Fine Structure of *Vibrio cholerae* During Toxin Production

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Received for publication 25 September 1969

The fine structural changes associated with cell growth and toxin production have been examined in *Vibrio cholerae* strain 569B. No morphological alterations in the cell envelope are apparent during logarithmic growth with thin-section techniques. However, internal swelling, suggesting alteration of cell envelope permeability, is evident particularly during the late logarithmic and early stationary phases of growth. Certain extracellular material demonstrable with negative-stain techniques does appear during the period of toxin production. The possible origin of this material is discussed. The effects of high temperature (37 C) and aeration on cell structure are also examined.

Since the first report of in vitro toxin production by *Vibrio cholerae* (4), a variety of growth conditions have been employed to obtain toxin for chemical and biological assay. In general, toxin has been harvested from late stationary-phase cultures grown for 48 hr at 30 C without aeration (3) or for 18 to 24 hr at 37 C with aeration (7, 8, 12). Recently, Coleman et al. (2) harvested toxin at the peak of logarithmic growth to minimize contamination due to cell autolysis. In studying the nutritional parameters of toxin production in *V. cholerae*, Richardson (13) has consistently demonstrated that in vitro toxin production, as measured by the permeability factor test, is completed by the time the population enters the stationary phase of growth. Thus, the question is raised as to the morphological condition of the population during logarithmic and stationary phases of growth. This study was undertaken to examine the relationship between cell growth, cell lysis, and toxin production and to determine, if possible, whether toxin production could be correlated with specific morphological changes in the cell wall, as suggested by Chatterjee and Das (1).

**MATERIALS AND METHODS**

The organism used in this study was *V. cholerae* strain 569B. All cultures were grown in 50 ml of TCY medium (6) in shaken 500-ml Erlenmeyer flasks (140 four-inch excursions/min) at 30 C. Samples were removed at various intervals for determination of growth, toxin production, and preparation for examination with an electron microscope. Culture turbidities (640 nm) and toxin vascular permeability factor (PF) assays (expressed as blueing doses/ml) were determined as previously described by Evans and Richardson. Determinations of proteinase, adenosine triphosphatase, and nucleic acid levels were as described by Richardson (13).

For electron microscopy, cell samples were fixed by the method of Kellenberger et al. (9) or were centrifuged at 1,500 X g for 15 min. The supernatant fractions from the latter preparations were assayed for toxin activity, and the pellets were fixed in a 1:1 mixture of cold glutaraldehyde (2.5%) and osmium tetroxide (1 part 5% OsO4 to 2 parts of 0.2 m sodium phosphate buffer) for 1 hr, according to Luft's modification of the technique of Trump and Bulger (15). All figures of sectioned material were fixed with the glutaraldehyde-osmium mixture. Samples were dehydrated and embedded in Epon 812 (10). Sections were cut on a Porter-Blum MT-1 microtome with diamond knives and stained with uranyl acetate and lead citrate.

For negative staining, samples of living organisms were suspended in 2% potassium phosphtungstate (pH 7.0) and placed on Formvar-coated grids. Excess stain was removed and the grids were set aside to dry. The material was examined with an EMU-3F microscope (RCA).

**RESULTS**

Cell surface. The basic cell wall structure of *V. cholerae* strain 569B is morphologically similar to that described for *Escherichia coli* (5). Thus, the same terminology will be employed. The outer surface of the cell is thrown into irregular folds (Fig. 3, 4). Upon close examination, a unit membrane organization (Fig. 1) is seen. This has
been called the L membrane by DePetris (5). The cytoplasmic membrane (CM) also shows the trilaminar configuration. Intermediate between these two membranes is a $G$ layer composed of a clear (g$_1$) and a granular (g$_2$) layer. The M layer, intermediate between g$_2$ and CM in E. coli, is not readily evident in our preparations. However, careful examination of V. cholerae under a variety of experimental conditions may reveal its existence. The densely staining portions of the outermost (L) and innermost (CM) surface membranes of V. cholerae, as well as the g$_1$ layer, appear to be composed of granules 4 to 5 nm in diameter. Cholera toxin formation has been attributed to vesiculation of large segments of the outer L membrane of V. cholerae (1).

**Changes in cell structure during the growth cycle (30°C).** Recently the nutritional parameters of cell growth and toxin production were closely examined (13). Under the conditions employed here for cell growth (30°C, vigorous aeration), an initial inoculum of cells reached logarithmic phase in about 3 to 4 hr (Fig. 2). By 8 hr, the culture was in early stationary phase with the death phase occurring shortly after. Toxin production (Fig. 2) was not measurable until the early logarithmic phase (titer 400 to 500 blueing doses/ml). By mid-logarithmic phase, toxin levels increased significantly to a titer of 4,000 to 5,000 and reached a peak of production by late logarithmic phase (8,000 to 20,000). No significant increase in toxin production was observed after late logarithmic phase of growth. Cultures examined at the various intervals during toxin elaboration revealed the following changes.

Early logarithmic-phase cells (optical density 1.0 at 640 nm) showed no alteration in either the outer L membrane or the cytoplasmic membrane (Fig. 3). The wavy appearance of the outer cell surface (L membrane) is quite evident (Fig. 3). Fine fibrous chromatin is distributed throughout the mid-region of the cell and is surrounded by
densely packed ribosomes. No significant change is seen in the cells at an optical density of 2.

By mid-logarithmic growth (optical density 3), little change occurred in cell structure (Fig. 4). No alterations in the surface membrane are evident, but an increase in the nuclear area and a closer packing of ribosomes seem to occur. Chromatin is still finely fibrous in nature and toxin production (Fig. 2) appears to be maximal. The excellent quality of fixation obtained with the glutaraldehyde-osmium fixative is evidenced (Fig. 4) by the preservation of the few spheroplasts present at this time. This is not true of cells fixed with the classical Kellenberger technique in which spheroplasts lyse during the extended fixation process.

During late logarithmic and early stationary phase, the only changes which occur are in the nuclear-cytoplasmic ratio. This seems to be most pronounced by 12 hr of culture growth, at which time there appears to be extensive increase in the nuclear area. In addition, the ribosomal content of the cells seems to be reduced when compared with both early and late logarithmic cells. By 24 hr of growth (Fig. 5), significant alteration in cell structure has occurred. The number of spheroplasts has greatly increased and numerous other changes in the cells are evident. However, cell lysis at 30 C is not extensive. The chromatin material tends to be more coarsely defined and the nuclear area is swollen. Ribosomes are less distinct and fewer in number, and significant separation of the cytoplasm and cytoplasmic membrane from the L membrane has occurred. Just as during all other intervals in the growth cycle, the 12- and 24-hr cells show no apparent vesiculation of the outer L membrane. However, cell samples fixed by the procedure of Kellenberger et al. (9) showed the same basic changes described above and also exhibited extensive alteration of the outer (L) membrane. These alterations appeared primarily as a blebbing of vesicles from the L membrane. This vesicle formation is evident throughout the growth cycle but appears to be most pronounced during early stationary phase and during the death phase (8 to 24 hr) of the culture, when toxin production is not occurring.

To further investigate the possibility that membrane vesiculation is responsible for toxin production as proposed by Chatterjee and Das (1), a logarithmic growth culture was prepared for electron microscopic examination as follows. Cells were grown as described above (30 C), and two samples were taken at optical density 3. One sample was centrifuged for 15 min at 1,500 × g (normal procedure for electron microscopy). The pellet was fixed for electron microscopic examin-
an artifact of the fixation employed and is not related to toxin production in *V. cholerae* strain 569B. However, the increased tendency for vesiculation during late logarithmic and early stationary phase, under suboptimal conditions of fixation, and the associated swelling of the nuclear area suggest an increased permeability of the outer membranes of the cell during these phases of the growth cycle.

**Effect of temperature on cell structure.** Observations by Craig (3) suggest that toxin production is greater at 30°C than at 37°C. Furthermore, he believes that toxin production is associated with cell lysis. Since lysis is more extensive at 37°C, we decided to examine the effects of the surface-to-volume ratio and growth at 37°C on cell structure and toxin production. A set of four 300-ml flasks containing 50, 50, 50, and 250 ml of medium were inoculated with *V. cholerae* (10⁹ colony-forming units/ml). Two 50-ml flasks and the 250-ml flask were incubated at 30°C with agitation. The remaining 50-ml flask was incubated at 37°C. At 7.5 hr, 9-ml samples were removed from each flask for electron microscopy and 5-ml samples were taken for toxin titer determination and biochemical analysis. One of the 50-ml flasks (shallow culture) and the 250-ml flask (deep culture) were transferred to 37°C, and all flasks were agitated for an additional 16 hr. At 24 hr, samples were taken for electron microscopy.

Certain differences are evident between 50-ml and 250-ml cultures grown for 7.5 hr at 30°C. The 50-ml culture appears comparable to a typical late logarithmic- or early stationary-phase
culture, whereas cells in the 250-ml culture are similar in appearance to early logarithmic-phase cells. The 37 C (shallow) culture was indistinguishable from the 30 C (shallow) culture after 7.5 hr of growth.

No apparent change was observed in the structure of cells grown in 250 ml of medium (deep culture) for 24 hr at 37 C when compared with samples of the same culture taken at 7.5 hr. On the other hand, the appearance of the 24-hr shallow culture at 30 C was entirely different. The cells were in poor morphological condition with abnormal nuclei. In most of the cells the cytoplasm was retracted from the outer (L) membrane, leaving an apparently empty area between it and the cell membrane, similar to the condition seen in Fig. 5. Large numbers of spheroplasts and some disrupted membrane material were also evident. The shallow culture incubated at 37 C for 16 hr showed considerably more disruption than the comparable 30 C culture. The only identifiable structures were greatly distended spheroplast-like bodies devoid of most of their cytoplasmic and nuclear elements. Lyed cell fragments were abundant, as was cytoplasmic debris. Biochemical data from these cultures (Table 1) indicate that vibrio adenosine triphosphatase activity (an indicator of cell lysis, reference 14) in the shallow culture increased roughly in proportion to the degree of lysis observed. Total nucleic acid concentrations in the supernatant fraction correlated well with adenosine triphosphatase activity and cell lysis. Proteinase activity decreased with time in all of the shallow cultures, particularly in the flask incubated at 37 C for 24 hr (Table 1). Toxin titers were significantly lower in the deep cultures as compared with the shallow. In one case, shallow culture titers appeared to increase and in two cases they seemed to decrease between 8 and 24 hr. However, the limits of the toxin bioassay techniques make these changes of questionable significance. Certainly, there is no significant increase between the end of logarithmic growth (8 hr) and 24 hr. Taken together these data suggest that, when cell lysis is excessive (as at 37 C for 24 hr), toxin may be destroyed or inactivated, and the degree of supernatant fluid contamination with nontoxic cellular components is greatly increased.

Surface changes of toxin-producing cells visible with negative staining. To determine whether some morphologically identifiable substance
**Table 1. Supernatant components from Vibrio cholerae strain 569B (as a function of time and temperature)**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Growth conditions</th>
<th>ATPase*</th>
<th>Nucleic acid (%)</th>
<th>Proteinase activity (relative)</th>
<th>Titer (blueing dose/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deep</td>
<td>7.5 hr, 30 C</td>
<td>1.53</td>
<td>8.0</td>
<td>5.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>+16.5 hr, 37 C</td>
<td>10.30</td>
<td>2.5</td>
<td>25.4</td>
<td>1,000</td>
</tr>
<tr>
<td>Shallow</td>
<td>7.5 hr, 30 C</td>
<td>2.93</td>
<td>13.0</td>
<td>16.9</td>
<td>8,000</td>
</tr>
<tr>
<td></td>
<td>24 hr, 30 C</td>
<td>33.30</td>
<td>13.0</td>
<td>16.9</td>
<td>16,000</td>
</tr>
<tr>
<td></td>
<td>+16.5 hr, 37 C</td>
<td>36.70</td>
<td>13.0</td>
<td>16.9</td>
<td>4,000</td>
</tr>
<tr>
<td>Shallow</td>
<td>7.5 hr, 37 C</td>
<td>10.00</td>
<td>14.0</td>
<td>14.7</td>
<td>2,000</td>
</tr>
<tr>
<td></td>
<td>24 hr, 37 C</td>
<td>18.90</td>
<td>14.0</td>
<td>14.7</td>
<td>8,000</td>
</tr>
</tbody>
</table>

* Adenosine triphosphatase. Results expressed as micromoles of P₄ per hour per milliliter.

...might be released from *V. cholerae* but not preserved by standard fixation and sectioning techniques, whole cells were negatively stained during various intervals of the growth cycle. Unaerated cultures, which might be equated with deep cultures with regard to toxin production (Table 1), were first examined to determine the basic surface configuration of cells low in toxin elaboration. On the surface of these cells are filamentous extensions which have an average diameter of 15 nm and appear several nanometers in length (Fig. 6), although actual length is difficult to estimate. These filamentous processes appear to have a double-layer organization and arise directly from the cell surface (Fig. 6). Upon close examination, each layer appears to be composed of a row of granules, each granule measuring about 4 to 5 nm in diameter. Although in some areas these projections appear to have a unit membrane organization, they are often much narrower at their point of origin on the cell surface (Fig. 6). Furthermore, cells fixed and...
dehydrated according to the procedure for preparation of cells for thin sectioning and negative staining show no surface material, only the basic L membrane waviness. Thus, we feel that the cell surface structures revealed by negative staining but not preserved by the procedures used for thin sectioning constitute something other than shedding of the L membrane.

Certain surface changes occur during logarithmic growth of toxin-producing cells. During early logarithmic growth, the amount of surface material present is small, similar in degree to that seen in still cultures (Fig. 6). By mid- to late logarithmic growth, when toxin production is at its peak, a large amount of surface material is evident. This material forms large projections from the cell surface (Fig. 7). Examination of stationary-phase cells, after toxin production has reached maximum, reveals that elaboration of this surface material has dropped to a level comparable to that seen in early logarithmic growth. Thus, the amount of surface material visible with negative staining is small in early logarithmic growth. It becomes most pronounced during the period of maximal toxin production and is again reduced when the population reaches stationary

![Image](http://jb.asm.org/)  
**Fig. 7.** Extensive material given off from the cell surface during periods of maximal toxin production (logarithmic growth). This material is reduced in both early logarithmic- and stationary-phase cells. ×63,000.
phase. These changes correlate well with the timing of toxin production in \textit{V. cholerae}. If these surface changes were due to cell lysis, one would expect them to be greatest during the late logarithmic and stationary phases of growth, when membrane permeability seems to be greatest.

**DISCUSSION**

Our morphological and biochemical data support previous observations (2, 13) that cell lysis is not a factor in toxin production for \textit{V. cholerae} strain 569B. In fact, failure to harvest toxin prior to the onset of stationary phase will result in an increase in the liberation of certain cellular enzymes into the medium, owing to cell lysis. This appears to be particularly true for cells grown at 37 C for 24 hr. During logarithmic growth, the ribosome content of the cells seems to be reduced and the nuclear area increased, especially during late logarithmic growth. No other changes are visible at this time in sectional material.

The major changes which occur during the growth cycle of \textit{V. cholerae} are on the surface of the cells. These changes correspond in timing to the events of toxin (PF) production described previously (13) and in these studies. The increased appearance of this extracellular material can be observed only in whole-mount preparations and is not preserved in the routine preparations for thin sectioning. In addition, cells actively producing toxin do not show any change in L-membrane structure comparable to the vesicle formation reported by Chatterjee and Das (1). When employing suboptimal fixation, vesicle formation can be observed to some degree throughout the growth cycle. However, the presence of these membranous outpocketings increases as late logarithmic growth is attained, and they reach their greatest number in stationary phase, well after toxin production has ceased. This increased vesicle formation may indicate increased permeability of the L membrane during the latter part of logarithmic growth, but it is felt that the appearance of vesicles is a fixation artifact in \textit{V. cholerae} strain 569B. This is supported by the absence of any vesicles in toxin containing supernatant fractions.

The material observed on the surface of toxin-producing cells seems to be composed of granules 4 to 5 nm in diameter. The cell surface of \textit{V. cholerae} is composed of a series of membranes (L membrane, g2 layer, and cytoplasmic membrane) or layers which appear to be made up of 4- to 5-nm subunits. In view of the proposed lipid content of cholera toxin (2), Richardson (13) recently suggested that toxin may be related to some component of the cell envelope. Morphological data presented here seem to support this hypothesis. The size (4 to 5 nm) of the subunits which make up the extracellular material is comparable to the morphological subunits comprising the membranes of the cell envelope. Furthermore, that toxin production may represent a failure of the cell to properly incorporate this subunit (possibly into the L membrane) is suggested by the apparent change in permeability of this membrane as reflected by vesicle formation in a hypotonic fixative. Failure to obtain a 4- to 5-nm granule fraction upon ultracentrifugation may indicate that the toxic moiety is a subfraction of the structure initially released by the cell. In fact, Coleman et al. (2) report that the toxin has a molecular weight of about 12,000. This is smaller than would be expected for a particle 4 to 5 nm in size. The extracellular surface material described in this study is distinct from bacterial endotoxin in size and time of production. For example, endotoxin extracted from \	extit{Veillonella parvula} (11) consists of vesicles ranging in size from 25 to 140 nm. Also, in vivo production of extracellular lipopolysaccharide in \textit{E. coli} (16) is most pronounced during stationary phase, resulting in the formation of globules 12 to 200 nm in diameter, considerably larger than the 4- to 5-nm granules reported here for \textit{V. cholerae}. The vesicles observed by Chatterjee and Das (1) more approximate those described during endotoxin production in \textit{E. coli} (16). In our studies, cholera toxin production (PF) is completed by stationary phase, at which time cell autolysis and thus the appearance of extracellular lipopolysaccharide would be expected to be most pronounced. Whether this extracellular material is in fact cholera toxin (PF) or a precursor of the toxic moiety and whether it represents an improperly incorporated subunit of the cell envelope liberated during imbalanced growth (6) is presently being investigated.

**ACKNOWLEDGMENTS**

This research was supported by Public Health Service grant AI 07772 from the National Institute of Allergy and Infectious Diseases and grant K3-00018 to S. H. Richardson.

We express our appreciation to Ellen B. Hirsh and Carolyn Benz for technical assistance during the course of these studies.

**LITERATURE CITED**