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A Novel Phage Element of *Salmonella enterica* Serovar Enteritidis P125109 Contributes to Accelerated Type III Secretion System 2-Dependent Early Inflammation Kinetics in a Mouse Colitis Model

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***Salmonella enterica* subsp. I serovar Enteritidis exhibits type III secretion system 2 (TTSS2)-dependent early colonization and inflammation kinetics faster than those of closely related *S. enterica* serovar Typhimurium. To investigate the accelerated TTSS-2-dependent pathogenic potential of *S.* Enteritidis, we focused on its genome. Results of a previously published comparative genomic study revealed the presence of mutually exclusive genes in both serovars. In this study, we investigated the roles of six *S.* Enteritidis-specific genes *in vivo* by using differential fluorescence induction (DFI) through putative gene-specific promoters. The promoter construct associated with the gene locus *SEN1140* induced green fluorescent protein (GFP) expression in the gut lumen, lamina propria, mesenteric lymph nodes, and related systemic organs. To further investigate the potential role of *SEN1140*, we compared a *SEN1140* deletion mutant with *S.* Typhimurium in a TTSS1-deficient background. Interestingly, the *S.* Enteritidis mutant lacking *SEN1140* did not show the unique TTSS-2-dependent early colonization and inflammation kinetic phenotype of *S.* Typhimurium. Consistent with this result, complementation of *SEN1140* restored the TTSS-2-dependent accelerated inflammatory potential of *S.* Enteritidis. This report presents a suitable screening strategy that uses a combination of DFI, fluorescence-activated cell sorting, quantitative PCR, and wild-type isogenic tagged-strain techniques to explore the unique roles of *S.* Enteritidis-specific genes in bacterial pathogenesis.**

Salmonella enterica bacteria are pathogenic organisms that cause an array of diseases. A few of the members of this species show host specificity, while others cause diseases in a wide range of hosts (10). Two of the main serovars of *S. enterica*, which cause gastroenteritis in humans and systemic and lethal infections in genetically susceptible mice, are *S. enterica* serovars Enteritidis and Typhimurium (2). These pathogenic bacteria and the diseases they cause are major public health concerns in many countries. Studies have shown that the vast majority of *Salmonella* virulence factors and associated pathogenicities result from multiple pathogen-host interactions. The deletion of known virulence determinants could be advantageous for identifying and analyzing novel interactions or virulence factors involved in bacterial adaptiveness. Indeed, this technique has facilitated the identification of various SPI-1 effector proteins (18, 29, 45) and the characterization of the roles of SPI-1, SPI-2 (19, 20), and many other *Salmonella* virulence factors. Surprisingly, rare cases of human *Salmonella* infection with SPI-1-deficient strains have been reported (22) and the prevalence of such mutants has also been identified in animal husbandry (35). Thus, mutants lacking major known virulence determinants are also of importance for the study of health and disease. The mode and mechanism of *S.* Typhimurium pathogenesis have been extensively studied, and recently, many studies have been focusing on the comparison of the infection profiles of *S.* Typhimurium and *S.* Enteritidis, as the genomes of both of these serovars have been sequenced and made available (27, 44). The comparative analysis of both genomes shows the presence of many sequences in *S.* Enteritidis that are not present in the chromosome of *S.* Typhimurium. These gene sequences originated primarily from prophages or genomic islands that were acquired through horizontal gene transfer and are designated *S.* Enteritidis-specific "regions of differentiation." Presumably, differences between the contents of the genomes of the two serovars might also reflect differences between their infection profiles.

S. Enteritidis-specific gene sequences represent approximately 2% of the total size of the *S.* Enteritidis genome, and many of these sequences are still not annotated. We have previously reported that *S.* Enteritidis has accelerated invasion kinetics and shows early SPI-1-independent inflammation, compared with SPI-1-deficient *S.* Typhimurium, in specific-pathogen-free, streptomycin-pretreated C57BL/6 mice (43). Thus, it would be interesting to identify the genetic factors in *S.* Enteritidis that contribute to its early infection process. It has been recently reported that *S.* Enteritidis infection involves additional genes that are absent from *S.* Typhimurium (3). A comparative analysis of *S.* Enteritidis and *S.* Typhimurium shows 98% similarity in their genome sequences (44), suggesting that the extra gene elements present in *S.* Enteritidis (2% of the genome) are most likely associated with its accelerated inflammation kinetics. Gene disruption, followed by *in vitro* or *in vivo* analysis of the mutant generated, is an established conventional way of identifying the roles of genes in the infection process, but this method is laborious when used to explore multiple mutations. Here we present a method that uses a simple screen for the identification of *S.* Enteritidis-specific promoters

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype	Background or resistance	Reference
Strains			
M1511	TTSS-1$^-$ (*invC*::*aphT*); Sm^r Km^r	M1525	43
M1525	*S.* Enteritidis 125109 wild type; Sm^r	Wild type	42
SB566	TTSS-1$^-$ (*invC*::*aphT*); Sm^r Km^r	SB300	38
Z290	SPI-1-deficient *SEN1140* mutant (*invC*::*aphT SEN1140*::*cat*); Sm^r Km^r Cm^r	M1511	This study
Z291	*SEN1140* mutant (*SEN1140*::*cat*); Sm^r Cm^r	M1525	This study
Z292	Z290 complemented with *SEN1140* through pCH112; Amp^r	Z290	This study
Plasmids			
pM968	Promoterless *GFPmut2* in promoterless pBAD24 vector	Amp^r	41
pM975	*bla* P_{ssaG} *GFPmut2* plasmid with ori_{pMB1}	Amp^r	28
pM973	*bla* P_{ssaH} *GFPmut2* plasmid with ori_{pMB1}	Amp^r	40
pCH112	*hilA* ORF cloned into P_{BAD}/*myc*-His; ori_{pBR322}	Amp^r	28
pKD3	*bla* FRT *cat* FRT PS1 PS2 ori_{R6K}	Cmp^r	25
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 ori_{TS}	Amp^r	25
pM2152	450 bp upstream (P_{0999}) of *SEN0999* cloned into pM968 between BamHI and XbaI	Amp^r	This study
pM2153	500 bp upstream (P_{4250}) of *SEN4250* cloned into pM968 between BamHI and XbaI	Amp^r	This study
pM2154	550 bp upstream (P_{1013}) of *SEN1013* cloned into pM968 between BamHI and XbaI	Amp^r	This study
pM2155	750 bp upstream (P_{1140}) to *SEN1140* cloned into pM968 between BamHI and XbaI	Amp^r	This study
pM2156	650 bp upstream (P_{1975}) to *SEN1975* cloned into pM968 between BamHI and XbaI	Amp^r	This study
pM2157	700 bp upstream (P_{1994}) to *SEN1994* cloned into pM968 between BamHI and XbaI	Amp^r	This study
pM2158	*SEN1140* ORF with 750-bp upstream region cloned into pCH112 between NcoI and XbaI; ori_{pBR322}	Amp^r	This study

and their corresponding genes that might contribute to the accelerated infection process of *S.* Enteritidis. The screen represents a promising combination of bioinformatic analysis, differential fluorescence induction (DFI), wild-type isogenic tagged-strain (WITS), fluorescence-activated cell sorting (FACS), and quantitative real-time PCR (qRT-PCR) techniques to evaluate the role of *S.* Enteritidis gene products. This combinatorial method is based on the *in vivo* expression of green fluorescent protein (GFP) in differentially tagged wild-type isogenic strains using an *S.* Enteritidis-specific promoter cloned into a promoterless GFP plasmid. The spatiotemporal endogenous promoter-driven expression of GFP in different host tissues might indicate the involvement of its respective gene(s) in various host-pathogen interactions *in vivo*. Subsequently, a small mutant library of selected genes could be created on the basis of the promoters screened instead of developing an exhaustive mutant library to conduct cumbersome *in vivo* analyses of individual mutants. The techniques proposed in this study are superior to RNA sequencing, as this approach works precisely even in a background of numerous other bacterial species and high loads of host-derived cells.

In the present study, our findings demonstrated that the involvement of an uncharacterized *S.* Enteritidis gene, *SEN1140*, in the invasion process resulted in accelerated SPI-1-independent inflammation. *SEN1140* was likely acquired through genetic transfer from a prophage to the *S.* Enteritidis genome and encodes a hypothetical phage protein whose function is not yet known. It is well known that prophages contain additional cargo genes called morons or lysogenic conversion genes that play insignificant roles in the phage life cycle. Interestingly, these morons get incorporated into the chromosomes of bacteria infected with moron-bearing prophages (5). The morons in many pathogenic bacteria have been studied and shown to encode proven or suspected virulence factors; however, *S.* Enteritidis P125109 has been poorly studied in this regard. The results of this study suggest a role for a moron element such as *SEN1140* in *S.* Enteritidis virulence and pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth condition. Bacterial strains (Table 1) were grown for 12 h at 37°C in Luria-Bertani (LB) medium supplemented with 0.3 M NaCl, diluted 1:20 in fresh LB medium, and subcultured for another 4 h under mild aeration until an optical density of 0.6 was obtained. The bacteria were washed in ice-cold phosphate-buffered saline (PBS), and 5×10^7 CFU were suspended in 50 μl cold PBS for use in the *in vivo* experiments. Prior to the start of the *in vivo* experiments, all strains were tested for growth attenuation for 16 h in 10 ml of culture medium at 37°C at 150 rpm under aerated conditions.

Ethics statement. The appropriate committee (Kantonales Veterinäramt, Zürich, Switzerland; license number ZH201/2007) approved all animal work. All animals were maintained in strict accordance with the good animal practice guidelines of the relevant national and/or welfare bodies.

Mouse infection experiment. The animal experiments were performed in compliance with all relevant federal guidelines and the institutional policies of ETH Zürich. The infection experiments were performed in individual ventilated cages as described previously (4). All C57BL/6 mice were pathogen free and bred at the Rodent Center HCI, ETH Zürich. Mice were pretreated intragastrically with 50 mg of streptomycin and infected 24 h later with 5×10^7 CFU (oral gavage) of the corresponding bacterial strain. When needed for bacterial coinfection experiments, freshly grown cultures of bacterial strains were mixed in equal proportions and a dose of 5×10^7 CFU of a mixed inoculum was administered to streptomycin-pretreated C57BL/6 mice. Fecal samples were collected at 12 and 24 h for estimation of lipocalin 2 as a marker of early inflammation. Bacterial loads in the cecum, mesenteric lymph nodes (MLN), liver, and spleen were determined upon plating of the homogenates on MacConkey agar plates supplemented with appropriate antibiotics. The tissues were collected in an optimum cutting temperature (OCT) compound (Sakura Finetek Inc.) for cryosectioning, snap-frozen in liquid nitrogen, and

TABLE 2 Primers used in this study

Primer	Sequence (5′–3′)
p999-Fw	GGGTCTAGA AATGAACATAAAAAAAATTC
p999-Rw	GCCGGATCCAATAACCTCTATATATAATC
p4250-Fw	GTTTCTAGAATATCGGATACTGCTGCACT
p4250-Rw	GCGGGATCCACTCCATTTAAAAAGCTAAT
p1013-Fw	GGGTCTAGATTGCTCATAAAATGATATCA
p1013-Rw	GGCGGATCC ATGTTTTCCTGAAGTGTGTA
p1975-Fw	GCGTCTAGA AGTTTCCAATATATCATTTG
p1975-Rw	GCGGGATCCCAGATTAACTCCCTGAGATA
p1994-Fw	GCGTCTAGAAGTTTCAAAAGTTATGATCA
p1994-Rw	CGGGGATCCATGTTAGGTTAATGTTAGTT
p1140-Fw	AATTCTAGA CAGCAGCTCATCGTGGCGAC
p1140-Rw	GGCGGATCC TTCCTATTGCTGATTAATAC
pM968-Fw	TCCGCTTACAGACAAGCTGTG
pM968-Rw	TACATAACCTTCGGGCATGGC
WITS-1	ACGACACCACTCCACACCTA
WITS-2	ACCCGCAATACCAACAACTC
WITS-11	ATCCCACACACTCGATCTCA
WITS-13	GCTAAAGACACCCCTCACTCA
WITS-17	TCACCAGCCCACCCCCTCA
WITS-19	GCACTATCCAGCCCCATAAC
WITS-21	ACAACCACCGATCACTCTCC
ydgA-Fw	GGCTGTCCGCAATGGGTC
SEN1140-Fw	GCAAGCCTGCTGAGGAACGGGATTTTTGTATTAATCA GCAATAGGAATGTGTAGGCTGGAGCTGCTTC
SEN1140-Rw	GATGTGTGGCAATTCTTACCGAGGGTGTTGAGCAA TCCTCTCTAAAATATGAATATCCTCCTTAGTT
SEN1140-comp-Fw	TTA ACC ATG GAG CAG CTC ATC GTG GCG AC
SEN1140-comp-Rw	GTT CTC TAG ACT ATT TCT GCG AGG CTA TAT

stored at −80°C. For fluorescent imaging and GFP counting, the tissues were collected in a 4% paraformaldehyde (PFA) solution (in PBS) and processed separately.

Preparation of WITS of *S.* Enteritidis. *S.* Enteritidis P125109 wild-type strain M1525 was tagged with specific barcode sequences to generate WITS (14). Insertion of tags into the *S.* Enteritidis genome was achieved by a conventional phage transduction method. Briefly, a small aliquot of the recipient wild-type *S.* Enteritidis strain (M1525) grown overnight was transduced with phage lysate obtained from individual donor WITS for 15 min at 37°C. The transduced colonies were selected on LB agar plates supplemented with kanamycin (50 μg/ml) and screened for the integration of individual tags into the chromosome by PCR using individual WITS tag-specific primers in combination with the *ydgA*-Fw common primer (Table 2).

Plasmid cloning. The genomes of *S.* Enteritidis and *S.* Typhimurium have already been compared (44). For this study, six *S.* Enteritidis-specific genes were selected on the basis of their putative encoded protein domains. Putative promoters for the selected *S.* Enteritidis-specific genes were PCR amplified from the genomic DNA of *S.* Enteritidis P125109 using promoter-specific primer pairs (Table 2) as described earlier. Briefly, the PCR mixture contained 1× reaction buffer, 0.5 mM deoxynucleoside triphosphate mix, 1.25 mM magnesium chloride, 0.5 μM promoter-specific forward and reverse primers, 1 ng of *S.* Enteritidis P125109 genomic template DNA, and 1 U of *Pfu* DNA polymerase. The PCR products were obtained after 30 PCR cycles of 95°C for 1 min, followed by 53°C for 45 s and 72°C for 1 min. The amplicons were gel eluted and column purified. The fragments were introduced between the BamHI and XbaI sites of promoterless *GFPmut2* plasmid pM968 to obtain recombinant plasmids (Table 1) and transformed into individual WITS of *S.* Enteritidis P125109 (wild-type) strain M1525. The transformants were selected on LB agar plates supplemented with ampicillin (100 μg/ml) and kanamycin (50 μg/ml). Transformants were screened by colony PCR; the clones were subjected to sequencing of plasmid DNA for confirmation of the incorporation of selected promoter regions into plasmid pM968 using primer pM968-Fw (Table 2). These manipulations generated the following combinations of chromosomal tags with plasmid constructs in strain M1525: WITS1-pM2152, WITS2-pM2153, WITS11-pM2154, WITS13-pM2156, WITS19-pM2157, and WITS21-pM2155.

Sample preparations for FACS analysis. To examine GFP expression through the control of the individually cloned promoters, the recombinant plasmid-bearing WITS of *S.* Enteritidis (M1525) were grown overnight in LB medium supplemented with 0.3 M NaCl and appropriate antibiotics. Strain M1525 with the WITS17 tag and plasmid pM975 (*ssaG* promoter in pM968) was used as the positive control for this set of experiment. The cultures were subcultured to an optical density of 0.6 and subsequently mixed in equal proportions (1:1:1:1:1:1:1) representing equal numbers of CFU of all of the strains in a mixed pool. Approximately 10^7 CFU of the mixed-inoculum pool was administered orally to a group of streptomycin-pretreated C57BL/6 mice; the cecal contents were collected from each mouse at 2 days postinfection (p.i.) and diluted in 500 μl of PBS. The cecal suspensions were FACS sorted to analyze the GFP expression in the bacterial strains.

FACS analysis. *S.* Enteritidis (wild-type) strain M1525 harboring the promoterless plasmid pM968 was used as the negative control for FACS analysis using a BD Biosciences FACSAria system. The side scatter (SSC-A) on the *y* axis and GFP fluorescence (FL1-A GFP) on the *x* axis were used to gate on single bacterial cells. A total of 10^5 cells for each GFP-positive or GFP-negative fraction were collected from each sample suspension and enriched overnight in LB medium supplemented with ampicillin (100 μg/ml) and kanamycin (50 μg/ml). The genomic DNA was isolated from each bacterial culture and subjected to qRT-PCR using WITS-specific primers in combination with the *ydgA*-specific common primer to determine the WITS copy numbers in a bacterial population. The relative WITS tag proportion directly represented the ratio of the individual recombinant plasmid present with respect to the specific WITS in the FACS-sorted samples.

Analysis of GFP-expressing *Salmonella* in the lamina propria. Streptomycin-pretreated C57BL/6 mice were infected with GFP reporter plasmid (pM973)-bearing *Salmonella* M1511, Z290, and SB566 mutant strains. Cecal tissues were collected at day 2 p.i., incubated overnight in a 4% PFA solution (in PBS) at 4°C, incubated for 8 h in 20% sucrose (in PBS) at 4°C, and then snap-frozen in OCT compound (Sakura Finetek Inc.). Cryosections 20 μm thick were obtained on a clean glass slide and air dried for a minimum of 4 h. Subsequently, the cryosections were blocked in the dark for 1 h in 10% goat serum prepared in PBS. The GFP-expressing recombinant plasmid-bearing *S.* Enteritidis bacteria were enumerated in the mucosa as described earlier. Briefly, the sections were stained with anti-Armenian hamster αCD54 antibody (clone 3E2; BD Biosciences) for 1 h and then incubated in a mixture of phalliodin-A647 (FluoProbes) (1:100), anti-Armenian hamster–Cy3 (Jackson ImmunoResearch Laboratories; 1:200) antibodies, and 4′,6-diamidino-2-phenylindole (DAPI) stain (Sigma-Aldrich; 1:1,000). After incubation, the sections were washed repeatedly with PBS and mounted with Vectashield (Vector Laboratories). Six to nine sections per cecum sample were analyzed for the enumeration of GPF-expressing bacteria in the lamina propria.

Chromosomal gene disruption and plasmid-based complementation. Chromosomal deletion of the *SEN1140* gene was done by using the standard lambda-red recombinase system with a pKD3 template plasmid (9, 23). The phage lysate of the mutant developed was prepared, and the stable double mutant strains Z290 and Z291 were obtained by transducing the mutation into recipient strains M1511 (*S.* Enteritidis *invC*::*aphT*) and M1525 (*S.* Enteritidis, wild type), respectively. For complementation of the *SEN1140* gene, plasmid pM2158 was constructed by manipulating plasmid pCH112 (28). Briefly, the *SEN1140* gene along with its 750-bp upstream sequence was PCR amplified using primers *SEN1140*-comp-Fw and *SEN1140*-comp-Rw (Table 2). The resulting amplicon was digested with nucleases NcoI and XbaI. The *hilA* open reading frame (ORF) between the NcoI and XbaI sites in plasmid pCH112 was replaced with the *SEN1140* ORF and its corresponding 750-bp upstream promoter sequence to obtain plasmid pM2158. Subsequent introduction of the recombinant plasmid (pM2158) into mutant strain Z290 to rescue the function of *SEN1140* resulted in complemented strain Z292. Plasmid pM2158

also contained a P_{BAD} promoter, which remained inactive in the absence of arabinose; however, the plasmid could express *SEN1140* under the control of its cloned promoter.

Histopathological evaluation. Segments of the ileum, cecum, and colon were fixed and embedded in OCT compound (Sakura Finetek Inc.), snap-frozen in liquid nitrogen, and stored at −80°C. The cryosections (5 μm) were obtained on glass slides and stained with hematoxylin and eosin (H&E) after drying for at least 2 h at room temperature. The stained histopathological sections were evaluated on the basis of a previously described scoring system for the quantitative analysis of cecal inflammation (4, 43). The H&E-stained sections (5 μm) were scored independently on the basis of the following criteria.

(i) Submucosal edema. Submucosal edema was observed at ×100 magnification for the distance between the tunica muscularis and lamina mucosalis mucosae. The feature was scored as follows: no significant pathological changes in the submucosal features, score 0; mild edema when the submucosa accounts for <50% of the diameter of the entire intestinal wall from the tunica muscularis to the epithelium, score 1; moderate edema when the submucosa covers nearly 50 to 80% of the intestinal diameter, score 2; significant edema when the submucosa covers more than 80% of the diameter of the entire intestinal wall, score 3.

(ii) PMN cell infiltration of the lamina propria. Polymorphonuclear (PMN) cells in the lamina propria were enumerated at ×400 magnification, and the average number of PMN cells/high-power field (HPF) was graded by using the following criteria: 0 to 5 PMN cells/HPF was assigned a score of 0, 5 to 20 PMN cells/HPF was assigned a score of 1, 21 to 60 PMN cells/HPF was assigned a score of 2, 61 to 100 PMN cells/HPF was assigned a score of 3, and more than 100 PMN cells/HPF was assigned a score of 4.

(iii) Goblet cells. Nearly 10 different regions of the cecal epithelium were observed at ×400 magnification to determine the average number of goblet cells per HPF. The appearance of more than 28 goblet cells/HPF was scored 0, considering that they are a normal feature of specific-pathogen-free mice and the disappearance of goblet cells would increase the score. A score of 1 was assigned for 11 to 28 cells/HPF, a score of 2 was assigned for 1 to 10 cells/HPF, and a score of 3 represented <1 goblet cell or the complete disappearance of goblet cells from the cecum as observed in the cryosections.

(iv) Epithelial integrity. Epithelial integrity was observed at ×400 magnification, and a score of 0 was assigned when there were no detectable pathological changes in the cecal epithelial layer. Epithelial desquamation was categorized grade 1, and erosion of the epithelial layer characterized by a gap of 1 to 10 epithelial cells/lesion was considered grade 2. However, a gap of more than 10 epithelial cells/lesion is a characteristic of epithelial ulceration, which was categorized as grade 3.

The independent scores for submucosal edema, PMN cell infiltration, goblet cells, and the epithelial integrity of each tissue sample were averaged and combined. The combined pathological scores ranged from 0 to 13 arbitrary units covering the following inflammation levels: intact intestine without any sign of inflammation, a pathoscore of 0; minimal sign of inflammation (which is commonly found in the ceca of specific-pathogen-free mice and generally not considered a pathological feature), a pathoscore of 1 or 2; slight inflammation (a minimal sign of tissue pathology), a pathoscore of 3 or 4; moderate inflammation, a pathoscore of 5 to 8; significant inflammation, a pathoscore of 9 to 13.

Lipocalin 2 estimation. Lipocalin 2 was measured using an enzyme-linked immunosorbent assay (ELISA) of 12- and 24-h fecal samples using a commercially available kit (R&D Systems). Briefly, for the early detection of cecal inflammation at an intermittent time point, fecal samples were collected from the mice at 12 and 24 h. The samples were weighed and suspended in 500 μl of sterile PBS. For the lipocalin 2 ELISA, the wells were coated with an anti-lipocalin 2 capture antibody (diluted 1:200 in PBS), washed with ELISA wash buffer (0.05% Tween 20 in PBS [PBST]), and blocked with 2% bovine serum albumin solution in PBS. The diluted fecal samples and lipocalin 2 standards were added to the respective wells and incubated overnight at 4°C. The wells were washed with wash buffer; subsequently, 100 μl of biotin-labeled anti-lipocalin 2 antibody diluted 1:200 in blocking solution was added to each well and incubated at room temperature for 1 h. Horseradish peroxidase-labeled streptavidin (Biolegend) was diluted 1:1,000 in PBS and added to each well after washing with wash buffer. The wells were developed with 100 μl of substrate buffer containing 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; 0.01%) and hydrogen peroxide (0.1%). The absorbance at 405 nm was measured on an ELISA plate reader, and the lipocalin 2 values were expressed as nanograms of lipocalin 2 per gram of feces.

RESULTS

DFI coupled with qPCR identified promoter P_{1140} as overrepresented in the mouse gut. The streptomycin-pretreated C57BL/6 mice were coinfected with differentially tagged wild-type *S.* Enteritidis (M1525) strains containing GFP reporter plasmid constructs. These plasmids harbored putative promoter elements for unique *S.* Enteritidis genes (see Fig. S1 in the supplemental material) cloned upstream of the GFP ORF between the XbaI and BamHI sites. We proposed that GFP would be expressed through these plasmid constructs under the control of the cloned putative promoters. The analysis was limited to seven different promoters at a time, as there were only seven unique M1525 WITS (numbered WITS1, -2, -11, -13, -17, -19, and -21). The analysis included strain M1525 carrying intracellular GFP reporter plasmid pM975 as a control strain. Thus, the scheme allowed the analysis of six other putative promoters of *S.* Enteritidis. Initially, the genomic DNA isolated from the inoculum pool was subjected to relative quantification of the WITS tags using tag-specific primers (Table 2) by qPCR. All seven tags were present in equal ratios in the inoculum pool, indicating the presence of an equal population of differentially tagged *S.* Enteritidis strains in the inoculum (Fig. 1A). The relative equal ratio of WITS tags was also observed in the DNA obtained from the fecal samples of mice at day 1 p.i., indicating that all of the strains had the same opportunity for *in vivo* colonization; this result ruled out stomach acid-related bottlenecks and reiterated inoculum proportions (Fig. 1B). Subsequently, the cecal samples obtained at day 2 p.i. were FACS sorted on the basis of their GFP expression profile using DFI. The GFP-positive fraction obtained represented a net of 24% of the GFP-expressing bacterial population (Fig. 2A). However, at day 2 p.i., the promoter construct pM2155 (upstream region of *SEN1140*; P_{1140}) was statistically predominant ($P < 0.0001$) in the GFP-positive fraction, accounting for 76 to 78% of the GFP-expressing population (Fig. 2B). Alternatively, the ratio of GFP-positive to GFP-negative fractions (~80-fold) was statistically significant only for *S.* Enteritidis strains harboring P_{1140} of all of the promoters tested. Furthermore, marginal GFP expression was observed for the M1525 strain containing pM975 (Fig. 2B and C and 3A), which could be attributed to phagocytosis by luminally recruited PMN leukocytes and the subsequent release of *Salmonella* back into the lumen after destruction of the immune cell. Thus, the activation of the putative promoter P_{1140} through the promoter construct pM2155 in the mouse gut could suggest novel roles for *SEN1140* in *S.* Enteritidis interactions with the host, possibly influencing subsequent inflammation.

Wild-type *S.* Enteritidis with a P_{1140}-harboring plasmid exhibits GFP expression in mouse secondary lymphoid organs. DFI revealed the activation of *S.* Enteritidis-specific putative promoter P_{1140} through strain M1525/pM2155 in the intestinal lumen as mentioned above. However, the gut colonization capacity

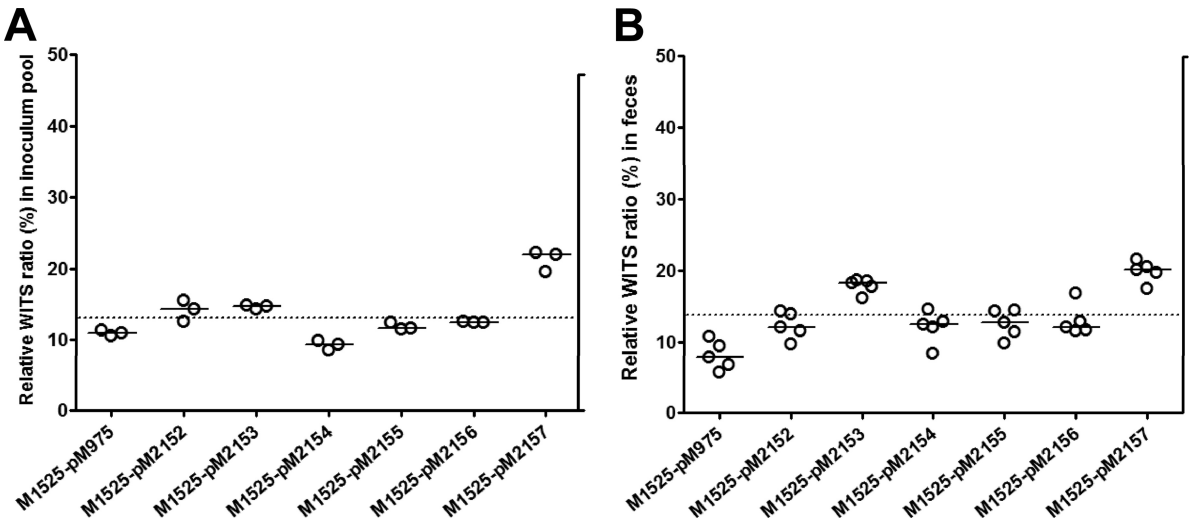

FIG 1 Recombinant promoter construct-bearing *Salmonella* strains colonize equally *in vivo*. Freshly grown cultures of seven differentially WITS of *S.* Enteritidis wild-type strain M1525 harboring different promoter constructs integrated into a GFP reporter plasmid were mixed in equal proportions (1:1:1:1:1:1:1) to create a mixed-inoculum pool. (A) Individual WITS tags from three independent replicates of the inoculum pool were quantified by qPCR. All WITS showed an average equal proportion of 11 to 19% of the inoculum pool. (B) At day 1 p.i., all seven strains were found to have colonized well and to be shed in equal ratios in the feces. All strains showed corresponding relative WITS ratios of about 14.28%, which represents the theoretical average WITS ratio (shown by the dashed line) of individual WITS in a pool of seven strains.

in C57BL/6 mice pretreated with streptomycin was equivalent in mutant strain deficient in *SEN1140* (strain Z290) and the parental M1525 strain (Fig. 4A). Therefore, we investigated the role of *SEN1140* in secondary lymphoid tissues. Interestingly, the P_{1140} promoter in strain M1525/pM2155 was active in gut-associated lymphoid tissues, including the lamina propria (Fig. 3A), with a mean count of 46.80 (standard error of the mean, ±3.184)/20-μm section (6 sections/mouse; 5 mice). Qualitatively, the draining MLN and systemic sites, i.e., the spleen and liver, also showed GFP expression through pM2155 reflecting activation of putative promoter P_{1140} (Fig. 3B). Furthermore, P_{1140} activation was twice as high as that of positive control pM975, which was harbored by wild-type strain M1525, in the observed lamina propria tissues ($P < 0.05$). Overall, the activation of the putative P_{1140} promoter in the secondary lymphoid tissues might indicate the involvement of its downstream gene *SEN1140* in multiple bacterial interactions inside the host. These interactions might also include virulence-specific roles of *SEN1140* genes unique to *S.* Enteritidis. However, the identification of key factors involved in the accelerated inflammation kinetics of TTSS1-deficient *S.* Enteritidis remains a challenge.

***SEN1140* contributes to TTSS2-dependent accelerated inflammation kinetics.** Our previous work comparing the virulence of *S.* Enteritidis and *S.* Typhimurium revealed significant differences in inflammation kinetics when invasion and subsequent inflammation were triggered via an alternative pathway in a TTSS2-dependent manner (43). However, the molecular mechanisms underlying this phenomenon remained largely unknown. In the present study, we have shown that the genetic deletion of *SEN1140* in a TTSS1-deficient background results in differences between the inflammation kinetic profiles of these two serovars. Streptomycin-pretreated C57BL/6 mice were infected with the *S.* Enteritidis double mutant Z290 (*invC*::*aphT SEN1140*::*cat*); parallel and independent infections were conducted with *S.* Enteritidis parent strain M1511 (*invC*::*aphT*) and the *S.* Typhimurium equivalent SB566 (*invC*::*aphT*), respectively. All of these strains were carrying GFP reporter plasmid pM973 to facilitate fluorescence microscopic screening of the lamina propria. Upon infection, all strains colonized efficiently *in vivo* and exhibited comparable gut colonization levels (Fig. 4). However, TTSS1-deficient *S.* Enteritidis parent strain M1511 was able to induce early inflammation. The degree of inflammation was assessed indirectly through the fecal levels of lipocalin 2, which is one of the many possible biomarkers of intestinal inflammation, reflecting PMN cell infiltration of the lamina propria and the subsequent luminal secretion of glycoprotein. Initially, the mean lipocalin 2 concentration in strain Z290-infected mice was similar to the levels observed in mice infected with strains M1511 and SB566 at 12 h p.i.; however, significant differences were observed at 24 h p.i. (Fig. 5A). The mean lipocalin 2 concentration produced by strain Z290 infection was approximately 2.893 (standard deviation [SD], ±0.643) ng/g feces, whereas the concentration produced by strain M1511 infection was 6.637 (SD, ±0.445) ng/g feces ($P < 0.05$) at 24 h p.i. Notably, the lipocalin 2 level produced by strain Z290 infection was comparable to that produced by strain SB566 infection, i.e., 2.453 (SD, ±0.673) ng/g feces. Furthermore, the cecal pathology score was consistent with the lipocalin 2 concentrations (Fig. 5A) and reflected a pronounced difference in the degree of inflammation at day 2 p.i. (Fig. 5B). The early inflammation profile observed in the TTSS1-deficient *S.* Enteritidis (M1511) strain and its difference from the *S.* Typhimurium equivalent strain (SB566) were consistent with our previously published results (43). However, the degree of inflammation from strain Z290 (M1511, *SEN1140*::*cat*) was comparable to that from strain SB566. It became clear that the deletion of *SEN1140* lowered the degree of inflammation expected from the SPI-1-deficient *S.* Enteritidis strain. This reduction in the inflammation profile could possibly be considered deferred inflammation kinetics. It was intriguing to observe that the deletion of a single gene, i.e., *SEN1140*, in a TTSS1-deficient background could explain the kinetic differences between these serovars, high-

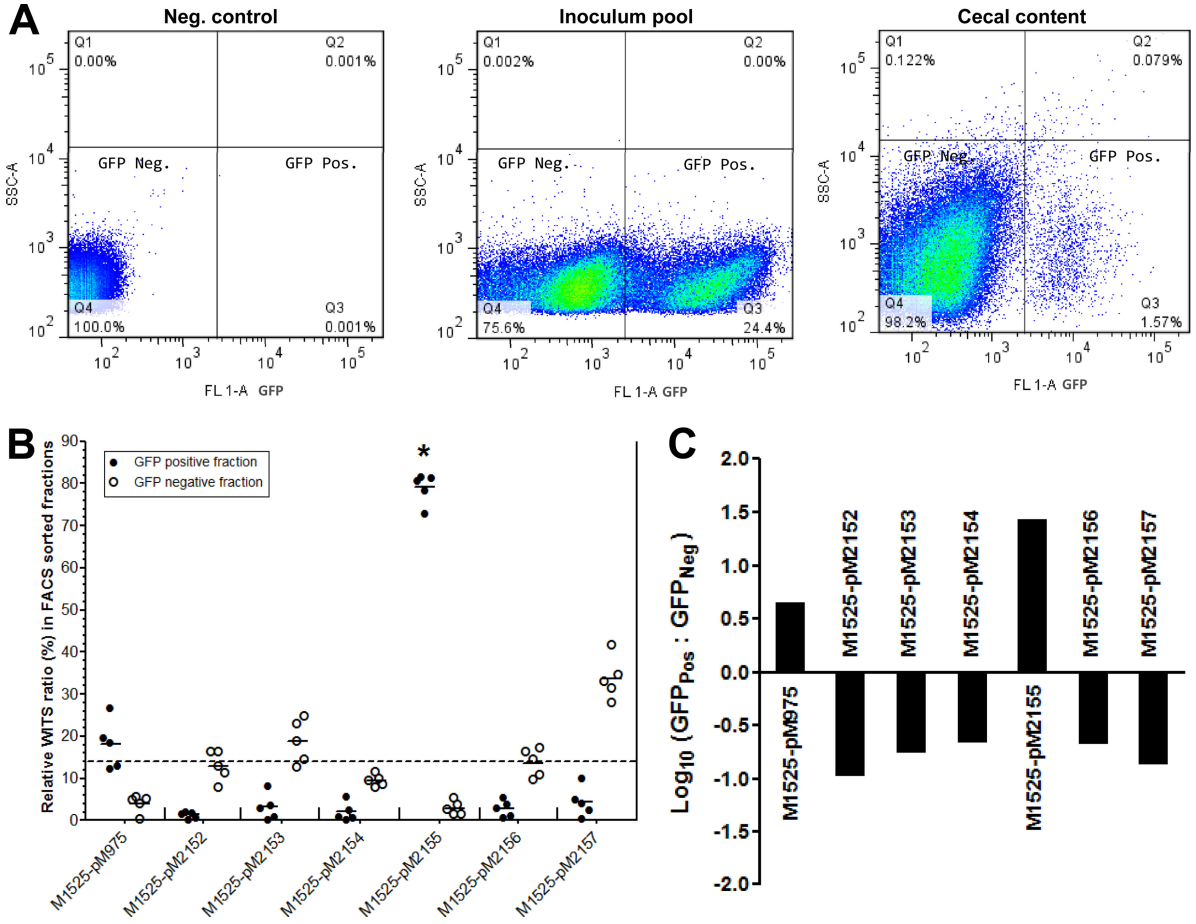

FIG 2 The GFP-positive fraction was represented mainly by one promoter construct. A mixed-inoculum pool (1:1:1:1:1:1:1:1) of WITS of *S.* Enteritidis having specific promoter constructs was used to infected a group of five streptomycin-pretreated C57BL/6 mice, whose cecal contents were analyzed at day 2 p.i. (A) Cecum samples were collected and suspended in 500 μl PBST solution. The suspension was diluted 1:100, passed through a 50-μm sieve, and FACS sorted to collect GFP_{Pos} and GFP_{Neg} bacterial fractions from the third and fourth quadrants, respectively, during FACS sorting. The FACS was calibrated with a negative-control strain bearing plasmid pM968. (B) Relative WITS counts of GFP_{Pos} (●) and GFP_{Neg} (○) fractions of cecal contents. Collected GFP fractions were enriched in LB medium supplemented with an appropriate antibiotic. Genomic DNA was isolated from the culture, and qPCR was performed for WITS. Most of the GFP_{Pos} fraction was represented by strain M1525/pM2155. *, $P < 0.001$ (Kruskal-Wallis test). (C) Statistics of FACS-sorted GFP_{Pos} and GFP_{Neg} fractions obtained from cecal contents on a logarithmic scale showing higher expression of GFP through promoter construct pM2155 than the positive control pM975 in the mouse cecum.

lighting the previously unknown roles of *S.* Enteritidis-specific genes.

In an independent experiment, we tested the differences in colonization and inflammation kinetics between *SEN1140* single mutant Z291 and its parental *S.* Enteritidis wild-type strain, M1525. We observed efficient colonization by these strains in all of the organs assessed (see Fig. S3A in the supplemental material). The cecal inflammation profiles of strains M1525 and Z291 were found to be comparable (see Fig. S3B in the supplemental material). Further, we could not observe any significant difference between the competitive indexes ($\log_{10}$) of M1525 and Z291 upon the execution of an independent coinfection experiment ($n = 7$); both strains had equal access to systemic organs (see Fig. S3C in the supplemental material). These data suggest that the disruption of *SEN1140* in an SPI-1-proficient strain could not address the differences in inflammation kinetics observed between M1511 and Z290 in previous experiments. When taken together, all of the data collectively suggest a possible role for *SEN1140* in *S.* Enteritidis-induced cecal inflammation in a TTSS-2-dependent manner.

***SEN1140* augments the mucosal tissue load in a TTSS2-dependent manner.** TTSS1-deficient *S.* Enteritidis demonstrated accelerated inflammation kinetics, as mentioned above; however, this phenotype could arise for several possible reasons, including efficient sampling of *S.* Enteritidis, faster replication, or increased resistance to host defenses, which result in a net increase in the *S.* Enteritidis mucosal tissue load compared with that of *S.* Typhimurium. Alternatively, the former could provoke a more sensitive cytokine response, resulting in accelerated inflammation despite similar colonization levels. However, a previous report confirmed that TTSS1-deficient *S.* Enteritidis produced a greater mucosal tissue load, i.e., 10-fold more CFU in the lamina propria on day 2 than did the *S.* Typhimurium equivalent (43). Therefore, we investigated whether the abrogated inflammation of Z290 results from differential mucosal colonization. Cecum tissues were analyzed by fluorescence microscopy (see Materials and Methods). As expected, strain M1511 reached a mean tissue density of 77.21 (SD, ±5.96) cells per tissue section, while that of the *S.* Typhimurium equivalent, SB566, was significantly lower at a mean of 21.20

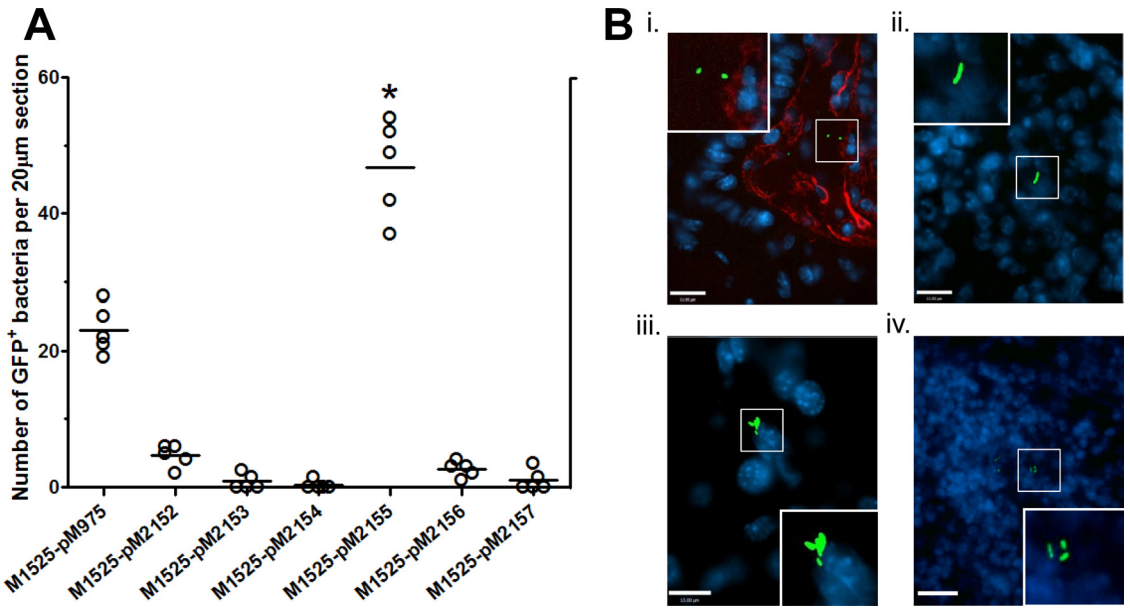

FIG 3 Construct pM2155 is expressed at systemic sites *in vivo*. All of the constructs were tested in C57BL/6 for GFP expression at the systemic sites monitored after cecal colonization. (A) Cecal PFA sections were stained for immunofluorescence microscopy of the lamina propria. Shown are the counts of all of the GFP-expressing bacteria in the lamina propria. *, $P < 0.05$ (unpaired *t* test with Welch's correction). (B) Immunofluorescence staining of the lamina propria (i), spleen (ii), liver (iii), and MLN (iv) showing GFP-expressing strain M1525/pM2155. Bacteria expressing GFP at the host tissue sites are shown in the insets of all immunofluorescence images. Bars, 15 μm.

(SD, ±4.76) cells per tissue section (Fig. 5C). Interestingly, strain Z290 had approximately 2-fold attenuated cecal lamina propria colonization compared with that of its parent strain, M1511, but was comparable to SB566. Thus, the inefficient lamina propria colonization of strain Z290 could further support its corresponding inflammation kinetics compared with strain M1511, which efficiently colonized the lamina propria and produced a high pathoscore at day 2 p.i. Furthermore, the complementation of *SEN1140* in strain Z290 rescued the accelerated inflammation phenotype, which was comparable to that of M1511 at day 2 p.i. ($P > 0.05$) (see Fig. S2 in the supplemental material), confirming the role of *SEN1140* in a TTSS2-dependent alternative pathway of *S.* Enteritidis-induced inflammation.

DISCUSSION

Since the beginning of evolution, bacteria have continuously adapted to survive under exposed conditions through the manipulation of their genome contents and protein expression profiles. In some cases, small changes in genome content led to the evolution of bacteria with altered phenotypes (6). In pathogenic bacteria, genetic changes might result in an altered *in vivo* phenotype and host adaptation. In some cases, this enhances the virulence and/or transmission of the bacterium (5, 33). *Salmonella* is a pathogenic organism that has undergone numerous changes in its genome content throughout the evolutionary process. Among other members of this species, *S.* Typhimurium (SL1344) and *S.* Enteritidis (P125109) have been sequenced and annotated. Although the average nucleotide sequence identity between the shared orthologs of these serovars was reported to be 98.98% (44), approximately 6.4% of the genes present in *S.* Enteritidis are specific to this group and not found in *S.* Typhimurium. Indeed, *S.* Enteritidis possesses additional gene sequences in its genome that are not present in the genome of *S.* Typhimurium or the closely related serovar *S.* Gallinarum (7, 44). The functions of most of the *S.* Enteritidis genes, those shared with extensively studied *S.* Typhimurium, are now know; however, the extra gene elements present in *S.* Enteritidis have yet to be explored for their functional importance. There are few reports addressing the roles of *S.* Enteritidis-specific gene elements in the invasiveness and pathogenicity of the serovar (39). The *SEN1975* gene has been reported as a novel virulence factor of *S.* Enteritidis that modulates the host immune response, as it bears close homology with the mammalian Toll/interleukin-1 (IL-1) receptor family of proteins (30). Similarly, the deletion of prophage-like element ϕSE12 severely attenuates the strain and promotes protective immunity against *S.* Enteritidis (1), suggesting the involvement of this element in the enhancement of *S.* Enteritidis virulence.

S. Enteritidis and *S.* Typhimurium are identical in gene distribution and alignment in the key pathogenicity islands, SPI-1 and SPI-2, of their genomes. These serovars colonize host tissues equally during the course of infection. Surprisingly, in a comparison of these serovars in a TTSS1-deficient background, *S.* Enteritidis showed accelerated invasion and inflammation patterns via an alternative pathway in streptomycin-pretreated mice (43). The reported difference in the inflammation patterns was attributed to additional gene elements present in the genome of *S.* Enteritidis (43). However, the identification of virulence factors remains a technical challenge for many researchers and many have reported on modern methods of screening for genes expressed *in vivo*. Phenotypic study upon the deletion of specific genes is still an influential and widely accepted approach. Alternatively, screening for promoters could ultimately indicate the expression of a related gene (36). In this regard, one of the most widely practiced approaches is to develop a promoter trap library for analysis of the differential expression of the reporter protein under variable con-

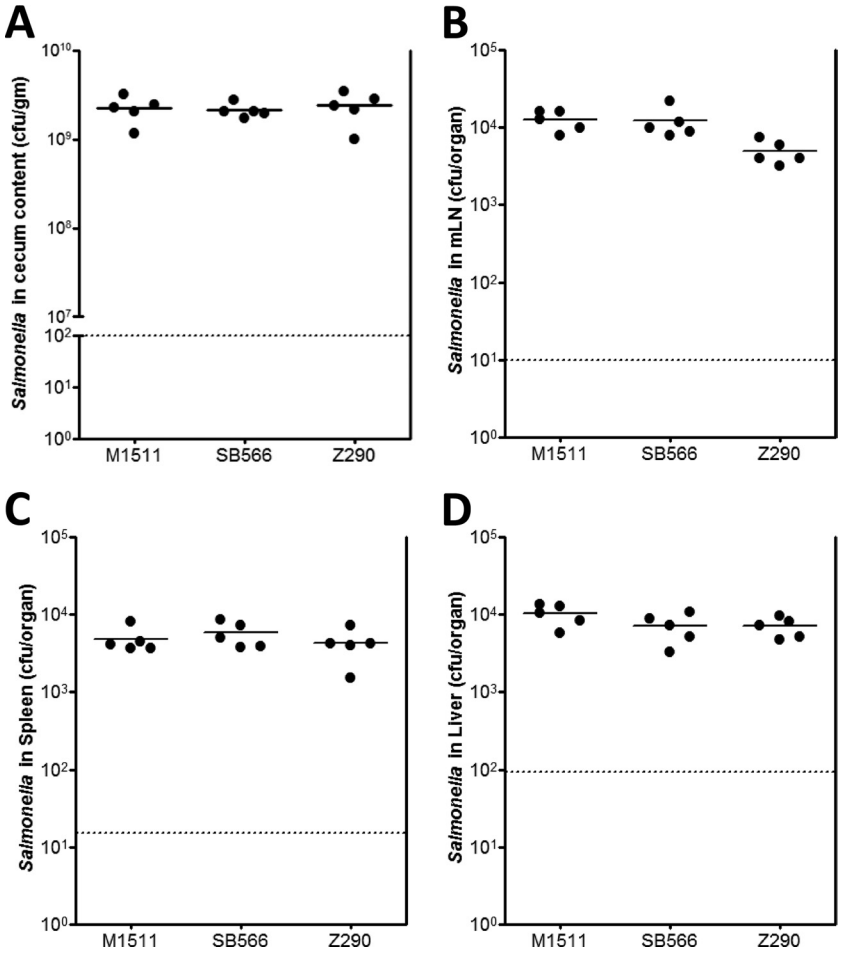

FIG 4 Strain Z290 colonizes efficiently *in vivo*. Different groups of streptomycin-pretreated C57BL/6 mice were infected with *S.* Enteritidis strain M1511 (SPI-1 deficient), *S.* Typhimurium strain SB566 (SPI-1 deficient), and S. enteritidis strain Z290 (SPI-1 deficient, *SEN1140*::*cat*). The cecum (A), MLN (B), spleen (C), and liver (D) were isolated at day 2 p.i., and the CFU in each organ were enumerated. $P > 0.05$ for Z290 versus the other strains.

ditions at different host sites; this method seems more promising and convenient than other more expensive methods, such as microarray and transcriptome analysis (8, 46). In this study, the putative promoter regions of six distinct *S.* Enteritidis-specific genes were studied. These genes were chosen on the basis of their presence in the *S.* Enteritidis chromosome or relative similarity to some domains within mammalian proteins as assessed with SMART and Pfam tools (12, 24). Of the experimental promoters selected, the putative promoter upstream of *SEN1140* induced the expression of GFP *in vitro* and *in vivo*. Often in highly sensitive and advanced screening systems, such as microarrays, genes that are expressed both *in vitro* and *in vivo* show reduced expression upon data analysis, indicating that these genes are constitutively expressed; however, this could be misleading. It has been reported that many constitutive genes are differentially expressed under various host conditions and in various infection types. In addition, the altered expression of a few constitutive genes was reported in a comparison of enteritis and typhoid models of infection (37). This provides an adequate platform for the selection of a widely expressive promoter associated with the *SEN1140* gene for the assessment of its possible role in *in vivo S.* Enteritidis infection. The use of the WITS technique in this study mediated the indirect identification of individual promoter constructs and facilitated the quantification of exclusive promoter-bearing strains directly from *in vitro* cultures or *in vivo* cecal samples. The WITS technique has been developed for the design of phenotypically identical strains bearing different signature tags in noncoding regions of the chromosome (26). This technique has been demonstrated to facilitate a comprehensive view of the spatial and stochastic nature of the isogenic tagged strains within a host (14).

In this study, we demonstrated that the additional in-frame deletion of *SEN1140* in an SPI-1-deficient *S.* Enteritidis strain (Z290) lowers the inflammation profile, compared with that of a TTSS1-deficient parental strain (M1511), in streptomycin-pretreated mice. The concentration of lipocalin 2 in the feces of infected mice directly reflected the severity of the inflammation. Lipocalin 2 is a Th17-induced antimicrobial peptide produced by host epithelial cells against luminal bacteria (15). The secretion of lipocalin 2 is a secondary antimicrobial response, whereas the primary response involves the recruitment of neutrophils to the site of inflammation, which is induced through the action of IL-13/IL-17. Lipocalin 2 inhibits the growth of intestinal bacteria by preventing their acquisition of iron, an essential nutrient element. It has been reported that *Salmonella* infection results in marked IL-

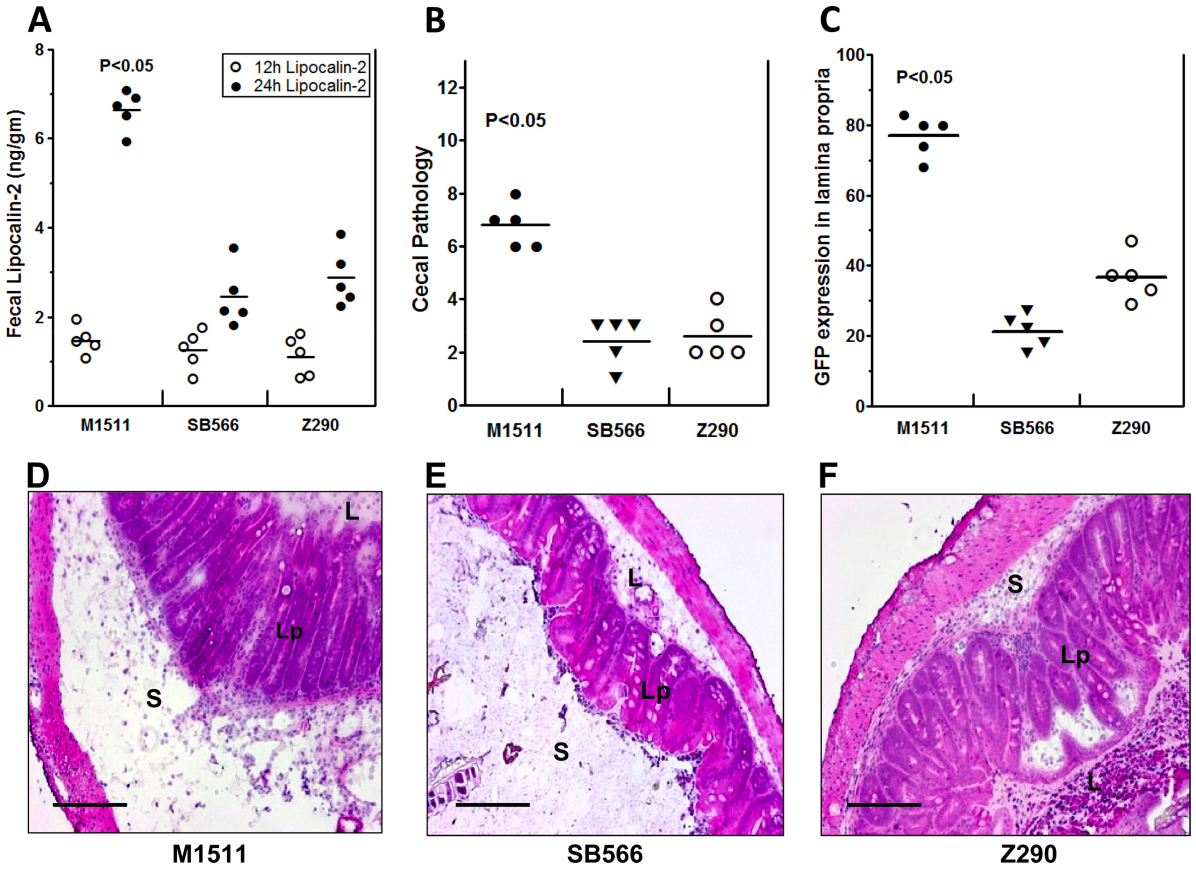

FIG 5 Deletion of the *SEN1140* gene lowers inflammation in the absence of TTSS1. The mouse groups shown in Fig. 4 were also analyzed for secretion of lipocalin 2 as a marker of cecal inflammation. (A) Fecal samples were collected from each group of mice at 12 and 24 h p.i. and suspended in sterile PBS, and the lipocalin 2 concentrations in the supernatants of fecal samples were estimated by ELISA. (B) Cecum sections from each group of mice were examined to determine pathoscores. (C) PFA sections of mouse cecum were stained for immunofluorescence microscopy, and the number of CFU of each strain expressing GFP in the lamina propria was determined. (D to F) H&E-stained representative cecum sections from each group of mice showing induced cecal inflammation: D, *S.* Enteritidis strain M1511 (SPI-1 deficient); E, *S.* Typhimurium strain SB566 (SPI-1 deficient; F, *S.* Enteritidis strain Z290 (SPI-1 deficient, *SEN1140*::*cat*). L, lumen; Lp, lamina propria; S, submucosal edema. Bars, 200 μm.

17- and IL-22-dependent intestinal epithelial induction and luminal accumulation of the antimicrobial peptide lipocalin 2 (34). Determination of the concentration of the highly stable lipocalin 2 protein is convenient for evaluation of the severity of diarrheal inflammation using the cecal or fecal contents of an infected host.

The restoration of the inflammation rate through complementation of *SEN1140* confirms its contribution to the accelerated inflammation kinetics of *S.* Enteritidis pathogenicity *in vivo*. *SEN1140* encodes a hypothetical phage protein with an unknown function. *SEN1140* homologs have been observed in *S.* Gallinarum/Pullorum (strain RKS5078), *S.* Dublin (strains CT_02021853 and 3246), and *S.* Gallinarum (strain 287/91); however, some *S. enterica* serovars, such as *S.* Typhimurium (strains DT104, SL1344, DT2, and D23580), *S.* Bongori (strain 12419), *S.* Hadar, and other related serovars, did not show the presence of any such homolog, as observed in the available online database of typed *Salmonella* strains. Analysis of the primary sequence showed the presence of a YxxL/I–$(x)_{6-8}$–YxxL/I (x denotes any amino acid) consensus sequence for an immunoreceptor tyrosine-based activation motif (ITAM) between residues 40 and 60 and a consensus sequence (I/V/L/S)xYxx(L/V) for its inhibition motif (ITIM) between residues 155 and 162 of the *SEN1140*-encoded protein. These motifs have been reported to actively modulate immune responses by activating signaling cascades (3) or by counterbalancing certain immune processes (16, 17, 30, 31). Moreover, it has been established that the signals derived from ITAM-containing receptors specifically control interstitial neutrophil migration toward the site of bacterial infection (13).

The majority of *S.* Enteritidis-specific gene clusters range in size from >3 to >40 kb. These unique gene clusters are called regions of difference of *S.* Enteritidis, which are involved primarily in prophage-related functions (44). Studies utilizing *Escherichia coli* K-12 and *Shigella* models have shown that differences between the DNA contents of related bacterial genomes are attributable to differences between the DNA sequences of the associated prophages (6). In the *S.* Enteritidis genome, the *SEN1140* gene is present in the ϕSE12 prophage element (44). Studies have indicated that some prophages contain additional cargo genes, termed morons, which are not required for the phage cycle. However, many morons from prophages in pathogenic bacteria might encode additional virulence factors that change the phenotype of the lysogen (6). Such moron elements are also present in the *S.* Enteritidis genome (6, 21, 32), contributing to its virulence and pathogenesis; therefore, the phage element *SEN1140* could also be con-

sidered a moron that influences the virulence of *S.* Enteritidis. The identification of morons that encode fitness factors in several of the *S.* Typhimurium prophages has led to the hypothesis that the phage-mediated reassortment of virulence and fitness factors is a key driving force in the optimization of the *Salmonella*-host interaction and the emergence of new epidemic clones (11). Some of the well-studied examples of moron-encoded functions include superoxide dismutases, enzymes for lipopolysaccharide modifications, and bacterial effector proteins secreted into host cells through specialized type III secretion systems (21). The effector protein SopE represents another appropriate model of the involvement of immunomodulatory morons in bacterial pathogenesis (32). Thus, it would be interesting and essential to evaluate the rationale for the presence of such morons within the *S.* Enteritidis genome and to identify any domains in the proteins that these genetic elements, such as *SEN1140*, encode. This analysis will be extended to investigate the role of other *S.* Enteritidis-specific genes in invasion or virulence to obtain a better understanding of bacterial adaptiveness. We hypothesize that phage-encoded morons with immunomodulatory functions might facilitate the adaptation of *Salmonella* strains to new hosts and provide an interesting topic for future research.

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