), Abortusovis in lambs (47), or Gallinarum in chickens (9). Indeed, strains of serovars that are systemically avirulent in these hosts following oral inoculation, including serovar Typhimurium, exhibited increased invasion (9, 34, 47) and elicited greater enteropathogenic responses (34, 47). Furthermore, although the natural resistance-associated macrophage protein is important in the control of systemic serovar Choleraesuis infection in pigs (48), strains of serovar Choleraesuis do not exhibit an ability to survive better in porcine primary alveolar macrophages compared to serovar Typhimurium and no significant differences in the induction of proinflammatory cytokines in cultured porcine alveolar macrophages by the serovars could be detected (53). It has been noted that host-restricted serovars persist better in mesenteric lymph nodes, indicating that bacterial net growth at intestinal sites may be an important determinant of systemic virulence (34, 47).

Here, we used the temperature-sensitive replicon pHSG422 (23) to study the net replication of *S. enterica* serovar Typhimurium and Choleraesuis strains of well-defined virulence following oral inoculation of pigs. Plasmid pHSG422 has a temperature-sensitive origin of replication that permits replication at 30°C or below but results in segregation of the plasmid between dividing cells at 37°C or higher. Thus, during infection at body temperature the plasmid is titrated out of the bacterial population with each round of replication. For a strain with a fast in vivo replication rate, more bacteria will be recovered but fewer will be plasmid bearing, whereas a slow-replicating strain will yield fewer bacteria, more of which would harbor pHSG422. While the proportion of bacteria carrying pHSG422 is an indication of the growth rate, the absolute number of cells carrying pHSG422 provides a measurement of killing (see reference 22). Plasmid pHSG422 has been used to study intracellular growth of *Salmonella* in murine dendritic cells (26) and the impact of mutation of SPI-2 (37), *spv* genes (21, 22), prior infection with mouse hepatitis virus (19), and the host Ity locus (2) on *Salmonella* replication in mice. The fate of the serovar

Choleraesuis and Typhimurium strains in vivo was further dissected by analysis of intestinal invasion, induction of enteritis, and analysis of the transcription of host and bacterial genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. Serovar Typhimurium 4/74 and serovar Choleraesuis A50 have been described previously, and their virulence in pigs is well defined (53). The behavior of these strains in cultured cells and animals is comparable to that of other strains of the same serovar (3, 34, 53), and the clinical symptoms they elicit are typical of field isolates. Plasmid pHSG422 (23) was kindly provided by T. Hashimoto-Gotoh, Kyoto, Japan, and was electroporated into the *Salmonella* strains with selection for resistance to ampicillin (100 μg/ml) and kanamycin (50 μg/ml) at 25°C. Plasmid carriage did not impair the growth of the strains at 25°C in vitro or their ability to invade cultured INT-407 cells or bovine ligated ileal mucosa (data not shown). Additional strains of serovars Typhimurium (12/75 and TML) and Choleraesuis (14/74) were used for analysis of *sipC* transcript and protein levels (53). Serovar Typhimurium strains 4/74 and 12/75 show nearly identical replication kinetics in porcine alveolar macrophages (53) and invasion and enteropathogenicity in calves (34). The serovar Choleraesuis A50 and 14/74 strains also behave similarly to each other in these models, albeit that they invade, persist, and induce enteritis at lower levels than the serovar Typhimurium strains. Serovar Typhimurium 4/74 *sipB* (1) and *sipC* (32) mutant strains have been described previously. Strains were cultured in Luria-Bertani (LB) medium with agitation and antibiotics as appropriate. Derivatives harboring pHSG422 were cultured to maintain a low copy number as previously described (21).

Construction of an *aroA* mutant of serovar Choleraesuis A50. To establish that partitioning of pHSG422 can report differences in the net growth of *Salmonella* in pigs, a Tn*10* insertion in *aroA* was transferred into serovar Choleraesuis A50 by phage P22 HT/int-mediated transduction as previously described (31). The mutant was verified by analysis of growth on M9 minimal medium with and without aromatic amino acids and examined for nonagglutination with 5% acriflavine-HCl to confirm normal expression of lipopolysaccharide.

Infection of pigs. All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 (license number 30/1998) with the approval of the local Ethical Review Committee. Large-White × Landrace male pigs aged ca. 8 weeks were obtained from a commercial supplier and housed in containment level 2 accommodations with access to antibiotic-free irradiated weaner pellet and water ad libitum. Pigs were confirmed to be culture negative for *Salmonella* prior to infection by overnight enrichment of rectal swabs in Rappaport broth (at 37°C) and selenite brilliant green broth (at 42°C), followed by plating to modified brilliant green agar (Oxoid, Basingstoke, United Kingdom). Stationary-phase 25°C LB-grown cultures of serovar Typhimurium 4/74/pHSG422, Choleraesuis A50/pHSG422, or Choleraesuis A50 *aroA*/pHSG422 were diluted to contain approximately 1×10^9 CFU and suspended in 10 ml antacid [5% (wt/vol) Mg(SiO$_3$)$_3$, 5% (wt/vol) NaHCO$_3$, 5% (wt/vol) MgCO$_3$ in H$_2$O] and administered directly into the stomach of each pig immediately before the morning feed with a 10_{FG} catheter. To validate the use of pHSG422 for analysis of net replication, seven pigs were infected with serovar Choleraesuis A50/pHSG422 and three pigs were infected with serovar Choleraesuis A50 *aroA*/pHSG422. To compare the net replication of the serovars, a total of 22 pigs were inoculated with serovar Typhimurium 4/74 and sacrificed at 24 h (9 pigs), 36 h (3 pigs), 48 h (7 pigs), and 72 h (3 pigs). Net replication was compared to that of serovar Choleraesuis A50 in a total of 20 pigs killed at 24 h (7 pigs), 36 h (3 pigs), 48 h (7 pigs), and 72 h (3 pigs). Rectal temperatures were recorded, and animals were monitored for clinical signs of disease twice daily. At postmortem examination, samples of mid-ileum, mid-colon, and associated mesenteric lymph nodes were aseptically excised and viable bacteria were enumerated by plating of serial 10-fold dilutions of homogenates of triplicate 1-g biopsy samples from each site onto MacConkey agar with and without ampicillin and kanamycin, as appropriate. Tissue samples were also recovered from the same sites for analysis of host and bacterial mRNAs (see below). The limit of accurate quantification was 2.0 log$_{10}$ CFU/g. Samples containing fewer bacteria were enriched in Rappaport broth and selenite brilliant green broth as described above. The difference in the log$_{10}$ total number of CFU/g and the log$_{10}$ number of pHSG422-bearing CFU/g was calculated and provided a measurement of the titration of the plasmid from the bacterial population and hence the rate of replication (21, 22).

Quantification of intestinal invasion and induction of enteritis. Ligated ileal loops were constructed in ca. 8-week-old Large-White × Landrace pigs following sedation by intramuscular administration of 2 mg/kg Stresnil (Janssen Animal Health, High Wycombe, United Kingdom), induction of anesthesia by intrave-

TABLE 1. Primers and probes used for detection of porcine 28S rRNA and TNF-α, IL-8, and IL-18 mRNAs by real-time RT-PCR

Primer or probe	Sequence 5′–3′[a]
28S rRNA.fwd	GCTCCACGGGAGGTTTCTG
28S rRNA.rev	GGTACACCTGTCAAACGGTAACG
28S rRNA.probe	(FAM)-CTCCCTGAGCTCGCCTTAGGACACCT-(TAMRA)
TNF-α.fwd	AAGGACTCAGATCATCGTCTCAAAC
TNF-α.rev	CGGCTTTGACATTGGCTACA
TNF-α.probe	(FAM)-CGTGGGCGACGGGCTTATCTGA-(TAMRA)
IL-8.fwd	AGTTTTCCTGCTTTCTGCAGCT
IL-8.rev	TGGCATCGAAGTTCTGCACT
IL-8.probe	(FAM)-ACTCTTGCCAGAACTGCAGCCTCACA-(TAMRA)
IL-18.fwd	TCCTTTTCATTAACCAGGGACATC
IL-18.rev	GGTCTGAGGTGCATTATCTGAACA
IL-18.probe	(FAM)-CAGAATCAGGCATATCCTCAAACACGGCT-(TAMRA)

[a] FAM, 6-carboxyfluoroscein; TAMRA, 6-carboxytetramethylrhodamine.

nous administration of 18 mg/kg Saffan (Schering-Plough Animal Health, Welwyn Garden City, United Kingdom), and maintenance of anesthesia with isoflurane in oxygen via an endotracheal tube. A laparotomy was performed, and sequential 9-cm loops (invasion assays) or 6-cm loops (enteritis assays) with 1-cm spacers were constructed with surgical silk. Approximately 1×10^9 CFU of LB-grown mid-logarithmic-phase test strains were injected into appropriate loops (triplicate determinations in a semirandomized order), and the wound was repaired.

Invasion was quantified essentially as previously described (3), by injection of 4.5 ml of 300 μg/ml gentamicin in mucosal medium into distal ileal loops 2 h after inoculation with bacteria. Viable gentamicin-protected bacteria were then enumerated in tissues recovered after incubation in situ for a further 1 h (initial invasion) or 10 h (survival postinvasion) by plating of serial 10-fold dilutions of homogenates of triplicate 1-g biopsy samples from each loop blended separately for 30 s in 1% (vol/vol) Triton X-100 in phosphate-buffered saline onto MacConkey agar. The conditions used have been confirmed to result in efficient killing of an adherent noninvasive *Escherichia coli* strain (3, 52), and control loops were included to enumerate indigenous gentamicin-resistant bacteria.

Secretory responses were measured in triplicate mid-ileal loops for each strain and are defined by the ratio of the volume of fluid accumulated (V, ml) to loop length (L, cm). Neutrophil recruitment was quantified as a measurement of the induction of intestinal inflammatory responses by [^{111}In]oxinate labeling of neutrophils purified from blood sampled from the vena cava at the outset of the experiment and reinjected within 1 to 2 h of loop inoculation, essentially as previously described (51). Neutrophil influx is defined as the ratio of gamma emission from ^{111}In-labeled neutrophils within the combined mucosa and contents of test loops to that from loops filled with sterile medium as a negative control.

Analysis of porcine cytokine responses. Samples of ileal and colonic mucosa were obtained at postmortem examination from the same sites at the same intervals postinoculation as plasmid partitioning was assessed. Tissues were collected and snap-frozen in liquid nitrogen with minimal delay after death and stored at −70°C until required. Total RNA was prepared from 1-g samples with TRI REAGENT (Sigma, St. Louis, MO) with an on-column DNase treatment step. RNA was confirmed to be intact and free of genomic DNA by agarose gel electrophoresis. Purified RNA was eluted in 100 μl RNase-free water and stored at −70°C. Primers and probes for the detection of porcine tumor necrosis factor alpha (TNF-α), interleukin-8 (IL-8), and IL-18 mRNAs and 28S rRNA by real-time reverse transcriptase PCR (RT-PCR) were designed by using the *Sus scrofa* genome sequence at intron-exon boundaries such that amplicons could only derive from mRNA (Table 1). RT-PCR was performed with the ABI PRISM 7700 sequence detection system (PE Applied Biosystems, Warrington, United Kingdom) with quantitative RT-PCR Mastermix (Eurogentec, Seraing, Belgium) and an initial cycle of 50°C for 2 min, 60°C for 30 min, and 95°C for 5 min, followed by 40 cycles of 94°C for 20 s and 59°C for 1 min. To generate standard curves for the cytokine- and 28S rRNA-specific reactions, RNA was serially diluted from 10^{-1} to 10^{-5} in sterile, RNase-free water. Each RT experiment contained three no-template controls, test samples, and a $\log_{10}$ dilution series and was performed in triplicate with replicates prepared on different days. Results are expressed as the threshold cycle value (C_t), the cycle at which the change in reporter dye (Rn) passes a significant threshold. To account for variation in sampling and RNA preparation, the C_t values for the cytokine-specific product for each sample were standardized by using the C_t value of the 28S rRNA product for the same sample. Regression analysis of the mean values

of six replicate RT-PCRs for the $\log_{10}$-diluted RNA was used to generate standard curves. Corrected C_t values were calculated, and results were then expressed as $40-C_t$ values as previously described (25), 40 being the maximum number of amplification cycles in the assay. Hence, the higher the $40-C_t$ value, the greater the amount of specific mRNA in a particular sample.

Preparation and infection of porcine alveolar macrophages. Alveolar macrophages were isolated from healthy ca. 8-week-old Large-White × Landrace pigs by bronchoalveolar lavage as previously described (53). Macrophages were cultured in RPMI 1640 medium buffered with 2 g/liter sodium bicarbonate and supplemented with 18 mM HEPES buffer, 2 mM L-glutamine, and 5% (vol/vol) fetal calf serum. They were inoculated with a multiplicity of infection of 100:1 of nonopsonized, LB-grown stationary-phase bacteria. This relatively high multiplicity of infection was used in studies to define the serovar Typhimurium transcriptome in murine macrophage-like cells and was required to yield adequate bacterial mRNA for subsequent analysis (18). The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere and harvested for RNA extraction at 4, 8, and 24 h after infection.

Analysis of the transcription of T3SS-1 and -2 genes. Total RNA from LB-grown bacteria and infected macrophages was extracted with TRI REAGENT and treated in solution with Turbo DNase (Ambion, Inc., Austin, TX), followed by on-column DNase treatment with RNase-free DNase. A conserved housekeeping gene (*yejA*) was used as an internal standard and is expressed at constant basal levels in mid-logarithmic- and stationary-phase LB cultures following exposure to pH 3.0 and 10.0 low magnesium and phosphate levels and upon infection of epithelial and murine macrophage-like cells (18; J. Hinton, personal communication). Primers and probes used for amplification of *sipC*, *sseC*, and *yejA* are listed in Table 2. RT-PCR was performed as described for porcine cytokine mRNAs. Control reaction mixtures omitting RT or RNA were included, and three independent biological replicates were performed. The data from repeated experiments were pooled and analyzed as previously described (30). The C_t values for test genes were normalized to the C_t value of the internal standard (*yejA*) amplified from the corresponding sample. The sequences of the target genes are 100% identical in the sequenced genomes of serovars Typhi-

TABLE 2. Primers and probes used for detection of *mgtC*, *sipC*, *sseC*, and *yejA* mRNAs by real-time RT-PCR

Primer or probe	Sequence 5′–3′[a]
sipC.fwd	GGACGAAGCCCGTGAAAGT
sipC.rev	TGCTCTCCATTGTTTTCAGCATT
sipC.probe	(FAM)-CCTGAATCAGGCTGGTCGATTTACG TG-(TAMRA)
sseC.fwd	GCAGGTTGTGCAGGAATGGT
sseC.rev	TGGTCAGCACCGCACATC
sseC.probe	(FAM)-TGCCGTTTCGGCTCCGGCT-(TAMRA)
yejA.fwd	TCAGCGATCCGCTTTCAAC
yejA.rev	GCCCATTTTCCACTGAGTAATCC
yejA.probe	(FAM)-CCGCCCTTAGCCAGCGGGCCA AT-(TAMRA)

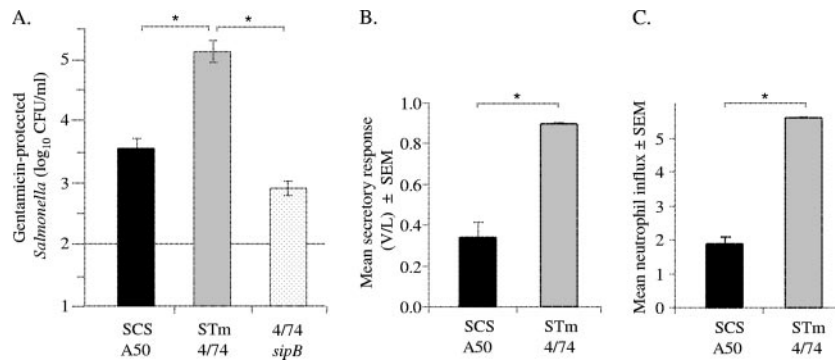[a] FAM, 6-carboxyfluoroscein; TAMRA, 6-carboxytetramethylrhodamine.

FIG. 1. Systemic virulence of serovar Choleraesuis (SCS) A50, relative to that of serovar Typhimurium (STm) 4/74, in pigs is not associated with enhanced intestinal invasion or enteritis. Panel A shows the magnitude of intestinal invasion by strains of *S. enterica* serovars Choleraesuis and Typhimurium 3 h after inoculation of porcine distal ileal loops, with the data representing the mean of triplicate determinations in two pigs $\pm$ the standard error of the mean (SEM). The dotted line represents the limit of accurate bacterial quantification. The magnitudes of secretory and inflammatory responses induced by the strains 12 h after inoculation of porcine mid-ileal loops are shown in panels B and C, respectively. Data in panels B and C represent the means of triplicate determinations in two pigs $\pm$ the standard errors of the means. The secretory response is reported as the ratio of the volume of fluid accumulated (V, ml) to the loop length (L, cm). Neutrophil influx is the ratio of the total gamma radiation emitted from ^{111}In-labeled neutrophils from test loops to that emitted from control loops. $*$, $P < 0.05$.

murium and Choleraesuis; therefore, differences in detection of the transcripts are likely to be real, rather than due to sequence polymorphisms in the primer or probe targets or template accessibility owing to secondary-structure variations. To quantify transcription, the $2^{\Delta\Delta-Ct}$ method (30) was used for data analysis and transcription was reported as *n*-fold induction normalized to the internal standard and relative to the uninfected control at time zero. The limit of detection of transcripts from LB-grown bacteria was determined by comparing the mean C_t value against the number of viable bacteria. Bacterial mRNAs could be detected over a linear range of 10^8 to 10^1 CFU/ml for both serovar Choleraesuis A50 and serovar Typhimurium 4/74, with C_t values ranging from 13 to 28.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Total and secreted *Salmonella* proteins were prepared as previously described (55) and were resolved by 4 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were either stained with Gel Code Blue (Pierce Biotechnology, Rockport, IL) or transferred to nitrocellulose membrane and probed with SipC-specific murine monoclonal antibody (clone F569AC6; E. Galyov, Institute for Animal Health, Compton, Newbury, Berkshire, United Kingdom) detected with horseradish peroxidase-conjugated anti-mouse immunoglobulin G as previously described (55).

Statistical analysis. A general linear model (Minitab) was used to determine whether the mean $\log_{10}$ total number of CFU/g or the difference between the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of plasmid-bearing CFU/g was statistically significant between time intervals and tissues sampled. RT-PCR data were analyzed by Student's *t* test with $P < 0.05$ taken to be significant.

RESULTS

Systemic virulence of serovar Choleraesuis A50 in pigs is not associated with enhanced intestinal invasion, secretory responses, or neutrophil recruitment. We previously described the outcome of oral infection of age-matched pigs with strains of serovars Choleraesuis and Typhimurium (53); however, it is not known if the systemic virulence of serovar Choleraesuis in pigs is associated with enhanced invasion of mucosal surfaces or intestinal pathology. With a porcine ligated ileal loop model, the number of viable gentamicin-protected bacteria was determined at 3 h postinoculation of distal ileal loops with serovar Choleraesuis A50, serovar Typhimurium 4/74, and an isogenic serovar Typhimurium 4/74 *sipB* mutant. The magnitude of invasion by the *sipB* mutant was significantly lower than that of invasion by the parent strain (Fig. 1A; $P < 0.05$), confirming the key role of T3SS-1 in invasion of porcine intestinal mucosa and indicating that recovered bacteria are not

derived from the intestinal lumen ($P < 0.05$). The number of gentamicin-protected serovar Typhimurium 4/74 CFU was significantly higher than that of serovar Choleraesuis A50 CFU (Fig. 1A; $P < 0.05$). Serovar Typhimurium 4/74 also elicited significantly higher levels of fluid accumulation and neutrophil recruitment 12 h postinoculation of mid-ileal loops compared to serovar Choleraesuis A50 (Fig. 1B and C; $P < 0.05$), indicating that the systemic virulence of the latter strain is not associated with enhanced invasion or induction of enteritis.

Plasmid partitioning detects reduced net replication of a serovar Choleraesuis A50 *aroA* mutant following oral inoculation of pigs relative to the parent strain. To confirm that partitioning of pHSG422 could accurately report the replication rate of *Salmonella* in the porcine intestines, pigs were inoculated orally with serovar Choleraesuis A50/pHSG422 (seven pigs) or serovar Choleraesuis A50 *aroA*/pHSG422 (three pigs) grown under conditions permissive for plasmid replication. Forty-eight hours postinoculation, plasmid-bearing and -nonbearing salmonellae were enumerated in homogenates of ileal and colonic mucosa and associated mesenteric lymph nodes. The difference in the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of pHSG422-bearing CFU/g was calculated and provides a measurement of the titration of the plasmid from the bacterial population and hence the rate of replication (21, 22). The serovar Choleraesuis A50 parent strain was found in overall higher numbers than the isogenic *aroA* mutant at the tissue sites examined (Fig. 2; total bar height; P values denoted by asterisks). In addition, the difference between the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of pHSG422-bearing CFU/g was greater with the wild type at the sites examined than with the *aroA* mutant (Fig. 2; difference in height of solid bar compared to total bar height; P values denoted by triangles), indicating that the plasmid had been titrated out of the population at a faster rate owing to the increased net replication of the wild-type strain.

Analysis of the net replication of serovar Choleraesuis A50 and serovar Typhimurium 4/74 following oral inoculation of pigs. To measure the net replication of strains representing the
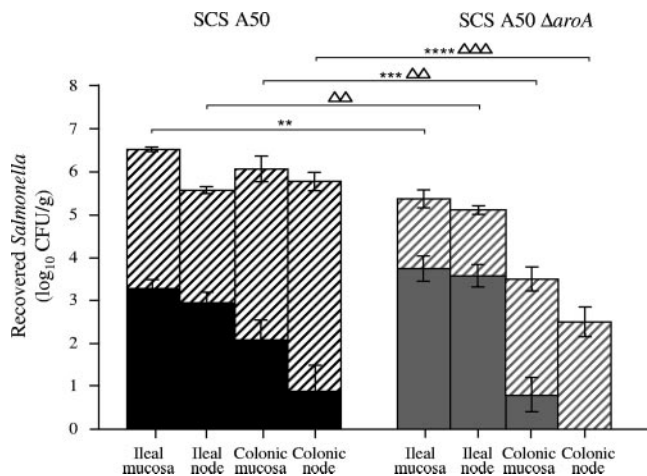
FIG. 2. Analysis of plasmid partitioning detects differences in the net replication of serovar Choleraesuis (SCS) A50 *aroA* mutant and parent strains 48 h after oral inoculation of pigs. Triplicate samples of each tissue type were taken, and means were calculated to give a value per animal. Each bar represents the mean bacterial count derived from seven pigs infected with serovar Choleraesuis A50 harboring pHSG422 and three pigs infected with serovar Choleraesuis A50 *aroA*/pHSG422 ± the standard error of the mean. The solid bars represent the number of bacteria carrying pHSG422, and the hashed bars represent total bacterial numbers. Triangles denote the statistical significance of the difference between the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of pHSG422-bearing CFU/g between the two strains ($P < 0.1$, 0.05, and 0.01 for one, two, and three triangles, respectively), and asterisks denote statistically significant differences in the $\log_{10}$ total number of CFU/g between strains ($P < 0.1$, 0.05, 0.01, and 0.001 for one, two, three, and four asterisks, respectively).

two serovars following oral inoculation of pigs, a total of 42 pigs were challenged with pHSG422-bearing derivatives of serovar Typhimurium 4/74 (22 pigs) or Choleraesuis A50 (20 pigs) cultured at 25°C. Pigs were then sacrificed in groups of three to nine at 24, 36, 48, or 72 h postinoculation, and the plasmid-bearing and -nonbearing salmonellae were enumerated as described above. In the ileal and colonic mucosa, the difference between the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of pHSG422-bearing CFU/g was significantly greater at 36 and 48 h postinoculation for serovar Typhimurium 4/74 than for serovar Choleraesuis A50, indicating that the replication rate was higher (Fig. 3; P values denoted by triangles). Significantly higher numbers of serovar Choleraesuis A50 CFU were recovered from ileal lymph nodes at all time points and from colonic lymph nodes at 36 to 72 h postinoculation compared to serovar Typhimurium 4/74 (Fig. 3; P values denoted by asterisks). Fewer plasmid-bearing cells of serovar Choleraesuis A50 were recovered from colonic lymph nodes compared to ileal lymph nodes, and it is possible that this reflects differences in the rate of replication at the two sites; however, at both sites for all but one time point, greater numbers of serovar Choleraesuis pHSG422-bearing cells were recovered compared to serovar Typhimurium 4/74-bearing cells (Fig. 3). Thus, the enteric virulence of serovar Typhimurium 4/74 following oral inoculation of pigs is associated with rapid replication in the intestinal mucosa whereas the systemic virulence of serovar Choleraesuis A50 is associated with enhanced persistence in mesenteric lymph nodes. Despite

inoculation with comparable doses, by 24 h postinoculation of pigs a significant difference in the level of fecal excretion of the two strains was observed, with serovar Typhimurium 4/74 being excreted at 7.3 ± 0.4 $\log_{10}$ CFU/g compared to serovar Choleraesuis A50/pHSG422 at just 4.1 ± 0.4 $\log_{10}$ CFU/g ($P < 0.05$).

Serovar Typhimurium 4/74 replicates more rapidly than serovar Choleraesuis A50 in ileal mucosa early after bacterial invasion. To probe replication rates earlier in the course of infection, porcine mid-ileal loops were inoculated with pHSG422-bearing derivatives of serovar Choleraesuis A50 and serovar Typhimurium 4/74 cultured at 25°C. Gentamicin was added 1 h after loop inoculation to kill extracellular bacteria, and the plasmid-bearing and -nonbearing salmonellae were enumerated at 12 h postinoculation as described above. The data indicate that serovar Typhimurium 4/74 reached statistically significantly higher numbers than serovar Choleraesuis in the ileal mucosa in the 12 h after loop inoculation ($P < 0.01$; Fig. 4), thereby confirming observations in orally challenged animals (Fig. 3). Though comparable numbers of plasmid-bearing cells of the two strains were recovered, the difference between the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of pHSG422-bearing CFU/g was significantly greater for serovar Typhimurium 4/74 than for serovar Choleraesuis ($P < 0.05$), implying a faster rate of replication. The data also define the growth kinetics of the strains in the same model and for the same duration as inflammatory and secretory responses were measured (above).

Faster replication of serovar Typhimurium in the intestinal wall is associated with enhanced induction of porcine proinflammatory cytokines early after infection. The transcription of genes encoding the porcine proinflammatory cytokines TNF-α, IL-8, and IL-18 was assessed 24, 48, and 72 h after oral inoculation of pigs by using biopsy samples of the same ileal mucosa as used to assess net replication by plasmid partitioning (as described above). Real-time RT-PCR assays were developed, and they revealed strong induction of transcription of each cytokine relative to 28S rRNA at each time interval compared to ileal mucosa from age-matched control uninoculated pigs (Fig. 5). At 24 h postinoculation, serovar Typhimurium 4/74 elicited significantly greater levels of transcription of TNF-α, IL-8, and IL-18 compared to serovar Choleraesuis A50 (Fig. 5; $P < 0.01$ for TNF-α and IL-8 and <0.05 for IL-18). By 48 and 72 h postinoculation, there were no significant differences in the levels of TNF-α, IL-8, or IL-18 mRNA in ileal mucosa infected with either strain.

Enhanced epithelial invasion and intracellular proliferation of serovar Typhimurium 4/74 may be associated with elevated expression of T3SS-1 and -2 genes relative to serovar Choleraesuis A50. To probe the possible basis of enhanced invasion and net growth of serovar Typhimurium 4/74 in porcine ileal tissue relative to serovar Choleraesuis A50, we examined the transcription of genes encoding translocon components of T3SS-1 (*sipC*) and -2 (*sseC*), since both systems are known to play key roles in the colonization of porcine intestines by serovar Typhimurium (8). The role of the T3SS-1 translocon was confirmed in this study by the reduced invasion of a serovar Typhimurium 4/74 *sipB* mutant in porcine ligated ileal loops (Fig. 1). Real-time RT-PCR assays for *sipC* and *sseC* mRNA were developed, and *n*-fold induction was measured
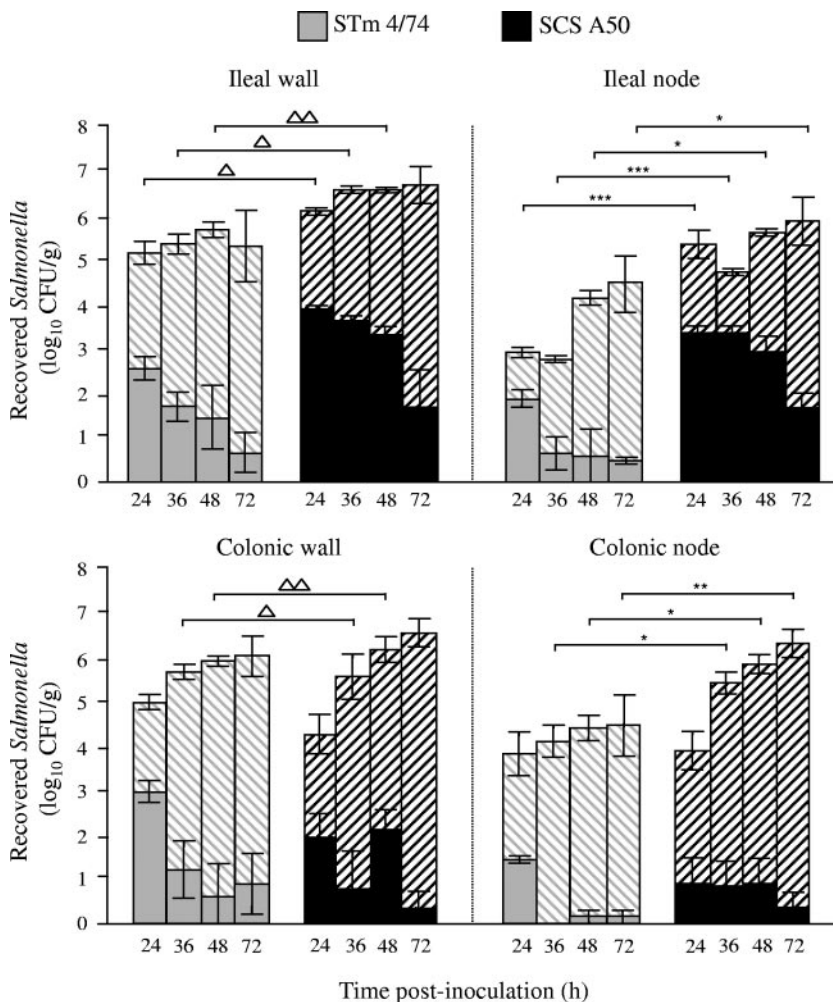
FIG. 3. Net replication of serovar Typhimurium (STm) 4/74 and serovar Choleraesuis (SCS) A50 in intestinal mucosa and lymph nodes associates with their differential virulence. Recoveries of total and plasmid-bearing serovar Choleraesuis A50/pHSG422 and serovar Typhimurium 4/74/pHSG422 at intervals of 24, 36, 48, and 72 h after oral inoculation of pigs are shown. Triplicate samples were taken from each tissue, and means were calculated to give a value per animal. Each bar represents the mean bacterial count derived from three to nine pigs per strain (see Materials and Methods) at each time point $\pm$ the standard error of the mean. The solid bars represent the number of bacteria carrying pHSG422, and the hashed bars represent the total bacterial numbers. Gray bars represent serovar Typhimurium 4/74, and black bars serovar Choleraesuis A50. Triangles denote the statistical significance of the difference between the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of pHSG422-bearing CFU/g between the two strains ($P < 0.1$, 0.05, and 0.01 for one, two, and three triangles, respectively), and asterisks denote statistically significant differences in total bacterial numbers between strains ($P < 0.1$, 0.05, and 0.01 for one, two, and three asterisks, respectively).

relative to the housekeeping gene *yejA*. Despite being able to detect mRNA of porcine cytokines in RNA extracted from ileal mucosa from pigs inoculated orally with serovar Typhimurium 4/74 or Choleraesuis A50, we were unable to amplify bacterial RNA from the same samples (data not shown). However, during mid-logarithmic-phase growth in LB broth and following infection of porcine primary alveolar macrophages, the *n*-fold induction of *sipC* and *sseC* (relative to *yejA*) was significantly greater in serovar Typhimurium 4/74 compared to that in serovar Choleraesuis A50 (Fig. 6A and B; $P < 0.05$). Furthermore, the level of transcription of *sipC* was found to be consistently higher in three serovar Typhimurium strains compared to two serovar Choleraesuis strains and the elevated level of *sipC* mRNA was mirrored by the presence of higher levels of SipC protein in the secreted fraction of serovar

Typhimurium strains, as detected by Western blotting with a SipC-specific monoclonal antibody (Fig. 6C).

DISCUSSION

Differences in the outcome of infection of pigs by *S. enterica* serovars Typhimurium and Choleraesuis are well defined in the field (reviewed in reference 50) and in experimental studies (3, 53). Toward an understanding of the differential virulence of these serovars, we dissected aspects of serovar Typhimurium 4/74 and serovar Choleraesuis A50 infections in pigs. These strains have been extensively characterized following oral inoculation of pigs (53) and infection of porcine alveolar macrophages (53) and in bovine oral challenge and ligated ileal loop models (34). Their behavior in such models is comparable
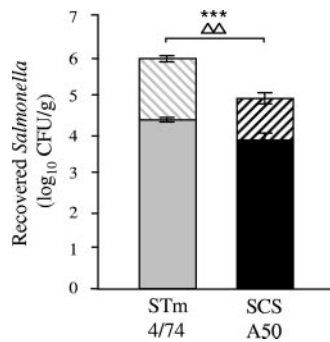
FIG. 4. Serovar Typhimurium (STm) 4/74 replicates more rapidly than serovar Choleraesuis (SCS) A50 in porcine ileal mucosa early after infection. Recoveries of total and plasmid-bearing serovar Choleraesuis A50/pHSG422 and serovar Typhimurium 4/74/pHSG422 12 h after intraluminal inoculation of porcine mid-ileal loops are shown. Gentamicin was added to each loop after 1 h to kill extracellular bacteria. Each bar represents the mean bacterial count derived from triplicate biopsy samples in triplicate loops from two pigs ± the standard error of the mean. The solid bars represent the number of bacteria carrying the pHSG422, and the hashed bars represent total bacterial numbers. Gray bars represent serovar Typhimurium 4/74, and black bars represent serovar Choleraesuis A50. Triangles denote the statistical significance of the difference between the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of pHSG422-bearing CFU/g between the two strains ($P < 0.05$), and asterisks denote significant difference between the total bacterial numbers ($P < 0.01$).

to that of other strains of the same serovar; thus, as far is reasonably practicable given the costly nature of large-animal experimentation, they may be considered representative of the serovar.

Consistent with studies on host-restricted *S. enterica* serovar Dublin in cattle (34), serovar Abortusovis in lambs (47), and serovar Gallinarum in chickens (9), systemic virulence of serovar Choleraesuis in pigs was not associated with enhanced invasion of ileal mucosa or greater induction of intestinal inflammatory responses compared to serovar Typhimurium 4/74. These data support previous observations that serovar Choleraesuis A50 causes less destruction of porcine intestinal mucosa than does serovar Dublin 3246, as assessed by electron microscopy of ileal mucosa 3 h after loop inoculation (3), even though serovar Choleraesuis A50 is more virulent than serovar Dublin 3246 following oral inoculation of pigs (53). We therefore hypothesized that the differential virulence of serovar Choleraesuis A50 and serovar Typhimurium 4/74 may be a function of distinct replication or killing kinetics in the intestinal mucosa and associated mesenteric lymph nodes.

Net bacterial replication in vivo was measured by analysis of the partitioning of a plasmid that exhibits temperature-sensitive replication. The system was confirmed to be suitable for
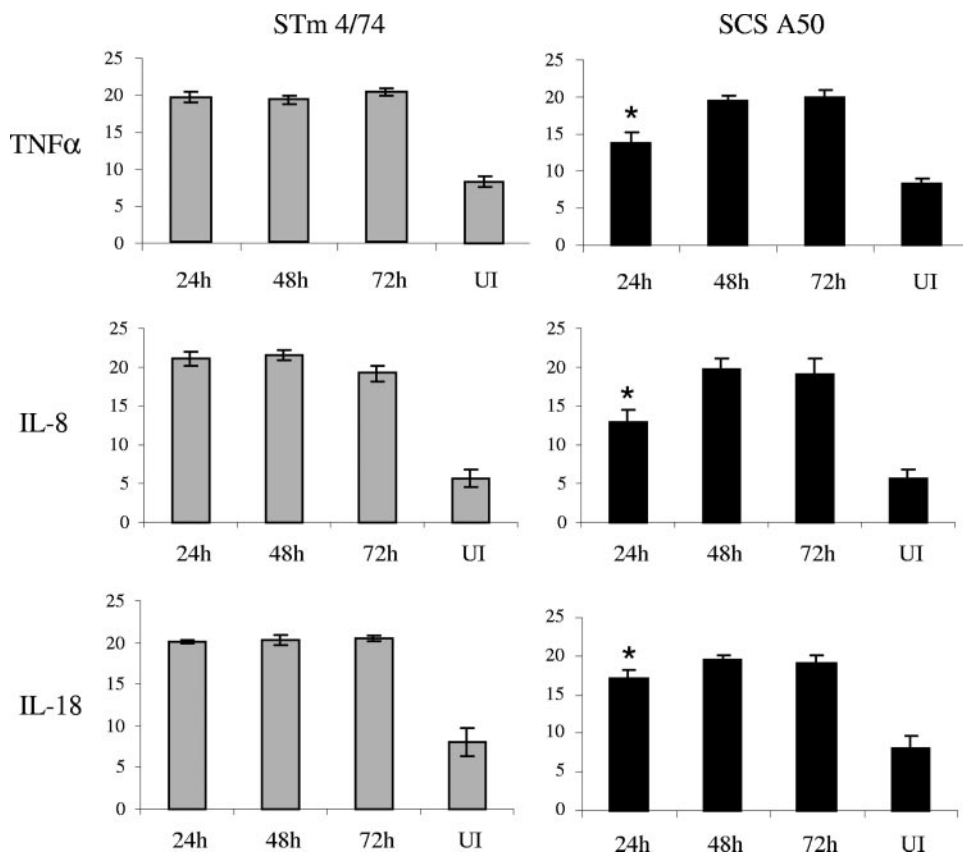

FIG. 5. Serovar Typhimurium (STm) 4/74 induces elevated levels of proinflammatory cytokine mRNAs compared to serovar Choleraesuis (SCS) early after oral inoculation of pigs. Data represent the mean $40\text{-}C_t$ values (a measurement of the abundance of cytokine mRNAs) for porcine TNF-α, IL-8, and IL-18 mRNAs in ileal mucosa 24, 48, and 72 h after oral inoculation of pigs with serovar Typhimurium 4/74 or serovar Choleraesuis A50 or mock infection relative to that for 28S rRNA. The mean was derived from independent determinations from at least three pigs at each time interval ± the standard error of the mean. ∗, $P < 0.05$ for elevated transcript levels induced by serovar Typhimurium 4/74 compared to those induced by serovar Choleraesuis A50 at 24 h postinfection. UI, uninfected.
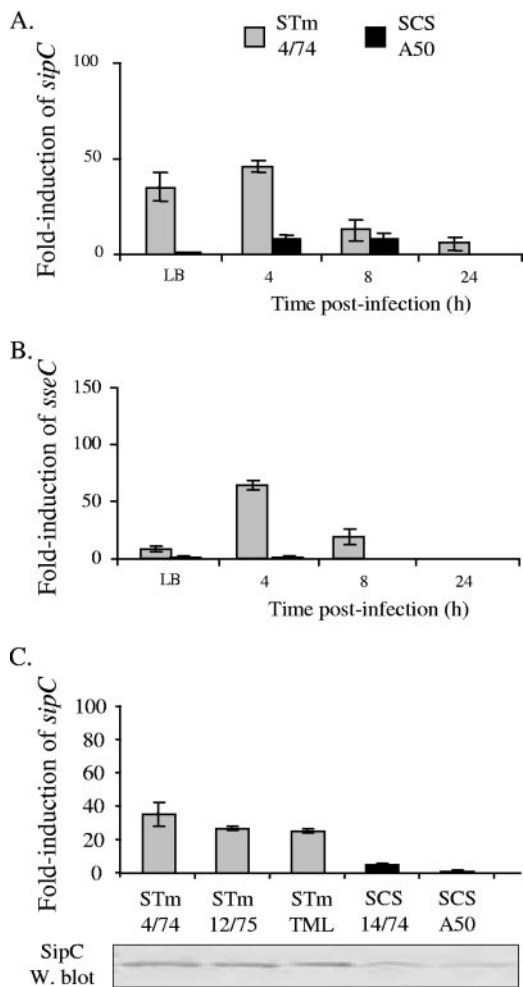
FIG. 6. Type III secretion-associated genes are expressed at a greater level in serovar Typhimurium (STm) 4/74 compared to serovar Choleraesuis (SCS) A50 during batch culture and infection of porcine alveolar macrophages. *n*-Fold induction of *sipC* (panel A) and *sseC* (panel B) relative to *yejA* in serovar Typhimurium 4/74 (gray bars) and serovar Choleraesuis (black bars) during mid-logarithmic-phase growth in LB broth (A_{600}, ca. 0.7) and 4, 8, and 24 h postinfection of primary porcine alveolar macrophages is shown. The *n*-fold induction of *sipC* mRNA relative to *yejA* during mid-logarithmic-phase growth in LB was also measured for different serovar Typhimurium and Choleraesuis strains (panel C). Secreted proteins prepared from the same cultures as used for RNA extraction were probed for SipC protein by Western (W.) blotting with a SipC-specific monoclonal antibody (panel C, bottom). All *P* values for *n*-fold differences, <0.05.

this purpose by comparison of a serovar Choleraesuis *aroA* mutant and its parent strain bearing pHSG422 following oral inoculation of pigs. Significantly fewer *aroA* mutant bacteria were recovered from most intestinal sites, and the difference between the $\log_{10}$ total number of CFU/g and the $\log_{10}$ number of plasmid-bearing CFU/g was significantly lower for the *aroA* mutant than for the parent strain by 48 h postinoculation, implying a slower rate of replication. These data are consistent with the known role of aromatic amino acid biosynthesis in *Salmonella* replication in vivo (33). Since the system had been validated, plasmid partitioning was used to measure the net growth of serovar Typhimurium 4/74 and serovar Choleraesuis

A50 following oral inoculation of pigs by sacrificing groups of at least 3 pigs at intervals of 24, 36, 48, and 72 h postinfection. The data indicated that serovar Typhimurium 4/74 replicates faster in the ileal and colonic mucosa than does serovar Choleraesuis A50, whereas strain A50 reached higher numbers in draining mesenteric lymph nodes than did strain 4/74. The faster replication rate of serovar Typhimurium 4/74 in intestinal mucosa was associated with elevated levels of porcine proinflammatory cytokines at 24 h after oral inoculation, elevated secretory and inflammatory responses in mid-ileal loops, and elevated fecal excretion relative to that of serovar Choleraesuis A50. In contrast, replication at a slower rate and elevated persistence in mesenteric lymph nodes are associated with the systemic virulence of serovar Choleraesuis A50.

The differences in the magnitudes of induction of proinflammatory cytokine mRNAs detected herein following oral inoculation of pigs with strains of serovars Typhimurium and Choleraesuis are broadly in agreement with those detected in porcine mesenteric lymph nodes in a recent study by Uthe et al. (45). Compared to uninfected control animals, serovar Typhimurium significantly induced IL-8 transcription in ileocecal nodes early after oral infection (24 and 48 h) whereas IL-8 was initially repressed at 8 to 24 h after serovar Choleraesuis infection and then observed to be induced at later times (2 and 7 days) (45). In that study and in the present one, it is possible that differences in earlier transcriptional responses to the strains that may influence the outcome of infection were missed. Studies using the porcine jejunal epithelial cell line IPEC-J2 have indicated that a strain of serovar Typhimurium elicits significantly higher levels of IL-8 protein and mRNA than serovar Choleraesuis at 3 and 6 h after invasion (39, 40). Conflicting data exist on the induction of IL-8 mRNA at just 1.5 h after invasion of IPEC-J2 cells, with one study detecting no difference between the serovars (39) and another reporting significantly elevated levels of IL-8 in response to serovar Typhimurium (40). Cytokine responses were not correlated with the magnitude of invasion or net bacterial replication in these studies.

One may speculate that the rapid replication of serovar Typhimurium would trigger inflammatory responses earlier following infection and of a greater magnitude, which may confine the infection locally to the intestinal mucosa, whereas slow replication of serovar Choleraesuis may enable it to evade the activation of host innate immunity, thus facilitating dissemination by stealth. Differences in the level of induction of proinflammatory responses likely also explain why serovar Typhimurium 4/74 elicits stronger enteropathogenic responses in ligated loops and following oral inoculation than does serovar Choleraesuis A50. It remains unclear if the elevated fecal excretion of serovar Typhimurium 4/74 compared to serovar Choleraesuis A50 shortly after oral inoculation of pigs with comparable doses results from faster replication in the intestinal wall and/or lumen or reflects differences in the frequency and/or consistency of passed fecal matter.

The connection between the bacterial replication rate and disease outcome is reinforced by the identification of serovar Typhimurium mutants that overgrow in macrophages because of their inability to stimulate host nitric oxide synthase but are attenuated in BALB/c mice (17). These and other findings (reviewed in reference 43) imply that control of net replication

by *Salmonella* may confer a survival advantage. In an attempt to dissect the molecular mechanisms that dictate the differential growth rates of serovar Typhimurium 4/74 and serovar Choleraesuis A50, we examined the transcription of genes encoding translocon components of T3SS-1 and T3SS-2. Both systems are known to play key roles in colonization of the porcine intestines by serovar Typhimurium (5, 8; Fig. 1A), and their activity has been correlated with the progression of typhoid fever in murine models. T3SS-1-injected effector proteins stimulate rearrangements of the subcortical actin cytoskeleton that facilitate bacterial invasion; however, T3SS-1 also contributes to bacterial growth in epithelial cells (42) and the T3SS-1 effector SopB stimulates nitric oxide production and remains active long after bacterial entry (16). T3SS-1 expression in vivo is also correlated with induction of a lysosomal repair response that can control *Salmonella* infection (35). T3SS-2 facilitates intracellular proliferation by modulating the trafficking of the *Salmonella*-containing vacuole. It is induced in the intestinal lumen (6), and contextual regulation of T3SS-2 is important in the progression of typhoid fever following oral dosing of mice with serovar Typhimurium (14).

In the strains of well-defined virulence used, transcription of *sipC* and *sseC* was consistently higher in serovar Typhimurium 4/74 than in serovar Choleraesuis A50 during mid-logarithmic-phase growth in vitro and following infection of porcine primary alveolar macrophages. We were unable to measure transcript levels in the same tissues as plasmid partitioning was assessed, presumably as they were present below the limit of detection since RNA extracted from the tissues yielded amplicons for porcine cytokine mRNA. Immunohistochemistry with antibodies directed against T3SS-1 and -2 effector proteins or epitope-tagged variants thereof also failed to detect expression of the proteins in vivo, presumably owing to the small quantities present (data not shown). Transcription of *sipC* was consistently higher in serovar Typhimurium strains that show behavior comparable to that of strain 4/74 in porcine macrophages (53), and the difference from serovar Choleraesuis strains was also reflected in the quantities of SipC protein produced.

In addition to differences in the levels of transcription of the genes for T3SS-1 and -2, our analysis of the genomes of sequenced *S. enterica* serovars reveals that the genes encoding the effector proteins SopA, SlrP, SseI, and SspH2 are pseudogenes in serovar Choleraesuis SC-B67 and SseK2 is absent, yet the genes are intact in serovar Typhimurium LT2. Of these, SopA is known to be important in the induction of enteritis by serovar Dublin in bovine ligated ileal loops (54). We confirmed that SopA is a pseudogene in serovar Choleraesuis A50 by nucleotide sequencing and Western blot analysis with a SopA-specific monoclonal antibody (data not shown). Such polymorphisms in the repertoire, sequence, and expression of T3SS-related genes may explain the reduced invasion and inflammatory potential of serovar Choleraesuis; however, it is likely that other factors are involved. Of 151 pseudogenes in the serovar Choleraesuis SC-B67 genome, 64 were predicted to be metabolism related (12). Furthermore, the *shdA* gene, which encodes a fibronectin-binding protein required for Peyer's patch colonization and fecal shedding in mice (27) and enteric virulence early after oral inoculation of pigs (4), is a pseudogene in SC-B67 relative to LT2. It is equally possible

that such defects may explain the reduced replication rate of serovar Choleraesuis compared to serovar Typhimurium in intestinal epithelia; however, a requirement exists to confirm that the polymorphisms also exist between the two strains examined herein. Mutational attrition of several predicted regulators has also been described previously in SC-B67 (12); however, the PhoPQ two-component system known to play a key role in intracellular proliferation (reviewed in references 20 and 43) appears to be intact in the sequenced genomes. Polynucleotide phosphorylase, which has been proposed to alter the balance between acute infection and persistence by modulating SPI-1 and -2 genes (13), is also identical in strains SC-B67 and LT2.

The importance of T3SS in the translocation of serovar Choleraesuis from the porcine intestines remains ill defined. A serovar Choleraesuis *hilA* mutant defective in the regulation of T3SS-1 was less able to colonize the intestines and disseminate to the liver and spleen following oral inoculation of pigs, but not following intraperitoneal infection (29). The role played by T3SS-2 in dissemination from the porcine intestines is unknown. In recent studies with calves, early translocation of serovar Dublin from the intestines to mesenteric lymph nodes, the liver, and the spleen was found to occur independently of T3SS-2 (G. D. Pullinger et al., submitted for publication); thus, caution is required in interpreting the importance of variations in the expression or sequence of T3SS-related genes until their precise contribution to pathogenesis is known.

As elegantly reviewed in reference 43, the rate of intracellular proliferation of *Salmonella* is strictly dependent on the cell type, occurring rapidly in immortalized epithelial and macrophage lines, whereas in dendritic cells and fibroblasts a homogeneous population in a nonreplicating state exists. It is unclear if the differences in net replication of the *S. enterica* strains examined here reflect the targeting of different cell populations. Understanding of cell tropism is of key importance when probing the roles of bacterial factors in net growth, since mutation of some systems can have different effects in different cell types. For example, SPI-2 is dispensable for survival and intracellular proliferation in dendritic cells even though it is induced (26) and mutation of PhoPQ enhances growth in fibroblasts but inhibits it in other cell types (7). The data reported here indicate that differences in replication rate and host innate immune activation by *S. enterica* serovars may be under bacterial control and have profound implications for disease progression.

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