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Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*

(cholera toxin/nicking/protein export)

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ABSTRACT **A genetically engineered *Vibrio cholerae* strain from which the cholera toxin genes had previously been deleted was used as a host in which to study the expression and secretion of related toxins and their subunits. Recombinant plasmids encoding heat-labile enterotoxins (LTs) from *Escherichia coli* of human and porcine origin were expressed in the *V. cholerae* host, and this resulted in the secretion of the LTs into the extracellular milieu. The secreted LTs were isolated and it was found that the A subunits of human and porcine LT were "unnicked" polypeptides, which indicates that nicking is not obligatory for toxin secretion. *V. cholerae* strains were also constructed that harbored plasmids encoding either the A or the B subunits of human LT (A^+B^-, or A^-B^+). Approximately 90% of the B subunits were secreted from the A^-B^+ strain, while all of the A subunits expressed by the A^+B^- strain remained cell associated. This implies that strains synthesizing both subunits assemble the A and B subunits prior to their secretion. We propose that the entry of the toxin into the secretory step of the export pathway is mediated by a secretory apparatus that recognizes structural domains within the B subunit of LT.**

Cholera is a severe and at times fatal diarrheal disease of man (1). It is caused by *Vibrio cholerae*, which secrete a potent cholera enterotoxin (2, 3), consisting of five identical B subunits (4) that bind to G_{M1} ganglioside receptors (5, 6) and a single A subunit that subsequently activates adenylate cyclase (7, 8). Many *Escherichia coli* strains that cause diarrhea in man and domestic animals, such as pigs, produce heat-labile enterotoxins (LTs) that are similar to cholera toxin in immunological (9, 10), structural (11, 12), and functional (13, 14) properties. However, several studies have revealed distinctive features between cholera toxin and the LTs. First, the A subunit of cholera toxin is activated by a proteolytic "nick," which gives rise to two polypeptides (A_1 and A_2) that are joined by a cystine bridge (15, 16). The A subunits of the LTs are "unnicked" (17, 18). Second, the B subunits of LTs bind to glycoprotein receptors as well as to G_{M1} ganglioside (19). Third, cholera toxin is secreted from *V. cholerae* (2, 20), while the LTs are cell associated (21, 22) and are located within the periplasmic space of *E. coli* (23, 24).

Our present investigations concern the export and secretion of cholera toxin and the LTs. Many of the steps in the export of these toxins are presumably common in both *V. cholerae* and *E. coli*, including, for example, synthesis of subunit precursors, translocation across the cytoplasmic membrane, maturation and subunit assembly (25–27). The capacity of *V. cholerae* to secrete cholera toxin into the extracellular milieu is, however, an additional step not found in the export of LT by *E. coli*. This is unlikely to be due solely to differences between cholera toxin and LT, because the cloning of cholera toxin genes in *E. coli* resulted in the accumulation of cell-associated cholera toxin (28, 29). Therefore, it may be reasoned that *V. cholerae* possesses physiological factors or mechanisms that recognize and secrete cholera toxin. In this paper, we report that *V. cholerae* can also mediate the secretion of human and porcine LT derived from *E. coli*. Neill *et al.* have recently described a toxinogenic *V. cholerae* strain harboring an *ENT* plasmid that secreted porcine LT into the extracellular milieu (30). These LT-producing *V. cholerae* strains thus provide a new approach for studying the export and secretory mechanisms of this bacterium.

Using various recombinant plasmids that encode either the A or B subunits of LT, we have investigated the role of these subunits in the secretory process. A model is presented for the secretion of toxins by *V. cholerae*.

MATERIALS AND METHODS

Cholera toxin, tetracycline, polymixin B, and trimethoprim were purchased from Sigma. Ampicillin was obtained from Astra, Södertalje, Sweden, and thymine was from Mann Research Laboratories, New York. A cyclic AMP assay kit was purchased from the Radiochemical Centre. G_{M1} cellulose was donated by J. L. Tayot (Institut Merieux, Lyon, France).

Bacterial Strains and Plasmids. *V. cholerae* JBK70 (ΔCT, polymixin B resistant) was constructed as described by Kaper *et al.*, by deleting the cholera toxin genes (31). A thymine auxotroph of this strain was selected in the presence of trimethoprim (10 μg/ml) and thymine (20 μg/ml) and was designated *V. cholerae* TRH7000.

E. coli HB101 ($Pro^-Leu^-Thi^-$ $RecA^-$) harboring the conjugative plasmid pRK2013 (km^r) (32) was transformed with various plasmids; EWD299 (porcine LT, A^+B^+, ap^r) (33), pWD600 (human LT A^+B^+, tc^r) (34), and pWD600 derivatives, pWD605 (human LT A^+B^-) (34), and pWD615 (human LT, A^-B^+) (34). Plasmids were the gift of W. Dallas (Wellcome Lab, Triangle Park, North Carolina). Transformants of *E. coli* HB101 (pRK2013) were then mated with *V. cholerae* TRH7000 and transconjugants were selected on blood agar plates containing polymixin B (50 units/ml)/trimethoprim (10 μg/ml)/thymine (50 μg/ml), and an appropriate additional antibiotic; ampicillin (100 μg/ml) or tetracycline (2 μg/ml). All transconjugants of TRH7000 were checked for the loss of pRK2013. These experiments were carried out

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Abbreviation: LT, heat-labile enterotoxin.

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E. coli K-12 G6 (Hfr, His^-, Thi^-) was also transformed with plasmids EWD299, pWD600, pWD605, and pWD615.

Toxin Localization During Cell Growth. *V. cholerae* TRH7000 (EWD299) was grown at 37°C with continuous shaking in syncase broth (35) supplemented with thymine (50 μg/ml)/trimethoprim (10 μg/ml)/ampicillin (100 μg/ml). *E. coli* G6 (EWD299) was grown in syncase supplemented with ampicillin at 100 μg/ml. At various times during the growth of these strains, 1-ml samples were removed and the cells were separated from the medium by centrifugation (8000 × *g* for 2 min in an Eppendorf centrifuge) and resuspended in the same volume of cold syncase broth and sonicated twice (15 sec each). The concentration of porcine LT in the cell sonicates and medium fractions was determined by G_{M1} ELISA as described (36), using cholera toxin as a standard.

Localization of Human LT and Its Subunits. *V. cholerae* TRH7000 strains harboring plasmids pWD600, pWD605, or pWD615 were grown in syncase broth supplemented with thymine, trimethoprim, and tetracycline (2 μg/ml). The control strain TRH7000 was grown under identical conditions in the absence of tetracycline. *E. coli* G6 strains harboring pWD600, pWD605, and pWD615 were grown in syncase supplemented with tetracycline (20 μg/ml), while the control G6 strain was grown in the absence of tetracycline. All the strains were shaken at 37°C until they reached the optical densities at 600 nm shown in Table 1, and the cells were separated from the medium and sonicated as before. The concentrations of human LT A and B subunits in the various fractions were determined using the pigeon erythrocyte lysate assay (37) and G_{M1} ELISA, respectively, with cholera toxin as the standard in each assay.

Table 1. Cellular location of LT and its subunits in *V. cholerae* and *E. coli*

Strain	Toxin produced	A_{600}	Toxin concentration, μg/ml: Extracellular	Toxin concentration, μg/ml: Cell associated
V. cholerae TRH7000*	—	0.95	<0.067	<0.067
V. cholerae TRH7000†	—	0.95	0.0	0.0
V. cholerae TRH7000 (pWD600)*	hLT	1.10	1.10 (100%)‡	<0.067
V. cholerae TRH7000 (pWD605)*	hLT-A	1.00	<0.067	<0.067
V. cholerae TRH7000 (pWD605)†	hLT-A	1.10	0.0	1.2 (100%)
V. cholerae TRH7000 (pWD615)*	hLT-B	0.90	1.38 (90%)	0.17 (10%)
V. cholerae TRH7000 (EWD299)*	pLT	1.70	0.29 (100%)	<0.036
E. coli G6*	—	1.20	<0.038	<0.038
E. coli G6 (pWD600)*	hLT	1.05	<0.060	0.76 (100%)
E. coli G6 (pWD615)*	hLT-B	0.95	<0.060	0.67 (100%)
E. coli G6 (EWD299)*	pLT	1.25	<0.034	0.43 (100%)

hLT, human LT; hLT-A, human LT A subunit; hLT-B, human LT B subunit; pLT, porcine LT
*Assayed by G_{M1}-ELISA (36).
†Assayed by pigeon erythrocyte lysate assay (37).
‡Percent toxin or toxin subunit in each fraction.

Purification of LTs Secreted from *V. cholerae*. *V. cholerae* strains TRH7000 (EWD299) and TRH7000 (pWD600) were each cultured in 250 ml of syncase broth with the appropriate supplements for 7 hr. Cells were removed from the culture medium by centrifugation at 16,000 × *g* for 20 min, and the medium was subjected to ammonium sulfate precipitation. The proteins that precipitated between 0% and 70% saturated ammonium sulfate were pelleted at 16,000 × *g* for 10 min and dissolved in 5.0 ml of 0.01 M sodium phosphate/0.15 M sodium chloride (P_i/NaCl), pH 7.2. The fraction (0.5 ml) was added to a slurry of 20 μl of cellulose to which G_{M1} ganglioside had been coupled covalently. After occasional shaking for 40 min at 4°C, the G_{M1} cellulose was washed twice with P_i/NaCl. LT bound to the cellulose by virtue of its affinity for G_{M1} was released by boiling in sample buffer containing $NaDodSO_4$ with or without 1% 2-mercaptoethanol. The boiled samples were analyzed by $NaDodSO_4$/14% polyacrylamide gel electrophoresis.

Purification of Porcine LT from *E. coli*. Porcine LT was purified from the periplasmic space of *E. coli* G6 (EWD299) using a method that will be described in detail elsewhere. In summary, a periplasmic fraction was prepared by treating the cells with EDTA and lysozyme and subjected to ammonium sulfate fractionation. The proteins that precipitated between 45% and 70% saturated ammonium sulfate were dissolved and dialyzed against 0.001 M sodium phosphate (pH 7.6). During dialysis, aggregation of porcine LT occurred, and the aggregates were collected by centrifugation and found to contain essentially pure LT.

RESULTS

JBK70 is a genetically engineered *V. cholerae* strain from which the cholera toxin genes had previously been deleted (31). This organism is a most convenient host in which to study the expression of related toxin genes because of the lack of contaminating cholera toxin. We crippled JBK70 further by introducing a thymine auxotrophic requirement to generate strain TRH7000 and then transferred into it various recombinant plasmids encoding the LT of *E. coli*.

Secretion of LT by *V. cholerae*. Plasmid EWD299 that encodes both porcine LT and β-lactamase was transferred into *V. cholerae* TRH7000. TRH7000 harboring EWD299 was then cultured in syncase broth and at various times during its growth, samples were removed and the cells were separated from the medium and sonicated. Both the disrupted cell and medium fractions were assayed for their concentrations of LT by G_{M1} ELISA. Almost 90% of the LT was found in the

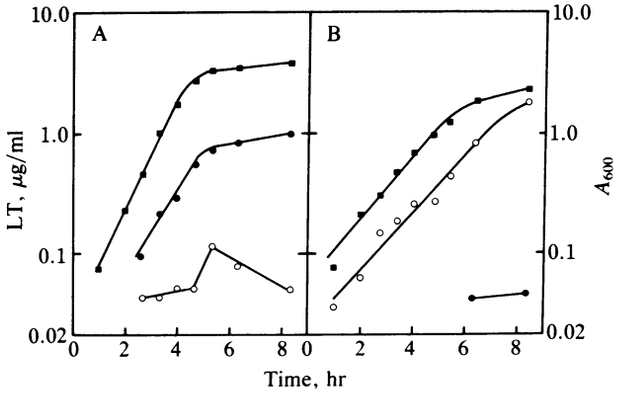

FIG. 1. Location of porcine LT in *V. cholerae* and *E. coli*. (*A*) *V. cholerae* TRH7000 (EWD299) and (*B*) *E. coli* G6 (EWD299) were cultured in syncase broth and growth was followed by measuring A_{600} (■). The concentration of porcine LT in the medium (●) and the cells (○) was determined by G_{M1} ELISA.

medium throughout the growth of the strain in syncase broth (Fig. 1*A*). The concentration of LT in the medium increased exponentially at a rate similar to the increase in cell density. As the cells entered stationary phase, the remaining toxin was secreted until >99.5% of the total LT was extracellular. Expression of the same plasmid in *E. coli* G6 resulted in the synthesis but not the secretion of LT, all of which remained cell associated (Fig. 1*B*).

Plasmid pWD600 that encodes human LT was also transferred into *V. cholerae* TRH7000, and the resulting strain both produced human LT and secreted it extracellularly (Table 1). A corresponding *E. coli* G6 strain harboring the same plasmid failed to secrete human LT into the medium (Table 1). This demonstrates that porcine and human LTs are cell-associated proteins in their normal host, *E. coli*, but they become extracellular proteins when synthesized by *V. cholerae*.

Structure of the Secreted LTs. The A subunits of porcine and human LT isolated from *E. coli* are linear unnicked polypeptide chains (18, 19, 27), whereas the A subunit of cholera toxin secreted by *V. cholerae* is proteolytically nicked (4, 15). We isolated the LTs secreted from the constructed strains of *V. cholerae* and determined their structures with special regard to the A subunit.

$NaDodSO_4$/polyacrylamide gel electrophoretic analysis of porcine LT isolated from the medium of TRH7000 (EWD299) showed that the A subunit migrated with an apparent molecular weight of 28,000 both in the presence (Fig. 2, lane 2) and absence (lane 5) of 2-mercaptoethanol. This behavior is identical to that of porcine LT purified from *E. coli* G6 (EWD299) (compare with lanes 3 and 6) but contrasts with the electrophoretic migration of cholera toxin. The A subunit of cholera toxin migrates with an apparent molecular weight of 28,000 in the absence of 2-mercaptoethanol (lane 4) and as two fragments of molecular weights 22,000 (lane 1) and 6000 (not shown) when reduced. We conclude that the A subunit of porcine LT is not proteolytically nicked when secreted by *V. cholerae*. A similar analysis of human LT secreted by *V. cholerae* TRH7000 (pWD600) showed that the A subunit was unnicked and migrated with an apparent molecular weight of 28,000 in presence of 2-mercaptoethanol (lane 7).

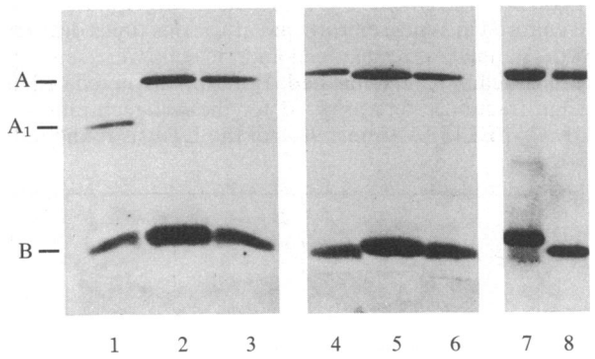

FIG. 2. A subunit structure. Porcine and human LT from the medium of *V. cholerae* TRH7000 (EWD299) and *V. cholerae* TRH7000 (pWD600), respectively, and porcine LT from *E. coli* G6 (EWD299) were purified. Toxins were boiled in electrophoresis sample buffer containing 1% 2-mercaptoethanol (lanes 1–3, 7, and 8) and without 1% 2-mercaptoethanol (lanes 4–6) and then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Lanes: 1, cholera toxin (Sigma); 2, porcine LT from TRH7000 (EWD299); 3, porcine LT from G6 (EWD299); 4, cholera toxin; 5, porcine LT from TRH7000 (EWD299); 6, porcine LT from G6 (EWD299); 7, human LT from TRH7000 (pWD600); 8, porcine LT from G6 (EWD299). Molecular weights of the proteins are as follows: cholera toxin B subunit (B), 11,500 (38); cholera toxin peptide A_1 (A_1), 22,000 (39); and porcine LT A subunit (A) from *E. coli*, 28,000 (17, 18).

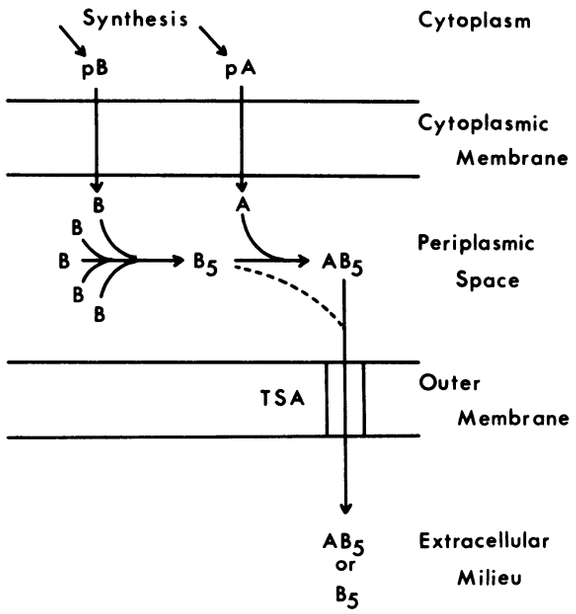

FIG. 3. Model for toxin secretion by *V. cholerae*. It is proposed that toxin secretion proceeds via synthesis of A and B subunits as precursors (pA and pB) that are translocated across the cytoplasmic membrane and released into the periplasmic space as processed mature subunits (A and B). B subunits assemble into pentamers (B_5) and associate with A subunits to give holotoxin (AB_5). Either holotoxin or B pentamers can be translocated across the outer membrane by a toxin secretory apparatus (TSA) and released into the extracellular milieu.

Role of the A and B Subunits in Toxin Secretion. Dallas (34) has constructed two recombinant plasmids carrying the LT genes, one with a 4-base-pair insertion in the A gene (pWD615) and the other with a 4-base-pair deletion in the B gene (pWD605). Strains carrying pWD605 or pWD615 express only the A subunit or only the B subunit, respectively.

We transferred these plasmids into *V. cholerae* TRH7000 and investigated the role of the A and B subunits in promoting toxin secretion. It was found that all of the holotoxin synthesized by TRH7000 (pWD600) and 90% of the B subunits synthesized by TRH7000 (pWD615) were secreted into the medium (Table 1). In contrast, all of the A subunits synthesized by TRH7000 (pWD605) remained cell associated. Corresponding *E. coli* strains G6 (pWD600) and G6 (pWD615) failed to secrete holotoxin or B subunits, respectively (Table 1).

DISCUSSION

A variety of Gram-negative bacteria, including *Vibrio*, *Aeromonas*, and *Pseudomonas* species, secrete proteins through their cell envelopes into the extracellular environment. Other Gram-negative bacteria, including *E. coli*, rarely exhibit this phenomenon. The mechanisms that mediate protein translocation have been studied for a few truly secreted proteins. For example, *Pseudomonas aeruginosa* exotoxin A has been postulated to be secreted via zones of inner and outer membrane adhesion (40), while *E. coli* hemolysin (41) and several proteins from *Aeromonas hydrophila* (42) have been proposed to transiently enter the periplasmic space during their export and secretion. In the case of hemolysin, specific outer membrane proteins, which are important for its secretion, have also been identified (41).

In this report, we describe the synthesis of LT by both *E. coli* and *V. cholerae*, and demonstrate that while *E. coli* retain LT in a cell-associated compartment (periplasm; see ref. 24) *V. cholerae* secrete it into the extracellular milieu. Thus,

LT is exported to two different locations, depending on the organism in which it is synthesized, suggesting that bacterial–physiological factors as well as the structural properties of LT are clearly important in determining its location.

Previous studies on the export of LT by *E. coli* have shown that the toxin subunits are synthesized as precursors (12, 25, 43, 44) that are translocated across the cytoplasmic membrane and are proteolytically processed (25) before being assembled into holotoxin in the periplasmic space (27, 45). We propose that a similar export pathway operates in *V. cholerae*, with an additional secretory step that releases the toxin into the medium (Fig. 3). The important aspects of this model include the transient periplasmic location of the subunits, their assembly into holotoxin, and the nature of the secretory step. Because of the close similarity between cholera toxin and LTs, we have assumed that these are secreted from *V. cholerae* via identical export pathways.

Studies on the synthesis and location of cholera toxin and LT in *V. cholerae* imply that these toxins are secreted via a periplasmic pool. Levner *et al.* showed that treatment of *V. cholerae* with lincomycin caused the accumulation of cholera toxin in the periplasm (46). Furthermore, we show that a small amount of LT (<10%) is cell associated during the exponential growth of *V. cholerae* TRH7000 (EWD299), which is subsequently secreted from the cells upon their entry into stationary phase. Similar studies on *V. cholerae* 569B showed that the small amount of cell-associated cholera toxin found in exponentially growing cells was released, without cell lysis, after a short exposure to polymixin B, EDTA, and lysozyme (unpublished results). These observations, together with the finding that both cholera toxin and LT are periplasmically located proteins in *E. coli* (23, 24, 29), suggest that these toxins enter a periplasmic pool during their secretion from *V. cholerae* (Fig. 3).

The site of assembly of A and B subunits during their export is clearly relevant to the mechanism of toxin secretion, because this governs whether *V. cholerae* secrete the subunits separately or as an assembled holotoxin. We demonstrate in this report that A subunits of LT are only secreted when they are synthesized in a strain that also produces B subunits. This implies that the two subunits must associate or assemble prior to their secretion in order to facilitate an extracellular location for the A subunit. However, in another strain that produces only B subunits, it was found that these were efficiently secreted into the medium even though no A subunits were synthesized. Thus, an association between the A and B subunits is not a prerequisite for the B subunits to enter the secretory step of the export pathway. In strains synthesizing both subunits, it might therefore be expected that assembly is well coordinated in order to avoid the secretion of B toxoid (Fig. 3).

The nature of the secretory step of enterotoxins in *V. cholerae* is unknown. However, several physiological features may be responsible for this event and could include, for example, (*i*) a "leaky" outer membrane that permits the passive diffusion of LT into the medium, (*ii*) modification enzymes that alter the structure of LT and cause it to adopt a secretory proficient conformation, or (*iii*) a "secretory apparatus" that recognizes and mediates the secretion of LT through the outer membrane.

It seems unlikely that *V. cholerae* has a leaky outer membrane because associated A and B subunits are secreted while the synthesis of only the smaller A subunit results in it remaining cell associated. In addition, we found that β-lactamase (a periplasmic enzyme) was primarily cell associated in both *V. cholerae* TRH7000 (EWD299) and *E. coli* G6 (EWD299) (unpublished results).

A modification of LT that we considered might be relevant to toxin secretion was the nicking of the A subunit, because cholera toxin had previously been shown to have nicked A subunits when secreted from *V. cholerae* (3) but unnicked A subunits when cell associated in *E. coli* (28). Furthermore, lincomycin induced synthesis of cell-associated unnicked cholera toxin A subunits in *V. cholerae* (46). However, in this paper we show that nicking is unimportant in the secretory process, because the holotoxins of both porcine and human LT were completely secreted without their A subunits being nicked. In addition, B subunits were secreted in the absence of A subunit synthesis.

V. cholerae may contain a "secretory apparatus," which mediates the secretion of LT, and this would presumably interact with domains of the toxin molecule. Our finding that B subunits are secreted in the absence of A subunits indicates that B subunit domains are important. It is not known whether these putative domains reside within unassembled monomeric B subunits or result from their assembly into pentamers. We propose that only the assembled B subunits contain the structural information for interacting with the secretory apparatus and that the A subunit is only secreted by virtue of its association with the B subunit pentamer (Fig. 3). The nature of the interaction between the B subunits and the secretory apparatus and the identity of the latter remain to be elucidated.

Our observation that the A subunits of porcine and human LT are unnicked after secretion from *V. cholerae* suggests either that these toxins are not susceptible to the protease(s) produced by *V. cholerae*, which nicks cholera toxin or that *V. cholerae* TRH7000 is a low producer of such proteases. The *V. cholerae* protease that nicks cholera toxin has been proposed to be soluble hemagglutinin/protease, which has been shown to have the capacity to nick *in vitro* purified human LT from *E. coli* (47–49). The fact that the A subunit of human LT expressed by *V. cholerae* was unnicked, even though it could be nicked by the addition of purified *V. cholerae* soluble hemagglutinin (unpublished results), suggests that *V. cholerae* TRH7000 does indeed produce insufficient soluble hemagglutinin to catalyze the nicking of LT.

In conclusion, it is clear that *V. cholerae* exploits various bacterial physiological processes in its synthesis of enterotoxins. These include an efficient secretory mechanism and nicking proteases that deliver activated toxins into the milieu where they interact with the host and cause disease. The construction of *V. cholerae* strains that secrete LT and its subunits has enabled aspects of these important processes to be elucidated. In addition, these strains may also be of use in vaccine development by providing an extracellular source of LT and LT-B subunits from which these proteins could be purified, by adapting methods previously developed for cholera toxin. We describe in this paper a one-step isolation of LTs using G_{M1} cellulose affinity chromatography, a procedure that could easily be scaled up to yield gram quantities of purified toxin or B subunits (50). Also, it has not escaped us that it may be possible to develop a generalized secretory system in *V. cholerae* based on the secretion of LT-B by engineering gene fusions in which *elt-B* is a component.

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1. Ouchterlony, O. & Holmgren, J., eds. (1980) *Cholera and Related Diarrheas* (Karger, Basel).
2. Finkelstein, R. A. & LoSpalluto, J. J. (1970) *J. Infect. Dis.* **121**, S63–S72.
3. Lönnroth, I. & Holmgren, J. (1973) *J. Gen. Microbiol.* **76**, 417–427.
4. Gill, D. M. (1976) *Biochemistry* **15**, 1242–1248.

5. Cuatrecasas, P. (1973) *Biochemistry* **12,** 3558–3566.
6. Holmgren, J., Lönnroth, I. & Svennerholm, L. (1973) *Infect. Immun.* **8,** 208–214.
7. Gill, D. M. (1977) *Adv. Cyclic Nucleotide Res.* **8,** 85–118.
8. Moss, J., Stanley, S. J. & Lin, M. C. (1979) *J. Biol. Chem.* **254,** 11993–11996.
9. Clements, J. D. & Finkelstein, R. A. (1978) *Infect. Immun.* **21,** 1036–1039.
10. Holmgren, J. & Svennerholm, A.-M. (1979) *Curr. Microbiol.* **2,** 55–58.
11. Gill, D. M., Clements, J. D., Robertson, D. C. & Finkelstein, R. A. (1981) *Infect. Immun.* **33,** 677–682.
12. Dallas, W. S. & Falkow, S. (1980) *Nature (London)* **288,** 499–501.
13. Holmgren, J. (1973) *Infect. Immun.* **8,** 851–859.
14. Moss, J. & Richardson, S. H. (1978) *J. Clin. Invest.* **62,** 281–285.
15. Holmgren, J. & Lönnroth, I. (1975) *J. Gen. Microbiol.* **86,** 49–56.
16. VanHeyningen, S. (1976) *J. Infect. Dis.* **133,** S5–S13.
17. Kunkel, S. L. & Robertson, D. C. (1979) *Infect. Immun.* **25,** 586–596.
18. Clements, J. D. & Finkelstein, R. A. (1979) *Infect. Immun.* **24,** 760–769.
19. Holmgren, J., Fredman, P., Lindblad, M., Svennerholm, A.-M. & Svennerholm, L. (1982) *Infect. Immun.* **38,** 424–433.
20. Richardson, S. H. (1970) *J. Bacteriol.* **100,** 27–34.
21. Kunkel, S. L. & Robertson, D. C. (1979) *Infect. Immun.* **23,** 652–659.
22. Wensink, J., Gankema, H., Jansen, W. H., Guinee, P. A. M. & Witholt, B. (1978) *Biochem. Biophys. Acta* **514,** 128–136.
23. Evans, D. J., Jr., Evans, D. G. & Gorbach, S. L. (1974) *Infect. Immun.* **10,** 1010–1017.
24. Hirst, T. R., Randall, L. L. & Hardy, S. J. S. (1984) *J. Bacteriol.* **157,** 637–642.
25. Palva, E. T., Hirst, T. R., Hardy, S. J. S., Holmgren, J. & Randall, L. L. (1981) *J. Bacteriol.* **146,** 325–330.
26. Mekalanos, J. J., Swartz, D. J., Pearson, G. D. N., Harford, N., Groyne, F. & deWilde, M. (1983) *Nature (London)* **306,** 551–561.
27. Hirst, T. R., Hardy, S. J. S. & Randall, L. L. (1983) *J. Bacteriol.* **153,** 21–26.
28. Pearson, G. D. N. & Mekalanos, J. J. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2976–2980.
29. Gennaro, M. L., Greenaway, P. L. & Broadbent, D. A. (1982) *Nucleic Acids Res.* **10,** 4883–4890.
30. Neill, R. J., Ivins, B. E. & Holmes, R. K. (1983) *Science* **221,** 289–291.
31. Kaper, J. B., Lockman, H., Baldini, M. M. & Levine, M. M. (1984) *Nature (London)* **308,** 655–658.
32. Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7347–7351.
33. Dallas, W. S., Gill, D. M. & Falkow, S. (1979) *J. Bacteriol.* **139,** 850–858.
34. Dallas, W. S. (1983) *Infect. Immun.* **40,** 647–652.
35. Finkelstein, R. A., Sobocinski, P. Z., Atthasampunna, P. & Charunmethee, P. (1966) *J. Immunol.* **97,** 25–33.
36. Svennerholm, A.-M. & Holmgren, J. (1978) *Curr. Microbiol.* **1,** 19–23.
37. Kunkel, S. L. & Robertson, D. C. (1979) *Infect. Immun.* **25,** 586–596.
38. Lai, C. Y., Cancedda, F. & Chang, D. (1979) *FEBS Lett.* **100,** 85–89.
39. Lai, C. Y. (1977) *J. Biol. Chem.* **252,** 7249–7256.
40. Lory, S., Tai, P. C. & Davis, B. D. (1983) *J. Bacteriol.* **156,** 695–702.
41. Wagner, W., Vogel, M. & Goebel, W. (1983) *J. Bacteriol.* **154,** 200–210.
42. Howard, S. P. & Buckley, J. T. (1983) *J. Bacteriol.* **154,** 413–418.
43. Spicer, E. K. & Noble, J. A. (1982) *J. Biol. Chem.* **257,** 5716–5721.
44. Yamamoto, T., Nakazawa, T., Miyta, T., Kaji, A. & Yokota, T. (1984) *FEBS Lett.* **169,** 241–246.
45. Hirst, T. R. (1983) Dissertation (York University, United Kingdom).
46. Levner, M. H., Urbano, C. & Rubin, B. A. (1980) *J. Bacteriol.* **143,** 441–447.
47. Finkelstein, R. A. & Hanne, L. F. (1982) *Infect. Immun.* **36,** 1199–1208.
48. Finkelstein, R. A., Boesman-Finkelstein, M. & Holt, P. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1092–1095.
49. Booth, B. A., Boesman-Finkelstein, M. & Finkelstein, R. A. (1983) *Infect. Immun.* **42,** 639–644.
50. Tayot, J.-L., Holmgren, J., Svennerholm, L., Lindblad, M. & Tardy, M. (1981) *Eur. J. Biochem.* **113,** 249–258.