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RESEARCH ARTICLE

Loss of Multicellular Behavior in Epidemic African Nontyphoidal *Salmonella enterica* Serovar Typhimurium ST313 Strain D23580

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ABSTRACT **Nontyphoidal *Salmonella enterica* serovar Typhimurium is a frequent cause of bloodstream infections in children and HIV-infected adults in sub-Saharan Africa. Most isolates from African patients with bacteremia belong to a single sequence type, ST313, which is genetically distinct from gastroenteritis-associated ST19 strains, such as 14028s and SL1344. Some studies suggest that the rapid spread of ST313 across sub-Saharan Africa has been facilitated by anthroponotic (person-to-person) transmission, eliminating the need for *Salmonella* survival outside the host. While these studies have not ruled out zoonotic or other means of transmission, the anthroponotic hypothesis is supported by evidence of extensive genomic decay, a hallmark of host adaptation, in the sequenced ST313 strain D23580. We have identified and demonstrated 2 loss-of-function mutations in D23580, not present in the ST19 strain 14028s, that impair multicellular stress resistance associated with survival outside the host. These mutations result in inactivation of the KatE stationary-phase catalase that protects high-density bacterial communities from oxidative stress and the BcsG cellulose biosynthetic enzyme required for the RDAR (red, dry, and rough) colonial phenotype. However, we found that like 14028s, D23580 is able to elicit an acute inflammatory response and cause enteritis in mice and rhesus macaque monkeys. Collectively, these observations suggest that African *S.* Typhimurium ST313 strain D23580 is becoming adapted to an anthroponotic mode of transmission while retaining the ability to infect and cause enteritis in multiple host species.**

IMPORTANCE **The last 3 decades have witnessed an epidemic of invasive nontyphoidal *Salmonella* infections in sub-Saharan Africa. Genomic analysis and clinical observations suggest that the *Salmonella* strains responsible for these infections are evolving to become more typhoid-like with regard to patterns of transmission and virulence. This study shows that a prototypical African nontyphoidal *Salmonella* strain has lost traits required for environmental stress resistance, consistent with an adaptation to a human-to-human mode of transmission. However, in contrast to predictions, the strain remains capable of causing acute inflammation in the mammalian intestine. This suggests that the systemic clinical presentation of invasive nontyphoidal *Salmonella* infections in Africa reflects the immune status of infected hosts rather than intrinsic differences in the virulence of African *Salmonella* strains. Our study provides important new insights into the evolution of host adaptation in bacterial pathogens.**

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Prior to the advent of the HIV pandemic, invasive *Salmonella* infections in sub-Saharan Africa primarily afflicted young children, often with malaria coinfection, and were rarely seen in adults (1, 2). However, as HIV began to sweep across sub-Saharan Africa in the 1980s, *Salmonella* was increasingly identified as the most common cause of bacterial bloodstream infections in HIV-infected adults (3–5). In contrast to invasive salmonellosis elsewhere in the world, which consists primarily of typhoid or paratyphoid fever, bloodstream *Salmonella* infections in sub-Saharan Africa are nontyphoidal and most often caused by *Salmonella enterica* serovar Typhimurium, *S.* Enteritidis, and *S.* Dublin (2, 6–8). Of note, while inflammatory enteritis is the most common clinical

manifestation of *S.* Typhimurium and *S.* Enteritidis infections among immunocompetent hosts in the developed world, diarrhea may be minimal or absent in as many as 20 to 50% of children and HIV-infected adults with invasive *Salmonella* infections in Africa (9, 10).

Initial reports emphasized the genetic diversity of nontyphoidal *Salmonella* (NTS) isolates causing bloodstream infections in Africa and their similarity to strains responsible for uncomplicated gastroenteritis (11). However, multilocus sequence typing of more than 50 *Salmonella* isolates from blood or cerebrospinal fluid specimens from patients in Malawi and Kenya found that many belong to a dominant sequence type, ST313 (12). Subsequent analysis has shown that the majority of invasive *S.* Typhimurium strains from sub-Saharan Africa fall within 2 closely related ST313 lineages that emerged during the HIV pandemic (13). These isolates are genetically distinct from well-characterized gastroenteritis-inducing ST19 strains, such as 14028s and SL1344. Sequencing of a representative ST313 isolate, D23580, revealed a large number of pseudogenes and deletions (12). As genomic degradation is a hallmark of host-adapted or -restricted *Salmonella* serovars, like *Salmonella enterica* serovar Typhi, in contrast to most strains of generalist serovars, like *S.* Typhimurium (14, 15), *S.* Typhimurium ST313 is thought to be evolving to become more host-adapted to humans (12). This suggestion is consistent with earlier reports that failed to detect *Salmonella* strains resembling invasive human isolates in the food and household animals of index patients with NTS infections (16) and suggests that *S.* Typhimurium in sub-Saharan Africa might be transmitted from person-to-person rather than from common source vehicles (17). The latter investigation analyzed fecal samples from relatives, animals, and the home environments of Kenyan index patients with NTS bloodstream infections (17). A majority of isolates from human fecal samples, but not from animals or the environment, matched the index patient isolates.

Since those initial studies, it has been demonstrated that *S.* Typhimurium ST313 strains are not host-restricted, as they have retained the ability to infect a variety of hosts, including chickens and mice (18–20). The absence of diarrhea in some patients with invasive *S.* Typhimurium ST313 infections has been attributed to a reduced propensity of these strains to elicit host inflammatory responses and suggested to result from the absence of *pipD* and *ratB* in the D23580 genome, genes associated with diarrhea and colonization of the mammalian intestine by *S.* Typhimurium ST19 strains (12, 21–25). However, we show in the present study that *S.* Typhimurium ST313 strain D23580 is comparable to the conventional *S.* Typhimurium ST19 strain 14028s in its ability to cause invasive disease and inflammation in murine and primate models of intestinal and systemic infection. However, *S.* Typhimurium D23580 is distinctive in its loss of oxidative stress resistance and the ability to form RDAR (red, dry, and rough) colonies that are required for multicellular development (26–29). These phenotypes result from point mutations in the *katE* and *bcsG* genes, which encode stationary-phase catalase and a cellulose biosynthetic protein, respectively. These observations suggest that, in contrast to *S.* Typhimurium ST19 strain 14028s, although *S.* Typhimurium D23580 appears to have adapted to an anthroponotic mode of transmission by losing traits associated with environmental persistence, it remains typical of other *S.* Typhimurium strains in its virulence characteristics and ability to cause enteritis in multiple host species.

RESULTS

Construction of a drug-susceptible ST313 derivative. *S.* Typhimurium ST313 isolates are resistant to multiple commonly used antibiotics, including chloramphenicol, ampicillin, kanamycin, streptomycin, sulfonamides, and trimethoprim, making genetic manipulation difficult. To facilitate the construction of targeted gene mutations in *S.* Typhimurium ST313 strain D23580, a multidrug-susceptible (MDS) version of this strain was constructed. The multidrug resistance (MDR) of D23580 is conferred by the plasmid pSLT-BT, which harbors a Tn*21*-like mobile element containing the aforementioned antibiotic resistance genes. The plasmid pSLT-BT is closely related to the virulence plasmid pSLT of *S.* Typhimurium ST19 strain 14028s, henceforth referred to as pSLT-14028s. Exploiting the similarity of these 2 plasmids, a tetracycline resistance locus was inserted into pSLT-14028s at a location identical to that of the MDR cassette in pSLT-BT. This insertion left all native plasmid virulence genes on pSLT-14028s intact. This new plasmid, named pSLT-14028s::*tetRA*, was then conjugally transferred into a spontaneous nalidixic acid-resistant derivative of D23580. Transconjugants capable of growth on tetracycline/nalidixic acid-containing medium were tested for sensitivity to ampicillin, kanamycin, and chloramphenicol to ensure selection of bacteria that had gained pSLT-14028s::*tetRA* and displaced the MDR plasmid pSLT-BT. The *S.* Typhimurium D23580 derivative with resistance to only 2 antibiotics (tetracycline and nalidixic acid) was used as a background for subsequent genetic manipulations.

D23580 is invasive and cytotoxic for mammalian cells. Clinical descriptions of *S.* Typhimurium ST313 and ST19 human infections have suggested that these NTS strains differ in their interactions with the intestinal tract. *S.* Typhimurium ST313 infections appear to be less frequently associated with diarrheal symptoms than ST19 infections (9). We compared the interaction of *S.* Typhimurium ST313 strain D23580 and ST19 strain 14028s with cultured human epithelial cells. Confluent layers of HeLa human cervical epithelial cells were infected with late-logarithmic-phase bacteria at a multiplicity of infection (MOI) of ~100:1. Ten minutes postinvasion, cell monolayers were washed and then lysed to enumerate intracellular bacteria. D23580 was approximately 3 times as invasive for HeLa cells as strain 14028s (Fig. 1A). Strains lacking the *Salmonella* pathogenicity island 1 (SPI1) type 3 secretion system (T3SS) subunit InvA were completely noninvasive, confirming that invasion by either strain is dependent upon the SPI1 T3SS. We next compared the ability of D23580 and 14028s to induce early macrophage pyroptosis (proinflammatory cell death), another SPI1-dependent phenotype. Early *Salmonella*-induced pyroptosis occurs when flagellin is translocated through the SPI1 T3SS into the host cell cytoplasm (30). One hallmark of pyroptotic cell death is the loss of membrane integrity and subsequent leakage of intracellular contents into the extracellular milieu, including the host cell protein lactate dehydrogenase (LDH). RAW 264.7 murine macrophage-like cells were infected with late-logarithmic-phase bacteria at an MOI of ~10:1. Following infection, macrophages were washed to remove extracellular bacteria and incubated at 37°C for 4 h. Supernatant samples were then assayed for the release of LDH. In agreement with the epithelial cell invasion experiments, D23580 was more than 3 times as cytotoxic for macrophages as 14028s (Fig. 1B). Uptake of D23580 by macrophages was 3 times more efficient than uptake of 14028s,

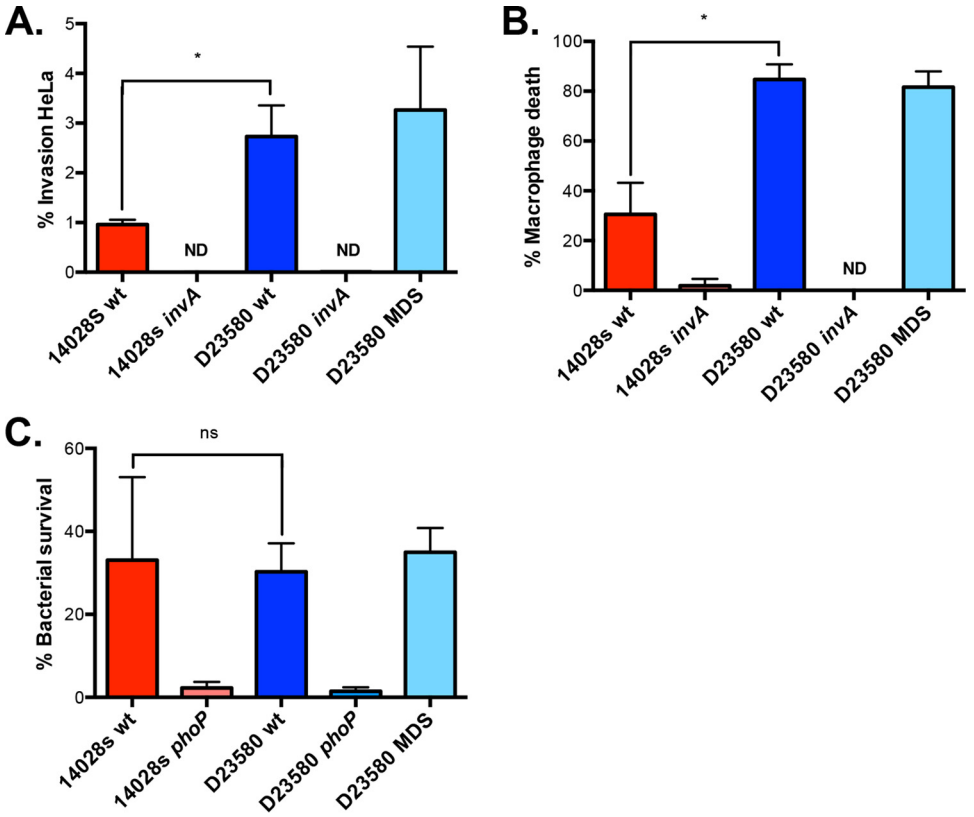

FIG 1 D23580 is more invasive and cytotoxic for cultured cells than 14028s (A) Confluent monolayers of HeLa human cervical epithelial cells were infected with late-logarithmic-phase *S.* Typhimurium ST313 strain D23580 or ST19 strain 14028s cells at an MOI of ~100:1 for 10 min. Intracellular CFU were enumerated 1 h after internalization by lysis and plating. Bacterial invasion is expressed as the proportion of the starting inoculum. *invA* mutants were included as controls for reduced invasion. (B) RAW 264.7 murine macrophage-like cells were infected with late-logarithmic-phase *S.* Typhimurium D23580 or 14028s cells at an MOI of ~10:1. Four hours postinfection, culture supernatants were assayed for released lactate dehydrogenase as a measure of macrophage death. *invA* mutants were included as SPI1-deficient controls. (C) RAW 264.7 murine macrophage-like cells were infected with opsonized stationary-phase *S.* Typhimurium D23580 or 14028s cells at an MOI of ~10:1. Eighteen hours postinfection, macrophages were lysed and intracellular CFU were enumerated. Bacterial survival is expressed as a proportion of internalized bacteria remaining at 18 h postinfection. *phoP* mutants were included as controls with reduced intramacrophage survival. Strain D23580 MDS was included as a control to exclude effects from deletion of plasmid-borne antibiotic resistance determinants. Bars represent the means ± standard deviations. ND, not detected. Statistical significance was determined using a paired *t* test (*, $P < 0.05$; ns, not significant).

which may account for the enhanced cytotoxicity. This phenotype was dependent upon the SPI1-encoded T3SS, as isogenic *invA* mutants of each strain failed to induce a significant degree of macrophage pyroptosis (Fig. 1B). Despite the differences in early pyroptosis, both D23580 and 14028s exhibited similar levels of long-term survival within murine macrophages (Fig. 1C). Collectively, these results demonstrate that D23580 is invasive and cytotoxic for mammalian cells, and these phenotypes are dependent on the SPI1 T3SS, as observed in other *Salmonella* strains.

D23580 is highly invasive following oral infection. To better understand how *S.* Typhimurium ST313 interacts with the mammalian intestine, D23580 and IR715 (a spontaneous nalidixic acid-resistant mutant of ST19 strain 14028s) were orally administered to mice and monitored in the feces, mesenteric lymph nodes, and Peyer's patches. D23580 has previously been shown to cause systemic disease in mice following intragastric (i.g.) or intraperitoneal (i.p.) inoculation (19, 20, 31). However, early interactions between D23580 and the mammalian gut and the subsequent course of infection have been less well studied. CBA/J mice were administered streptomycin i.g. prior to *Salmonella* infection to disrupt the gut microbiota and allow *Salmonella* to establish colonization of the intestinal tract. Following streptomycin treatment, 10^8 CFU of an ~1:1 mixture of D23580 and IR715 were administered i.g. At the indicated time points, mice were sacrificed and their organs homogenized prior to plating on selective medium to enumerate CFU of each strain. Values greater than 1 indicate a competitive advantage of D23580 over IR715. Similar to the observations in cultured human epithelial cells, D23580 was able to invade the murine intestinal tract to a greater degree than IR715 on the first day postinfection (Fig. 2A). In the small intestine, *Salmonella* cells gain access to lymphoid immune follicles known as Peyer's patches via specialized epithelial cells called M cells, or they can be taken up by dendritic cells in the lamina propria (32). The former pathway is SPI1-dependent, whereas the latter is SPI1-independent (32, 33). Following translocation across the intestinal epithelium by either route, the bacteria are able to reach the mesenteric lymph nodes. On day 1 postinfection, D23580 was found in 10-fold-greater abundance than IR715 in the mesenteric lymph nodes and nearly 100-fold-greater abundance in the colonic contents (Fig. 2A). In parallel, D23580 was shed at 10- to 100-fold-higher levels in fecal pellets (Fig. 2B). However, by day 4 postinfection, levels of D23580 in the Peyer's

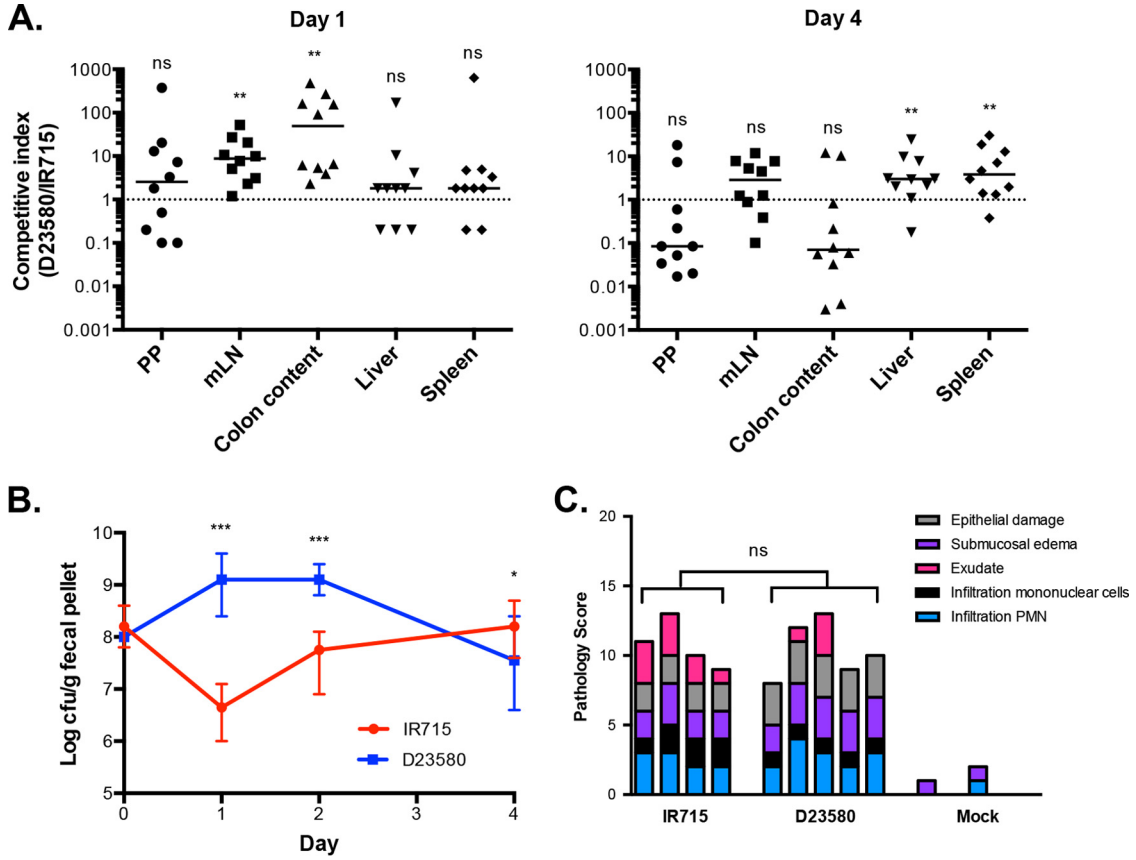

FIG 2 D23580 efficiently colonizes the murine intestine. CBA/J mice were pretreated with streptomycin for 24 h and infected i.g. with an ~1:1 mixture of *S.* Typhimurium ST313 strain D23580 or ST19 strain IR715. (A) On days 1 and 4 postinfection, coinfected mice ($n = 10$) were euthanized and CFU in Peyer's patches (PP), mesenteric lymph nodes (mLN), colonic contents, liver, and spleen were enumerated. Values greater than 1 indicate a competitive advantage of D23580 over IR715. Bars represent the median values. Each symbol represents the result for 1 mouse. Statistical significance was determined using an unpaired Wilcoxon signed rank test (**, $P < 0.01$; ns, not significant). (B) Fecal pellets of i.g.-infected mice ($n = 10$ per group) were collected and homogenized, and CFU were enumerated on days 1, 2, and 4 postinfection. Values represent the medians ± ranges. Statistical significance was determined using a Mann-Whitney test (*, $P < 0.05$; ***, $P < 0.001$; ns, not significant). (C) Streptomycin-treated CBA/J mice were infected i.g. with *S.* Typhimurium D23580 or IR715. At 24 h postinfection, mice were euthanized, followed by removal of the cecum for histopathological analysis. Blinded detailed cecal pathology scores for D23580-, IR715-, and mock-infected mice ($n = 4$) were recorded. PMN, polymorphonuclear leukocyte. Each bar represents the result for 1 mouse. Statistical significance was determined using a Mann-Whitney test of the total histopathology scores (ns, not significant).

patches, mesenteric lymph nodes, colon, and fecal pellets were comparable to those of IR715, or even somewhat reduced in certain sites (Fig. 2A and B). Despite rapid transit from the small intestine lumen to deeper lymphoid tissues, D23580 was not found in higher abundance in the spleen or liver on day 1 and was only found in moderately higher abundance on day 4 postinfection (Fig. 2A).

D23580 induces inflammation in the intestinal tract. Having determined that *S.* Typhimurium D23580 is able to colonize and disseminate from the murine intestinal tract, we next sought to determine whether D23580 differs from the ST19 strain IR715 (a spontaneous nalidixic acid-resistant mutant of 14028s) in its ability to elicit mucosal inflammation. As mentioned previously, the absence of significant diarrhea in some patients with invasive ST313 infections and the reduced inflammatory responses observed in tissue culture (21, 22) have suggested that D23580 might elicit less inflammation than IR715. Previous studies in mice and cattle have also suggested that D23580 elicits less inflammation than ST19 strains in the intestine (21, 23), although the strain of *S.* Typhimurium ST19 used in those studies (SL1344) is recognized to be particularly proinflammatory (34). In contrast to these findings, Parsons et al. reported that D23580 elicits more inflammation in the chicken intestine early in infection (18). In an attempt to clarify these disparate findings, we performed histopathological examinations of infected cecal tissue from streptomycin-pretreated mice inoculated i.g. with *S.* Typhimurium. The results of these experiments revealed no significant difference in intestinal pathology, with both D23580- and IR715-infected samples exhibiting moderate to severe levels of inflammation (Fig. 2C). Further, similar degrees of cecal exudate, epithelial damage, submucosal edema, and infiltration of the lamina propria by mononuclear and polymorphonuclear cells were noted (Fig. 2C).

To determine whether D23580 and IR715 differ in their early interaction with the intestinal mucosa, we compared inflammatory responses in a rhesus macaque ligated ileal loop assay after intraluminal inoculation with *S.* Typhimurium. Comparable quantities of fluid accumulation, a correlate of intestinal inflammation, were measured following inoculation of D23580 or IR715 (Fig. 3A). Similarly, no significant differences in the expression of

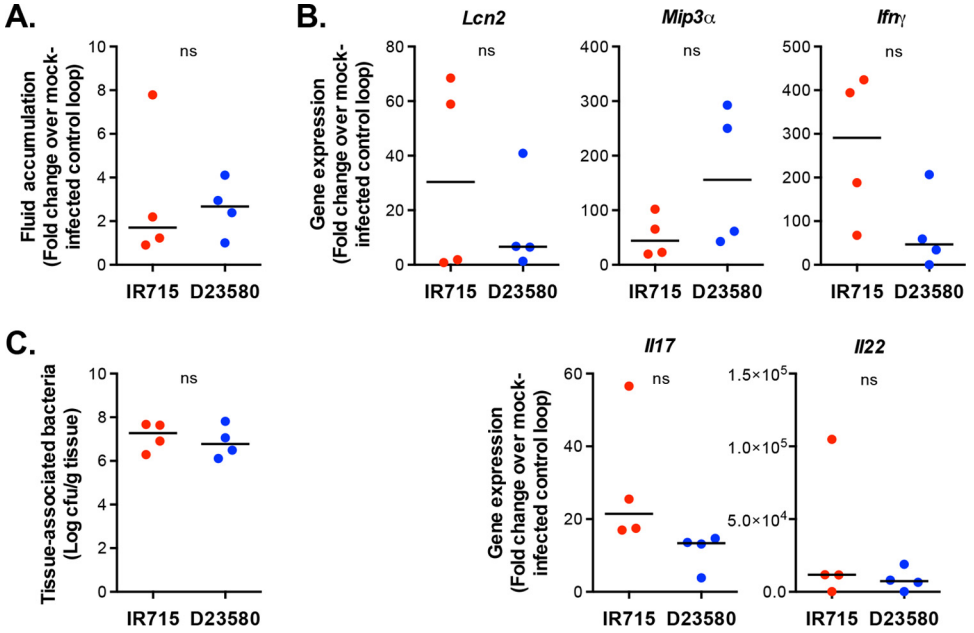

FIG 3 D23580 elicits acute inflammation in the rhesus macaque intestine. Ligated ileal loops of anesthetized rhesus macaques ($n = 4$) were infected with *S.* Typhimurium ST313 strain D23580, ST19 strain IR715, or an equivalent volume of sterile LB broth. (A) Fluid accumulation in the infected ileal loops was measured 5 h after injection. Results are expressed as the volume of fluid that accumulated during infection compared to the volume of fluid that accumulated following injection of sterile LB broth into a loop from the same animal. wt, wild-type. (B) Expression of inflammatory cytokines in the ileal mucosa of *S.* Typhimurium D23580- or IR715-infected ileal loops 5 h after injection. Results shown are the levels of expression in infected ileal loops compared to the amount of expression in loops injected with sterile LB broth from the same animal. (C) Tissue-associated bacteria were measured 5 h after injection of the ileal loops. Bars represent the median values. Each symbol represents the results for 1 animal. Statistical significance ($P > 0.05$) was determined using a Wilcoxon matched-pairs signed rank test. ns, not significant.

inflammatory cytokine genes *Lcn2* (lipocalin-2), *Mip3α* (macrophage inflammatory protein-3α), *IFN-γ* (gamma interferon [IFN-γ]), *Il17* (interleukin-17), and *Il22* (interleukin-22) were measured following inoculation of D23580 or IR715 into ligated ileal loops (Fig. 3B). The numbers of tissue-associated IR715 and D23580 bacteria in infected ileal loops were also similar (Fig. 3C). In summary, *S.* Typhimurium ST313 strain D23580 is capable of eliciting an inflammatory response in the mammalian intestine and does not appear to differ in this regard from the well-characterized ST19 strain IR715.

D23580 exhibits enhanced susceptibility to oxidative stress due to mutation of the *katE* catalase gene. Following investigation of host-related phenotypes, we evaluated D23580 for traits associated with environmental stress resistance, which may be lost during host adaptation of pathogens. First we evaluated the enzyme catalase, which converts hydrogen peroxide (H_2O_2) to oxygen and water. H_2O_2 detoxification by catalase is dispensable for *Salmonella* survival in phagocytes or during infection but is required during high-density or multicellular colonial growth (26, 35). Catalase activity can be detected by adding H_2O_2 to cultured bacteria and monitoring the elaboration of oxygen bubbles over time. *S.* Typhimurium ST313 strain D23580 was found to exhibit decreased catalase activity by this assay compared to the ST19 strains 14028s and SL1344, a phenotype that has also been recently reported by others (20) (Fig. 4A, left panel). The ST19 strains LT2, 14028s *rpoS**, and 14082 s Δ*rpoS* carry mutations in *rpoS*, rendering them extremely sensitive to H_2O_2. Strain 14208 s *rpoS** has an *rpoS* mutation that results in reduced expression of the σ^S (RpoS) regulon and enhanced sensitivity to H_2O_2. These strains were included as known catalase-deficient controls (36) (Fig. 4A, left panel). Often, laboratory-passaged bacteria may appear catalase negative due to the loss of a fully functional RpoS, prompting us to determine whether this was a universal characteristic of ST313 strains. Nine additional ST313 isolates were tested, and all were found to be catalase-deficient, in contrast to the ST19 strains 14028s and SL1344 (Fig. 4A, right panel).

The reduced catalase expression of *S.* Typhimurium ST313 strain D23580 correlated with enhanced susceptibility to H_2O_2 (Fig. 4B). The lag phase of D23580 cultured in the presence of H_2O_2 was prolonged in comparison to 14028s (Fig. 4B). Growth of D23580 in the presence of H_2O_2 was similar to that of *rpoS* mutant strain 14028s *rpoS** (Fig. 4B). As *rpoS* mutations are common among archival *Salmonella* isolates (37) and result in reduced catalase expression, the possibility of an *rpoS* mutation in D23580 was investigated. However, sequencing of the D23580 *rpoS* promoter and coding region yielded no mutations compared to 14028s. Moreover, expression levels of the σ^S-dependent *spvB* (encoding an ADP-ribosylating toxin), *otsA* (trehalose biosynthesis), and *katE* (catalase) genes were found to be comparable in *S.* Typhimurium D23580 and 14028s (Fig. 4C). In contrast, the *rpoS** 14028s derivative showed 5- to 10-fold-lower expression of *spvB*, *otsA*, and *katE* in comparison to wild-type 14028s (Fig. 4C). Thus, the reduced catalase activity of D23580 could not be attributed to a defect in σ^S regulation.

We next examined the sequence of *katE*, which encodes stationary-phase catalase, in D23580 and 14028s. A single amino acid change distinguishing KatE in the 2 sequence types was identified at position 117, with glutamic acid in 14028s corresponding

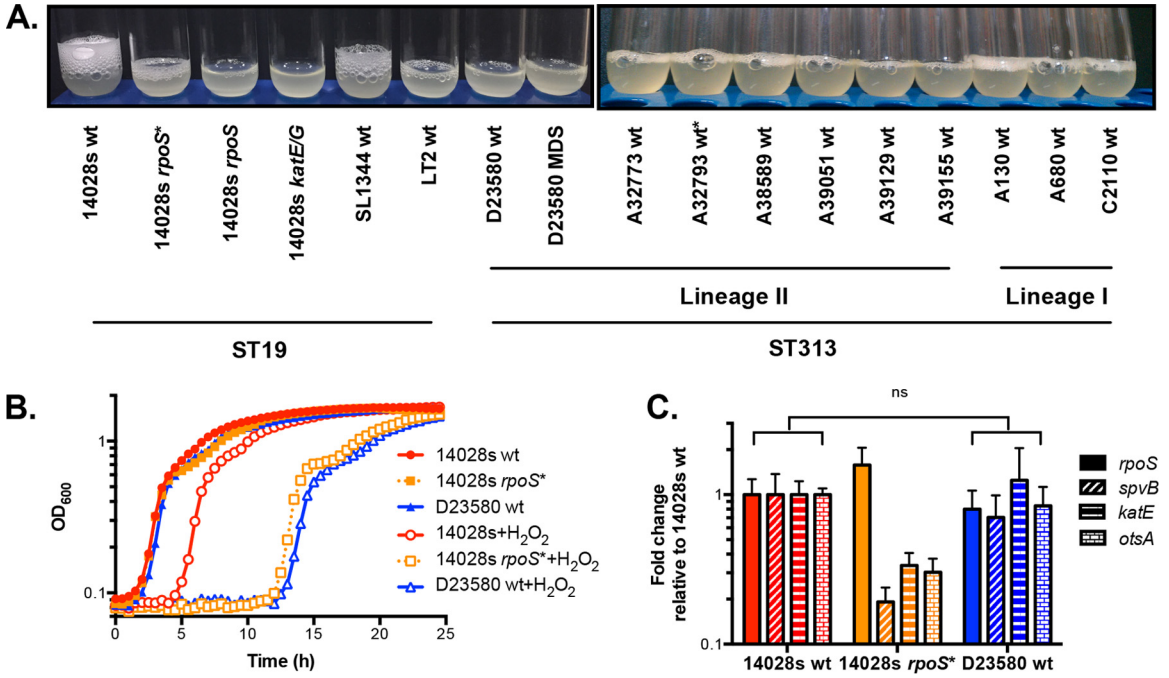

FIG 4 D23580 exhibits deficient detoxification of hydrogen peroxide despite retaining σ^S-dependent gene expression. (A) Stationary-phase cultures of *S.* Typhimurium were grown for ~16 to 18 h before 20 μl of hydrogen peroxide was added to 1 ml of each culture. The height of the oxygen bubble column correlates with the ability of each strain to detoxify H_2O_2. *S.* Typhimurium strains 14028s *rpoS**, 14028s Δ*rpoS*, and LT2 are known *rpoS* mutants and exhibit enhanced sensitivity to H_2O_2 (left). Multiple independent ST313 strains exhibit deficient H_2O_2 detoxification (right). D23580 MDS was included as a control to exclude effects from deletion of plasmid-borne antibiotic resistance determinants. wt, wild-type. *, the ST313 isolate A32793 was not sequenced and is of unknown lineage. (B) Stationary-phase cultures of *S.* Typhimurium were grown in the presence (open symbols) or absence (closed symbols) of 0.5 mM H_2O_2, and optical density was measured over time. A delay in the time to log phase indicates an enhanced susceptibility to H_2O_2. The data shown are average results for 3 biological replicates. (C) *S.* Typhimurium strains were grown to stationary phase prior to harvesting of RNA. Quantitative PCR (qPCR) was performed to determine relative levels of mRNA for the σ^S (RpoS)-regulated genes *spvB* (an ADP-ribosylating toxin), *katE* (stationary-phase catalase), and *otsA* (trehalose biosynthetic gene). Absolute qPCR values were normalized to values for the bacterial housekeeping gene *rpoD* and are expressed as the fold change over results for wild-type 14028s cells. Strain 14028s *rpoS** was included as a control for the effects of reduced σ^S-dependent gene expression. Statistical significance was determined using a paired *t* test (ns, not significant).

to glycine in D23580. We also examined the KatE sequence of 8 additional ST313 isolates for which the sequence was available and found the E117G mutation present in all strains analyzed (see Table S2 in the supplemental material). The 14028s allele of *katE*, including its promoter, was cloned onto a plasmid and transformed into D23580, thereby restoring catalase activity (Fig. 5). In contrast, replacement of the 14028s *katE* allele with the D23580 *katE* allele abolished catalase activity (Fig. 5). As a control, to exclude involvement of the heat-labile *Salmonella* catalase KatG, samples were heat treated for 15 min and catalase activity was measured. No difference in catalase activity before or after heat treatment was observed (Fig. 5). We concluded that D23580 has lost catalase activity as the result of an E117G KatE mutation.

D23580 is unable to form RDAR colonies due to a mutation of the *bcsG* cellulose biosynthetic gene. At room temperature, many strains of *Salmonella* are able to form RDAR (red, dry, and rough) colonies, which result from the formation of thin aggregative fimbriae (curli) and the production of cellulose and other polysaccharides (27, 38, 39). RDAR colonies represent a form of multicellular behavior that enhances *Salmonella* stress resistance in the environment and allows biofilm formation (40). To observe RDAR colony formation, *Salmonella* strains were grown on no-salt, Congo red-containing medium at room temperature (~25°C) for 7 days (27). D23580 was unable to form RDAR colo-

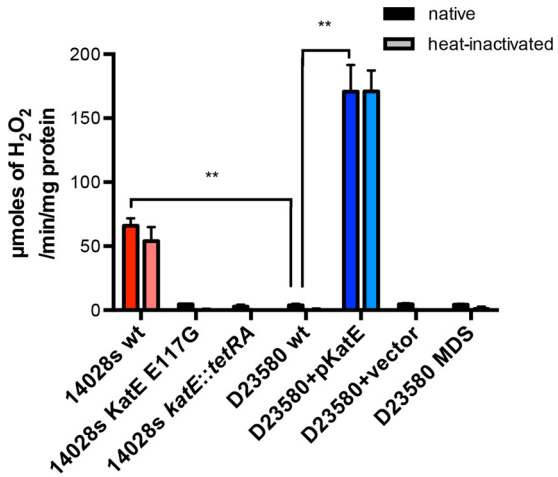

FIG 5 D23580 fails to detoxify hydrogen peroxide due to a mutation in the stationary-phase catalase KatE. Stationary-phase cultures of *S.* Typhimurium were sonicated over ice to release intracellular KatE before the addition of hydrogen peroxide. Decay of H_2O_2 was monitored colorimetrically (based on the A_{240}) over time. Heat-treated lysates were incubated at 55°C for 15 min to inactivate KatG, the heat-sensitive *Salmonella* catalase, before assaying for KatE activity. The strain 14028s *katE*::*tetRA* contains a targeted mutation with an inactive *katE*. 14028s *katE*::*tetRA* is a KatE-null strain, a control for reduced stationary-phase catalase activity. D23580 MDS was included as a control to exclude effects of deleting plasmid-borne antibiotic resistance determinants. Statistical significance was determined using a paired *t* test (**, $P < 0.01$).

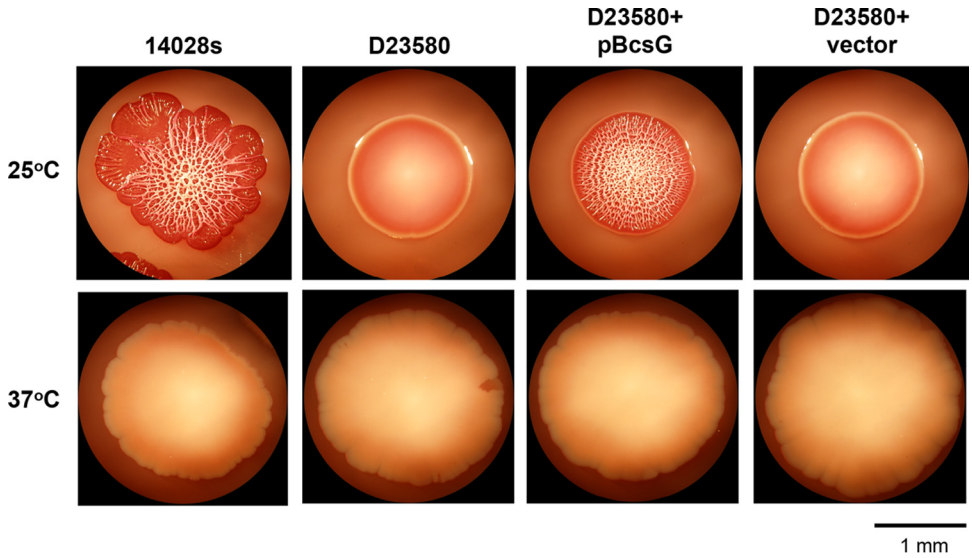

FIG 6 D23580 is unable to form RDAR colonies due to a mutation in the cellulose synthetic enzyme BcsG. *S.* Typhimurium strains were grown overnight in LB broth and plated onto LB agar containing the dyes Congo red and Coomassie blue without salt. Colonies were grown for 7 days at 25°C or 37°C. RDAR colonies characteristically form at 25°C and not 37°C. The images shown are representative examples.

nies, in contrast to the ST19 strain 14028s, which exhibited the typical RDAR colonial phenotype (Fig. 6, top panel). RDAR colony formation was not observed at 37°C in strains 14028s or D23580 because RDAR gene expression was repressed at this temperature (Fig. 6, bottom panel). Three genes implicated in RDAR colony formation were found to differ between D23580 and 14028s: *csgE*, required for the production of curli fimbriae, and 2 genes required for cellulose biosynthesis, *bcsC* and *bcsG* (41). CsgE in D23580 contains the amino acid mutation Q36H, whereas the only difference in *bcsC* between the 2 sequence types is a synonomous single nucleotide change from T to G at position 1461. The most impactful sequence change between 14028s and D23580 occurs in BcsG, with the introduction of a premature stop codon approximately halfway through the nucleotide sequence at position 247 (W → Stop). The 14028s *bcsG* allele, with promoter, was cloned into a plasmid and transformed into D23580. Transformants were plated onto nutrient-rich, no-salt Congo red medium and incubated for 7 days at 25°C or 37°C. The 14028s *bcsG* gene rescued the RDAR-deficient colonial phenotype of D23580 (Fig. 6), confirming that D23580 has lost the ability to form RDAR colonies and biofilms as the result of a *bcsG* nonsense mutation. The difference in appearance of the *bcsG*-complemented colony from 14028s is likely due to enhanced expression of *bcsG* from a multicopy plasmid (42). Overexpression of cellulose synthetic genes has previously been shown to enhance the RDAR phenotype (43). Eight additional ST313 isolates were tested for the RDAR morphology. Five isolates, all of lineage II, were negative for the RDAR morphology and harbored the mutant *bcsG* allele identical to lineage II isolate D23580 (see Table S2 in the supplemental material). The remaining 3 isolates, of lineage I, had weak or intermediate RDAR morphologies and contained the wild-type *bcsG* allele (see Table S2). The basis of the defective RDAR morphology of lineage I isolates is not known.

DISCUSSION

This study examined *S.* Typhimurium ST313, an NTS lineage responsible for invasive infections in young children and HIV-infected adults throughout sub-Saharan Africa, to test the hypothesis that *S.* Typhimurium ST313 represents an evolutionary transition to a more typhoid-like pathogen that is host-adapted to humans and preferentially causes systemic infection rather than inflammatory enteritis (12). We did not find that *S.* Typhimurium ST313 strain D23580 has become host-restricted for humans, in contrast to *S.* Typhi, nor impaired in its ability to cause inflammatory enteritis in multiple infection models, including rhesus macaques. However, we found that D23580 has lost its ability to exhibit multicellular behaviors associated with environmental persistence, which is consistent with epidemiological evidence of person-to-person transmission (16).

Clinical reports have suggested that *S.* Typhimurium ST313 has a reduced propensity to cause diarrhea in comparison to conventional *S.* Typhimurium strains (9, 10). However, we found that D23580 has retained the capacity to elicit inflammatory responses in the mammalian intestine. Inflammation-associated pathology and levels of inflammatory cytokines elicited by *S.* Typhimurium D23580 or the ST19 strain IR715, a spontaneous nalidixic acid-resistant mutant of 14028s, were similar in a murine and a primate colitis model (Fig. 2C and 3B). These findings, the latter of which represents the first report of ST313 virulence in nonhuman primates, are consistent with recent reports that D23580 is able to cause enteritis and invasive infections in chickens (18). However, our observations contrast with those of *in vitro* studies suggesting that D23580 elicits reduced inflammatory responses relative to *S.* Typhimurium ST19 strain SL1344 when infecting cultured cells (21, 22) or in murine or bovine models of intestinal infection (23). We suggest that this may in part be related to heterogeneity among ST19 strains with regard to SPI1 expression and related proinflammatory sequelae (34). ST19 *S.* Typhimurium strain SL1344 exhibits particularly high levels of SPI1 expression, and this was one of the comparator strains used in previously published *in vitro* and *in vivo* studies. It is also important to point out differences in the genetic backgrounds of the mice used in each study, C57/BL6 (*Salmonella* susceptible, Nramp1^{-}) versus CBA/J and 129P2/olaHsd (*Salmonella* resistant, Nramp1^{+}), which may account for some of the

differences observed. Regardless of the ST19 comparator strain examined, our observations suggest that strain D23580 has not in fact lost the ability to induce inflammation in the mammalian intestine and cause enteritis, in contrast to host-restricted *S.* Typhi (44–46). The systemic virulence of D23580 also appears comparable to that of IR715 (Fig. 2A). It is therefore likely that the reduced incidence of diarrhea and the high frequency of invasive disease associated with *S.* Typhimurium ST313 in Africa are attributable to differences in host innate immune responses rather than solely to intrinsic properties of ST313. This conclusion is supported by results of recent studies in which experimental malarial coinfection with increased expression of IL-10 was associated with enhanced susceptibility to invasive NTS infection and blunted intestinal inflammation (31), as well as epidemiological data showing an increased incidence of NTS bacteremia in HIV-positive adults, children with malaria, and children less than 1 year of age (31, 47, 48).

The enhanced invasiveness of *S.* Typhimurium D23580 in cultured epithelial cells (Fig. 1A) correlated with increased CFU in the mesenteric lymph nodes of infected mice (Fig. 2A) and with enhanced fecal shedding at early time points following infection (Fig. 2B). Both epithelial cell invasion and fecal shedding of *Salmonella* are influenced by the SPI1 type 3 secretion system (49). Previous studies have reported differential expression of the SPI1 effector *sopE2* between ST19 and ST313 strains (21). The observations described here further support that SPI1 genes may be differentially expressed in the ST313 strain D23580 and the ST19 strain 14028s, which might be investigated in future studies.

As corroboration of findings reported in a recent publication (20), we found that multiple *S.* Typhimurium ST313 strains express reduced catalase activity. Although reduced catalase activity is typically a result of reduced activity of the alternative sigma factor σ^S (RpoS) (50–52), we found that activation of the σ^S regulon was intact in ST313 strain D23580 (Fig. 4C). Instead, an E117G mutation in the stationary-phase catalase KatE was found to be responsible for the reduced catalase activity of D23580 (Fig. 5) and other ST313 strains. Other investigators previously reported D23580 to be "weakly positive" for catalase activity (20), while our data indicate that D23580 lacks catalase activity (Fig. 5, lighter bars). This is most likely because we assayed catalase during stationary phase, in which catalase activity is KatE dependent, whereas the earlier study assayed catalase from growing colonies, in which both KatE and KatG catalases are expressed. Catalase is not required for *Salmonella* virulence (35) but is required for oxidative stress resistance during multicellular colonial growth (26). This suggests that *S.* Typhimurium D23580 has lost a functional *katE* gene by mutation, because it no longer requires multicellular behavior for transmission and/or survival in the environment. It is interesting that the human-adapted *Salmonella* serovar *S.* Typhi also exhibits reduced catalase activity. However, in the case of *S.* Typhi, the reduced catalase activity is more often a consequence of reduced σ^S activity (52). This difference may reflect the requirement of NTS serovars to retain σ^S in order to preserve expression of *spv* virulence genes carried by the plasmid pSLT (50), whereas the negative regulatory influence of σ^S on Vi polysaccharide expression (53) may favor the accumulation of *rpoS* mutations in *S.* Typhi.

We also found that D23580 no longer exhibits a RDAR (rough, dry, and red) colonial phenotype, a correlate of biofilm formation (Fig. 6). RDAR colonies are resistant to a variety of environmental stresses, including desiccation and nutrient deprivation (27, 28, 39, 54). The failure of D23580 to form RDAR colonies results from a nonsense mutation in the cyclic-di-GMP-sensitive cellulose biosynthetic gene *bcsG* (38, 41). As with catalase, the RDAR phenotype is not required for *Salmonella* virulence but is required for environmental stress resistance during multicellular growth (55). The RDAR colonial phenotype is seen in a majority of generalist *S.* Typhimurium isolates but is variable in chicken-adapted *S. gallinarum* and absent in human-adapted *S.* Typhi or swine-adapted *S.* Choleraesuis (56). In addition, the loss of RDAR in *S.* Typhimurium ST313 strain D23580 contrasts with other *S.* Typhimurium isolates associated with infection in areas in Africa where it is endemic (57), for which it may still require the ability to survive outside the host for effective transmission to susceptible hosts. Alternatively, the loss of RDAR colony formation has also been reported to increase the fitness of *Salmonella* in plants (58), and the involvement of an as-yet-unidentified food vector cannot be excluded. Interestingly, the premature stop codon in *bcsG* is only found in ST313 lineage II and not in lineage I isolates (see Table S2 in the supplemental material). In contrast, the KatE E117G mutation was present in all ST313 isolates tested (see Table S2). This suggests that the KatE mutation was acquired prior to lineage divergence, while the *bcsG* mutation was subsequently acquired by lineage II isolates only.

In conclusion, the studies described herein provide novel insights into the evolution of host-specific strains of the enteric pathogen *Salmonella enterica*. Specifically, the robust early fecal shedding and loss of environmental resistance phenotypes observed in *S.* Typhimurium ST313 strain D23580 are consistent with epidemiological evidence suggesting anthroponotic transmission (17). Furthermore, we have shown that D23580 has retained its ability to induce inflammatory colitis in mammalian models of infection, suggesting that the reduced incidence of diarrhea and increased incidence of invasive disease associated with these strains most likely reflect differences in host susceptibility rather than in intrinsic virulence properties of the pathogen.

MATERIALS AND METHODS

Additional information regarding our materials and methods is provided in Text S1 in the supplemental material, as is a table of strains, plasmids, and primers used (see Table S1 in the supplemental material).

Bacterial growth conditions. Unless otherwise stated, all strains were routinely cultured in Luria-Bertani (LB) medium with shaking at 250 rpm at 37°C. Antibiotic supplementation was as follows, unless otherwise stated: kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), tetracycline (20 μg ml^{-1}), and nalidixic acid (50 μg ml^{-1}).

Animals. All animal experiments were approved by the University of California at Davis Institutional Animal Care and Use Committee. Animal experiments were performed in accordance with guidelines regarding animal welfare.

Epithelial cell invasion assay. Cell invasion assays were adapted from methods described elsewhere (59). *Salmonella* strains were grown overnight in LB broth supplemented with sodium chloride to a final concentration of 0.3 M. Overnight cultures of *Salmonella* were subcultured 1:33 in 10 ml of LB supplemented with sodium chloride and grown with shaking at 37°C for 3.5 h, when bacteria reached late logarithmic phase (optical density at 600 nm [OD_{600}] of ~1.0). Bacteria were used to infect 3×10^4 HeLa cells/well (96-well plate) at a multiplicity of infection of ~100:1. Infected cells were incubated at 37°C for 10 min to promote invasion. Following invasion, epithelial cells were washed 3 times with phosphate-buffered saline (PBS) and then incubated at 37°C for 20 min in antibiotic-free growth medium. After 20 min, the medium was supplemented with gentamicin at a final concentration of 50 μg ml^{-1}. Cells were incubated for 40 min at 37°C and then lysed with 1% Triton X-100 to enumerate internalized bacteria. Results are expressed as the percentage of the bacterial inoculum internalized.

***Salmonella*-induced murine macrophage cytotoxicity assay.** *Salmonella* strains were grown overnight in LB broth supplemented with sodium chloride to a final concentration of 0.3 M. Overnight cultures of *Salmonella* were adjusted to an OD_{600} of ~2.5 and then subcultured 1:100 in LB supplemented with sodium chloride and grown with shaking at 37°C for 4 h, when bacteria reached late logarithmic phase. Bacteria were then used to infect 1.4×10^5 RAW 264.7 murine macrophage-like cells/well (96-well plate) at a multiplicity of infection of ~10:1. Infected monolayers were incubated at 37°C for 30 min to promote internalization. Following internalization, monolayers were washed twice with Dulbecco's modified Eagle's medium supplemented with 20 μg ml^{-1} gentamicin to kill extracellular bacteria. Infected monolayers were incubated at 37°C for 4 h before supernatant levels of lactate dehydrogenase were assayed using the CytoTox96 cytotoxicity kit (Promega, Madison, WI) per the manufacturer's protocol. Percent cytotoxicity was calculated as follows: [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

***Salmonella* intramacrophage survival assay.** *Salmonella* strains were grown overnight in LB broth. One milliliter of bacterial culture was centrifuged, supernatant was removed, and pellets were resuspended in 100 μl of 100% normal mouse serum. Opsonized bacteria were incubated at 37°C for 15 min. A total of 8×10^5 RAW 246.7 murine macrophage-like cells/well were infected with opsonized *Salmonella* at a multiplicity of infection of ~10:1, centrifuged at 1,000 rpm for 5 min, and then incubated at 37°C for 15 min. Following internalization, macrophages were washed with RPMI 1640 1x medium containing 20 μg ml^{-1} gentamicin to kill extracellular bacteria and then lysed with 1% Triton X-100 to enumerate intramacrophage bacteria at 0 and 18 h postinfection.

Mouse infections. Mouse infections were performed similarly to those previously described, with modifications as noted (47). Male and female CBA/J mice were treated with 20 mg streptomycin Q1 i.g. 24 h prior to *Salmonella* infection. These mice were then infected by gavage with 100 μl of 10^8 bacteria in LB broth. For competitive infections, a 50/50 mixture of strains D23580 and IR715 was used to infect mice. To determine the numbers of viable *Salmonella*, colon contents, Peyer's patches (3 per mouse), mesenteric lymph nodes, livers, and spleens were homogenized in PBS by using an Ultra Turrax T25 basic mixer (IKA). Homogenates were serially diluted and plated on LB agar plates containing nalidixic acid (0.05 mg ml^{-1}) for IR715 or chloramphenicol (0.03 mg ml^{-1}) for D23580. After overnight growth at 37°C, the CFU per gram of tissue were enumerated. Competitive indices (CI) were determined as the CFU of D23580 divided by the CFU of IR715, normalized to the input inoculum. Histopathological samples were scored in a blinded fashion by a trained pathologist (M.X.B.).

Rhesus macaque ileal loop infections. Rhesus macaque ileal loop infections were performed as described elsewhere (47, 60). Briefly, 4 healthy male rhesus macaques were anesthetized prior to a laparotomy to expose the ileum. Ileal loops averaging 4 cm in length were created by ligation and then infected by intralumenal injection of 1 ml of 1×10^9 CFU of *Salmonella* or sterile LB broth. Five hours after infection, loops were collected and the change in intralumenal fluid accumulation or RNA expression of inflammatory cytokines compared to levels in mock-infected loops was measured. Monkeys were designated 37248, 36171, 38498, and 36782. Loops used in this study were loop 11 (LB medium), loop 13 (strain IR715), and loop 14 (D23580).

Hydrogen peroxide susceptibility assays. To measure the ability of *Salmonella* strains to detoxify hydrogen peroxide, 20 μl of ~8.8 M H_2O_2 was added to 1 ml of an overnight culture (~16 to 18 h of growth) of *Salmonella* and incubated at room temperature for 5 min. The height of the oxygen bubble column correlates with the ability of a strain to detoxify H_2O_2. To measure growth in the presence of H_2O_2, overnight cultures of *Salmonella* were diluted to a final OD_{600} of 0.002 in LB broth with or without 0.5 mM H_2O_2. The OD_{600} was measured at 15-min intervals in a Bioscreen C microplate reader (Labsystems, Helsinki, Finland).

Catalase activity assay. *Salmonella* catalase activity was measured as described elsewhere, with modifications as noted (61). Briefly, 2 ml of overnight culture was washed twice and resuspended in a final volume of 400 μl wash buffer to reduce frothing during sonication. Bacteria were subjected to 4 rounds of sonication at power level 2 for 5 s each (Mixonix, Farmingdale, NY). Lysates were centrifuged, and the protein concentration of the supernatant was determined using a Coomassie (Bradford) protein assay kit (Pierce, Waltham, MA). Twenty microliters of lysate were combined with H_2O_2, and decay was measured colorimetrically as the change in the A_{240} (ΔA_{240}) over time. The specific activity of catalase was calculated using the following formula: (1,000 × average ΔA_{240}/min)/(43.6 × milligrams of protein/milliliter of reaction mixture). Heat-stable KatE activity was measured by heating cell extracts to 55°C for 15 min prior to assaying catalase activity.

Detection of the RDAR colonial morphotype. For data regarding the RDAR colonial morphotype, bacteria were grown overnight in LB broth and then diluted to a concentration of ~100 CFU/ml. Aliquots of 100 μl were plated onto LB agar without salt and supplemented with 40 μg ml^{-1} Congo red and 20 μg ml^{-1} Coomassie blue. Plates were incubated at 25°C or 37°C for 7 days without inversion (27).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02265-15/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.

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