Prolonged Colonization of Mice by *Vibrio cholerae* El Tor O1 Depends on Accessory Toxins

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Cholera epidemics caused by *Vibrio cholerae* El Tor O1 strains are typified by a large number of asymptomatic carriers who excrete vibrios but do not develop diarrhea. This carriage state was important for the spread of the seventh cholera pandemic as the bacterium was mobilized geographically, allowing the global dispersion of this less virulent strain. Virulence factors associated with the development of the carriage state have not been previously identified. We have developed an animal model of cholera in adult C57BL/6 mice wherein *V. cholerae* colonizes the mucus layer and forms microcolonies in the crypts of the distal small bowel. Colonization occurred 1 to 3 h after oral inoculation and peaked at 10 to 12 h, when bacterial loads exceeded the inoculum by 10- to 200-fold, indicating bacterial growth within the small intestine. After a clearance phase, the number of bacteria within the small intestine, but not those in the feces or colon, stabilized and persisted for at least 72 h. The ability of *V. cholerae* to prevent clearance and establish this prolonged colonization was associated with the accessory toxins hemolysin, the multifunctional autoprocessing RTX toxin, and hemagglutinin/protease and did not require cholera toxin or toxin-coregulated pili. The defect in colonization attributed to the loss of the accessory toxins may be extracellularly complemented by inoculation of the defective strain with an isogenic colonization-proficient *V. cholerae* strain. This work thus demonstrates that secreted accessory toxins modify the host environment to enable prolonged colonization of the small intestine in the absence of overt disease symptoms and thereby contribute to disease dissemination via asymptomatic carriers.

*Vibrio cholerae* O1 is the causative agent of pandemic cholera, a severe diarrheal disease still prevalent worldwide. Cholera pandemics that occurred from the 18th through the early 20th centuries were caused by nonhemolytic O1 strains of the classical biotype. In 1963, the hemolytic El Tor O1 biotype emerged as the most prevalent agent of cholera, leading to the seventh cholera pandemic that continues today (4, 38). The epidemiology of El Tor cholera is distinct from that of the classical disease, as El Tor cholera epidemics are characterized by lower ratios of symptomatic infections to total infections (3, 4, 21, 29, 31, 33, 38, 39, 51). This reduced virulence of El Tor strains is demonstrated by a ratio of all infected persons to infected persons requiring hospitalization of 4.7:1 to 17:1, compared to the 1.3:1 to 2.6:1 ratio for classical infections (21). The El Tor vibrio carriers then excrete bacteria for a longer period of up to 10 days, with an average of 3 to 5 days, than classical strain carriers, who excrete bacteria for an average of 1.5 days (21, 31, 33, 51). It has been postulated that this prolonged period of vibrio excretion contributed to the pandemic dissemination of El Tor vibrios by facilitating person-to-person transmission (51), although the factors carried by El Tor strains that allow the establishment of this persistent asymptomatic infection have not been previously identified.

Both classical and El Tor strains colonize the human small intestine, where they secrete the major virulence factor cholera toxin (CT), an ADP-ribosylating toxin that causes increased cyclic AMP synthesis by intestinal epithelial cells, leading to the severe diarrhea that is the hallmark of cholera disease (19). Although CT is the most important virulence factor for pandemic cholera, naturally occurring CT-negative strains have been implicated in localized outbreaks of enterocolitis, as well as septicemia and extraintestinal infections (1, 5, 30). In addition, CT-deficient vaccine candidates based on El Tor strains still elicit mild to severe diarrhea and evidence of inflammation in human volunteers (9, 42–45, 47). By comparison, CT-deficient classical strains were safe when tested in human volunteers, and a live, attenuated vaccine based on the classical strain 569B has been developed (18, 20, 25). Thus, El Tor strains produce additional virulence factors absent from classical strains that cause mild gastroenteritis and vaccine reactivity.

In order to colonize and cause disease, *V. cholerae* first has to attach to the mucosal surface of the small intestine. The toxin-coregulated type IV pili (TCP) are essential for disease in humans since Δtcp mutants of either classical or El Tor strains do not cause diarrhea and the bacteria are not shed by human volunteers (48, 49). Yet the role of these pili during infection is unclear since TCP are not required for adherence to epithelial cells (13, 46). Rather, it has been proposed that individual bacteria adhere to the intestinal epithelial cells, possibly through chitin binding proteins, and that TCP then mediate the bacterium-to-bacterium adhesion that leads to the formation of microcolonies (22).
Although compelling, this model developed from studies of classical strains may not thoroughly explain colonization by El Tor strains. Although TCP are required for the efficient growth of El Tor strains within the small intestines of infant mice, TCP are not absolutely essential. Indeed, El Tor ΔtcpA mutants persist in numbers only 1 to 2 log units below the initial inoculum at 24 h postinfection (hpi), a dramatic difference from the complete clearance of classical ΔtcpA mutants by 10 hpi (2). Hence, there may be additional stages or factors important for the colonization of the intestine by El Tor strains.

*V. cholerae* El Tor strains secrete at least three proteins with cytopathic effects in vitro that are absent from or poorly expressed in classical strains (6, 26, 35). These accessory toxins include the *V. cholerae* multifunctional autoprocessing RTX (MARTX<sub>vp</sub>) toxin that causes both actin cross-linking and Rho inactivation, the nonspecific zinc metalloprotease hemagglutinin (HA)/protease that degrades occludin in tight junctions, and the pore-forming hemolysin that has both hemolytic and cytolytic activities (11, 41). The contributions of these toxins to pathogenesis in vivo have not been well established since the deletion of the accessory toxin genes in a previous study either had no effect or caused only modest changes in virulence in suckling mice (11). As further barriers to the use of infant mice for the study of accessory toxins, the intestines are not fully developed, either anatomically or immunologically, and experiments are often limited to less than 24 h since animals are removed from their mothers’ care (10, 17, 23). Thus, infant mice are not ideal for analyses of alterations in host immune responses, tissue damage, or long-term colonization—all processes that may be affected by the accessory toxins (14, 16).

Previous studies using the lung as a model mucosal tissue showed that the accessory toxins may cause serious tissue damage and inflammation (12). Indeed, a strain in which the genes for CT and all three accessory toxins were deleted was avirulent after intranasal infection, and the colonizing bacteria were cleared from the lung (12). Yet this multitoxin-deficient strain stimulated the innate immune response in a classical fashion through multiple pathways both dependent upon and independent of Toll-like receptor 4 (16). These results suggested that accessory toxins function coordinately to enable the bacteria to evade the host immune response and prevent clearance of the colonizing bacteria during the early stages of infection. Although compelling, these results were obtained from experiments with the lung, an organ that is not the natural site of infection.

A study of the role of accessory toxins in the process of colonization by El Tor strains in an intestinal infection model would clearly be more appropriate. In 1975, it was reported that the gastrointestinal tracts of adult mice can be colonized with classical *V. cholerae* strains but that the bacteria are eliminated in less than 2 h by an unknown mechanism (24). However, more recent studies have shown that El Tor *V. cholerae* is shed in the stools of adult Swiss mice raised under germfree conditions for 7 to 11 days (37) or treated with streptomycin to remove the normal flora for up to 48 h after oral inoculation (28). Based on these findings, we surmised that intestinal colonization of adult mice with *V. cholerae* El Tor does occur, and in the present study, we developed a new model of *V. cholerae* intestinal infection in adult C57BL/6 mice to investigate the role of the accessory toxins during *V. cholerae* infection.

Using this model, we demonstrated that *V. cholerae* El Tor colonizes the mucus layer of the mouse small intestine and forms microcolonies within crypts. The accessory toxins were found to be important for the ability of *V. cholerae* El Tor strains to establish a stable infection independent of TCP and CT. Thus, the presence of these toxins in El Tor strains may increase the fitness of these strains in an era of improved sanitation and disease surveillance by allowing the bacteria to establish sustained infections in asymptomatic carriers, possibly accounting for the emergence of the El Tor O1 strains as the primary agent of cholera in the modern era.

**MATERIALS AND METHODS**

**Bacterial strains.** All strains were derived from the streptomycin-resistant isolate of *V. cholerae* El Tor O1 P27459, originally isolated in Bangladesh in 1979 (27). Strain P4 has an engineered mutation replacing the genes for ctxA and ctxB in P27459 with a kanamycin resistance cassette (15). KVF101 is a derivative of P4 genetically engineered to carry unmarked internal deletions in toxin genes tcpA, hlyA, and hapa (12). Strains VOV27 (P4 ΔtcpA) and SAV1 (P27459 ΔtcpA) were generated using plasmids pDLT (pCVD442 Δ tcpA) and pHT3 (pCVD442 ΔtcpA) (49) for sacB-counterselective mutagenesis in *V. cholerae* as previously described (13).

**Bacterial cultures.** Overnight cultures of *V. cholerae* strains were diluted 1:1,000 in fresh Luria-Bertani (LB) medium supplemented with 100 µg of streptomycin/ml with or without 50 µg of kanamycin/ml as necessary and grown at 30°C with shaking to the mid-log phase (ΔOD<sub>600</sub> = 0.5). Bacteria were pelleted and washed twice with phosphate-buffered saline (PBS), and then the suspensions were adjusted to the desired number of CFU per milliliter as previously described (12). The actual dose (CFU per milliliter) delivered was determined by plating dilutions of the inocula onto LB agar.

**Mouse inoculation.** All experiments were done according to protocols approved by the Northwestern University institutional animal care and use committee. Unless otherwise noted, 4- to 5-week-old female specific-pathogen-free C57BL/6 mice (Harlan, Indianapolis, IN) were treated with streptomycin (1 mg/ml in drinking water) for 4 to 7 days. The evening before an experiment, food but not water was removed from the cage. Mice were anesthetized intraperitoneally with 60 to 70 mg of ketamine/kg of body weight and 12.5 mg of xylazine/kg, and were fed 50 µl of 8.5% (wt/vol) sodium bicarbonate intragastrically, immediately followed by 50 µl of the bacterial suspension in PBS, by using a 22-gauge animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY). After inoculation, mice had free access to food and sterile water without streptomycin.

**Bacterial colonization.** Mice were inoculated with a sublethal dose of *V. cholerae* strain and at specified time points, mice were sacrificed and small intestines were collected. For studies of the colonization dynamics, mice were sacrificed after 1, 3, 6, 12, 24, 48, and 72 h and small intestines, ceca, and colons were collected. The small intestines were dissected by length into proximal, middle, and distal segments. All organs were homogenized in 5 ml of PBS, and serial dilutions were plated onto LB agar for CFU counting.

**Coinfection experiment.** Mice were inoculated with a mixture of the CT-deficient mutant VOV27 (P4 ΔtcpA) and the multitoxin-deficient mutant strain KVF101. After 48 h, the small intestines were collected, homogenized, and plated onto LB agar with streptomycin, kanamycin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for CFU counting.

**FISH.** Segments (1 cm long) from the ileocecal junction were fixed in Carnoy’s fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid), embedded, deparaffinized by incubations in xylene followed by 100% ethanol, heated at 60°C, and were fed 50 µl of 8.5% (wt/vol) sodium bicarbonate intragastrically, immediately followed by 50 µl of the bacterial suspension in PBS, by using a 22-gauge animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY). After inoculation, mice had free access to food and sterile water without streptomycin.

**Materials and Methods.** Bacterial strains. All strains were derived from the streptomycin-resistant isolate of *V. cholerae* El Tor O1 P27459, originally isolated in Bangladesh in 1979 (27). Strain P4 has an engineered mutation replacing the genes for ctxA and ctxB in P27459 with a kanamycin resistance cassette (15). KVF101 is a derivative of P4 genetically engineered to carry unmarked internal deletions in toxin genes tcpA, hlyA, and hapa (12). Strains VOV27 (P4 ΔtcpA) and SAV1 (P27459 ΔtcpA) were generated using plasmids pDLT (pCVD442 Δ tcpA) and pHT3 (pCVD442 ΔtcpA) (49) for sacB-counterselective mutagenesis in *V. cholerae* as previously described (13).
RESULTS

Dynamics of colonization of the C57BL/6 gastrointestinal tract after oral infection with El Tor P27459. For this study, appropriate conditions for the colonization of 4- to 6-week-old streptomycin-fed C57BL/6 mice inoculated with the wild-type El Tor O1 strain P27459 were established in pairwise experiments to generate an infection protocol that resulted in the recovery of CFU from the small intestines of 100% of 5- to 6-week-old C57BL/6 mice 20 to 24 hpi (see Materials and Methods for the inoculation protocol). We did not observe the elimination of bacteria from the small intestine 2 hpi, indicating that the El Tor strain used was able to colonize the small intestine, in contrast to the classical strain 569B used in previous studies (24). Further experimentation revealed that colonizing El Tor P27459 bacteria persisted in the small intestine to 24 and 48 hpi, but, consistent with the results of previous studies (24), classical strain O395 could not be recovered. After the determination of colonization parameters, the 50% lethal dose was determined to be 10^7 CFU (32).

To establish a profile of the interaction of V. cholerae with the adult mouse intestine over time, mice were inoculated with a sublethal dosage of 1.5 × 10^6 CFU of wild-type V. cholerae P27459. After 1, 3, 6, 12, 24, 48, and 72 h of infection, mice were sacrificed and the small intestines, ceca, and colons were collected. The small intestines were divided into three equal sections, the proximal, middle, and distal segments. Tissues were homogenized and plated for CFU counting. These studies were performed with six to seven mice per group examined at each time point, and two separate experiments were performed.

As shown in Fig. 1A, after intragastric inoculation, wild-type V. cholerae P27459 rapidly reached the proximal small intestine and was detected as early as 1 hpi. At about 3 hpi, the bacteria reached the middle section of the small intestine and then, after 3 h, the distal region. The numbers of CFU within the middle and distal sections increased rapidly, exceeding the inoculation dose at 3 to 6 hpi, and reached a maximum level of colonization at 12 hpi. At this time point, we also observed a preference for the distal portion of the small intestine, where the maximum bacterial load increased to up to 3 log units above the inoculation dose, demonstrating that the V. cholerae population expanded within the small intestine. After 12 h of infection, the colonizing bacteria went through a rather dramatic clearance, and the population stabilized at a colonization index (calculated as the number of CFU recovered from the small intestine divided by the number of CFU inoculated) of about 10^-3 by 24 hpi. This value was maintained for at least 72 h.

V. cholerae bacteria were also recovered from the lower intestinal tract. The bacteria reached the cecum and colon 3 h after inoculation and achieved the peak level of colonization between 6 and 12 hpi. During this time, the bacterial load exceeded the inoculum. After 12 h, the level of colonization decreased almost linearly such that by 72 hpi, the median colonization index for the colon was 45-fold lower than the index for the distal small intestine (P = 0.05). These results show that V. cholerae bacteria preferably colonized the small intestines of the mice, especially the distal portion approximating the ileum, where they established colonization that persisted for at least 72 h.

V. cholerae forms microcolonies in the crypts of the mouse small intestine. To determine whether V. cholerae attaches to the intestinal mucosal surfaces, FISH was performed with segments of small intestines of mice inoculated with wild-type P27459. Mice for this experiment were inoculated with 10^8 CFU to ensure bacterial numbers high enough to be detectable by FISH, and mice were sacrificed only 6 h after inoculation for the examination of early events in colonization as well as the avoidance of animal death after the delivery of a lethal dose. Segments (1 cm long) of the distal small intestines were fixed in Carnoy’s fixative, and 3-μm-thick paraffin sections were hybridized simultaneously with a fluorescein isothiocyanate-labeled probe against Vibrio 16S RNA (Fig. 2A, C, and E) and a universal eubacterial probe (Fig. 2B, D, and F). Small microcolonies detected by the Vibrio-specific probe were observed in the crypts of the distal small intestinal villus epithelium (Fig. 2A and B). Many bacteria were found in the intestinal lumen or confined to the mucus layer (Fig. 2C and D). Some microcolonies were also found in the proximity of the follicle-associated epithelium on top of structures appearing to be Peyer’s patches (Fig. 2E and F).

TCP are not required for colonization of adult mouse intestines. To investigate whether TCP are necessary for the infection of mice in this model system, mice were inoculated with 5 × 10^6 to 10 × 10^6 CFU of wild-type V. cholerae P27459 or 4 × 10^6 to 6 × 10^6 CFU of the isogenic ΔtcpA mutant. The small intestines were collected after 12, 20, and 48 h, and the level of colonization was determined by plating the intestinal homogenates for CFU counting. At 12 and 20 hpi, the numbers of CFU recovered from the small intestines were 2 log units higher than the respective inocula, and at 48 hpi, the numbers were close to the inocula (Fig. 3). However, no difference in colonization levels between the wild type and the ΔtcpA mutant was observed at any time during the infection, indicating that the ΔtcpA mutant did not have a colonization defect in this model. To determine whether the deletion of tcpA had an influence on the site of infection, especially on the ability to form microcolonies, FISH was performed as described above. Microcolonies of bacteria identified as V. cholerae were found deep in the intestinal crypts both of mice inoculated with the wild type and of those inoculated with the ΔtcpA mutant (Fig. 4). Overall, there was no obvious difference in the ability to form microcolonies between the wild type and the ΔtcpA mutant. Both strains were found within the crypts of the distal small intestines.

The absence of CT has only a minimal influence on colonization. To investigate if CT plays a role in colonization, a colonization profile similar to that shown for P27459 in Fig. 1A was developed for strain P4, an isogenic derivative of P27459 with a kanamycin resistance cassette replacing the ctxAB genes.
Carnoy’s fixative. Bacteria were labeled by FISH and identified as the intestines were collected and 1-cm-thick sections were fixed by using a probe specific for Vibrio cholerae. Preference was not significant (was below the value for P27459-inoculated mice, but the differences were not statistically significant (Table 1). Therefore, CT may be required for the maintenance of colonization beyond 48 h. This conclusion is consistent with a role for CT in modulating the immune response at the bridge between innate and adaptive immunity, as it has been shown to do when functioning as an adjuvant (40, 50).

The levels of colonization of the large intestines (ceca and colons) of mice inoculated with P4 were lower than those of P27459-inoculated mice. The differences at 12, 24, and 48 hpi were significant. These data demonstrate that CT may be required to sustain a long-term infection but that it does not affect the establishment of the infection within the small intestine.

Accessory toxins play an important role in colonization. To investigate the role of the accessory toxins in colonization, mice were inoculated with a multitoxin-deficient strain. Strain KFV101 is an isogenic derivative of P4 that has additional unmarked deletions that remove the rtxA, hlyA, and hpa genes, which encode MARTXVc toxin, hemolysin, and HA/protecte, respectively. This strain was previously shown to colonize the lung infection model, but the bacteria were rapidly cleared (12). To determine if KFV101 would exhibit a similar difference in the rate of clearance from the intestine, a colonization profile was developed as described above for P27459 and the CT-deficient strain P4. The profile revealed a dramatic variation in colonization (Fig. 1C). Although bacteria colonized the small intestine within 3 to 6 h at levels similar to those of wild-type and P4 colonization, the maximum level of colonization was reduced such that the bacterial load attained in the small bowel exceeded the inoculation dose of 1×10^6 to 2×10^6 by 6 hpi in only 23% (3 of 13) mice, compared to 93% (13 of 14) of wild-type and 79% (11 of 14) of P4-inoculated mice. The level of KFV101 colonization at 12 h was significantly below both those in both P27459- and P4-inoculated mice (P < 0.05). These data show that, in the absence of toxins,
there was a small defect in the initial colonization of the small intestine but that there was a more dramatic defect in the establishment of colonization that persisted beyond 24 h. As shown in Table 1, KFV101 underwent much more rapid clearance than the other strains, with the majority of mice clearing the distal small intestine by 24 h, and no mouse showed detectable CFU by 48 h. The levels of colonization of the large intestines (ceca and colons) of mice inoculated with KFV101 were significantly lower at all time points than those of P27459-inoculated mice.

A multitoxin-deficient mutant strain can overcome the colonization defect when inoculated with P4. The colonization profiles of both wild-type and CT-deficient V. cholerae showed prolonged colonization lasting up to at least 72 hpi. However, mice inoculated with the multitoxin-deficient strain had completely cleared the infection by 48 hpi, indicating that the accessory toxins play an important role in establishing and maintaining a stable level of colonization of the small intestine in the early phase of V. cholerae infection. To determine if the accessory toxins act as colonization factors, possibly at the surfaces of the bacteria, or if they affect colonization by altering host cells at the site of infection, mice were coinoculated with the ΔctxAB strain P4 and the multitoxin-deficient strain KFV101. To distinguish between colonies of the multitoxin-deficient mutant and those of P4, the lacZ gene in P4 was partially deleted, resulting in blue KFV101 colonies and white P4 colonies when the strains were plated onto LB agar containing X-Gal. All mice inoculated with P4 alone were still colonized after 48 h (Fig. 5; Table 2), in contrast to only 2 (18%) of 11 KFV101-inoculated mice, a statistically significant difference (P < 0.0001). However, when mice were infected with a 1:1 mixture of P4 and KFV101, the level of colonization with KFV101 in the coinfected mice was significantly higher than the level in mice infected with KFV101 alone (P < 0.05), while the level of P4 colonization remained constant (Table 1). The level of colonization by KFV101 was also increased by inoculation with P4 at P4/KFV101 ratios of 1:3, 1:8, and even 1:255, indicating that only a few CFU of P4 are necessary for the extracellular complementation of the KFV101 colonization defect (Table 2). These results indicate that the accessory toxins are not factors that affect the bacteria that secrete the toxins but rather factors that modulate the host environment to enable prolonged colonization by the bacteria.

### DISCUSSION

Extensive research on the effects of the V. cholerae accessory toxins hemolysin, MARTXVc, and HA/protease has been conducted in vitro, and it is clear that these secreted factors have...
cytopathic and cytotoxic effects on host cells. However, little is known about their role in vivo as no overt defects in mutants in relevant animal models have been observed. A role for accessory toxins in pathogenesis in a pulmonary infection model was indicated previously (12, 16); however, as the lung is not the natural site of infection, the relevance of these studies was questioned. In this study, we established a new model for the intestinal infection of 4- to 6-week-old C57BL/6 mice with V. cholerae. This model was then used to demonstrate a role for accessory toxins in the establishment of prolonged colonization of the small intestine by altering the host environment to prevent the clearance of V. cholerae early in infection.

An animal model is most useful when it mimics human infection or when at least certain characteristics of human infection are observed. Infections in both our adult mice and the infant mice used in previous studies have been found to mimic some, but not all, aspects of human infection, with different benefits associated with each model. In both models, obvious diarrhea is not detected, consistent with reports that the accessory toxins are responsible for localized cell defenses. However, unlike the wild-type strain, the multitoxin-deficient mutant strain did not ultimately achieve a stable population within the small intestine decreased rapidly between 12 and 24 after infection, presumably due to the movement of bacteria out of the lumen by peristalsis and by the initiation of innate immune defenses. However, unlike the wild-type strain, the multitoxin-deficient mutant strain did not ultimately achieve a stable population in the distal small intestine but was cleared entirely by 48 hpi.

A second explanation for the persisting colonization may be that the bacteria not only colonize, but also divide and grow within the host. Indeed, at 10 to 12 h after infection, the small intestines of adult mice are colonized by numbers of bacteria 4 log units below the inocula. However, the colonization of adult mice does not require TCP, an important distinction from disease in humans (49). It is also notable that TCP are not absolutely essential for the intestinal colonization of infant mice by El Tor strains, as ΔtcpA strains established colonization at 1 to 2 log units below the inocula at 20 hpi (2), similar to the level of colonization in adult mice (Fig. 1A). Thus, we suggest that there is a defect in TCP production in adult mouse intestines. Since TCP functions in infant mice to enhance the growth rates of El Tor V. cholerae, we may predict that the increased expression of TCP in adult mice would have an enhancing role in growth in the intestine and that even higher numbers of CFU would be recovered from the small intestines (2). However, if this is the case, then we believe that the advantage of the adult mouse model for studies of accessory toxins is that the absence of TCP reveals the role of accessory toxins in establishing a low level infection that may otherwise be masked by the influence of TCP.

Having established parameters for the infection of adult mice, we sought to determine if secreted toxins were critical for pathogenesis. Since studies with the pulmonary model predicted a role for accessory toxins in protection from innate immune clearance (16), we determined the dynamics of infection during the first 3 days of infection. All strains colonized rapidly within the first 6 h, and the populations within the small intestine expanded beyond the inoculation dose. Regardless of the infecting strain, the number of colonies recovered from the small intestine decreased rapidly between 12 and 24 after infection, presumably due to the movement of bacteria out of the lumen by peristalsis and by the initiation of innate immune defenses. However, unlike the wild-type strain, the multitoxin-deficient mutant strain did not ultimately achieve a stable population in the distal small intestine but was cleared entirely by 48 hpi.

The mechanism behind the persisting colonization facilitated by the presence of the accessory toxins needs to be elucidated. One potential mechanism is that the accessory toxins either are adhesion molecules or act as such. This proposed mechanism is not supported by the results of our experiment demonstrating extracellular complementation of the loss of the accessory toxins. If these toxins were acting as adhesion molecules, only P4 would have been recovered from the intestines.

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death in the epithelium of the small intestine, which then enhances bacterial adhesion due to greater accessibility of receptor molecules. Histopathology of the sectioned ilea, jejunum, and duodena revealed no severe tissue damage (32), although sloughed cells of unidentified origin did accumulate in the intestinal lumina of mice inoculated with strains that expressed accessory toxins. The presence of these cells suggests that the accessory toxins may cause localized cell death throughout the small intestine, opening new attachment sites or improving the accessibility of adherin receptors. Such a model is consistent with the findings of studies of Listeria monocytogenes, which preferentially binds polarized epithelial layers at sites of extrusion of cells that have undergone apoptosis (34). In such a model, the accessory toxins may increase localized cell death and thus increase turnover rates in the intestinal epithelial cells, revealing sites of colonization at cell junctions.

A third possibility is that the accessory toxins specifically target immune cells, including neutrophils, at the site of infection. In the presence of accessory toxins, innate immune cell counts would be kept low and the bacterial infection would persist at a constant level. In the absence of the accessory toxins, innate immune clearance would continue unimpaired and the infection would clear entirely.

A final key question regarding the accessory toxins is whether the presence of these toxins in El Tor strains contributed to their emergence as the predominant agent of cholera. El Tor strains have long been recognized as having reduced virulence compared to classical strains. Infections with these strains are also characterized by a large number of asymptomatic carriers. In the era of poor public health during which the first six cholera pandemics occurred, more-virulent strains of V. cholerae were likely selected as the number of susceptible hosts consuming contaminated water was large and the bacteria were able to spread quickly from host to host. Thus, classical strains likely evolved to favor rapid spread by up-regulating the expression of CT and TCP while genes for hemolysin, HA/protease, and MARTX.vo toxin either accumulated mutations or were down-regulated (6, 26, 35).

In modern times, cholera is monitored in most countries and epidemics can be localized and often contained. Under these circumstances, less virulent strains that have increased mobility within asymptomatic carriers would likely emerge. Thus, V. cholerae strains able to establish prolonged asymptomatic infection would be selected as the predominant agent. Our studies suggest that the ability to produce secreted accessory toxins may enhance the fitness of El Tor strains over that of classical strains by allowing the bacteria to establish more-persistent infections in the intestine, even when the major virulence factors that would induce severe symptomatic disease are not expressed.

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