

***In situ* characterization of *Clostridium botulinum* neurotoxin synthesis and export**

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J.E. CALL, P.H. COOKE AND A.J. MILLER. 1995. A monoclonal antitoxin/colloidal gold probe and sequential centrifugation were used to study synthesis, translocation and export of *Clostridium botulinum* strain 62A neurotoxin (NT). Exponential growth occurred after 5 h of anaerobic incubation of spores and continued for 15–16 h. NT was detected at 15 h using the probe and transmission electron microscopy (TEM), 2 h earlier than the first detection by the mouse bioassay. During exponential growth, the probe localized NT primarily in the cytoplasm, on the inner side of the cytoplasmic membrane and in the cell wall. During stationary and death phases, the NT was located within the cytoplasm, cell wall and extracellularly. NT was released from the cell during cell wall exfoliation. Cells retained NT after repeated gelatin-phosphate washes and sequential centrifugations, consistent with the TEM observation that the NT is bound to the cell wall. These observations indicate that the process of *Cl. botulinum* type A NT production follows a sequence of synthesis, translocation across the cytoplasmic membrane and export through the cell wall.

INTRODUCTION

Dormant spores of *Clostridium botulinum* are ubiquitous in soil and aquatic sediments and these contaminate many foods. Outgrowth of the spores in foods is associated with the synthesis of a neurotoxin (NT) that is one of the most potent biological substances known (Lamana 1959). Ingestion of foods contaminated with preformed NT can lead to foodborne botulism, a paralytic disease which, if left untreated, can result in death (Lund 1990).

The mode of action of the NT at the peripheral cholinergic nerve ending has been suggested to be a three-step process, involving extracellular binding, membrane penetration and intracellular acetylcholine blockade (Simpson 1989). The poisoning mechanism was recently characterized by Shiavo *et al.* (1992) as proteolytic cleavage of synaptobrevin. The neurotoxin light chain cleaves a membrane-bound protein found on synaptic vesicles, resulting in the neurotransmitter blockade.

On the other hand, there is little information concerning the mechanism of synthesis and export of the botulin NT. Seven antigenically-different types of the NT (A–G) are synthesized by *Cl. botulinum*. These seven strains can be

distinguished further on the basis of their proteolytic activity. NT is synthesized as a 150 000 Da single chain protein which lacks a signal sequence on the *N*-terminal end (Dasgupta and Dekleva 1990). There is evidence that bacteriophages mediate the toxigenicity of some or all types of *Cl. botulinum*. Dolman and Chang (1972), for example, observed that small numbers of phage particles were found in the majority of young toxic cultures, regardless of NT type. After synthesis, the intracellular NT remains inactive as a single chain (Krysinski and Sugiyama 1981). NT is activated by protease nicking to form a dichain molecule held together by disulphide bonds across cysteine residues and by non-covalent forces. Proteolytic strains of *Cl. botulinum* produce endogenous proteases, but non-proteolytic strains require exogenous proteases, such as trypsin in the animal intestinal tract. The primary structure alteration results in NT activation (Dasgupta 1990).

Early studies on NT production with type A strains suggested that cell lysis, which occurs after exponential growth, was necessary for NT release from the cell (Bonventre and Kempe 1960). Subsequently, Siegle and Metzger (1979) established that this was not necessary and there was a steady increase in NT concentration during logarithmic growth of the cell culture. Maximum NT concentration was obtained before cell lysis, and extracellular NT concentration did not increase following autolysis. Differential centrifugation studies with types C and E strains

indicated that NT is bound to cell wall peptidoglycan, and is gradually released into the surrounding medium by cell wall exfoliation (Hirata 1987; Hyun and Sakaguchi 1989).

In this experiment a monoclonal antitoxin type A was conjugated to colloidal gold particles to localize *Cl. botulinum* NT in thin sections of spores and vegetative cells over time to determine if there is an ordered mechanism of toxin synthesis, cell membrane translocation and export.

MATERIALS AND METHODS

Bacterial spores

Spores of *Cl. botulinum* 62A were acquired from T.A. Roberts (Institute for Food Research, Reading, UK). They were maintained in distilled water at 4°C, and periodically checked for viability and NT production. A working stock was prepared by transferring 100 µl of the approximately 10⁶ spores ml⁻¹ permanent stock to 500 ml of botulinum assay medium (BAM) broth, pH 7.3 (Huhtanen 1975). The culture was incubated anaerobically for approximately 2 weeks at 35°C, in a flexible anaerobic chamber (Coy Laboratories, Ann Arbor, MI), using a gas mixture of 5% hydrogen, 10% carbon dioxide, 85% nitrogen. A palladium catalyst was used to maintain anaerobiosis. Spores were collected, cleaned and purified following a procedure for *Bacillus subtilis* spores (Todd *et al.* 1986). After purification the spore stock was heated to 80°C for 10 min and enumerated by serial dilutions in 0.1% peptone water, plated onto BAM agar using a spiral plater (Model D, Spiral Systems Inc., Cincinnati, OH). Petri dishes were incubated anaerobically at 35°C for *Cl. botulinum* enumeration, and, also aerobically for detection of possible contaminants. A Gram stain, catalase test and a mouse NT bioassay and neutralization procedure were performed on grown-out cultures to confirm the purity of the spore preparation (Kautter *et al.* 1992). The purified spore crop was maintained in distilled water at 4°C. Quantitative mouse bioassays were performed throughout the study to determine NT presence. Decimal dilutions of each sample were prepared in 0.1% gelatin phosphate, pH 7.4, and 1 ml injected into each of two 15–20 g Swiss-Weber mice. Mice were observed for 96 h for signs of botulism. All animal protocols were approved by an Institutional Animal Care and Use Committee.

Germination and growth

Freshly prepared BAM broth (250 ml) was inoculated with the purified 62A spore crop and grown anaerobically at 35°C. Periodically samples were withdrawn aseptically from the culture to determine growth, NT presence by mouse bioassay and *in situ* NT localization within vegetative cells. Samples were enumerated as described above. Vegetative

cell numbers were estimated in two trials and the growth kinetics were analysed by fitting experimental data to the Gompertz equation (Gibson *et al.* 1987).

Immunolocalization experimental procedure

For *in situ* localization of NT, cells were prepared first for electron microscopy using a modified rapid procedure of Altman *et al.* (1984). Each sample (3 ml) was centrifuged to form a pellet, fixed in a 1 ml mixture of 0.1% glutaraldehyde–2% formaldehyde (Electron Microscopy Services, Ft Washington, PA) and phosphate buffered saline (PBS) for 20 min at room temperature. The pellet was resuspended and washed twice in 1 ml of PBS. Unreacted aldehyde groups were quenched with 1 ml of 0.1 mol l⁻¹ glycine (Bio-Rad Laboratories, Hercules, CA)—0.1 mol l⁻¹ ammonium chloride (pH 8.5, J.T. Baker Chemical Co, Phillipsburg, NJ) for 30 min. After quenching the cell pellet was resuspended and washed three times in 1 ml of PBS. The fixed cell pellet was then dehydrated sequentially for 10 min each in 50, 75, 90, then 100% *N,N*-dimethylformamide (DMF, Aldrich Chemical Co, Milwaukee, WI). This was followed by infiltration with 1 : 2 Lowicryl K4M plastic medium : DMF (Chemische Werke Lowi D-8264, Waldkraisburg, Germany) for 10 min, 1 : 1 Lowicryl : DMF for 15 min, 100% Lowicryl K4M for 20 min, and 100% Lowicryl K4M for 25 min, using gentle agitation. Each cell pellet was transferred to fresh Lowicryl K4M plastic and polymerized by exposure to u.v. light (*ca* 360 nm) for 45 min at 4°C. Sections (*ca* 750 nm) were cut using a diamond knife (Diatome US, Ft Washington, PA) and mounted onto carbon-coated Formvar films (EMS, Ft Washington, PA) on 200 mesh nickel grids (EMS).

Uniform colloidal gold particles (20 nm) were prepared following a technique outlined by Geoghegan and Ackerman (1977).

BA11 monoclonal *Cl. botulinum* type A NT antitoxin (MA_t) was prepared by Dr Clifford Shone (PHLS Centre for Applied Microbiology and Research, Salisbury, UK). This MA_t showed no cross-reactivity, by ELISA, with NT types B and E, or with *Cl. tetani* NT, *Cl. perfringens* enterotoxin A, *Corynebacterium diphtheriae* toxoid, *Staphylococcus aureus* enterotoxins A and B, and *Vibrio cholerae* toxin. An amplified ELISA, using this MA_t, detected 5–10 Mouse Lethal Dose₅₀ ml⁻¹ of purified type A neurotoxin (Shone *et al.* 1985). Shone *et al.* (1985) also showed that ascites fluid from BA11 hybridoma cells neutralized 50 Mouse Lethal Dose₅₀ of type A NT. A mouse neutralization test (Kautter *et al.* 1992) and ELISA (Huhtanen *et al.* 1992) were performed in this laboratory to confirm the specificity of the BA11 MA_t to type A NT. The MA_t was diluted in sterile distilled water and dialysed against sterile distilled water for 6 h at 4°C. The dialysed MA_t was

adsorbed onto the surface of the colloidal gold particles to form a conjugate following Geoghegan and Ackerman (1977). This conjugate, which was stored at 4°C, was used to demonstrate the localization of *Cl. botulinum* neurotoxin.

Thin section immunolabeling

Sections of cell pellets were adsorbed with 0.1% bovine serum albumin (Sigma Chemical Co., St Louis, MO) in PBS (blocking buffer) for 1 h at 4°C. Sections were rinsed first with six drops (ca 300 µl) of blocking buffer, followed with six drops of sterile distilled water, then labelled with the MAT-colloidal gold conjugate by placing a drop of the conjugate onto the section and incubating at 4°C for 24 h in a moist chamber (Roth 1982). After incubation, the sections were rinsed with six drops each of blocking buffer and sterile distilled water, stained with 2% uranyl acetate and observed using a transmission electron microscope (Model 10B, Zeiss Inc, Thornwood, NY). A negative control was prepared by blocking binding sites by exposing the MAT-colloidal gold conjugate to diluted (44 µg protein ml⁻¹) purified type A toxin (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and adding the mixture to thin sections on grids, which were then incubated at 4°C for 24 h.

Immunogold probe distribution was quantified by selecting randomly cross-sectional profiles of *Cl. botulinum* cells. For each cross-section an area of 1.1 µm was counted. Quantification of gold particle distribution was performed by counting areas around 10 cells from each of three fields, for a total of 30 cells. Each field was a micrograph of thin sections of *Cl. botulinum* cells magnified 8000×. Particles in each were assigned to either the cytoplasm, cell wall or extracellular. Means of the distribution from 30 cells were determined.

Distribution of NT between *Cl. botulinum* cells and extracellular fluid medium

In addition to the immunolocalization study, the authors determined the distribution of NT between the vegetative cells and the supernatant fluid by measuring by mouse bioassay the NT in the cell pellet and supernatant fluid following six gelatin-phosphate washes and sequential centrifugations. The experiment was conducted using stationary phase (24 h) cells in which NT is maximal. Supernatant fluid and cell pellet samples were tested for presence of NT using the mouse bioassay. Freshly prepared BAM broth (20 ml) was inoculated with type 62A spores and incubated anaerobically at 35°C for 24 h. At the conclusion of incubation, 1.2 ml of the culture was removed, diluted decimally in 0.1% gelatin-phosphate and 1 ml of each dilution injected into each of two mice. The remaining culture

was centrifuged at 7710 g at 4°C for 20 min. The supernatant fluid was decanted, then passed through a 0.2 µm syringe filter (Nalge Co., Rochester, NY) to remove remaining vegetative cells or debris. The cell pellet was resuspended in 20 ml of 0.1% gelatin-phosphate and 1.2 ml of both the filtrate and cell suspension were diluted decimally in 0.1% gelatin-phosphate and 1 ml injected into each of two mice. The cell pellet was centrifuged and resuspended in 20 ml of gelatin-phosphate in the same manner as above five more times. After each centrifugation, both the cell pellet and the supernatant fluid were evaluated for NT as above.

RESULTS

Growth kinetics

The average growth and NT detection kinetics of two separate experiments are shown in Fig 1. Exponential growth occurred within 5 h and continued until 15–16 h. There was no distinct stationary phase; rather a decline in the number of viable vegetative cells from 16–120 h, during which time the population dropped log₁₀ 9 to log₁₀ 3 colony-forming units (cfu) ml⁻¹. The results were evaluated using the Gompertz equation. In the first trial the growth rate was 0.78 log₁₀ cfu h⁻¹, the generation time 0.39 h and the lag time 3.74 h. The corresponding values for the second trial were 0.59 log₁₀ cfu h⁻¹, 0.51 h and 6.11 h, respectively. Means and standard deviations for the two trials were 0.69 (0.09) log₁₀ cfu h⁻¹, 0.45 (0.06) h, and 4.95 (1.19) h for growth rate, generation time and lag duration, respectively. NT was first detected at 17 h (late-exponential) by bioassay, and reached its maximum concentration at 24–25 h.

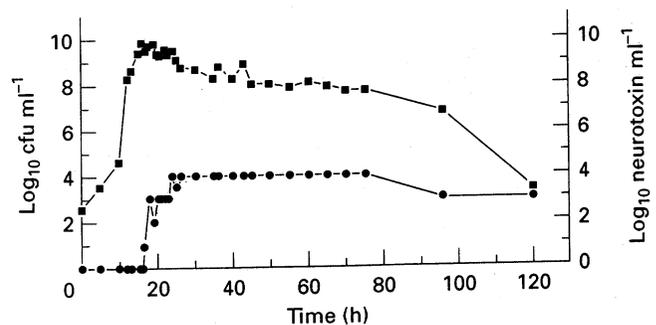


Fig. 1 Average growth and NT production of two experiments. ■, Population; ●, neurotoxin

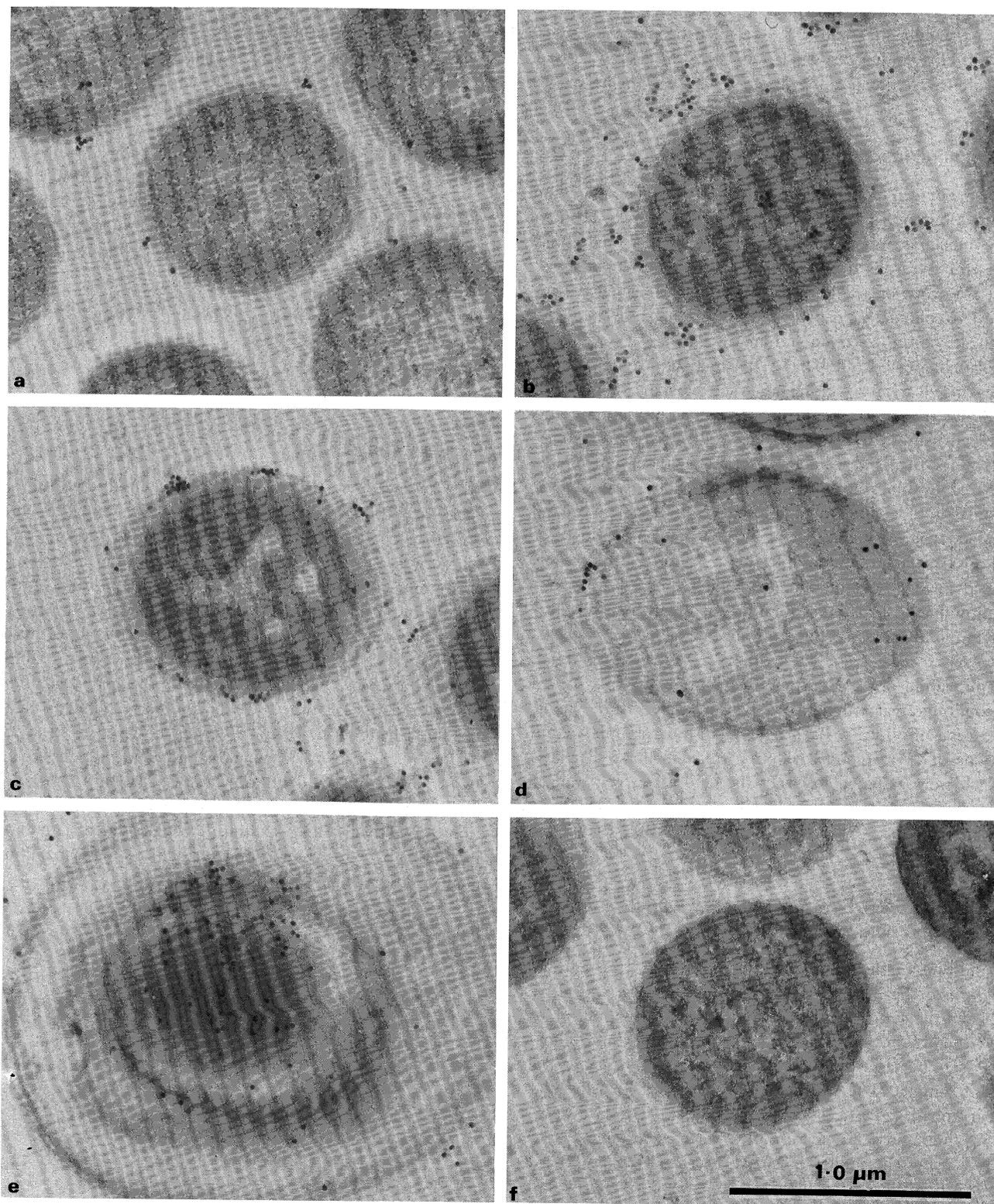


Fig. 2 Transmission electron micrographs of cross-sections of *Clostridium botulinum* cells showing the location of colloidal gold probe at (a) 15 h, (b) 25 h, (c) 45 h, (d) 120 h, (e) 120 h endospore, and (f) control

Table 1 The distribution and mean number of gold beads in relation to *Clostridium botulinum* cells at various sampling times

Sampling time (h)	Total viable count (\log_{10} cfu ml ⁻¹)	Mean number of gold beads cell ⁻¹	Distribution of gold beads (%)
15 Late exponential	7.01	17	Cytoplasm (59) Cell wall (23) Extracellular (18)
25 Early stationary	8.73	56	Cytoplasm (29) Cell wall (23) Extracellular strands (48)
45 Mid-late stationary	8.02	50	Cytoplasm (20) Cell wall (46) Extracellular strands (34)
120 Death	3.32	15	Cytoplasm (40) Cell wall (33) Extracellular strands (27)

NT detection by electron microscopy immunolocalization and mouse bioassay

The effect of sampling times and growth phase on the amount and distribution of colloidal gold beads during growth of *Cl. botulinum* is shown in Table 1 and Figs 2a-f. The 15 h sample (Fig 2a) was the earliest sample selected for electron microscopic examination because earlier samples lacked sufficient cell concentrations for processing. The majority (59%) of colloidal-MAt conjugate was located within the cytoplasm, smaller amounts were located within the cell wall (23%) and extracellularly (18%). At this time, NT was undetected by the mouse bioassay. Results from the early stationary phase sample (25 h, Fig. 2b) showed a threefold increase in gold beads indicating an increase in NT synthesis. Forty-eight per cent of the beads were bound to cell-associated extracellular strands, possibly exfoliated cell wall fragments. By 45 h (Fig. 2c) cell growth and NT levels stabilized. The greatest proportion of gold beads was located within the cell wall (46%). At 120 h (Fig. 2d) there was a 5 \log_{10} decrease in the viable count, but

there was no evidence of ruptured cells or cell debris, which would indicate lysis. Cells containing endospores were observed at death phase (Fig. 2e) and the average number of colloidal gold labels in these was 64 per cell. A high fraction of colloidal gold (54%) was associated with these spores and the remaining label was located primarily within the mother cell. Controls for all samples showed no evidence of non-specific colloidal gold label (Fig 2f).

As the culture progressed from log phase (Fig. 2a) to stationary phase (Figs 2b, c) the appearance of the cell walls changed from compact and well organized with a smooth surface to filamentous with a wavy surface. In some areas of the stationary phase cell wall extracellular strands were continuous with the surface. The cell walls of death phase cells (Fig. 2d) appeared similar to those seen in the log phase. The amount of extracellular strands increased with the transition from log phase to stationary phase, although at 45 h the number of strands were greatly reduced compared to 25 h, perhaps because all shedding had been completed earlier.

Another experiment using six sequential centrifugations was conducted to determine the relative NT amounts associated with the cell and extracellular medium. The effect of multiple washings on the association of NT is shown in Table 2. There was a decrease from 11 000 mouse lethal doses (MLD) ml⁻¹ of NT in the culture to 20 MLD ml⁻¹ after the final centrifugation. The cell pellet remained stable at 200 MLD ml⁻¹ until the fifth centrifugation when NT levels dropped 10-fold.

Table 2 Centrifugation effects on the distribution of *Clostridium botulinum* NT in 24 h vegetative cells*

Centrifugation/wash no.	MLD ml ⁻¹	
	Cell pellet	Supernatant fluid
1	200	11 000
2	200	200
3	200	20
4	200	20
5	200	20
6	20	20

* Initial NT levels were estimated to be 11 000 mouse lethal doses (MLD) ml⁻¹.

DISCUSSION

This study demonstrated that NT could be detected by immunolocalization prior to detection by mouse bioassay. This suggests that NT levels were either too low to be detected by the mouse bioassay or the majority of intracellular NT was immunogenically but not biologically active. It was not, however, possible to determine the earliest time

at which NT is produced, since it was unfeasible by centrifugation to obtain a cell pellet earlier than 15 h.

Using types A and B strains at 37°C, Sperber (1982) reported similar lag duration and generation times of 5 and 0.7 h, respectively, as those found in the current study. No growth curve was shown in Sperber (1982), and the study did not indicate if types A and B exhibited the same decline after maximum population density was achieved as in the current study.

The association of the colloidal gold probe with the cell wall and the extracellular strands indicated that the cell wall plays a role in NT export. Using Koch's model of Gram-positive cell wall formation as a basis (Koch 1990), it can be hypothesized that as the NT reaches the outer surface of the cytoplasmic membrane it becomes associated with the developing peptidoglycan layers of the cell wall. As the cell wall grows, the peptidoglycan layers, with associated NT, are pushed outward until at the outer surface, the peptidoglycan layers, with associated NT, are shed.

An experiment was conducted to test if NT was removed by repeatedly centrifuging growing cells. This study showed that the NT generally remains with the vegetative cell; however, the supernatant fluid contained some free NT as well as NT bound to cell wall fragments. If present, NT and wall fragments would pass through the 0.2 µm pore size filter because Fierle (1994) showed that bacterial endotoxin is not retained. These findings indicate that NT is closely associated with the vegetative cell. This is consistent with the reports by Hyun *et al.* (1989), who indicated that type C NT preferentially bound to cell wall peptidoglycan, and Hirata (1987), who indicated that cell wall associated NT was gradually released from the type E wall by wall ablation and fragmentation. A future experiment, in which the extracellular strands were labelled with antibody, would help determine if the NT-associated material is peptidoglycan.

The observation that lysis is not a requisite for NT release from the cell is consistent with the findings of Siegle *et al.* (1979). The shift in gold bead distribution from intracellular to extracellular with respect to time occurred in the absence of detectable cell lysis, and suggests that the NT is synthesized in the cell cytoplasm, translocated across the cytoplasmic membrane and then exported to the extracellular medium via cell wall exfoliation. Although there was no visual evidence of cell lysis, lysed cell fragments may have been removed during TEM processing.

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Multiplex polymerase chain reaction to detect toxigenic *Vibrio cholerae* and to biotype *Vibrio cholerae* O1

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Y.H. SHANGKUAN, Y.S. SHOW AND T.M. WANG. 1995. A multiplex polymerase chain reaction (PCR) was developed to identify cholera toxin-producing *Vibrio cholerae* and to biotype *V. cholerae* O1. Enterotoxin-producing *V. cholerae* strains were identified with a primer pair that amplified a fragment of the *ctxA2-B* gene. *Vibrio cholerae* O1 strains were simultaneously differentiated into biotypes with three primers specified for the *hlyA* gene in the same reaction. The *hlyA* amplicon in the multiplex PCR serves as an internal control when testing toxin-producing strains, as *hlyA* gene sequences exist in all tested *V. cholerae* strains. Enrichment of *V. cholerae* present on oysters for 6 h in alkaline peptone water before detection by a nested PCR with internal primers for *ctxA2-B* gene yielded a detection limit lower than 3 colony-forming units (cfu) per gram of food.

INTRODUCTION

Cholera epidemics are caused by cholera toxin-producing *Vibrio cholerae* O1. Cholera toxin (CT) belongs to enterotoxins that consist of two subunits (Spangler 1992). Subunit A is responsible for the toxic activity of CT, and subunit B is involved in binding the toxin to the ganglioside GM1 of the target cell (Guidolin and Manning 1987). The subunit A is generally nicked to form two disulphide-linked polypeptides named A1 and A2 by proteases. The chromosomal genes encoding the A and B subunits are designated *ctxA* and *ctxB*, expressed as a single transcriptional unit (Guidolin and Manning 1987). Toxin production does not correlate with serotype, as some strains of *V. cholerae* O1 may not produce CT (Kaper *et al.* 1981) and *V. cholerae* non-O1 may be enterotoxigenic (Craig *et al.* 1981). The non-O1 group is generally associated with sporadic cause of gastroenteritis and extraintestinal infections (Morris 1990) but has no epidemic potential. However, an epidemic of cholera, which emerged in India in 1992 and in Bangladesh in 1993, was caused by a strain of *V. cholerae* with a novel serogroup, O139. All tested serogroup O139 isolates can produce CT (Jesudason and John 1993), unlike the previous non-O1 serogroups which were known to produce the toxin with a minute frequency. As only CT-producing strains cause epidemic cholera, it is necessary to

examine *V. cholerae* isolates regularly for their ability to produce CT in order to assess their clinical significance.

In addition to toxin production, strains in serogroup O1 can be divided into two biotypes, E1 Tor and classical. Current tests to distinguish between biotypes are often difficult to interpret. Historically, the E1 Tor biotype was identified by its ability to haemolyse sheep erythrocytes, whereas classical strains were nonhaemolytic; more recent E1 Tor isolates are only poorly haemolytic on sheep erythrocytes (Gallut 1974). A genetic probe for the E1 Tor biotype based on the *hlyA* structural gene for the *V. cholerae* haemolysin was developed by Alm and Manning (1990). The probe was designed on the basis of the distinct sequences of *hlyA* genes between the two biotypes: in the classical strain the *hlyA* gene sequence has an 11-base-pair deletion but in the E1 Tor strain it remains intact. Their results showed that all E1 Tor isolates regardless of their haemolytic phenotype had homology to the 19-base-pair probe, whereas the classical isolates exhibited a negative result. This 19-base-pair probe is more effective in distinguishing the two biotypes of *V. cholerae* serogroup O1 than other commonly used methods.

The polymerase chain reaction (PCR) provides a sensitive and specific method to identify a known DNA sequence. Multiplex PCR, a modification of the basic PCR process, has the added advantage of having multiple primer pairs specific for varied targets in the same amplification reaction. PCR assays based on amplification of target DNA sequences in the *ctx* gene of *V. cholerae* were reported