Morphological Changes of *Vibrio cholerae* during Toxin Production

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Abstract : Morphological changes of *Vibrio cholerae* during toxin production were analyzed by electron microscopy. The appearance of large round cells, bulgings out (or buddings) of the cell surface, and membrane enveloped particles were observed. The particles were often seen on the cell surface, and their size ranged mainly from 500–1,000 Å in diameter. The same kind of particles were also seen in the widened periplasmic space, and seemed to originate from the cytoplasmic membrane. These changes were not generally observed in a non-toxigenic strain, nor in a toxigenic strain grown under conditions in which toxin was not produced.

INTRODUCTION

Since the beginning of the 7th Cholera pandemy in 1961, the *Vibrio cholerae* organism and its associated disease has been intensively investigated. The enterotoxin produced by *Vibrio cholerae* which is now known to be an exotoxin, was completely purified and crystalized by Finkelstein and LoSpalluto (1972). Although many theories on the mechanism for the development of choleraic diarrhea have been described, it is now generally thought the elevation of cyclic AMP in the intestinal epitherial cells by the action of cholera toxin plays a role in establishing this syndrome (Field *et al.*, 1968; Field, 1971; Chen *et al.*, 1971; Chen *et al.*, 1972). Although the role of cholera toxin has been well studied, the mechanism of its production by *V. cholerae* is still poorly understood. The relationship between the morphology of *V. cholerae* and its toxin production was discussed by Chatterjee and Das (1967), Kennedy and Richardson (1969), Fernandes and Smith (1977), and Fernandes *et al.* (1977), although contradictions exist in their observation and conclusions. This paper describes the morphology of highly toxigenic and non (or weakly) toxigenic *V. cholerae* during the presence or absence of toxin production under a variety of culture conditions.

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MATERIALS AND METHODS

Organism: *Vibrio cholerae* 569B, a highly toxigenic strain, and *V. cholerae* El Tor K-25, a non (or weakly) toxigenic strain were used in this study. Stocks of the strains were maintained by subculture in soft agar.

Culture: About 5×10^7 organisms from a stock grown overnight on meat extract agar slants were inoculated into either 200 ml Erlenmeyer flasks, or 50 ml bottles which contained 50 ml of media (pH 7.2) consisting of 0.5% NaCl, 0.5% yeast extract, and 3% Bacto peptone (Difco). The 200 ml Erlenmeyer flask cultures were shaken in a 30°C water bath, and the 50 ml bottles cultures were kept stationary at 37°C. Samples were then taken for viable cell counts, toxin assays, and morphological analysis.

Toxin assay: A cell free culture filtrate was prepared by centrifugation followed by filtration. The amount of toxin in the filtrate was measured by the rabbit skin test (Craig, 1965). Purified cholera toxin for the standardization was derived from The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan.

Electron microscopic technique: Cell pellets were obtained by centrifugation at 8,000 g for 30 minutes, fixed in 2% cold glutalaldehyde with 0.1 M phosphate buffer (pH 7.2) for 16−20 hours, and then postfixed in 1.5% osmium tetroxide in phosphate buffer for 2 hours. The fixed samples were serially dehydrated by ethanol and acetone, and embedded in Epon. Ultrathin sections were cut on a REICHERT Om U2 microtome with a glass knife, and stained with ulanyl acetate and lead citrate. Whole cell observations were carried out on living vibrio by placing a drop of culture on Formvar coated grids and allowing them to be air-dried. The grid was negatively stained by 2% potassium phosphotungstate, and observed with a JEOL-JEM 100B transmission electron microscope.

RESULTS

Growth curve and toxin production: As shown in Fig. 1, the culture of *V. cholerae* strain 569B had an initial cell concentration of 1.2×10^7/ml. The patterns of growth and toxin production observed differed according to the culture conditions used. When strain 569B was cultured in a shallow medium and shaken at 30°C, the resting phase was about 2 hours, followed by an 8 hour period of logarithmic growth. The culture reached a maximum viable cell density of 8.0×10^9/ml after 24 hours. On the other hand, the same strain cultured in stationary, deep medium at 37°C showed logarithmic growth in the initial 4 hours, and reached maximum viable cell count of 5.2×10^8/ml after 6 hours. The density of viable cells remaining after 24 hours was 1.5×10^7/ml. Toxin production correlated with cell growth when the organism was cultured with shaking at 30°C, but no production was seen in the stationary culture at 37°C. Under these same conditions, *V. cholerae* strain K-25 showed about the same growth curve kinetics as strain 569B, as seen in Fig. 2, but production of toxin was not observed.

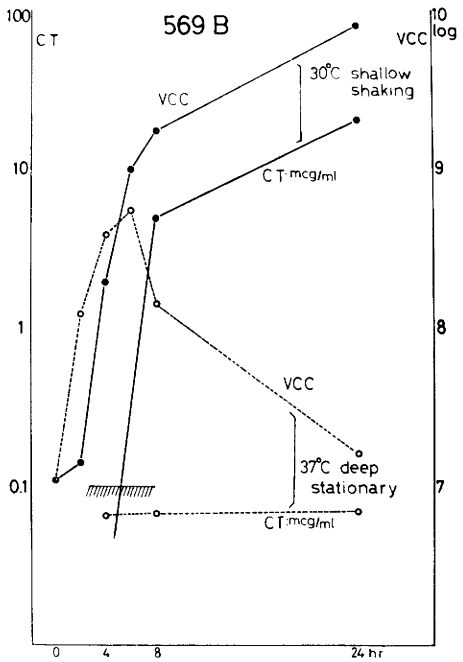


Fig. 1. Growth curves and toxin production in different culture conditions Strain 569B.

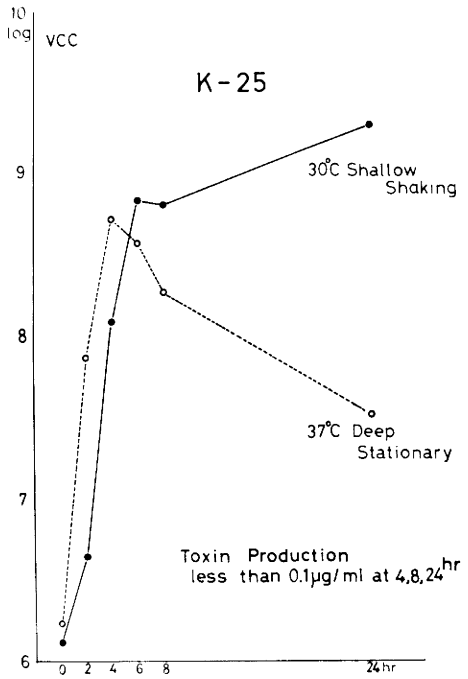
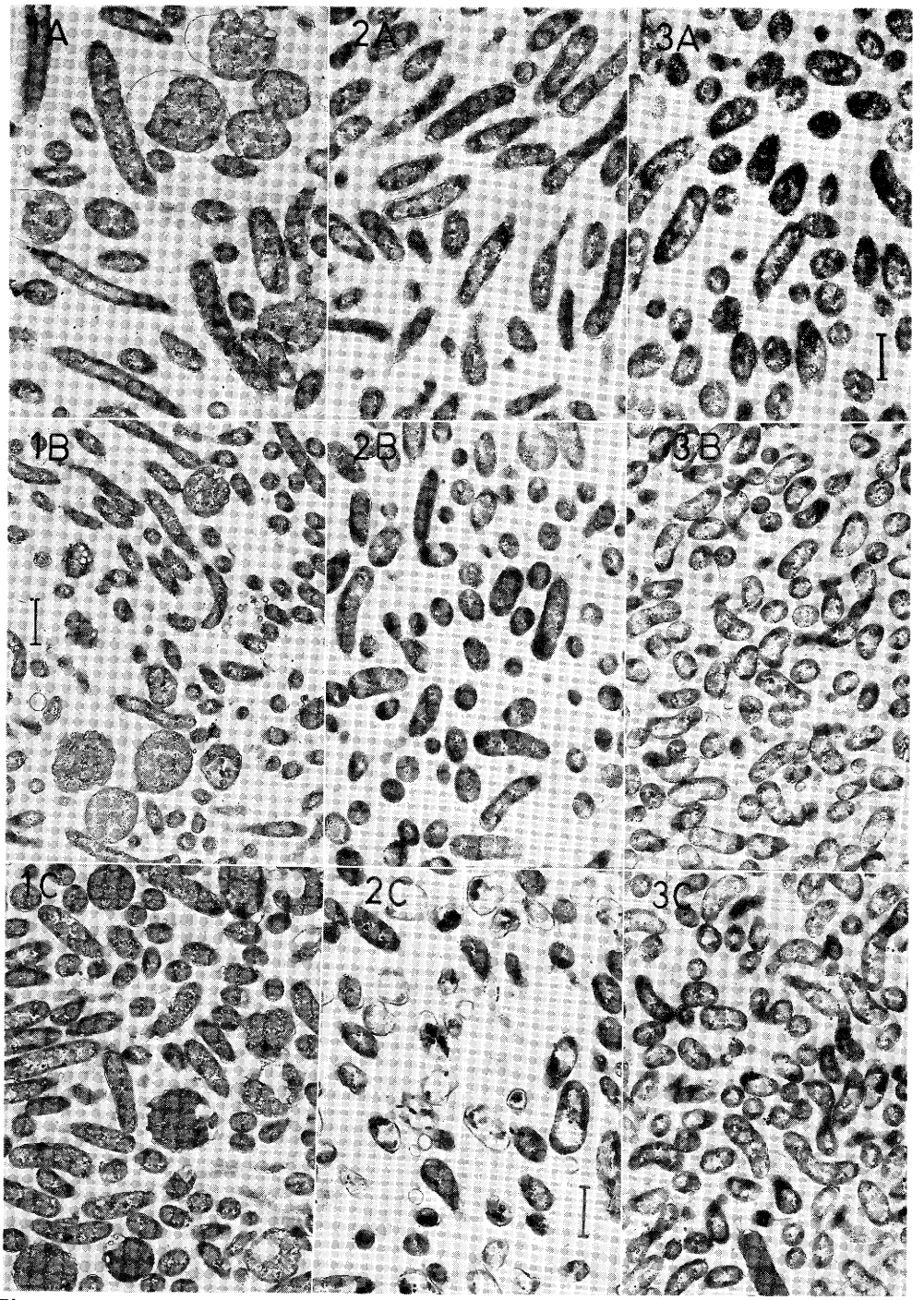


Fig. 2. Growth curves and toxin production in different culture conditions Strain K-25.

Cell morphology: Strain 569B, when grown in a shaking culture of shallow medium at 30°C, showed pleomorphic changes characterized by the presence of large round cells. This pleomorphism was already evident at 4 hours, and was still observed after 24 hours (Photos. 1-A, B, C). When the same strain was cultured in stationary deep medium at 37°C, pleomorphism was not observed. Although the cells exhibited a regular rod shape morphology at 4 and 8 hours (Photos. 2-A, B), many ghost cells appeared after 24 hours (Photo. 2-C). Strain K-25 did not show this pleomorphism, even when cultured at 30°C with shaking (Photos. 3-A, B, C).

An examination of pleomorphic cells at high magnification revealed a bulging out (or budding) of the cell surface, and the presence of membrane enveloped particles (Photos. 4,5,6). Widened periplasmic spaces which formed a vacuole-like pattern often compressed the cytoplasm (Photos. 4-A, C, D; Photos. 5-A, B). The bulging out of the cell surface seemed to originate from the cell wall, and this phenomena may be responsible for the production of the membrane enveloped particles (Photos. 4-A, B; Photos. 5-C, D). Some of the particles have a lamellar structure (Photos. 5-A, B). The membrane enveloped particles were also seen in the periplasmic space, and there they seemed to originate from the cytoplasmic membrane (Photos.5-A, B). The particles were of various sizes ranging mainly from 500−1,000 Å in diameter (Table 1). These morphological



Photos. 1, 2, 3. Morphologies of the cells at the time of sampling.
A, 4 hours culture; B, 8 hours culture; C, 24 hours culture.
1: The strain 569B cultured at 30°C with shaking in shallow medium. Large round cells appeared after 4 hours in cultures and were still observed at 24 hours. 2: The strain 569B cultured at 37°C in stationary deep medium. Large round cells are not seen. Cells have a regular rod-shape at 4 and 8 hours, but many ghost cells are seen at 24 hours. 3: The strain K-25 cultured at 30°C with shaking in shallow medium. The cells have a regular rod-shape in all three samples. Scales indicate one micron.

changes were rarely observed with the strain K-25, and in strain 569B when it was cultured in stationary deep medium at 37°C.

Whole cells visualized by negative staining were coated with a substance that made them appear ragged, regardless the culture of origin (Photo. 7-A). This substance was removed by washing the cells with a buffer solution, revealing bulgings of the cell wall (Photo. 7-B).

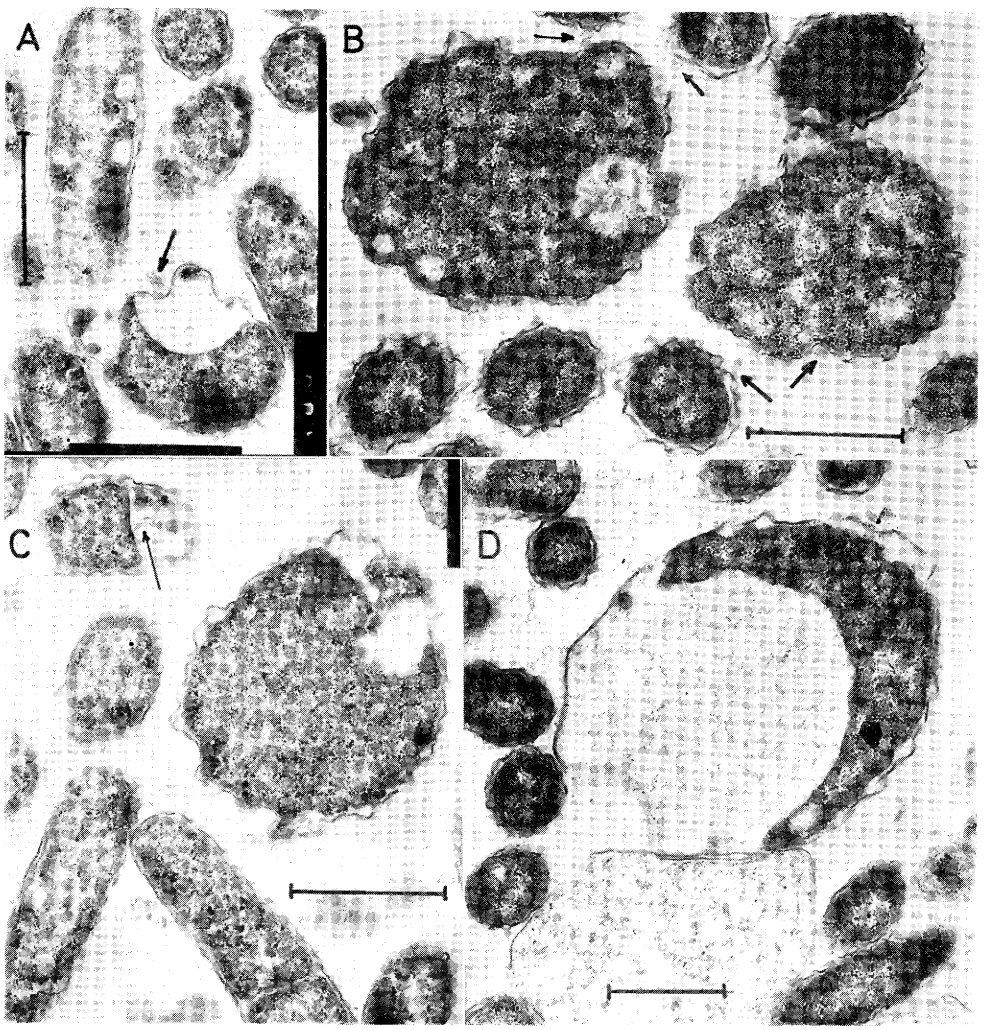


Photo. 4. High magnification of pleomorphic cells (569B 30°C).
Vacuole-like periplasmic spaces compress the cytoplasm (A, D). Note the small protrusion from the other membrane (A, B: arrows) and from the cytoplasmic membrane (C: arrow). Scales indicate one micron.

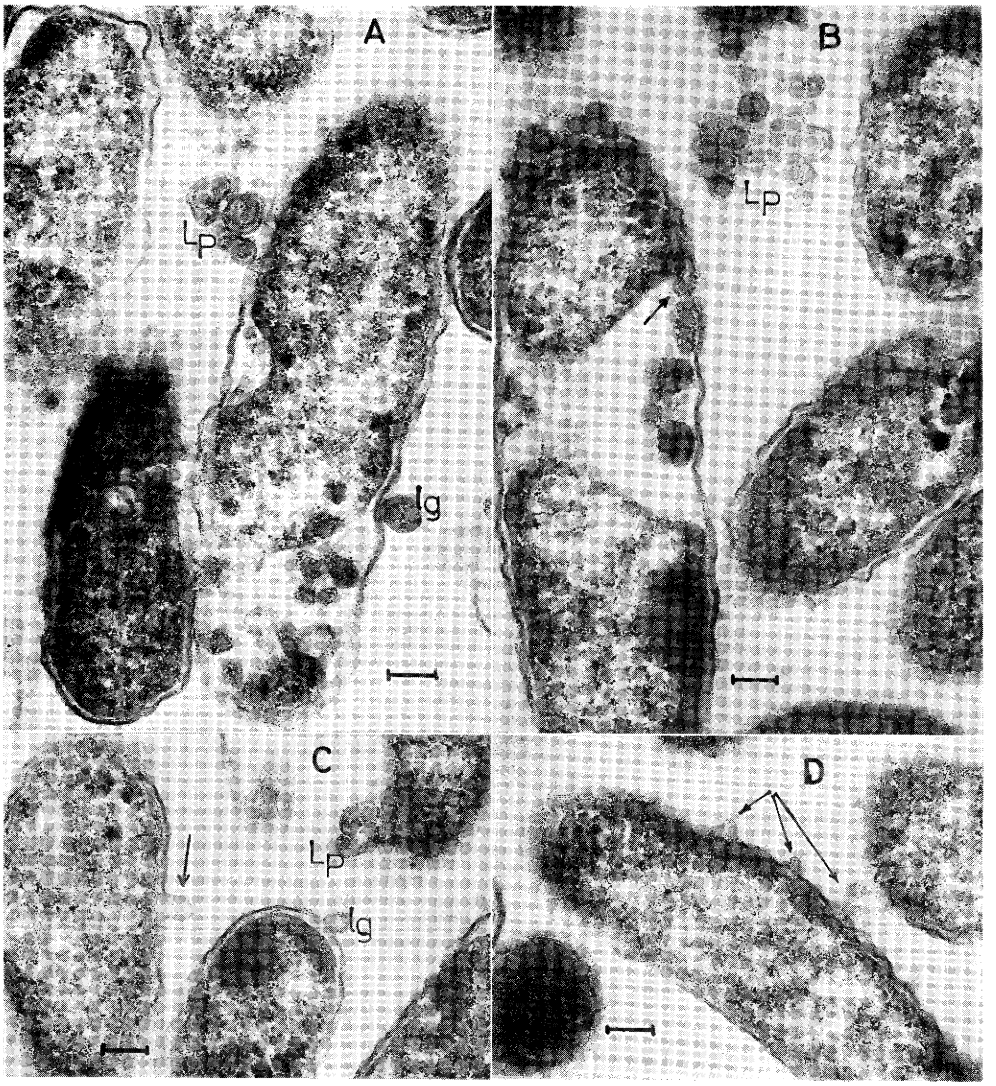


Photo. 5. Higher magnification of pleomorphic cells (569B 30°C).
Bulging out of the cell wall (C: arrow) and of the cytoplasmic membrane (B: arrow). The process of producing membrane enveloped particles, by the bulging out and pinching off of the cell membrane, is observed (D: arrows). Large particles are seen on the cell surface (A, C: lg). Small ones are seen elsewhere (A). The particles are also seen in the widend periplasmic space (A, B). Lamellar bodies are evident in pictures A and B, originating from the cell wall (C: Lp). Scales indicate 0.1 micron.

Table 1. Size distribution of particles 569B, 30°C, shaking

diameter \ phase	4 hours	8 hours	24 hours	Total
<500Å	19 (35)	32 (32)	40 (77)	91 (33)
500−1,000Å	19 (35)	41 (41)	66 (53)	126 (45)
1,000−1,500Å	8 (15)	17 (17)	14 (11)	39 (14)
1,500Å<	8 (15)	9 (9)	4 (3)	21 (8)
Total counts	54 (100)	99 (100)	124 (100)	277 (100)

Number of counted particles, (%).

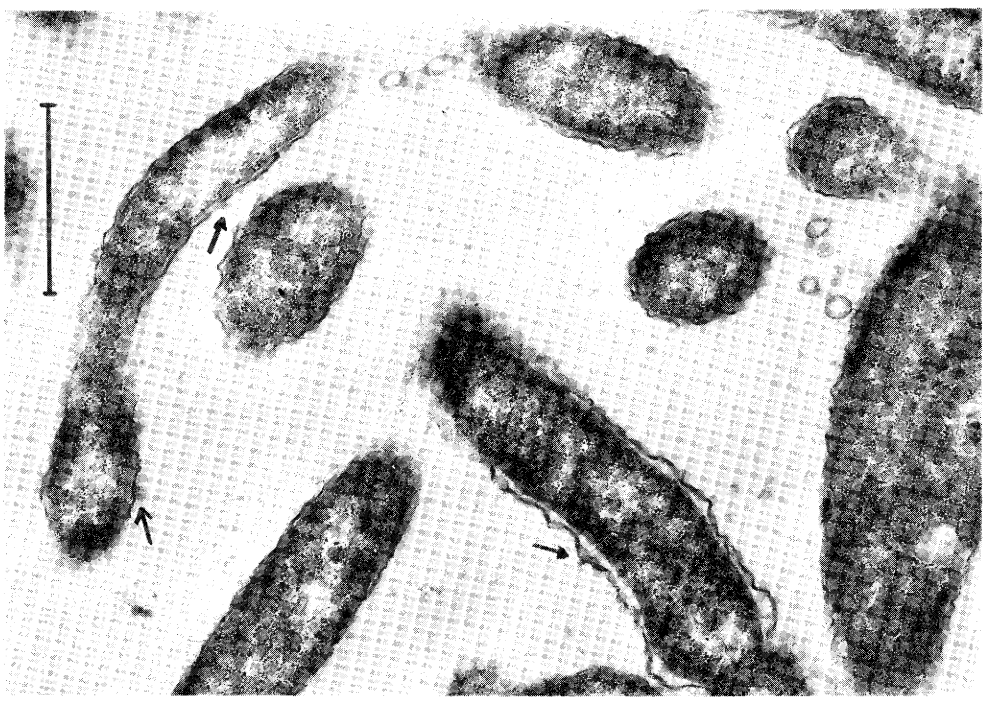


Photo. 6. Membrane enveloped particles detached from the cells. Bulgings of the cell surface are also present in places (arrows).

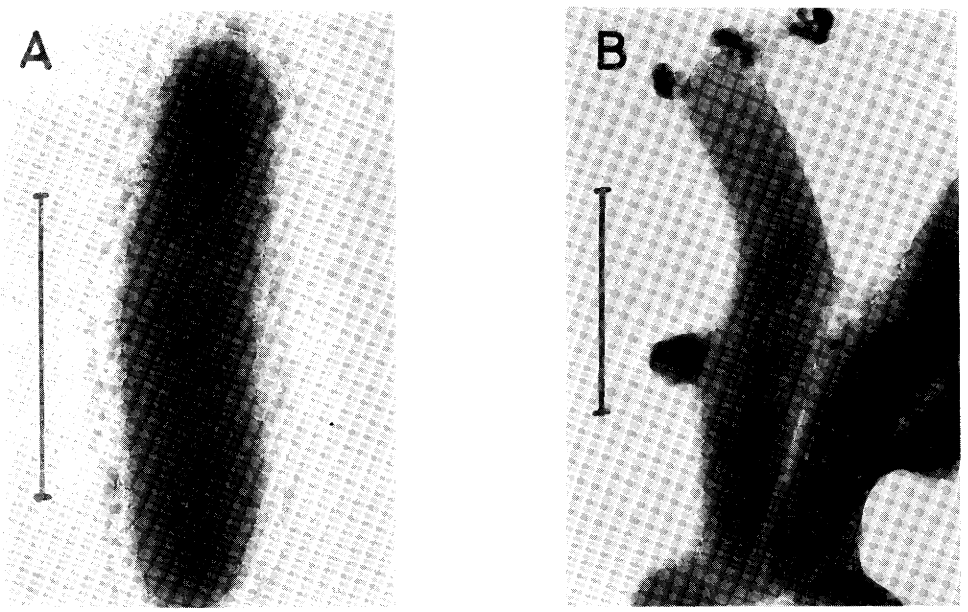


Photo. 7. Negative staining of the strains. A, K-25 cultured at 30°C for 8 hours. The surface of the organism appears ragged. B, 569B during toxin production, stained after washing by buffer solution. The ragged appearance at the surface of the organism is eliminated. Bulgings of the cell surface can now be clearly seen. Scales indicate one micron.

DISCUSSION

Vibrio cholerae produced many kinds of proteins other than enterotoxin during its growth, including enzymes such as protease, phosphatase, lecitinase, phospholipase and kitinase (Barua and Burrows, 1974). Previous studies have not substantially established a relationship between toxin production and morphological changes (Chatterjee and Das, 1967; Kennedy and Richardson, 1969; Fernandes *et al.*, 1977; Fernandes and Smith, 1977). In order to rule out the possibility that proteins other than toxin might be responsible for the morphological changes of *V. cholerae*, another strain of *V. cholerae* which does not produce toxin in vitro was employed as a control.

In this study, it was noted that toxin production was accompanied by the appearance of pleomorphism in the cell culture. The pleomorphism was mainly characterized by large round cells, and the release of membrane enveloped particles following budding of the cell surface. These changes were rarely seen in either the culture of strain K-25, or strain 569B cultured in stationary deep medium at 37°C.

Large round cells were reported by Kawata and Inoue (1963) and Fernandes *et al.* (1977), who observed this morphology during suboptimal culture condition. Kawata and Inoue made no reference to toxin production, but Fernandes *et al.* reported a possible

relationship between large round cell and toxin production only in anaerobic cultures.

Suprisingly, Chatterjee and Das (1967), and Kennedy and Richardson (1969) did not mention the large cell morphologies which can be seen in photographs presented in the paper of Kennedy and Richardson, as these were the most characteristic morphologies that appeared in our study.

The membrane enveloped particles appeared to originate from bulgings of the cell surface. These were similar to the sac-like structures reported by Chatterjee and Das (1967), who demonstrated that bulgings of cell walls were pinched off to produce particles. Although they speculated that this was a process of toxin excretion, they did not titrate the toxin, and had incubated the culture at 37°C. In our experience, toxin is almost never produced under such conditions.

Kennedy and Richardson (1969), contrary to the data of Chatterjee and Das (1967), reported that a large amount of surface materials formed large projections from the cell wall, and correlated this with toxin production. We report that these extracellular substances were seen in strains that did not produce toxin, and could not be observed after washing the cells with buffer and in regularly fixed thin sections. Therefore we believe that such a correlation to toxin production cannot be demonstrated.

The present study shows that the release of membrane enveloped particles is very likely to be related to toxin excretion, but further clarification of the relationship between the changes of cell surface and excretion of the toxin is needed perhaps by employing immunological techniques with electron microscopy.

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コレラ菌が毒素産生中に示す形態学的変化について

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毒素産生期にみられるコレラ菌の形態学的変化に基づいて，毒素放出の機序を推定しようとする試みが散見されるがまだ一致した見解は得られていない．そこで毒素強度産生性のコレラ菌569B株及び毒素非産生のK−25株を用いて，それぞれ増殖過程にみられる形態学的変化を比較し，毒素産生との関係について検討した．30°C の振盪培養を行うと569B株では毒素産生期に多形性が認められたが，K−25株では全く多形性を示さなかった．また569B株でも37°C 高層静置培養の場合には毒素も産生されず，多形性もみられなかった．これらの所見は多形性と毒素産生との間に何らかの関係があることを示すものといえる．この形態変化の中で特徴的なものは，大円形細胞の出現および細胞壁の突出とそれに続く被膜小体の遊出であり，被膜小体は大半が1,000A 以下であった．また拡大した Periplasmic space には細胞質膜に由来すると思われる被膜小体の存在が認められた．今回の観察によって，毒素非産生株は勿論，毒素産生株でもその産生が無視出来る条件下で培養すると多形性は認められないことが明らかとなり，コレラ菌におけるこのような変化は毒素の産生または放出に密接な関係を有するものと思われた．

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