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Differential expression of the ToxR regulon in classical and El Tor biotypes of *Vibrio cholerae* is due to biotype-specific control over *toxT* expression

(ToxT/virulence regulation/cholera)

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ABSTRACT **The two major disease-causing biotypes of *Vibrio cholerae*, classical and El Tor, exhibit differences in their epidemic nature. Their behavior in the laboratory also differs in that El Tor strains produce two major virulence factors, cholera toxin (CT) and the toxin coregulated pilus (TCP), only under very restricted growth conditions, whereas classical strains do so in standard laboratory medium. Expression of toxin and TCP is controlled by two activator proteins, ToxR and ToxT, that operate in cascade fashion with ToxR controlling the synthesis of ToxT. Both biotypes express equivalent levels of ToxR, but only classical strains appear to express ToxT when grown in standard medium. In this report we show that restrictive expression of CT and TCP can be overcome in El Tor strains by expressing *toxT* independently of ToxR. An El Tor strain lacking functional ToxT does not express CT or TCP, ruling out existence of a cryptic pathway for virulence regulation in this biotype. These results may have implications for understanding the evolution of El Tor strains toward reduced virulence with respect to classical strains.**

Vibrio cholerae causes the human diarrheal disease cholera. Epidemic strains of *V. cholerae* are classified into two biotypes, classical and El Tor. Much of the world is currently experiencing an El Tor pandemic, unlike at least the two previous cholera pandemics that were classical pandemics (1). In addition, strains of the atypical O139 serogroup, responsible for outbreaks of cholera beginning in 1993, are derivatives of El Tor strains (2). The biotype classification is based on biochemical differences including susceptibility to polymixin B, hemagglutination of chicken erythrocytes, hemolysis of sheep erythrocytes, and the Voges–Proskauer test, which measures the production of acetylmethylcarbinol (3). Although the biotype definition is essentially bacteriological, differences in the epidemic nature of the biotypes also exist. While both classical and El Tor strains of *V. cholerae* have epidemic potential, classical strains are associated with more severe infection. In one study it was estimated that a severe outcome (i.e., frank cholera) accounted for 11% of infection with classical strains, whereas only 2% of infections with El Tor strains resulted in severe disease (3).

The ability of *V. cholerae* to cause disease is due to production of the cholera toxin (CT), an ADP-ribosylating toxin that disrupts cAMP metabolism in intestinal cells leading to secretory diarrhea in cholera patients. Several other virulence genes have been identified in *V. cholerae* and many of these are expressed coordinately with the genes encoding the CT. Included among these are the genes encoding the toxin coregulated pilus (TCP), a fimbrial structure on the surface of the bacteria that is required for colonization (4, 5). While the pilus is made up primarily of a single subunit, TcpA, its assembly requires the products of several genes linked to *tcpA*, and these also are coordinately expressed with CT (6, 7).

Coordinate expression of CT and TCP is controlled by the ToxR regulatory protein. The current model for virulence regulation by ToxR is that of a cascade in which ToxR controls expression of another regulator, ToxT, and ToxT directly controls expression of virulence genes (8). The ToxR cascade is modulated by growth signals such that certain conditions result in high level expression of ToxR-regulated genes, whereas other conditions do not. There are marked differences in the regulation of the ToxR cascade between the two biotypes. Classical strains express high levels of toxin and TCP in standard laboratory medium when cultivated at 25–30°C (5, 9, 10). El Tor strains, by contrast, require growth under special conditions, termed AKI, for high-level expression of ToxR-regulated genes (11, 12). Under the AKI conditions, organisms are cultivated for several hours in bicarbonate without aeration, followed by several hours of growth with vigorous aeration (11, 12, 28).

The substantially different way that classical and El Tor strains regulate production of virulence factors in response to *in vitro* growth may in part account for the observations described above regarding their epidemic behavior. We hypothesize that differences in virulence regulation between the two biotypes are associated with the way that ToxT is regulated in the two biotypes. In this report, we show that high-level production of toxin and TCP by El Tor strains under normally nonpermissive conditions can be achieved if expression of the *toxT* gene is removed from ToxR control. This suggests that the basis for the regulatory difference between classical and El Tor *V. cholerae* is expression of the *toxT* gene.

MATERIALS AND METHODS

Bacterial Strains and Media. Strains were stored in 20% glycerol at −70°C and inoculated onto plate medium prior to growth in liquid culture. Luria broth (LB) was made with tryptone (10 g/liter), yeast extract (5 g/liter), and NaCl (5 g/liter). TCBS agar medium, which is selective for *Vibrio* spp. (Difco) was prepared according to supplier's instructions. Growth of *V. cholerae* in LB was done overnight at 30°C on a tube roller. AKI conditions were as described (11, 13). Ampicillin and kanamycin were used at final concentrations of 100 μg/ml and 30 μg/ml, respectively.

Immunoblot Analysis of TcpA and ToxR, CT ELISA, and Primer Extension. Preparation of samples for immunoblot analysis was done as described (14). Samples were subjected to

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Abbreviations: CT, cholera toxin; TCP, toxin coregulated pilus; IPTG, isopropyl β-D-thiogalactoside; MSHA, mannose-sensitive hemagglutinin.

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SDS/PAGE electrophoresis followed by blotting to nitrocellulose paper for 2 h using a Hoefer semi-dry blotting apparatus. Parallel gels loaded with the same sample volumes were electrophoresed and stained with Coomassie brilliant blue to ensure equal loading on the blotted gel. Blots were incubated for 2 h to overnight in 5% milk/20 mM Tris·HCl/0.5 M NaCl (pH 7; TBS) with a 1:2000 dilution of rabbit polyclonal anti-TCP serum or anti-ToxR serum (supplied by J. Mekalanos, Harvard Medical School). After washing for 15 min with three changes of TBS, blots were incubated for 2–4 h with 5% milk/TBS and a 1:5000 dilution of goat anti-rabbit IgG linked to alkaline phosphatase. After washing in TBS, blots were developed in 1 M Tris·HCl, pH 9.0/5-bromo-4-chloro-3-indolyl phosphate (Sigma; 40 μg/ml)/nitroblue tetrazolium (Sigma; 100 μg/ml).

Culture supernatant fractions were analyzed for CT production by GM_1-ELISA as described (15) using monoclonal or polyclonal sera supplied by M. Bagdasarian (Michigan State University). Values for toxin production are given as concentration of toxin per OD_{600} of the culture.

RNA was isolated using the Tri-Reagent (Molecular Research Center, Cincinnati) following the manufacturer's protocol. Cultures were grown overnight, subcultured at 1:10 into fresh medium, and grown for 2–3 h prior to harvesting the cells for RNA production. Ten micrograms of total RNA was used in the primer extension protocol with *toxT* primer as described (16).

Introduction of Plasmids into *V. cholerae*. Plasmid pMT5 is a broad host range plasmid in which a PCR-amplified fragment of *toxT*, generated from classical strain O395, was cloned as a *Sal*I–*Bam*HI restriction fragment into pMMB66HE (17) downstream of the isopropyl β-D-thiogalactoside (IPTG)-inducible *tac* promoter. pMT5 was mobilized from *E. coli* VM2 (18) into *V. cholerae* by triparental plate mating with *E. coli* MM294 carrying pRK2013, which supplies the mobilization apparatus. *V. cholerae* recipients of pMT5 were identified as ampicillin-resistant cells on TCBS agar (Difco).

RESULTS

LB and AKI Growth of Classical and El Tor *V. cholerae*. Production of CT and the TCP major subunit TcpA is readily achieved with classical strains of *V. cholerae* by growth in LB medium (pH 6.5–7.0) at 25–30°C. El Tor strains will not produce these virulence factors when grown under the same conditions but will do so under very strictly defined growth in conditions termed AKI (refs. 11 and 12; Fig. 1). In AKI growth, the organisms are cultured without aeration for several hours and then grown with vigorous aeration for several hours more. As shown in Fig. 1, classical strain O395 produced high levels of CT and TcpA when grown in either LB or AKI, although LB seemed the more favorable medium. In contrast, the El Tor strains E7946 and C6709 produced detectable CT and TcpA only under AKI conditions (Fig. 1).

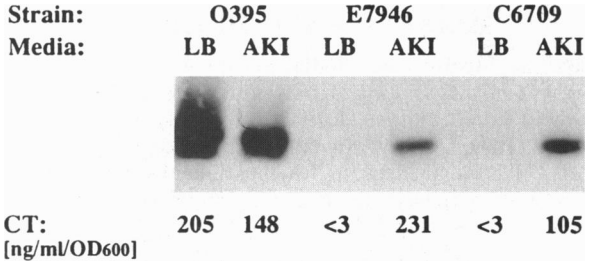

FIG. 1. TcpA and CT production by classical and El Tor strains. O395 is a classical strain and E7946 and C6709 are El Tor strains. Cultures were grown in the indicated media [LB represents cultures grown overnight in Luria broth at 30°C and AKI represents growth under conditions previously described (11).] Whole cell lysates were prepared by boiling cell pellets in SDS/PAGE sample buffer. Aliquots of the gels were subjected to SDS/PAGE in 15% gels and subsequently electroblotted to nitrocellulose paper. These filters were probed with anti-TCP antiserum as described (14). CT represents the amount of CT detected in the supernatants of the cultures as measured by GM_1-ganglioside ELISA (15).

Expression of ToxR in Classical and El Tor *V. cholerae*. Because El Tor and classical strains differ so greatly in their response to environmental signals, we considered it likely that there is a difference in the ToxR/ToxT regulatory cascade between the two biotypes. It is improbable that gross differences in ToxR account for biotype-specific virulence regulation because the predicted coding sequence of *toxR* from classical and El Tor strains differs at only a few residues, with the changes conferring only conservative replacements (ref. 19; Fig. 2). We therefore considered the possibility that expression of ToxR might be different in the two different biotypes.

To address this, we performed immunoblot analysis using ToxR-specific antiserum on extracts from cultures of *V. cholerae* classical and El Tor strains grown in LB or AKI media at 30°C. Fig. 3 shows that levels of ToxR in classical and El Tor strains are equivalent and independent of the growth conditions. An interesting point about this observation, which will be discussed further below, is that while *toxR* expression *per se* is sufficient to result in transcription of the *ctxAB* operon in the heterologous host *E. coli* (18), it does not appear to be sufficient for *ctxAB* expression in *V. cholerae*.

***toxT* Expression in Classical and El Tor Strains.** Under conditions permissive for virulence factor production by the classical strains, a ToxR-dependent promoter controlling *toxT* expression is active and appears to be required for subsequent virulence factor production (16, 20). We performed primer extension of mRNA from an El Tor strain of *V. cholerae* grown under these same conditions with a primer specific for *toxT* to determine whether the lack of CT and TcpA production in the El Tor background might be correlated with a lack of *toxT* expression. As seen in Fig. 4*A*, *toxT*-specific message is undetectable from the El Tor strains C6706 and C6709 grown in parallel with the classical strain O395, from which *toxT* mRNA can be observed. Analysis of the RNA used for the primer extension confirmed that equivalent amounts of RNA were analyzed in each reaction and that the El Tor mRNA is not subject to a generally higher level of degradation compared with the classical mRNA (Fig. 4*B*). We previously identified two primer-extension products from *toxT* RNA mapping experiments in wild-type classical *V. cholerae*. One of these, signified with an arrow in Fig. 3, is expressed from a ToxR-regulated promoter preceding the *toxT* gene in the *tcp* gene cluster (20). The other (Fig. 4*A*) can also be detected in *toxR* mutant classical *V. cholerae* (20) and its promoter has not been identified. Whatever the precise mechanisms governing the level of this transcript may be, it is clearly absent from the El Tor strains grown for the experiment shown in Fig. 4*A*.

The precise mechanism by which growth conditions influence ToxR-regulated gene expression is not fully worked out. In classical strains, elevated culture pH (8.5 versus 6.5) results in dramatically reduced expression of *toxT* and, consequently, of CT and TcpA (14, 16). This high-pH effect can be overcome, even in the absence of ToxR, by overexpression of *toxT* from a ToxR-independent promoter (14). By analogy we reasoned that if the main difference in virulence regulation in the El Tor strains is their lack of *toxT* expression when grown under conditions permissive for such expression in classical strains, then activation of *toxT* expression in the El Tor strains by an exogenously regulated promoter might lead to expression of ToxR regulated genes under non-AKI growth conditions.

This was tested by expressing *toxT* from the *tac* promoter, which is induced in the presence of IPTG. When cultured in the presence of IPTG, El Tor strains with the *tac–toxT* gene

MFGLGHNSKEISMSHIGTKFILAEKFTFDPLSNTLIDKEDSEEIIRLGSNESRILWLLA

QRPNEVISRNDLHDFVWREQGFEVDDSSLTQAISTLRKMLKDSTKSPQYVKTVPKRG

YQLIARVETVEEEMARESEAAHDISQPESVNEYAESSSVPSSATVVNTPQPANVVT

N (AAC - AGC) A (GCG - ACG)

NKSAPNLGNRLLILIAVLLPLAVLLLTNPSQTSFKPLTVVDGVAVNMPNNHPDLS

P (TTT - CTT) S (TCC - ACC)

NWLPSIELCVKKYNEKHTGGLKPIEVIATGGQNNQLTLNYIHSPEVSGENITLRIVAN

PNDAIKVCE

FIG. 2. Predicted protein sequence of ToxR from *V. cholerae* El Tor strain E7946. Positions that differ from the classical protein (3) are underlined and the codon change is indicated in parentheses.

fusion in pMT5 produced high levels of CT and TcpA under the same culture conditions favored by the classical strains (Fig. 5). Without addition of IPTG, expression of these factors was not observed (Fig. 5). To determine if the TcpA protein produced upon the induction of *toxT* expression was incorporated into pili, *tcpA*$^+$ and *tcpA* deletion strains expressing *toxT* from plasmid pMT5 were examined for the presence of TCP by electron microscopy. As seen in Fig. 6, pili resembling TCP were produced by the wild-type strain C6706str2 after overnight growth with induction of *toxT* expression from pMT5, while no such pili were seen in the *tcpA* deletion strain KHT52. Occasional single flexible fibrils that did not resemble the rigid structure of TCP were seen in the mutant. Thus, the production of TcpA as determined by the immunoblot appears to correlate with pilus production, suggesting that ToxT activates the expression of all the genes in the *tcp* biogenesis operon, similar to what is seen in classical biotype strains.

El Tor Strains Require *toxT* for Virulence Gene Expression. One possibility for the altered regulation of ToxR controlled genes in the El Tor background is that this biotype has a pathway for expression of these genes that is independent of ToxT. We investigated this possibility by analyzing CT and TcpA expression in a *toxT* mutant derivative of El Tor strain E7946. In this mutant, the wild-type copy of *toxT* has been replaced with a mutant copy carrying an in-frame deletion of 93 bp predicted to encode a helix–turn–helix DNA binding domain. (Complete characterization of this mutant will be described in a separate report.)

Wild-type El Tor strain E7946 and its *toxT* mutant derivative were grown in AKI conditions. Cell extracts were assayed by immunoblot for TcpA production and culture supernatants were assayed for CT (Fig. 7). Neither of these factors was detectably produced in the *toxT* mutant strain, showing that ToxT is required for their expression in El Tor *V. cholerae* as it is in classical *V. cholerae* (unpublished results).

DISCUSSION

Expression of CT and TCP by the two major biotypes of *V. cholerae*, classical and El Tor, appears to be regulated differently by environmental signals in each biotype. Only when grown in the specialized AKI growth medium is high-level CT and pilus production by the El Tor biotype achievable, whereas classical strains can produce these factors in a wide variety of growth media, including AKI. Although they exhibit this difference, both biotypes share a requirement for the ToxR and ToxT regulatory proteins for toxin and pilus production. In this report we have shown that removing *toxT* gene expression from its normal control is sufficient to broaden the range of growth conditions in which the El Tor biotype of *V. cholerae* can express toxin and pilus. This suggests that an important difference between El Tor and classical *V. cholerae* is their respective abilities to express the *toxT* gene. We assume that the normal control of *toxT* in this biotype involves ToxR, although this has not been formally proven. For the purposes of the discussion that follows, this assumption holds.

There are other differences between the biotypes beside the way they regulate toxin expression. El Tor strains produce a fimbrial structure termed the mannose-sensitive hemagglutinin (MSHA) that is not expressed by the majority of classical strains (21). Antibodies directed against MSHA can block colonization by El Tor strains in two different animal models, showing that this structure is important *in vivo* (22). In addition to MSHA, El Tor strains also produce a hemolysin not expressed by classical strains. The role of this factor in virulence has not been explained, but animal model experiments suggest that factors coordinately regulated with hemolysin may

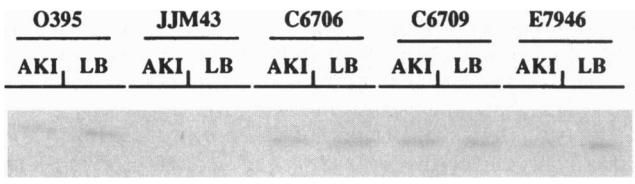

FIG. 3. ToxR immunoblot of extracts from classical and El Tor *V. cholerae* grown in LB or under AKI conditions. Strains O395(*toxR*+) and JJM43(*toxR*−) are classical biotype; strains C6706, C6709, and E7946 are El Tor biotype.

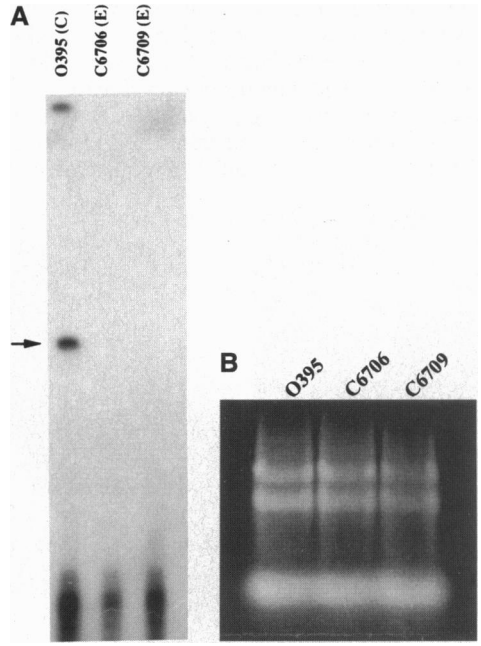

FIG. 4. (*A*) *toxT* primer extension of RNA from *V. cholerae* cultures grown under conditions for high-level expression of the ToxR regulon by classical strains (30°C, pH 7.0). The arrow points out the *toxT* transcript that initiates from a ToxR-dependent promoter preceding the *toxT* gene. Lanes: C, classical biotype; E, El Tor biotype. (*B*) Ethidium-stained gel of the same RNA preparation used for primer-extension reactions. Ten micrograms of RNA is loaded in each lane and the strain designation is given above each lane.

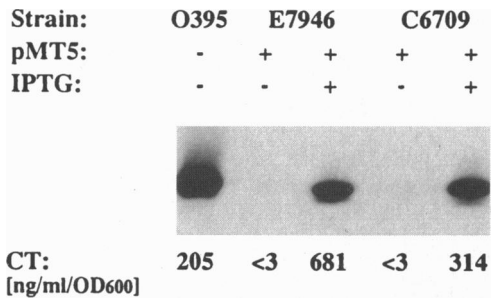

FIG. 5. TcpA and CT production in El Tor strains E7946 and C6709 expressing *toxT* under the control of the *tac* promoter in plasmid pMT5. TcpA and CT from classical strain O395 are shown as well. Cultures were grown in LB with or without addition of 1 mM IPTG, as indicated.

be important for successful colonization by El Tor strains (23, 24). Production of virulence-associated factors like MSHA and hemolysin by El Tor strains distinguishes them from classical strains in important ways and may play a role in the differences observed in the epidemic nature of El Tor and classical biotypes (see below), but the *sine qua non* of disease caused by either biotype is the ability to produce CT. Our results show that the control of toxin production by the ToxR/ToxT system in each biotype is different.

That ToxT is required for virulence gene expression in the El Tor biotype (Fig. 7) rules out the possibility that biotype-specific virulence control is associated with gross differences in the virulence regulatory schemes of the two biotypes. It also suggests that the previous demonstration of a requirement for ToxR in the El Tor biotype (13) most likely relates to its role in activating *toxT* expression, as postulated for the classical biotype (8). One question raised by our work, however, is the role of ToxR in *ctxAB* transcription in *V. cholerae*. Both ToxR and ToxT, independently of each other, can activate a *ctx*::*lacZ* fusion in *E. coli* to high levels (14, 18). Transcription of *ctx–lacZ* in *E. coli* initiates at the same site with each activator, and this is the site at which *ctxAB* transcription initiates in *V. cholerae* (G. Champion, M.N., and V.J.D., unpublished work). Nevertheless, El Tor strains do not produce CT under non-AKI growth conditions even though ToxR is expressed at levels similar to what is observed under AKI growth conditions (Fig. 3). That this is not simply due to a requirement for a signal

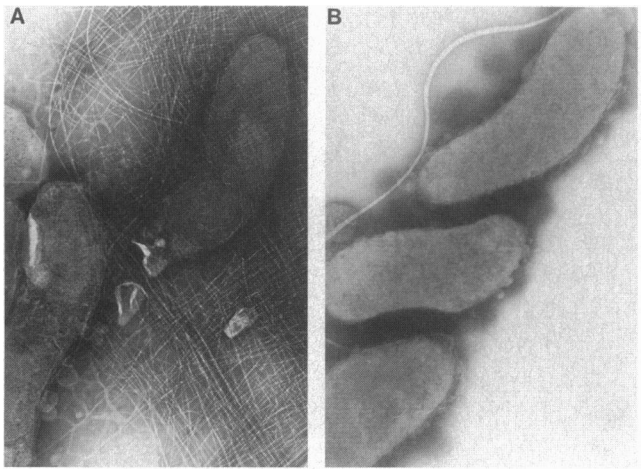

FIG. 6. Comparison of pilus production after induction of *toxT* expression in wild-type and *tcpA* deletion El Tor biotype strains. Parental strain C6706str2 carrying pMT5 (*toxT*$^+$) (*A*) and *tcpA* deletion strain KHT52 also carrying pMT5 (*B*) were grown overnight in LB with ampicillin at 37°C in the presence of 0.05 mM IPTG to induce *toxT* expression. Samples were visualized by electron microscopy after negative staining with 0.5% phosphotungstic acid (pH 6.5).

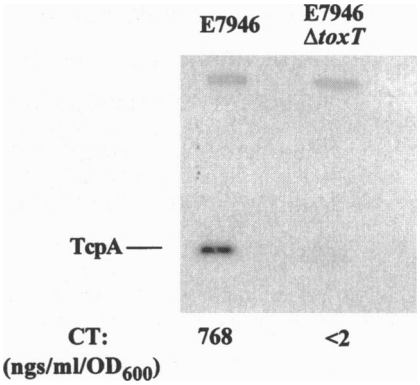

FIG. 7. CT and TcpA production in wild-type El Tor strain E7946 and its *toxT* mutant derivative grown under AKI conditions.

provided by AKI growth to cause ToxR to activate transcription of *ctxAB* is shown by the fact that the *toxT* mutant derivative of El Tor strain E7946 does not produce CT under AKI growth (Fig. 7). This result suggests that, at least *in vitro*, the activation of *ctxAB* expression is due solely to ToxT in El Tor *V. cholerae*. This also appears to be the case for classical biotype *V. cholerae* (G. Champion, M.N., and V.J.D., unpublished results).

The work in this report supports a model in which activation of *toxT* by ToxR is under biotype-specific control in *V. cholerae*. Previous work has demonstrated that ToxR alone is insufficient for activation of the classical *toxT* promoter (20). It therefore seems that another factor may be involved in activating *toxT*. If this were the case, then the most appealing model for biotype-specific gene regulation would be that this factor is active only under AKI conditions in the El Tor biotype but is active in a broader range of growth conditions in the classical biotype.

The level of asymptomatic *V. cholerae* infection is higher with El Tor strains than with classical strains, suggesting that the pathogenicity of El Tor strains is reduced compared with that of classical strains (3). Ewald (25, 26) suggests that displacement of classical *V. cholerae* by the El Tor biotype may have occurred due to improvements in the quality of drinking water in endemic areas. The basis for this is his hypothesis that a higher level of virulence should be seen in a gastrointestinal pathogen that is likely to be subject to waterborne transmission and that, conversely, a gastrointestinal pathogen less likely to be transmitted solely via contaminated drinking water should evolve to lower virulence (25, 26). This hypothesis is based on retrospective data showing a correlation between waterborne transmission and the virulence (measured by mortality rate) of the causative strain. When looked at in this retrospective way, the most virulent gastointestinal pathogen is the classical biotype of *V. cholerae* (26) and El Tor *V. cholerae* are considerably less virulent. As intriguing as this hypothesis is, it suffers from not being based on controlled prospective studies and it also does not account for the alternating displacement of classical and El Tor strains in one geographic locale such as has occurred in Bangladesh over the past 25 years (27). Nevertheless, if the history of interaction between *V. cholerae* and human populations indeed resulted in the decreased severity of the El Tor biotype compared with the classical biotype, it is logical to consider that the target of this natural selection might have been the control of toxin production. The results we report herein suggest that a difference in toxin regulation exists between the two biotypes; the selection pressures that may have caused this difference remain unclear.

This paper is dedicated to the memory of Gunhild Jonson, who made pioneering contributions toward understanding El Tor *V. cholerae*. We thank David Beattie for helpful discussions and critical

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