**Vibrio cholerae** produces a second enterotoxin, which affects intestinal tight junctions

(cholera/bacterial pathogenesis/toxins/diarrhea/zonula occludens)

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**ABSTRACT** Attenuated *Vibrio cholerae* vaccine strains specifically mutated in genes encoding cholera toxin (CT) are still capable of causing mild to moderate diarrhea. Culture supernatants of *V. cholerae* strains, both CT-positive and CT-negative, were examined in Ussing chambers, and a toxin was found that increases the permeability of the small intestinal mucosa by affecting the structure of the intercellular tight junction, or zonula occludens. The activity of this toxin is reversible, heat-labile, sensitive to protease digestion, and found in culture supernatant fractions containing molecules between 10 and 30 kDa in size. Production of this factor (named ZOT for *zonula occludens* toxin) correlates with diarrheagenicity of *V. cholerae* strains in volunteers and may represent another virulence factor of infectious diarrhea that must be eliminated to achieve a safe and effective live oral vaccine against *cholerae*.

*Vibrio cholerae* produces the copious diarrhea characteristic of cholera by means of a potent enterotoxin, cholera toxin (CT). The A subunit of CT, encoded by *ctxA*, stimulates adenylyl cyclase in intestinal epithelial cells, which results in net secretion of fluid into the intestinal lumen (1). Initial recombinant *V. cholerae* vaccine strains, attenuated by removal of the *ctxA* gene, were greatly reduced in their ability to induce diarrhea in volunteers (2). However, despite the absence of CT, these strains were still capable of inducing an unacceptable amount of diarrhea. One such strain, *V. cholerae* CVD101, is a *ctxA* deletion mutant of *V. cholerae* strain 395 in which 94% of the sequence encoding the A1 peptide of CT has been removed (3). When evaluated in volunteer studies, CVD101 caused mild to moderate diarrhea (mean stool volume of 0.9 liter with a range of 0.3–2.1 liters) in 54% of subjects ingesting this organism (2). While greatly attenuated compared with the parent strain 395 [which induces a mean diarreal stool volume of 5.5 liters with a range of 0.3–44 liters in >90% of volunteers (4)], the amount of diarrhea induced by CVD101 is still unacceptable for use of this strain as a vaccine.

Given the magnitude of the diarrhea induced in the absence of CT, we hypothesized that *V. cholerae* produced a second toxin, which was still present in strains deleted of the *ctxA* sequence. To investigate this hypothesis, we examined *V. cholerae* strains with and without intact *ctx* genes by using rabbit intestinal tissue mounted in Ussing chambers, a classic technique for studying the process of transport across intestinal tissue (5, 6). The results indicate that *V. cholerae* produces a toxin that increases intestinal tissue conductance by altering the structure of intercellular tight junctions. Production of this toxin correlates with diarrheagenicity of *V. cholerae* strains in volunteers and may represent another virulence mechanism of infectious diarrhea.

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions.** *V. cholerae* 395 is a classical Ogawa CT-positive strain that has been extensively studied in volunteers (4). *V. cholerae* CVD101 and 395N1 are *ctxA* deletion mutants of strain 395 (3, 7). Culture supernatants were prepared by centrifugation of overnight cultures (37°C, LB broth) followed by filtration through a 0.45-μm filter (Millipore).

**Ussing Chambers.** Experiments were performed according to Guandalini et al. (8). Adult male New Zealand White rabbits (2–3 kg) were anesthetized with methohexital anesthesia and then sacrificed by air embolism. A 15-cm segment of ileum was removed, rinsed free of intestinal contents, opened along the mesenteric border, and stripped of muscular and serosal layers. Four sheets of mucosa so prepared were then mounted in Lucite Ussing chambers (1.12-cm² aperture) and bathed by freshly prepared buffer containing (in mM) NaCl, 53; KCl, 5; NaSO₄, 30.5; mannitol, 30.5; NaH₂PO₄, 1.69; NaH₂PO₄, 0.3; CaCl₂, 1.25; MgCl₂, 1.1; NaHCO₃, 25. The bathing solution was maintained at 37°C with water-jacketed reservoirs connected to a constant-temperature circulating pump and gassed with 95% O₂/5% CO₂. Potential difference (PD) and short-circuit current (Isc) were measured and tissue conductance (Gt) was calculated (5). Once the tissue reached equilibrium, 300 μl of culture supernatant or unincubated medium was added to the mucosal side (time 0); 300 μl of the same sample was also added to the serosal side to preserve the osmotic balance. Variations in *Isc*, PD, and Gt were then recorded every 20 min. Because of tissue variability, data are presented as ΔIsc and ΔGt, where ΔIsc = (Isc at time x) − (Isc at time 0) and ΔGt = (Gt at time x) − (Gt at time 0). At the end of every experiment, 200 μl of 0.5 M glucose was added to the mucosal side of each chamber. Only those tissues which showed an increase in *Isc* in response to glucose (indicating tissue viability) were included in the analysis.

**Electron Microscopy (EM).** Rabbit ileal tissues exposed to culture supernatants of *V. cholerae* strains were examined by the wheat germ agglutinin–horseradish peroxidase (WGA–HRP) permeability assay (9). In brief, 0.5 ml of Eagle’s minimal essential medium containing WGA–HRP (100 μg/ml; Miles) was placed on the apical (luminal) surface of the

Abbreviations: CT, cholera toxin; WGA–HRP, wheat germ agglutinin–horseradish peroxidase (WGA–HRP) permeability assay (9). In brief, 0.5 ml of Eagle’s minimal essential medium containing WGA–HRP (100 μg/ml; Miles) was placed on the apical (luminal) surface of the...
tissues previously exposed to supernatants or broth control for 60 min and held in place by support pins of the Ussing chamber. After a 5-min incubation, the Ussing chambers were disassembled, the WGA–HRP solution was decanted, and the tissues were cut away from the support pins. The tissues were then prefixed with 3% glutaraldehyde/0.1 M sodium cacodylate, pH 7.4, for 60 min, washed twice with 0.9% NaCl for 10 min, and immersed for 30 min in 0.1 M imidazole/0.05 M Tris-HCl, pH 7.0, containing 3,3'-diaminobenzidine tetrahydrochloride (1.5 mg/ml; Polysciences) and 0.02% H2O2. The tissues were washed twice with 0.9% NaCl for 10 min and stored overnight at 4°C in 0.1 M sucrose/0.1 M sodium cacodylate, pH 7.4. The tissues were postfixed in 1% OsO4/0.1 M sodium cacodylate, pH 7.4, for 1 hr. The postfixed tissues were washed in 0.1 M sodium cacodylate (pH 7.4), dehydrated using a graded series of ethanol solutions (30–100%), and embedded in Epon by standard procedures. Toluidine blue-stained thick sections were prepared and areas selected for ultramicrotomy were trimmed, sectioned, and placed on 300-mesh grids coated with Formvar and carbon for electron microscopy.

Pieces of ileum from Ussing chamber experiments, taken 60 min after addition of culture supernatant or medium control, were prepared for complementary-replica freeze-fracture (10). Tissues were fixed in 3% glutaraldehyde/0.1 M sodium cacodylate buffer, pH 7.4, for 1 hr, washed in 0.1 M sucrose in the same buffer, and stored at 4°C. Fixed tissue was pinned, luminal surface up, to a thin layer of Sylgard (Dow). Rows of three to seven adjacent villi were dissected free, cryoprotected by immersion in 30% glycerol in PBS (145 mM NaCl/10 mM Na2HPO4/10 mM Na3PO4, pH 7.2) for 30 min, and sandwiched between two specimen carriers with a drop of 20% polyvinyl alcohol and 20% glycerol in PBS. The sandwich was frozen in melted Freon-22 followed by liquid N2, then fractured at −115°C and <10−7 torr (1 torr = 133 Pa) with the complementary-replica device in a Balzers apparatus. Surfaces were shadowed immediately with platinum at 45 degrees followed by carbon at 90 degrees. Replicas were stabilized with a drop of collodion and cleaned by successive flotation on bleach, chrome-sulfuric acid, and distilled water, then mounted on slot grids coated with Formvar and carbon for EM.

Statistical Analysis. The complexity of the tight junctions exposed to each strain supernatant was quantified by measuring the density of strand intersections. Because not all bacterial strains were tested in the same rabbits, and because rabbits differed with respect to the number of intersections per unit area, statistical analyses were separated by strain. For each strain, data from strain supernatants were compared with their paired medium controls. To overcome non-normality in the distribution of the number of junctions per unit area, data were converted to ranks within rabbits (11). Ranked counts in tissues exposed to the bacterial supernatant were compared with those in tissue exposed to medium, for each strain, by randomized complete-blocks analysis of variance. For strains tested in a single rabbit (i.e., 395 and 395N1), this analysis was analogous to a Mann–Whitney test. For CVD101, which was assayed in two animals, “mean” is the unweighted mean of the two animals; SD is the pooled within-animal standard deviation.

Sizing of the Active Fraction. Cultures were cleared by centrifugation and the supernatants were filtered through a 0.45-μm filter (Millipore). Culture filtrates were fractionated using an ultrafiltration system (Amicon) and the following filters: YM100 (M, 100,000 cutoff), YM30 (M, 30,000 cutoff) and YM10 (M, 10,000 cutoff). The retentates were washed with 10 volumes of PBS, reconcentrated twice, and reconstituted to the original volume in PBS.

RESULTS

Effect of V. cholerae Culture Supernatants on Gt. Culture supernatants of V. cholerae were added to rabbit ileal tissue mounted in Ussing chambers, and PD (the difference in voltage measured on the mucosal side versus the serosal side of the tissue) and Ic (the amount of current needed to nullify the PD) were measured (6). From these measurements, Gt was calculated from Ohm’s law: \[ I_c = PD \times G_t \].

We first studied the effect of supernatants of the wild-type strain 395, using un inoculated culture medium added to matched ileal tissue from the same animal as a negative control. Fig. 1A shows the Ic and Gt variations obtained. The initial peaks in Ic and PD that occurred in both negative controls and test samples were most likely due to the cotransport of Na+ and nutrients present in the medium (12–14). In the negative control, \( I_{c\text{neg}} \) and PD returned to baseline values after ~1 hr, and subsequently \( I_{c\text{neg}}, PD, \) and \( G_t \) remained unchanged for the rest of the experiment. In contrast, tissues exposed to strain 395 supernatant exhibited a significant increase in \( G_t \), reaching a maximum after 2 hr of incubation. In such samples, \( I_{c\text{neg}} \) never returned to the baseline, but a steady-state period for \( I_{c\text{neg}} \) was noted between 40 and 60 min. PD, however, returned to its baseline value after almost 50 min (data not shown). Since the observed PD after 60 min was similar to the initial value, the significant increase in \( I_{c\text{neg}} \) in 395-treated tissues at that time point can only be due to an increase in \( G_t \) (see Fig. 1A, 60 min) (15). After 60 min, \( I_{c\text{neg}} \) began to rise again along with PD in 395-treated tissues. This second phase probably reflects the effect of CT on ion fluxes,

![Fig. 1. Effect of V. cholerae culture supernatants on ileal Ic and Gt. For clarity, PD values are not shown but can be readily calculated using Ohm's law. Values are means for six animals at each time point; error bars show ±1 SE. Medium control consisted of uninoculated culture medium. (A) Effect of V. cholerae 395 supernatants on Ic (solid lines) and Gt (dashed lines). Arrow indicates time at which 5 mM theophylline was added to the serosal side of both 395- and medium-exposed tissue after Ic values reached a plateau. (B) Effect of V. cholerae 395, CVD101, and 395N1 supernatants on Gt.](image-url)
since purified CT increases \( I_{sc} \) in rabbit ileal tissue only after a lag time of at least 40 min (6). Addition of glucose at the end of the experiment showed equivalent responses in treated and untreated tissues, indicating that glucose–Na\(^{+}\) active-cotransport function was unaffected (data not shown). In some experiments, theophylline, a phosphodiesterase inhibitor, was added to examine the contribution of cAMP to the \( I_{sc} \) response. The difference between the \( \Delta I_{sc} \) values of 395-treated tissue and those of the medium control after addition of theophylline is similar to the difference in \( \Delta I_{sc} \) values at 60 min (Fig. 1A). This indicates that a cAMP-independent factor contributes to the total response. These data suggest that there are two factors expressed by \textit{V. cholerae} 395 that can alter transport processes across ileal mucosa mounted in Ussing chambers. One factor induces an immediate increase in tissue conductance that is observable within 20 min after addition of culture supernatant, while another factor, CT, induces a cAMP-dependent increase in \( I_{sc} \) and PD beginning \( \approx 60 \) min after addition of culture supernatant.

We next studied \( G_{i} \) variation induced by culture supernatants of the CT-negative \textit{V. cholerae} strains CVD101 and 395 and 395N1, both attenuated by deletion of \textit{ctxA}. Although both of these strains are \textit{ctxA} mutants of strain 395, they did not behave similarly in volunteers. In contrast to CVD101, 395N1 induced very mild diarrhea (0.3-liter stool volume) in only 1 of 21 volunteers (16) \((P = 0.002, \text{ compared with 13 of 24 volunteers with diarrhea after ingestion of CVD101})\). When culture supernatants of CVD101 were added to Ussing chambers, an immediate increase in \( G_{i} \) was observed that was indistinguishable from that seen with 395 (Fig. 1B). In contrast, 395N1 induced no immediate increase in \( G_{i} \); \( G_{i} \) variation in 395N1-treated tissues was similar to the negative broth control and significantly lower than that seen with 395 and CVD101 for almost 100 min of incubation. After this period, the \( G_{i} \) modifications in tissues exposed to 395, CVD101, and 395N1 were similar. These results suggest that 395N1 produces lower amounts or a less active form of the factor responsible for this increase in \( G_{i} \).

**Effect of \textit{V. cholerae} Culture Supernatants on Intestinal Tight Junctions.** Given the striking effect on intestinal \( G_{i} \) induced by \textit{V. cholerae}, we next examined the epithelial cell ultrastructure for a possible mechanism for this effect. Variation in transepithelial conductance reflects modification of tissue permeability through the intercellular space, since plasma membrane resistances are relatively high (17). Since tight junctions (zonulae occludentes, ZO) represent the major barrier in this paracellular pathway (17–20) and variation in \( G_{i} \) is the most sensitive measure of ZO function (21), we examined morphological modifications of ZO induced by \textit{V. cholerae} 395, CVD101, and 395N1 supernatants. WGA-HRP, a soluble, electron-dense marker normally incapable of passing beyond the ZO (22), was added to the mucosal side of intestinal tissue treated with culture supernatants of 395, CVD101, and 395N1 or with uninoculated broth (control) for 60 min. Tissues treated with uninoculated culture medium were not permeable to WGA-HRP (Fig. 2A), whereas 395- and CVD101-treated tissues showed the entry of the stain into the paracellular space (Fig. 2B and D). Tissues exposed to 395N1 supernatants were unaffected, inasmuch as the intercellular space remained tight enough to exclude the passage of WGA-HRP (Fig. 2C). These results were confirmed and extended using freeze–fracture EM, wherein ZO appear as an anastomosing network of strands. Tissues exposed to \textit{V. cholerae} culture supernatants showed a mixture of unaltered ZO (Fig. 3A) and altered ZO with decreased strand complexity (Fig. 3B). Strands lying perpendicular to the long axis of the ZO appeared to be preferentially lost, resulting in a decreased number of strand intersections. Since several studies have shown a correlation of the number of strands with transepithelial electrical conductance (21, 22), we quantified the complexity of the ZO exposed to each strain supernatant by measuring the density of strand intersections (Table 1). There was a significant overall decrease in the number of ZO strands, reflecting an increased number of altered ZO, in tissues treated with culture supernatants of 395 or CVD101 compared with tissues treated with uninoculated broth or culture supernatant of 395N1. Because of the striking effect on ZO, we have designated the vibrio factor responsible for this effect as ZOT (for zonula occludens toxin).

**Characterization of the Active Fraction.** Culture supernatants of \textit{V. cholerae} 395 were fractionated by ultrafiltration and the resulting fractions were tested in Ussing chambers. Increased tissue conductance activity was found in the \( M_{r} < 30,000 \) fraction but not in the \( M_{r} < 10,000 \) fraction (Fig. 4A). In addition, the increase in \( G_{i} \) was abrogated by treating culture supernatants with proteinase K (250 \( \mu \)g/ml, 37°C, 1 hr), boiling for 15 min, freezing, or lyophilization (data not shown). The increase in \( G_{i} \) was readily reversible; removal of the culture supernatant was quickly followed by a decrease in \( G_{i} \) (Fig. 4B).

**DISCUSSION**

Despite being highly efficacious in preventing cholera in experimental challenge studies, the initial recombinant \textit{V. cholerae} vaccine strains attenuated by removal of the \textit{ctxA} genes were unacceptably reactogenic. Ingestion of \textit{V. cholerae} 3BK70 or CVD101 caused diarrhea in more than half the
volunteers (2). While this diarrhea was not the severe, dehydrating diarrhea typical of cholera, it was, nevertheless, significant enough to preclude the further testing of these strains. In addition to diarrhea, many volunteers experienced abdominal cramps, anorexia, malaise, low-grade fever, and headache. Two hypotheses were advanced to explain this reactogenicity (2). One hypothesis suggested that the act of colonization of the proximal small bowel by adherent vibrios results in diarrhea. While there is some evidence to support this hypothesis for *Escherichia coli* (23), there is no convincing evidence that this is the case for *V. cholerae*. An alternative hypothesis to explain the reactogenicity of these strains is that *V. cholerae* possess an additional secretagogue(s), distinct from CT, which causes net intestinal secretion manifested clinically as mild diarrhea. Derivatives of JBK70 and CVD101 that were specifically mutated in a gene encoding a cytotoxin/hemolysin implicated as a secretagogue in animal studies (24) still caused mild diarrhea in volunteers (2). The possibility that a Shiga-like toxin was responsible for this diarrhea was also raised (25). We have been unsuccessful in our attempts to clone the gene encoding this factor and thereby address this issue by using isogenic strains. However, Morris et al. (26) have shown that a *V. cholerae* non-O1 strain (2076-79) expressing Shiga-like toxin can successfully colonize the bowel without causing diarrhea in volunteers.

In the present study, we examined culture supernatants of *V. cholerae* 395 and its derivatives in Ussing chambers for secretogogenic activity, having previously failed to demonstrate secretion induced by the vaccine strains in ligated rabbit ileal loops or suckling mouse assays (unpublished data). Both CT-positive and CT-negative *V. cholerae* strains were capable of inducing an immediate increase in $G_t$ in rabbit ileal tissue. This increase in $G_t$ was not due to cell death, since the effect was rapidly reversible, and glucose–Na⁺ active cotransport (a mechanism of transport that occurs only when the tissue is viable) was unaffected. We sought a correlation of this change in $G_t$ with diarrheagenicity; we examined two derivatives of strain 395 that do not produce active CT but differ in their ability to cause diarrhea in volunteers. *V.

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**Table 1.** Quantification of ZO complexity (mean number of strand intersections per $\mu$m²) in tissues exposed to culture supernatants or medium control

<table>
<thead>
<tr>
<th>Sample</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>395</td>
<td>30</td>
<td>244.2</td>
<td>107.9</td>
<td>0.002</td>
</tr>
<tr>
<td>Medium</td>
<td>14</td>
<td>373.7</td>
<td>129.0</td>
<td></td>
</tr>
<tr>
<td>CVD101</td>
<td>32</td>
<td>230.2</td>
<td>75.5</td>
<td>0.004</td>
</tr>
<tr>
<td>Medium</td>
<td>30</td>
<td>305.8</td>
<td>96.3</td>
<td></td>
</tr>
<tr>
<td>395N1</td>
<td>31</td>
<td>305.8</td>
<td>80.1</td>
<td>0.322</td>
</tr>
<tr>
<td>Medium</td>
<td>31</td>
<td>289.1</td>
<td>83.8</td>
<td></td>
</tr>
</tbody>
</table>

ZO complexity was quantified by counting the number of intersections between ridges or furrows in a representative area (0.1–0.25 $\mu$m²) of each ZO and dividing by that area. To avoid bias in selecting ZO due to variation in crypts versus villus tips, the borders of replicated villi were followed and 16–48 consecutive ZO were photographed from a given replica. n, Number of ZO examined.

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**Fig. 3.** Freeze-fracture studies of rabbit ileal tissue exposed to culture supernatants of *V. cholerae* for 60 min. (A) An intact ZO with numerous intersections (arrowheads) between junctional strands; MV, microvilli. (B) An affected ZO from ileal tissue exposed to *V. cholerae* 395; the reticulum appears simplified due to greatly decreased incidence of strand intersections.

**Fig. 4.** Sizing and reversibility of Ussing chamber activity of *V. cholerae* 395 supernatant. (A) Activity of different size fractions in Ussing chambers: □, medium control; △, unfractionated supernatant; ○, Mr 30,000–100,000 fraction; ●, Mr <30,000 fraction; •, Mr <10,000 fraction. (B) Reversibility of $G_t$ variations induced by 395 supernatant (△) added to Ussing chambers and removed at the times indicated by arrows; total time course was 100 min; □, unoinoculated medium control.
cholerae CVD101 and 395N1 were constructed by similar recombinant DNA methods, resulting in the deletion of ≈94% and ≈80%, respectively, of the ctxA gene (3, 7). Although volunteer studies showed that both strains colonized the small intestine, CVD101 caused diarrhea in 54% of volunteers and 395N1 caused little if any diarrhea (2, 16). When examined in Ussing chambers, CVD101 produced an immediate increase in Gt, similar to that seen with the parent strain 395, while 395N1 produced no significant change in Gt for nearly 100 min after addition of culture supernatants. Hence, the ability to increase conductance of rabbit ileal tissue paralleled the diarrheagenicity of these strains. Eventually, Gt in 395N1-treated tissue did increase, suggesting that 395N1 produces lower amounts or a less active form of the factor (ZOT) responsible for this effect.

Diarrhea is a result of an imbalance in the secretive and absorptive functions of the bowel. The ability of CVD101, but not 395N1, to cause a rapid increase in Gt may be sufficient to disrupt this balance and produce diarrhea. Since 395N1 and CVD101 were derived from the same parent strain, 395, by nearly identical methods, an additional, spontaneous mutation must have occurred in the construction of 395N1 to alter its effect on Gt. The increased Gt induced by 395 and CVD101 is accompanied by alterations in ZO morphology including a decreased ZO strand complexity and a decreased ability to exclude an electron-dense marker (WGA–HRP) from the intercellular spaces. The function of intestinal ZO is to restrict or prevent the diffusion of water-soluble molecules through the intercellular space (the paracellular pathway) back into the lumen (17). This diffusion is driven by concentration gradients created by the active transepithelial transport processes. As a consequence of alteration of the paracellular pathway, the intestinal mucosa becomes more permeable, and water and electrolytes, under the force of hydrostatic pressure, leak into the lumen, resulting in diarrhea (27). Interestingly, the ZO may be a major pathway of nutrient uptake (17). Since 395N1 and CVD101 produce symptoms other than diarrhea that are undesirable for a vaccine, such as abdominal cramps, vomiting, and headache, it is possible that high levels of ZO permeability may give rise to some of these symptoms by altering intestinal epithelial permeability. The alteration of the paracellular pathway induced by V. cholerae 395 and CVD101 is specific for the small intestine; substitution of rabbit cecal tissue for ileal tissue resulted in no variation in Gt induced by 395N1 (data not shown). Regulation of ZO function is complex and poorly understood but the permeability of ZO may be altered by intracellular levels of Ca2+ and cAMP, among other factors (17). The effect of theophylline on Gt (Fig. 1) suggests that the ZOT effect is not mediated by cAMP, but we do not know whether ZOT acts exclusively on the ZO or mediates a change in intracellular messengers that regulate ZO permeability.

Alterations of epithelial tight junctions is an unusual property for an infectious agent. Clostridium difficile toxin A (28) and influenza and vesicular stomatitis viruses (29) have been shown to loosen tight junctions in tissue culture monolayers, but correlations with diarrhea in infected patients have not been reported. C. difficile toxin A differs markedly from ZOT in size (≈300 kDa [30]), cytopathicity (31), and attenuation of glucose-stimulated Na+ cotransport (32). We do not know the prevalence of ZOT in other strains of V. cholerae. However, preliminary studies (A.F., unpublished data) show that ZOT is found in both classic and El Tor strains, including JBK70, but not in the CT-negative non-O1 strain 2076-79. We have cloned the gene encoding ZOT and mapped its location to a site immediately adjacent to ctx (unpublished data). Deletion of the ZOT gene from attenuated strains of V. cholerae may be essential to develop a live attenuated V. cholerae vaccine with optimal safety and immunogenicity.

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