Type III Secretion Is Essential for the Rapidly Fatal Diarrheal Disease Caused by Non-O1, Non-O139 *Vibrio cholerae*

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ABSTRACT **Cholera is a severe diarrheal disease typically caused by O1 serogroup strains of *Vibrio cholerae*. The pathogenicity of all pandemic *V. cholerae* O1 strains relies on two critical virulence factors: cholera toxin, a potent enterotoxin, and toxin coregulated pilus (TCP), an intestinal colonization factor. However, certain non-O1, non-O139 *V. cholerae* strains, such as AM-19226, do not produce cholera toxin or TCP, yet they still cause severe diarrhea. The molecular basis for the pathogenicity of non-O1, non-O139 *V. cholerae* has not been extensively characterized, but many of these strains encode related type III secretion systems (TTSSs). Here, we used infant rabbits to assess the contribution of the TTSS to non-O1, non-O139 *V. cholerae* pathogenicity. We found that all animals infected with wild-type AM-19226 developed severe diarrhea even more rapidly than rabbits infected with *V. cholerae* O1. Unlike *V. cholerae* O1 strains, which do not damage the intestinal epithelium in rabbits or humans, AM-19226 caused marked disruptions of the epithelial surface in the rabbit small intestine. TTSS proved to be essential for AM-19226 virulence in infant rabbits; an AM-19226 derivative deficient for TTSS did not elicit diarrhea, colonize the intestine, or induce pathological changes in the intestine. Deletion of either one of the two previously identified or two newly identified AM-19226 TTSS effectors reduced but did not eliminate AM-19226 pathogenicity, suggesting that at least four effectors contribute to this strain's virulence. In aggregate, our results suggest that the TTSS-dependent virulence in non-O1, non-O139 *V. cholerae* represents a new type of diarrheagenic mechanism.**

IMPORTANCE **Cholera, which is caused by *Vibrio cholerae*, is an important cause of diarrheal disease in many developing countries. The mechanisms of virulence of nonpandemic strains that can cause a diarrheal illness are poorly understood. AM-19226, like several other pathogenic, nonpandemic *V. cholerae* strains, carries genes that encode a type III secretion system (TTSS), but not cholera toxin (CT) or toxin coregulated pilus (TCP). In this study, we used infant rabbits to study AM-19226 virulence. Infant rabbits orally inoculated with this strain rapidly developed a fatal diarrheal disease, which was accompanied by marked disruptions of the intestinal epithelium. This strain's TTSS proved essential for its pathogenicity, and there was no diarrhea, intestinal pathology, or colonization in rabbits infected with a TTSS mutant. The effector proteins translocated by the TTSS all appear to contribute to AM-19226 virulence. Thus, our study provides insight into *in vivo* mechanisms by which a novel TTSS contributes to diarrheal disease caused by nonpandemic strains of *V. cholerae*.**

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Vibrio cholerae includes a genetically diverse group of organisms that have long been associated with human disease. To date, more than 200 *V. cholerae* serogroups have been identified (1), and of these, the most is known about *V. cholerae* O1 and O139, the serogroups responsible for epidemic cholera in the world at the present time. The canonical virulence factors of *V. cholerae* O1 and O139 include cholera toxin (CT), an enterotoxin that stimulates the secretion of chloride ions from intestinal epithelial cells, and toxin coregulated pilus (TCP), a factor essential for intestinal colonization. While O1 strains continue to be the major annual causes of epidemic cholera in the world, recent studies indicate that non-O1, non-O139 *V. cholerae* strains cause sporadic cases of gastroenteritis or extraintestinal infection (2–7). The clinical spectrum of gastrointestinal illness caused by non-O1, non-O139 *V. cholerae* is more variable than that caused by *V. cholerae* O1. Both groups of pathogens can cause a cholera-like illness, but individuals with non-O1, non-O139 infection can exhibit signs of invasive disease such as bloody diarrhea (8). Non-O1, non-O139 *V. cholerae* strains are heterogenous but are thought to cause disease via CT- and TCP-independent virulence mechanisms (1).

Previous work revealed that the non-O1, non-O139 *V. cholerae*

strain AM-19226, a clinical isolate from Bangladesh, colonized the infant mouse intestine (9) and caused disease in adult rabbits (10), phenotypes that are normally associated with the production of TCP and CT in O1 strains, respectively. However, genome sequence analysis revealed that AM-19226 lacks the genes that encode CT and TCP; instead, the AM-19226 genome encodes a type III secretion system (TTSS) (10). TTSSs enable Gram-negative bacteria to translocate effector proteins directly into the host cytosol (11–13). Translocated effectors manipulate host cellular processes such as those controlling the actin cytoskeleton (14–16). The pathogenicity of many bacteria, including *Salmonella*, *Pseudomonas*, *Yersinia*, *Shigella*, and enteropathogenic and enterohemorrhagic *Escherichia coli* (EPEC and EHEC, respectively), depends upon TTSSs (17–19). However, little is known about the role of the TTSS in the pathogenicity of non-O1, non-O139 *V. cholerae*.

To date, two effector proteins translocated by the *V. cholerae* AM-19226 TTSS have been identified (9, 20). One of these proteins, VopF, promotes actin nucleation and was found to be required for AM-19226 to efficiently colonize the suckling mouse intestine (9). The other characterized effector, VopE, promotes actin depolymerization (20). The importance of these and other as yet uncharacterized effectors in the diarrheal response and intestinal pathology elicited by strain AM-19226 is not currently known.

In contrast to strain AM-19226, which lacks CT and TCP, some non-O1, non-O139 strains have acquired the CTX phage and the pathogenicity island that encodes TCP (21). For example, the *V. cholerae* serogroup O141 strain V51 encodes both CT and TCP, as well as a TTSS (10). Whether and how all these virulence factors interact and contribute to the pathogenicity of V51 has not been explored.

Infant rabbits can serve as a useful model host to explore several aspects of *V. cholerae* pathogenicity (22–24). Orally infected 2- or 3-day-old rabbits routinely develop CT-dependent cholera-like diarrhea, and *V. cholerae* colonization of the infant rabbit small intestine requires TCP (22). These animals can also be used to study reactogenic diarrhea caused by live attenuated cholera vaccine candidates that contain deletions in the genes encoding cholera toxin (23). Approximately 24 hours after inoculation of infant rabbits with *V. cholerae* O1, they develop watery diarrhea which often results in their death (25). In contrast, inoculation of rabbits with *V. cholerae* O1 *ctxAB* deletion mutants does not result in watery diarrhea but instead causes self-limiting "fecal diarrhea," which appears to result from a host innate immune response to *V. cholerae* flagellins (23).

In this study, we used infant rabbits to explore the pathogenicity of *V. cholerae* AM-19226. Infant rabbits orogastrically inoculated with this non-O1, non-O139 *V. cholerae* strain lacking *ctx* and *tcp* developed severe diarrhea even more rapidly than infant rabbits infected with *V. cholerae* O1. In contrast to infant rabbits infected with *V. cholerae* O1, strain AM-19226 caused marked disruptions of the intestinal epithelium in infected rabbits. This strain's TTSS was essential for its pathogenicity. Deletion of individual TTSS effectors, including two newly identified effectors, reduced but did not eliminate AM-19226 virulence. Finally, we show that the TTSS in a *V. cholerae* strain that also encodes CT and TCP plays a key role in its pathogenicity. In aggregate, our findings provide insight into a new type of diarrheagenic mechanism used by non-O1, non-O139 *V. cholerae* strains and suggest that TTSS can lead to diarrheal illness.

RESULTS

A functional TTSS is required for *V. cholerae* AM-19226-induced diarrhea in infant rabbits. Since infant rabbits proved to be a useful model host to investigate intestinal disease caused by *V. cholerae* O1 strains (22, 23), we used 3-day-old rabbits to study the pathogenicity of non-O1, non-O139 *V. cholerae* strains. We were particularly interested to investigate whether the AM-19226 TTSS contributed to pathology in these animals. To assess the role of the TTSS in AM-19226 virulence, infant rabbits were orogastrically inoculated with either wild-type AM-19226 or a Δ*vcsN2* AM-19226 derivative. *vcsN2* encodes the putative ATPase component of the TTSS, and its deletion renders the strain deficient for type III secretion (9).

All rabbits orogastrically inoculated with ~10^9 CFU of the wild-type AM-19226 strain developed severe diarrhea, which was evident as extensive wetness on the rabbits' legs and perianal areas (Fig. 1A). Remarkably, the onset of diarrhea was even quicker than that observed with *V. cholerae* O1 infection of rabbits. Rabbits routinely exhibited diarrhea 12 to 15 h postinoculation with AM-19226 and died shortly thereafter. In most experiments, we euthanized rabbits ~12 h after infection, a point when all animals infected with wild-type AM-19226 exhibited watery diarrhea. At necropsy, the entire small intestine (proximal, mid, and distal regions) of infected rabbits appeared red, swollen, and filled with fluid (Fig. 1B). The AM-19226 TTSS proved to be critical for this strain to cause disease. In marked contrast to animals inoculated with wild-type AM-19226, rabbits inoculated with the Δ*vcsN2* mutant did not exhibit diarrhea (Table 1) for up to 3 days of observation. Furthermore, the appearance of the small intestines of infant rabbits inoculated with the Δ*vcsN2* strain appeared indistinguishable from those of the control rabbits that were given sodium bicarbonate buffer (Fig. 1B).

V. *cholerae* AM-19226 damages the small intestine. The intestinal histopathology associated with *V. cholerae* AM-19226 infection differed markedly from that observed in rabbits infected with *V. cholerae* O1. There was severe vascular congestion and multifocal hemorrhage of the mucosa observed in hematoxylin-and-eosin (H&E)-stained sections from the intestines of rabbits infected with AM-19226 (Fig. 1C). These abnormalities were most prominent in the upper two-thirds of the small intestine (see Table S2 in the supplemental material). Notably, AM-19226-infected rabbits exhibited disruption of the mucosal epithelial surface throughout the small intestine (Fig. 1C). In humans and infant rabbits infected with *V. cholerae* O1, the mucosal surface remains intact (22, 26). The AM-19226 TTSS appears to be critical for tissue damage induced by this non-O1, non-O139 strain. Histopathologic abnormalities were not readily apparent in the small intestines of rabbits inoculated with the Δ*vcsN2* strain; sections from the intestines of rabbits infected with this strain appeared similar to sections from control rabbits (Fig. 1C). AM-19226 did not appear to induce an influx of inflammatory cells into the intestine. Heterophils (the rabbit equivalent of neutrophils) or mononuclear cells were rarely observed in sections from the small intestines of either wild-type animals or animals infected with the Δ*vcsN2* mutant. Histological abnormalities were not detected in the lungs, kidneys, liver, or brain of rabbits infected with AM-19226, suggesting that the pathology caused by this strain is lim-

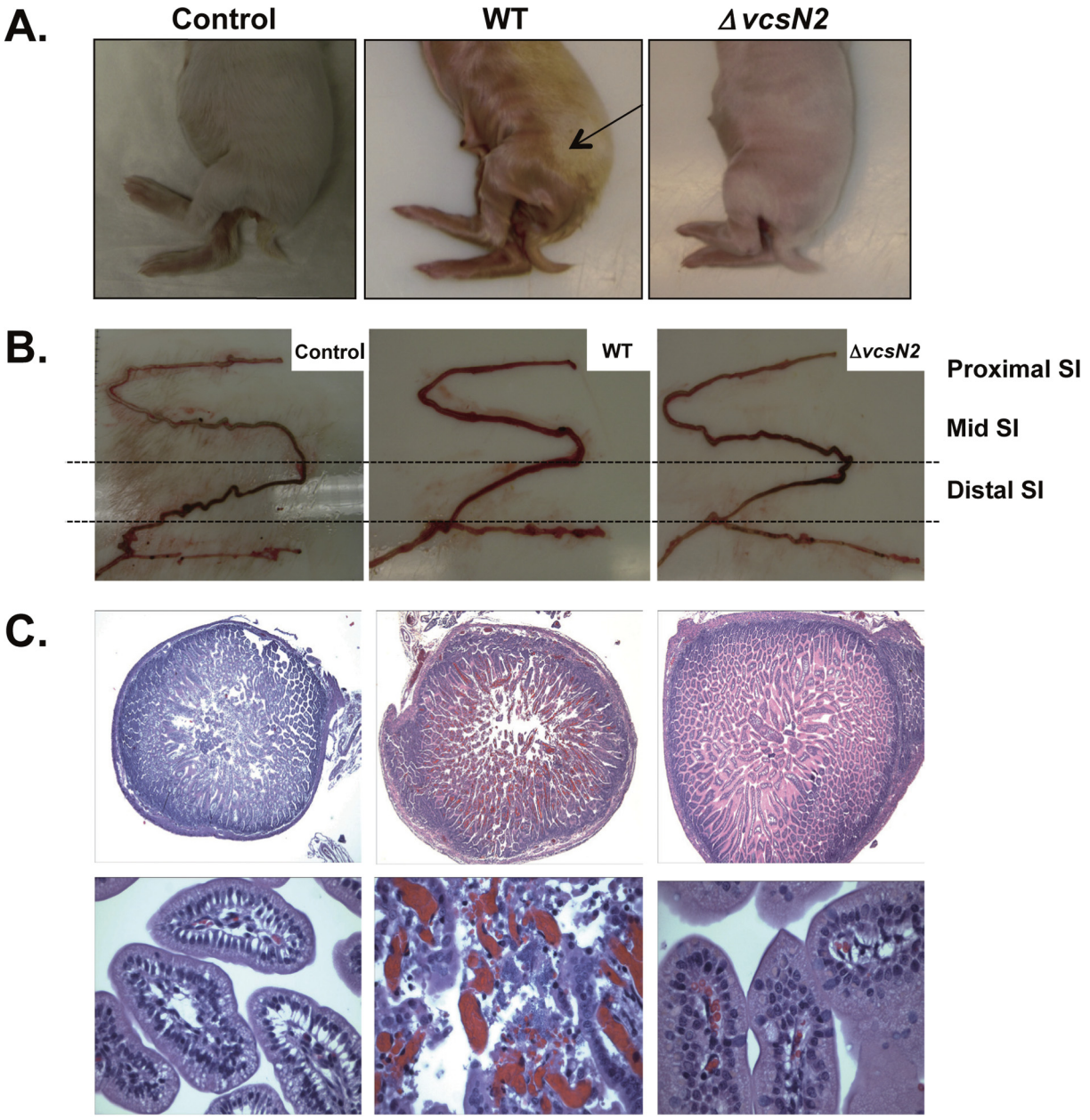

FIG 1 Gross and histologic features of infant rabbits infected with the wild-type or Δ*vcsN2 V. cholerae* AM-19226 strain. (A) Infant rabbits were orogastrically inoculated with either sodium bicarbonate buffer control or 10^9 CFU of the wild-type (WT) or Δ*vcsN2* AM-19226. All rabbits infected with wild-type AM-19226 developed severe watery diarrhea, which is evident as extensive wetness in the perianal region and hind legs. The arrow indicates the boundary between wet and dry fur. (B) Representative intestines from rabbits infected with the indicated strain or buffer control are shown (SI, small intestine). (C) Representative H&E-stained sections of the small intestines of infant rabbits are shown. Top images, ×10 magnification; bottom images, ×40 magnification.

ited to the intestine. Taken together, these findings suggest that the AM-19226 TTSS damages the epithelium of the small intestine and strongly supports the idea that this non-O1, non-O139 *V. cholerae* strain relies on a mechanism to cause diarrhea that is different from that of toxigenic *V. cholerae* O1.

Electron microscopic (EM) analysis of sections from the small intestines of rabbits infected with *V. cholerae* AM-19226 corroborated the destructive potential of this strain. Massive tissue disruption and severe blunting of epithelial cell microvilli, resulting in the reduced brush border, were observed in electron micrographs from rabbits infected with AM-19226 (see Fig. S1 in the supplemental material). This phenotype resembles attaching and effacing (A&E) lesions caused by A&E pathogens such as enteropathogenic *Escherichia coli* (EPEC) (14); however, unlike EPEC, AM-19226 did not appear to induce formation of actin pedestals.

The AM-19226 TTSS induces production of proinflammatory cytokines in the small intestines. We suspected that the tissue damage caused by *V. cholerae* AM-19226 might induce a host innate immune response with production of proinflammatory cytokines or chemokines. The amounts of transcripts for several

TABLE 1 Incidence of diarrhea in infant rabbits inoculated with *V. cholerae* AM-19226 and its derivatives[a]

AM-19226 strain	Incidence (% of rabbits with diarrhea)	No. of rabbits with the following diarrhea score:			Total no. of rabbits	*P* value[b]
		Severe	Mild	None		
WT	100	16	0	0	16	
Δ*vcsN2* mutant	0	0	0	16	16	<0.0001
Δ*vopE* mutant	57.1	4	4	6	14	0.05
Δ*vopF* mutant	80	9	3	3	15	0.12
Δ*mcf* mutant	50	3	4	7	14	0.0017
Δ*tcdB2* mutant	28.6	2	2	10	14	<0.0001

[a] This table shows the incidence of diarrhea (percentage of rabbits infected with the indicated strains exhibiting diarrhea) and the number of infant rabbits with the different diarrhea scores, and statistical analyses of these results are presented. At least two independent experiments were performed for each strain.
[b] The *P* value of the incidence for mutant strain compared to the value for the WT.

cytokines and chemokines (interleukin-8 [IL-8], tumor necrosis factor alpha [TNF-α], interleukin-6 [IL-6], and interleukin-1β [IL-1β]) in tissue homogenates from infant rabbits inoculated with either the wild-type or Δ*vcsN2* strain were measured with quantitative real-time PCR (RT-PCR). Compared to control rabbits, there was a significant increase in the levels of TNF-α, IL-8, IL-1β, and IL-6 transcripts in small intestinal homogenates from wild-type AM-19226-infected rabbits (Fig. 2). The levels of these transcripts did not differ between homogenates from the rabbits inoculated with the *vscN* mutant or the buffer control, suggesting

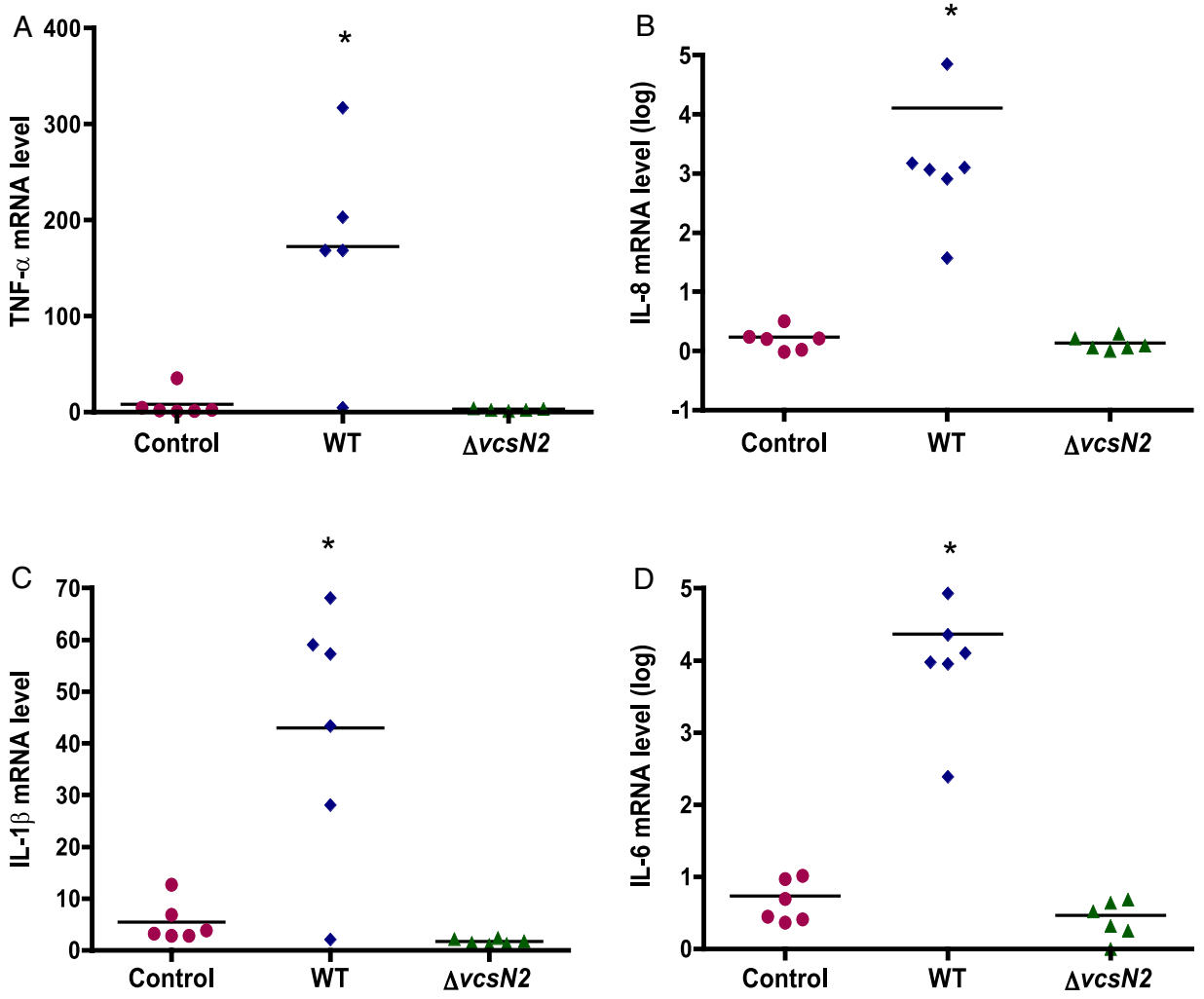

FIG 2 Transcriptional levels of proinflammatory cytokines and chemokines are elevated in the small intestines of rabbits infected with *V. cholerae* AM-19226. RNA was isolated from homogenates of small intestines of infant rabbits inoculated with buffer (control) or the indicated strains. The levels of transcripts of TNF-α (A), IL-8 (B), IL-1β (C), and IL-6 (D) were determined by quantitative real-time PCR, and all the values were normalized to the housekeeping gene HPRT. Each symbol represents the value for an individual rabbit, and the bar indicates the mean for the group. An asterisk indicates that the values for the WT samples were significantly different ($P < 0.05$) from those in the buffer control and Δ*vcsN2* samples.

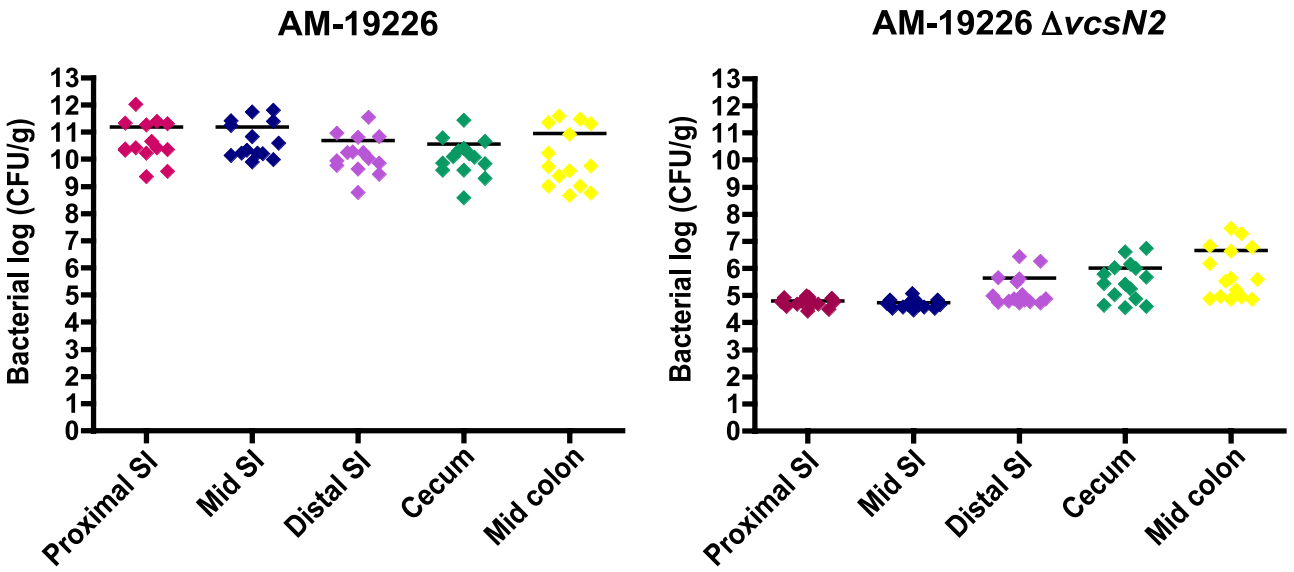

FIG 3 Intestinal colonization of WT and Δ*vcsN2* AM-19226 strains. The numbers of bacterial CFU recovered from sections taken from the proximal, mid, and distal small intestines (SI), ceca, and midcolons of rabbits infected with WT AM-19226 or the Δ*vcsN2* mutant are shown. Bars represent the mean values from 15 or 16 rabbits.

that the AM-19226 TTSS is critical for stimulating the rabbit innate immune response. However, it is possible that the absence of the innate immune response in rabbits infected with the TTSS-deficient strain is a consequence of the marked reduction in colonization of the rabbit small intestine by the AM-19226 Δ*vcsN* mutant (see below).

TTSS is required for intestinal colonization of infant rabbits. To assess the role of the AM-19226 TTSS in intestinal colonization, we compared the number of colony-forming units (CFU) of the wild-type and Δ*vcsN2* strains in tissue homogenates recovered from the proximal, mid, and distal small intestine, cecum, and midcolon. *V. cholerae* AM-19226 robustly colonized all regions of the infant rabbit small intestine as well as the cecum and midcolon (~10^{11} CFU/g in all regions [Fig. 3]). In contrast, *V. cholerae* O1 strains do not efficiently colonize the proximal small intestine (22). Furthermore, even in the mid and distal small intestine, where maximal *V. cholerae* O1 colonization occurs, there were ~1,000× more *V. cholerae* AM-19226 CFU recovered than *V. cholerae* O1 CFU (22). The *vcsN2* mutant was severely attenuated in its ability to colonize all regions of the infant rabbit intestine (Fig. 3). Thus, as Tam et al. found in studies of AM-19226 intestinal colonization of suckling mice (9), our results indicate that a functional TTSS is necessary to promote efficient colonization of the infant rabbit intestine.

Localization of *V. cholerae* AM-19226 within the small intestine. Recovery of *V. cholerae* in tissue homogenates reflects the ability of the organisms to grow and multiply in the host intestine but does not provide information about where bacterial cells localize within the intestine, e.g., whether cells are in the lumen or close to the epithelium. We constructed green fluorescent protein (GFP)-marked wild-type (WT) and *vcsN2* mutant AM-19226 derivatives to determine the localization of these strains within intestinal sections using confocal microscopy. The sections were counterstained with wheat germ agglutinin (WGA) (blue) and phalloidin (red) to allow us to simultaneously visualize mucin and F-actin, respectively. At 4 and 8 hours postinfection, there were too few bacteria to detect in the tissue sections. However, by 12 h postinfection, fluorescent AM-19226 bacteria were easily detectable (Fig. 4). At this point, bacteria were observed along the length of the villi and within crypt-like structures (Fig. 4 and data not shown). Most of the bacteria appeared to be clustered in aggregates, and many of the aggregates were closely apposed to the epithelial cells, apparently interrupting the epithelial cell border. Phalloidin staining showed that in areas where AM-19226 was observed there was disruption of the peripheral actin ring of the villi and the underlying actin cytoskeleton. Thus, these confocal images reinforce and extend our findings described above that AM-19226 causes extensive damage to the mucosal epithelium. Consistent with the severe colonization defect of the TTSS-deficient strain, few fluorescent bacteria were observed in sections taken from rabbits inoculated with the *vscN2* mutant. The intestinal epithelium of rabbits infected with the Δ*vcsN2* strain did not differ from buffer control rabbits.

The known AM-19226 TTSS effector proteins contribute to AM-19226 virulence. To date, two proteins, VopF and VopE, have been shown to be effectors translocated by the *V. cholerae* AM-19226 TTSS (9, 20). Infant rabbits were inoculated with either the Δ*vopE* or Δ*vopF* AM-19226 derivative to explore the contribution of VopE and VopF to AM-19226-induced disease and intestinal colonization. Both effectors appear to contribute to the diarrheal response caused by AM-19226. Compared to rabbits inoculated with WT AM-19226, rabbits infected with the *vopE* mutant strain showed reductions in the incidence and severity of diarrhea (Table 1). Rabbits infected with the *vopF* mutant also exhibited reduced severity and incidence of diarrhea, but these findings were not statistically significant (Table 1). Both effectors also proved to be important for AM-19226 intestinal colonization (Fig. 5). Each of the deletion mutants exhibited ~10- to 100-fold reductions in colonization throughout the intestines. Thus, the two characterized AM-19226 effectors contribute to the abilities of this pathogen to elicit diarrhea and to colonize the intestine. However, neither one of the two effectors is essential for either

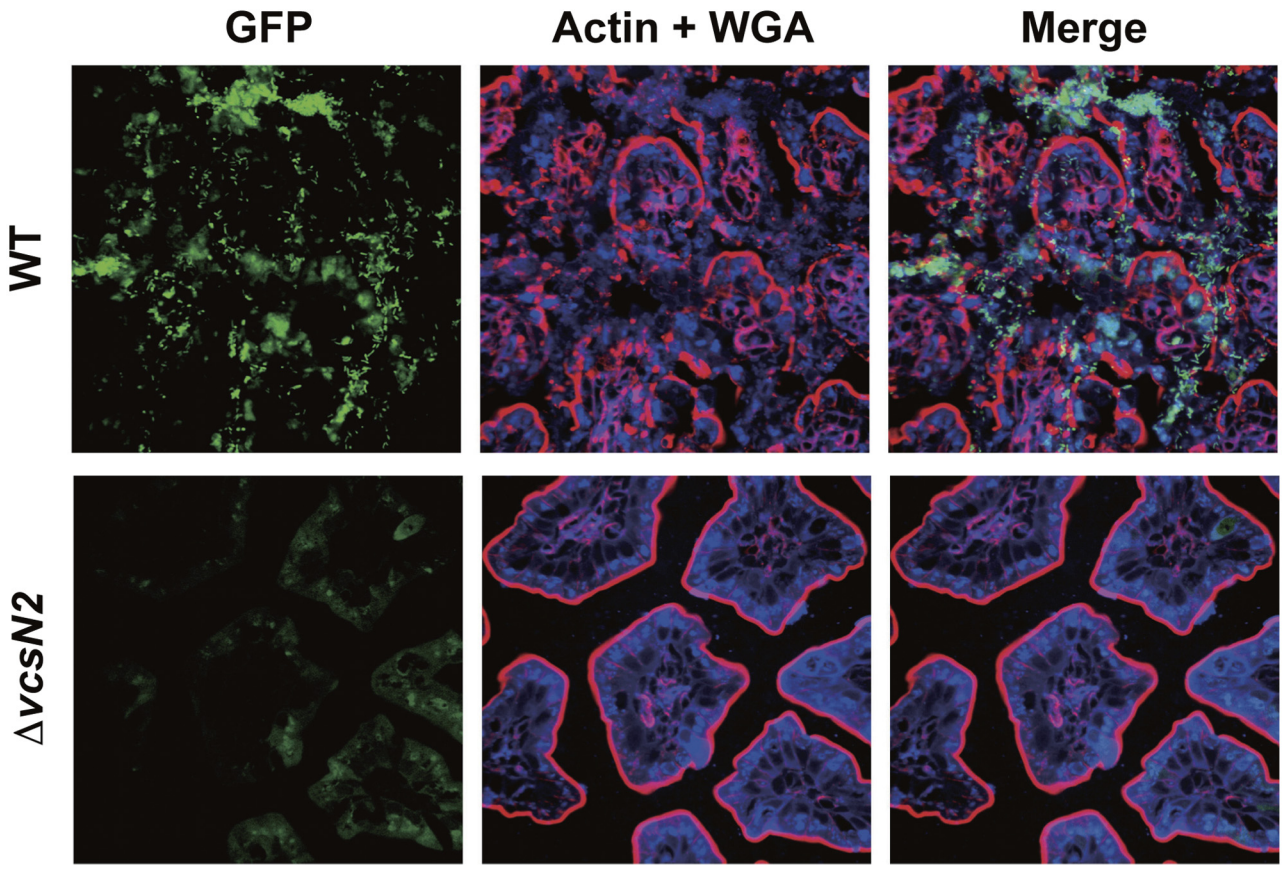

FIG 4 Representative confocal images of GFP-expressing WT and Δ*vcsN2* AM-19226 strains in the rabbit small intestine. Distal small intestinal sections from rabbits infected with GFP-labeled WT or Δ*vcsN2* AM-19226 were prepared at 12 hours postinfection. Tissues were stained with Alexa Fluor 568-labeled phalloidin (red) to visualize the F-actin and counterstained with wheat germ agglutinin (WGA) (blue) to visualize mucin.

phenotype. The reductions in diarrhea and intestinal colonization observed with these effector mutants were not nearly as dramatic as those observed with the *vscN2* mutant, suggesting that there may be redundancy in the requirements for the AM-19226 TTSS effectors to cause disease.

Newly identified AM-19226 TTSS effectors also contribute to this strain's virulence. Bioinformatic analyses of the open reading frames (ORFs) in the *V. cholerae* AM-19226 TTSS island suggested that two additional loci might encode previously unrecognized effectors. These loci, which are not present in the *Vibrio parahaemolyticus* TTSS2 island (see Fig. S2 and S4 in the supplemental material), encode homologs of the *Photorabdus* insecticidal toxins McfV (Mcf stands for Makes caterpillars floppy), a BH3 domain-containing protein that triggers apoptosis in insect hemocytes and midgut epithelium (27), and TcdB (Tc stands for toxin complex), a component of a complex that potentiates the toxicity of TcdA (28). AM-19226 locus *1699* (now designated *mcfV*) encodes a protein that is similar (29% identity, 45% positives) to a 136-amino-acid region preceding the BH3 domain in the *Photorhabdus* Mcf, which is a much larger protein. AM-19226 locus *1700* (now designated *tcdB*), which is transcribed convergently from *mcfV*, encodes a protein that is similar to *Photorhabdus* TcdB2 (41 identical amino acids [aa] and 60 positives/178 aa).

We fused *mcfV* and *tcdB* to a gene encoding β-lactamase to test whether these 2 putative toxins are translocated into eukaryotic cells with a β-lactamase translocation assay (9). In this assay, translocated fusion proteins cleave a fluorescent substrate in the eukaryotic cell, resulting in a change in emitted fluorescence. Both McfV and TcdB were translocated into Hep-2 cells in a *vscN2*-dependent fashion, suggesting that both proteins are TTSS substrates (see Fig. S3 in the supplemental material). We also constructed *V. cholerae* AM-19226 *mcfV* and *tcdB* deletion mutants and found that both strains were attenuated for virulence in infant rabbits. Each deletion mutant caused less severe diarrhea than the WT strain; the incidence of diarrhea in rabbits infected with these strains was also reduced (Table 1). Furthermore, both strains had a reduced capacity to colonize all regions of the intestine (Fig. 5). Thus, both *mcfV* and *tcdB* appear to encode AM-19226 TTSS effectors that promote this strain's virulence, and these effectors have redundant roles to cause diarrheal disease.

The TTSS contributes to virulence even when CT and TCP are present. Several non-O1, non-O139 *V. cholerae* strains encode TTSSs that are related to the *V. cholerae* AM-19226 TTSS (10). The DNA sequences of the TTSS islands in non-O1, non-O139 strain V51 and AM-19226 are extremely similar; in contrast, the TTSS island in AM-19226 has less homology to the *V. parahaemolyticus* TTSS2 island (see Fig. S4 in the supplemental material). Interestingly, V51, a serogroup O141 clinical isolate from the United States, encodes CT and TCP as well as the TTSS (10). Thus, this strain provides an opportunity to assess the relative contributions

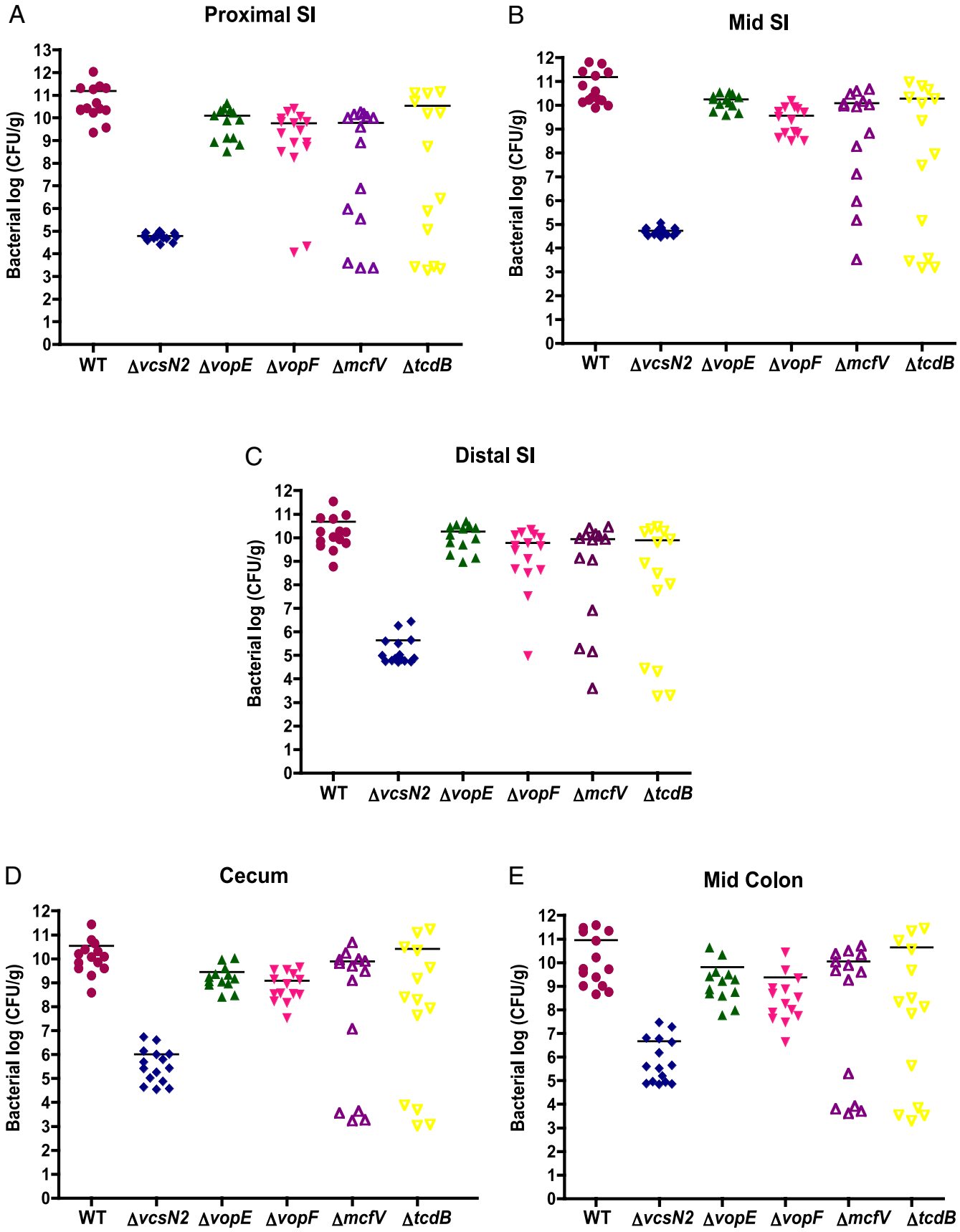

FIG 5 Intestinal colonization of *V. cholerae* AM-19226 TTSS effector mutants. Infant rabbits were inoculated with the indicated AM-19226 derivatives containing deletions of genes encoding TTSS effectors. The numbers of bacterial CFU recovered from sections taken from the proximal (A), mid (B), and distal small intestines (SI) (C), ceca (D), and midcolons (E) of infant rabbits are shown. Bars represent the geometric mean values.

TABLE 2 Incidence of diarrhea in infant rabbits inoculated with *V. cholerae* V51 strain and its derivatives[a]

V51 strain	Incidence (% of rabbits with diarrhea)	No. of rabbits with the following diarrhea score:			Total no. of rabbits	*P* value[b]
		Severe	Mild	None		
WT	100	7	0	0	7	
Δ*tcpA* mutant	62.5	7	3	6	16	0.12
Δ*vcsN2* mutant	53.8	3	4	6	13	0.05
Δ*tcpA vcsN2* mutant	0	0	0	6	6	0.006
Δ*ctxAB* mutant	77.7	5	2	2	9	
Δ*ctxAB tcpA* mutant	70	7	0	3	10	1.0
Δ*ctxAB vcsN2* mutant	16.7	2	0	10	12	0.01

[a] This table shows the incidence of diarrhea (percentage of rabbits infected with the indicated strains exhibiting diarrhea) and the number of infant rabbits with the different diarrhea scores, and statistical analyses of these results are presented. At least two independent experiments were performed for each mutant strain.
[b] For the Δ*tcpA*, Δ*vcsN2*, and Δ*tcpA vcsN2* mutants, the *P* value of the incidence for the mutant strain compared to the value for the WT is shown. For the Δ*ctxAB*, Δ*ctxAB tcpA*, and Δ*ctxAB vcsN2* mutants, the *P* value of the incidence for the mutant strain compared to the value for the Δ*ctxAB* mutant is shown.

of the TTSS versus CT/TCP to the pathogenicity of a single *V. cholerae* strain.

The severity and rapidity of the onset of diarrhea in rabbits inoculated with wild-type V51 were similar to the severity and kinetics of disease caused by strain AM-19226. All rabbits inoculated with wild-type V51 developed severe watery diarrhea 12 to 15 h postinfection (Table 2), faster than rabbits infected with *V. cholerae* O1 strains (22). *tcpA* or *ctxAB* V51 deletion mutants still caused severe diarrhea in most rabbits (Table 2); in contrast, deletion of either one of these two loci in pandemic O1 strains renders them avirulent (22). However, CT and TCP appear to contribute to V51 pathogenicity, since most rabbits infected with a *vscN2* V51 deletion mutant still developed at least mild diarrhea (Table 2), whereas the *vscN2* AM-19226 mutant strain was avirulent (Table 1). Only combined deletions of both *tcpA* and *vscN* rendered V51 avirulent. Intestinal colonization by V51 and the deletion mutants paralleled the clinical scores. Either TCP or TTSS appears to be sufficient to enable V51 to colonize the rabbit intestine (Fig. 6). Deletion of both *tcpA* and *vcsN2* was required to severely reduce V51's capacity to colonize the infant rabbit intestine (Fig. 6). In aggregate, these observations suggest that the TTSS and CT/TCP are at least partially redundant in enabling V51 pathogenicity.

DISCUSSION

Type III secretion systems (TTSSs) have long been known to be essential for the pathogenicity of several enteric pathogens, including *Salmonella enterica* serovar Typhimurium, enterohemorrhagic *E. coli*, and *Yersinia enterocolitica* (17–19). However, until recently, TTSSs were not thought to contribute to *V. cholerae* pathogenicity. Studies of the TTSS-positive ($TTSS^+$) TCP-negative (TCP^-) CT^- *V. cholerae* strain AM-19226 have refuted this notion. The AM-19226 TTSS was shown to be critical for this strain to colonize the intestine of the infant mouse and to cause diarrhea in adult rabbits (9, 10). Here, we used infant rabbits to further characterize the pathogenicity of AM-19226. Suckling rabbits orally inoculated with this strain rapidly developed fatal diarrhea. A functional TTSS was required for AM-19226 to colonize the small intestine and to cause histopathology and disease in this model host. Deletion of either *vopE* or *vopF*, two previously characterized AM-19226 TTSS effectors, or *mcfV* or *tcdB*, two effectors identified here, all reduced AM-19226 intestinal colonization and disease severity, suggesting that all of the known AM-19226 effectors contribute to virulence. In contrast to CT^+ TCP^+ *V. cholerae* strains lacking a TTSS, AM-19226 caused pronounced damage to the small bowel epithelium and elicited the production of high levels of transcripts for proinflammatory cytokines. Collectively, our findings indicate that AM-19226 and likely other vibrios that harbor closely related TTSSs elicit enteric disease via mechanisms that markedly differ from TCP^+ CT^+ (toxigenic) *V. cholerae*.

There are major differences in disease kinetics, pattern and extent of intestinal colonization, and pathology caused by toxigenic *V. cholerae* versus $TTSS^+$ *V. cholerae*. $TTSS^+$ *V. cholerae* (at least AM-19226) causes disease and death even more rapidly than toxigenic *V. cholerae*. $TTSS^+$ *V. cholerae* colonizes both the proximal and distal small bowel, the dominant site of colonization of TCP^+ *V. cholerae*; furthermore, the $TTSS^+$ strain reaches 100 to 1,000× the density (CFU/g) in the intestine compared to the density of TCP^+ strains. Finally, TCP^+ CT^+ *V. cholerae* is the paradigmatic nondestructive, noninvasive pathogen; disease caused by toxigenic *V. cholerae* is almost entirely attributable to the actions of CT and is not thought to have a significant inflammatory component. In contrast, $TTSS^+$ *V. cholerae* causes marked destruction of the epithelium and evokes secretion of proinflammatory cytokines. In aggregate, our observations suggest that $TTSS^+$ *V. cholerae* can overwhelm the innate capacity of the infant rabbit small intestine to resist colonization by bacteria. The AM-19226 TTSS enables the bacterium to create an extraordinarily permissive environment for its growth throughout the small bowel.

The marked damage to the epithelium of the small intestine, which accompanies *V. cholerae* AM-19226 colonization, is most likely caused by TTSS-dependent delivery of effectors into intestinal epithelial cells. Tissue culture-based studies have shown that VopF alters the organization of the eukaryotic actin cytoskeleton (9) and that VopF and VopE compromise the integrity of tight junctions by inducing cortical actin depolymerization and aberrant localization of ZO-1, a protein that promotes epithelial cell barrier function (20). The profound disruption of the villous structure seen in AM-19226-infected rabbits could in part be explained by the actions of these two effectors. In addition, the activities of McfV and TcdB, two effectors that we identified in this study, contribute to the AM-19226 virulence, since deletion of *mcfV* or *tcdB* reduced the severity and incidence of diarrhea. Both McfV and TcdB are homologous to toxins that have insecticidal properties (29), but their targets and mechanisms of action have not been established. Recently, a *Yersinia pestis* TcdB homolog was also shown to be translocated via TTSS (30). Also, it was reported that a domain within the *V. cholerae* actin-targeted MARTX toxin (named MARTX for multifunctional autoprocessing repeats-in-

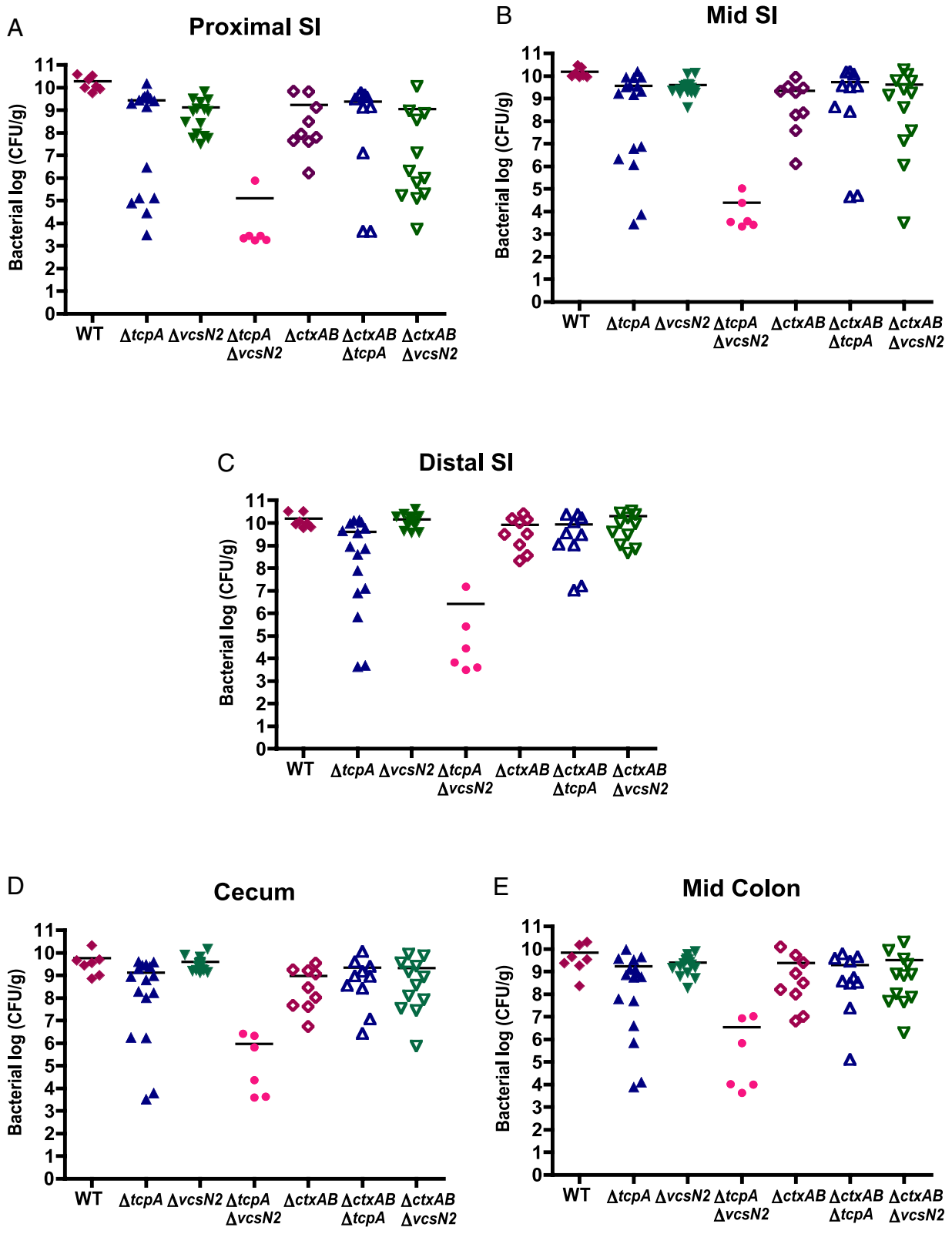

FIG 6 Importance of TCP and TTSS in *V. cholerae* V51 for intestinal colonization. Infant rabbits were inoculated with the indicated derivatives of the TCP$^+$ CT$^+$ TTSS$^+$ strain V51. The numbers of bacterial CFU were determined in sections taken from the proximal (A), mid (B), and distal (C) small intestines (SI), ceca (D), and midcolons (E) of infant rabbits. Bars represent the geometric mean values.

toxin toxins) is similar to a domain in McfV (31). Thus, the targets and actions of McfV and TcdB warrant future investigation. Another important future challenge will be deciphering how the activities of the AM-19226 TTSS effectors produce an environment that is so conducive to the pathogen's survival and proliferation.

The tissue damage caused by *V. cholerae* AM-19226 likely explains the marked elevation of transcripts encoding proinflammatory cytokines in intestinal homogenates from infected rabbits. Even though transcripts for IL-8, a chemokine that promotes neutrophil migration, were almost 1,000× greater in AM-19226-infected rabbits than in the *vcsN2* mutant, we did not observe many heterophils (rabbit neutrophils) in tissue sections from infected rabbits; the relative paucity of heterophils may be due to the rapidity of the course of infection in AM-19226-infected animals. In addition to the AM-19226 TTSS-induced damage to intestinal villous structures, the actions of the cytokines on the intestinal tissue likely promote the diarrheal response to this pathogen.

Our experiments with the serogroup O141 strain V51 revealed that this strain's TTSS, which is highly similar to the *V. cholerae* AM-19226 TTSS, contributes to intestinal colonization and disease even in the presence of the canonical *V. cholerae* virulence factors—CT and TCP. Both TCP and the TTSS contribute to V51's capacity to colonize the intestine. Deletion of genes required for production of both TCP and a functional TTSS was required to greatly reduce V51 intestinal colonization. Furthermore, the V51 TTSS appears to have a dominant role versus CT in producing diarrhea in rabbits, as the *vscN2* mutant exhibited less severe diarrhea than the *ctx* mutant. It should be possible to dissect how TCP and CT modulate the intestinal histopathologic response elicited by the V51 TTSS in future studies. Finally, the importance of all three virulence-associated elements in strain V51—TTSS, TCP, and CT—to mediate the full pathogenic potential of this strain provides a striking illustration of how distinct mobile elements can cooperate to cause disease.

MATERIALS AND METHODS

Ethics statement. The animal experiments were performed with protocols approved by the Harvard Medical School Office for Research Protection Standing Committee on Animals. The Harvard Medical School animal management program is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and meets National Institutes of Health standards as set forth in the *Guide for the Care and Use of Laboratory Animals* (32). The institution also accepts as mandatory the PHS Policy on Humane Care and Use of Laboratory Animals by Awardee Institutions (33) and NIH Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training (34). An approved Assurance of Compliance (A3431-01) is on file with the Office of Laboratory Animal Welfare (OLAW).

Bacterial strains. *V. cholerae* AM-19226, a serogroup O39 clinical isolate (10), and V51, a serogroup O141 clinical isolate (10), were used in this study. Strains were grown at 37°C in LB containing streptomycin (100 μg/ml). All the strains used in this study are listed in Table S1 in the supplemental material. In-frame deletion mutants of virulence genes were made as described previously (35). *V. cholerae* AM-19226 *lacZ*::*gfp* and Δ*vcsN2 lacZ*::*gfp* strains, which constitutively express green fluorescent protein (GFP) under the *lac* promoter, were constructed using a vector, pJZ111, a kind gift of Jun Zhu.

Infant rabbit model. Infant rabbits were infected with various strains as described previously (22). Briefly, 2- or 3-day-old infant rabbits were injected intraperitoneally with cimetidine (50 mg/kg of body weight) 2 to 3 h prior to orogastric inoculation with ~1×10^9 CFU of *V. cholerae* (suspended in sodium bicarbonate solution). The rabbits were monitored for signs of disease. Diarrhea was scored as follows: no diarrhea, no watery or fecal material evident around the perianal area, tail, or hind limbs and dry skin; mild diarrhea, limited area of wetness around perineum and tail; severe diarrhea, extensive area of wetness covering most of lateral surfaces around the perianal area, tail, or hind limbs. Rabbits were routinely euthanized at 12 to 15 h postinoculation, and intestinal samples were collected for histological and microscopic analyses, RNA isolation, and bacterial recovery from intestinal tissues.

V. *cholerae* intestinal colonization. The numbers of *V. cholerae* CFU in tissue samples were determined by plating. The samples were homogenized in sterile phosphate-buffered saline (PBS), serially diluted, and plated on LB-streptomycin (100 μg/ml). The detection limit was ~100 CFU/g. In samples where no bacterial colonies were detected at the lowest dilution, the mean values presented in figures were calculated using the lower limit of detection as a value.

Histological analysis. Intestinal tissues were fixed in 10% neutral-buffered formalin and stained with hematoxylin and eosin (H&E). Samples were scored for the levels of edema, vascular congestion, and overall mucosal damage by a comparative pathologist blinded to the sample identity.

Confocal microscopy. Infant rabbits were inoculated with the GFP-expressing wild-type AM-19226 strain or Δ*vcsN2* deletion mutant. Sections of small intestines were removed and prepared for confocal microscopy as described previously (22). The tissue sections were counterstained with Alexa Fluor 568-labeled phalloidin (1/50; Invitrogen, OR) to visualize F-actin and wheat germ agglutinin (WGA) to visualize mucin. Slides were examined using the confocal microscope in the Nikon Imaging Center at Harvard Medical School.

Transmission electron microscopy. Small intestinal samples for transmission electron microscopy were fixed in 2.5% glutaldehyde (pH 7.4) buffered in 0.1 M sodium cacodylate and visualized with a Tecnai G^2Spirit BioTWIN microscope.

Quantitative real-time PCR. RNA from the distal small intestines of infant rabbits was isolated with Trizol reagent (Invitrogen). RNeasy mini-columns (Invitrogen) were used to isolate RNA, and DNase I (Ambion) was added to the columns. First-strand synthesis of cDNA from total RNA was performed using ImProm-II (Promega) according to the manufacturer's instructions. Quantification of cDNA was performed by quantitative real-time PCR (qRT-PCR) (Applied Biosystems) using Sybr green PCR mix (Bio-Rad). Cycling parameters were 60°C for 5 min and 95°C for 15 min, followed by 40 cycles, with 1 cycle consisting of 30 s at 95°C and 1 min at 60°C. The primers used in this study are as follows: IL-8F (F for forward), ACTCTTTGTGAAGCTGCAGT; IL-8R (R for reverse), GTGT CTTTATGCACTGGCAT; IL-6F, GAGCATCCTGGAGACCATCAA; IL-6R, TGCCTCCTTTCTGTTCATGCA; TNF-αF, CATGAAGCTCACGG ACAACCA; TNF-αR, TTGACCGCTGAAGAGAACCTG; IL-1βF, CATC TCCTGCCAACCCTACAAC; IL-1βR, CAGAGCCACAACGACTGAC AAG; HPRT-F, TGATAGATCCATTCCTATGACTGTAGA; and HPRT-R, GGGTCCTTTTCACCAGCAG.

Expression of target genes was normalized to that of the housekeeping gene, hypoxanthine phosphoribosyltransferase (HPRT). Calculations of transcript levels were normalized using the $\Delta\Delta C_T$ method (36).

Effector translocation assay. Human epithelial HEp-2 cells (ATCC CCL-23) were cultivated in RPMI 1640 supplemented with 10% fetal calf serum (FCS), L-glutamine (2 mM), and penicillin or streptomycin at 37°C in a 5% CO_2 atmosphere. Translocation assays were performed as described previously (37). The *blaM* gene (encoding β-lactamase TEM-1) was cloned into pDSW204 to generate pVTM30. Genes encoding McfV and TcdB were cloned into pVTM30 to generate pVTM502 and pVTM503, respectively. HEp-2 cells were seeded at 5×10^4 cells per well in Lab-Tek eight-well chamber slides (Becton Dickinson) in 500 μl of RPMI 1640. Bacteria were inoculated in LB with streptomycin or ampicillin. On the following day, bacteria were subcultured 1:100 in LB with streptomycin, ampicillin, and isopropyl-β-D-thiogalactopyranoside

(IPTG) (0.8 mM) and grown at 37°C for 2 h. HEp-2 cells were washed and infected with 10^5 bacteria for 3 h. CCF2/AM was added for 1 h. The slides were covered with coverslips and observed using a Nikon inverted fluorescence microscope.

Statistical analysis. The paired differences of experimental groups were compared using the nonparametric Mann-Whitney U test. A *P* value of <0.05 was considered statistically significant (Prism software; GraphPad, San Diego, CA).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00106-11/-/DCSupplemental.

Table S1, DOC file, 0.061 MB.
Table S2, DOC file, 0.032 MB.
Figure S1, PDF file, 1.007 MB.
Figure S2, PDF file, 0.325 MB.
Figure S3, PDF file, 0.128 MB.
Figure S4, PDF file, 0.716 MB.

REFERENCES

1. **Faruque SM, et al.** 2004. Genetic diversity and virulence potential of environmental *Vibrio cholerae* population in a cholera-endemic area. Proc. Natl. Acad. Sci. U. S. A. **101**:2123–2128.
2. **Bag PK, et al.** 2008. Putative virulence traits and pathogenicity of *Vibrio cholerae* non-O1, non-O139 isolates from surface waters in Kolkata, India. Appl. Environ. Microbiol. **74**:5635–5644.
3. **Bagchi K, et al.** 1993. Epidemic of diarrhea caused by *Vibrio cholerae* non-O1 that produced heat-stable toxin among Khmers in a camp in Thailand. J. Clin. Microbiol. **31**:1315–1317.
4. **Chatterjee S, et al.** 2009. Incidence, virulence factors, and clonality among clinical strains of non-O1, non-O139 *Vibrio cholerae* isolates from hospitalized diarrheal patients in Kolkata, India. J. Clin. Microbiol. **47**:1087–1095.
5. **Dalsgaard A, et al.** 1995. Characterization of *Vibrio cholerae* non-O1 serogroups obtained from an outbreak of diarrhea in Lima, Peru. J. Clin. Microbiol. **33**:2715–2722.
6. **Dalsgaard A, Serichantalergs O, Pitarangsi C, Echeverria P.** 1995. Molecular characterization and antibiotic susceptibility of *Vibrio cholerae* non-O1. Epidemiol. Infect. **114**:51–63.
7. **Sharma C, et al.** 1998. Molecular analysis of non-O1, non-O139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera-like disease in Calcutta, India. J. Clin. Microbiol. **36**:756–763.
8. **Morris JG, Jr, et al.** 1981. Non-O group 1 *Vibrio cholerae* gastroenteritis in the United States: clinical, epidemiologic, and laboratory characteristics of sporadic cases. Ann. Intern. Med. **94**:656–658.
9. **Tam VC, Serruto D, Dziejman M, Brieher W, Mekalanos JJ.** 2007. A type III secretion system in *Vibrio cholerae* translocates a formin/spire hybrid-like actin nucleator to promote intestinal colonization. Cell Host Microbe **1**:95–107.
10. **Dziejman M, et al.** 2005. Genomic characterization of non-O1, non-O139 *Vibrio cholerae* reveals genes for a type III secretion system. Proc. Natl. Acad. Sci. U. S. A. **102**:3465–3470.
11. **Cornelis GR, Van Gijsegem F.** 2000. Assembly and function of type III secretory systems. Annu. Rev. Microbiol. **54**:735–774.
12. **Galán JE, Collmer A.** 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284**:1322–1328.
13. **Hueck CJ.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62**:379–433.
14. **Caron E, et al.** 2006. Subversion of actin dynamics by EPEC and EHEC. Curr. Opin. Microbiol. **9**:40–45.
15. **Parsot C.** 2009. *Shigella* type III secretion effectors: how, where, when, for what purposes? Curr. Opin. Microbiol. **12**:110–116.
16. **Patel JC, Galán JE.** 2005. Manipulation of the host actin cytoskeleton by *Salmonella*—all in the name of entry. Curr. Opin. Microbiol. **8**:10–15.
17. **Galán JE.** 2009. Common themes in the design and function of bacterial effectors. Cell Host Microbe **5**:571–579.
18. **Kim M, et al.** 2010. Bacterial interactions with the host epithelium. Cell Host Microbe **8**:20–35.
19. **Mattoo S, Lee YM, Dixon JE.** 2007. Interactions of bacterial effector proteins with host proteins. Curr. Opin. Immunol. **19**:392–401.
20. **Tam VC, et al.** 2010. Functional analysis of VopF activity required for colonization in *Vibrio cholerae*. mBio **1**(5):e00289-10.
21. **Dalsgaard A, et al.** 2001. Clinical and environmental isolates of *Vibrio cholerae* serogroup O141 carry the CTX phage and the genes encoding the toxin-coregulated pili. J. Clin. Microbiol. **39**:4086–4092.
22. **Ritchie JM, Rui H, Bronson RT, Waldor MK.** 2010. Back to the future: studying cholera pathogenesis using infant rabbits. mBio **1**(1):e00047-10.
23. **Rui H, et al.** 2010. Reactogenicity of live-attenuated *Vibrio cholerae* vaccines is dependent on flagellins. Proc. Natl. Acad. Sci. U. S. A. **107**:4359–4364.
24. **Zheng J, Shin OS, Cameron DE, Mekalanos JJ.** 2010. Quorum sensing and a global regulator TsrA control expression of type VI secretion and virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. U. S. A. **107**:21128–21133.
25. **Rudra S, Mahajan R, Mathur M, Kathuria K, Talwar V.** 1996. Cluster of cases of clinical cholera due to *Vibrio cholerae* 010 in east Delhi. Indian J. Med. Res. **103**:71–73.
26. **Nelson ET, Clements JD, Finkelstein RA.** 1976. *Vibrio cholerae* adherence and colonization in experimental cholera: electron microscopic studies. Infect. Immun. **14**:527–547.
27. **Dowling AJ, et al.** 2004. The insecticidal toxin Makes caterpillars floppy (Mcf) promotes apoptosis in mammalian cells. Cell. Microbiol. **6**:345–353.
28. **Waterfield N, Hares M, Yang G, Dowling A, Ffrench-Constant R.** 2005. Potentiation and cellular phenotypes of the insecticidal toxin complexes of *Photorhabdus* bacteria. Cell. Microbiol. **7**:373–382.
29. **Waterfield N, Kamita SG, Hammock BD, Ffrench-Constant R.** 2005. The *Photorhabdus* Pir toxins are similar to a developmentally regulated insect protein but show no juvenile hormone esterase activity. FEMS Microbiol. Lett. **245**:47–52.
30. **Gendlina I, et al.** 2007. Identification and type III-dependent secretion of the *Yersinia pestis* insecticidal-like proteins. Mol. Microbiol. **64**:1214–1227.
31. **Satchell KJ.** 2007. MARTX, multifunctional autoprocessing repeats-in-toxin toxins. Infect. Immun. **75**:5079–5084.
32. **National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, DC.
33. **National Institutes of Health.** 2002. Public Health Service policy on humane care and use of laboratory animals. Office of Laboratory Animal Welfare, National Institutes of Health, Bethesda, MD.
34. **U.S. Office of Science and Technology Policy.** 1985. Laboratory animal welfare; U.S. government principles for the utilization and care of vertebrate animals used in testing, research and training. Fed. Regist. **50**(97):20864–20865.
35. **Skorupski K, Taylor RK.** 1996. Positive selection vectors for allelic exchange. Gene **169**:47–52.
36. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods **25**:402–408.
37. **Charpentier X, Oswald E.** 2004. Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic *Escherichia coli* effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter. J. Bacteriol. **186**:5486–5495.