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Production and Characterization of Cholera toxin-like Enterotoxin by a Non-01 *Vibrio cholerae* Isolated from Food

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ABSTRACT

Non-01 *V. cholerae* caused small localized outbreaks of diarrhoeal diseases and produced cholera toxin-like enterotoxins, heat-stable enterotoxins and thermostable direct hemolysin-like hemolysins. In this paper, we reported on the production of a cholera toxin-like enterotoxin of a non-01 strain isolated form food in Taiwan and the bioactivity and thermostability of this toxin. Production of toxin was optimum by stationary culture in casamino acid yeast extract lincomycin medium incubated at 30°C. This toxin was heat-labile and its cytotonic activity as assayed by Chinese hamster ovary cell was neutralized by anti-cholera toxin antiserum. However, this toxin failed to produce lines of identity with the anti-cholera toxin antiserum in a double gel diffusion assay.

Key words : Cholera toxin, enterotoxin, *Vibrio cholerae,* food

INTRODUCTION

A number of *Vibrio* species have been involved in diarrhoeal diseases, e. g. *V. cholerae, V. parahaemolyticus, V. mimcus, V. fluvialis* I, *V. fluvialis* II, and *V. hollisae*[1,2]. *V. vulnificus, V. alginolyticus* and *V. damsela* involved in wound and other infections[2]. The 01 serotype *V. cholerae,* classical or El Tor biotypes, have been implicated in cholera. It has been recognized for many years that non-01 *V. cholerae* strains also caused small localized outbreaks of diarrhoeal diseases[2-4].

Closely related toxins have been identified in 01 or non-01 *V. cholerae* and *V. mimcus*[5,6], another non-marine *Vibrio* species. Cholera toxin (CT)-like enterotoxins have been most intensively studied[4,5,7,8]. Heat-stable enterotoxin[9] and thermostable direct hemolysin (TDH)-like hemolysin were also found in non-01 *V. cholerae* [9,10]. Some *V. cholerae* also showed cytotoxicity which could be neutralized by anti-shiga toxin antiserum[11].

In our previous study, we isolated a non-01 *V. cholerae* strain from aquacultured food in Taiwan[12] and this strain produced CT-like toxin as detected by reversed passive latex agglutination (RPLA) and hybridized to the CT nucleic acid probe[13]. In this paper, we reported on the production of CT-like toxin by this strain and the bioactivity and thermostability of this toxin.

MATERIALS AND METHODS

I . *Production of enterotoxin*

Non-01 *V. cholerae* strain 11100 was isolated from food in Taiwan[12]. This strain was stocked in 10% skim milk at -70°C and cultured on tryptic soy agar (TSA, Difco Lab., Detroit, Michigan) at 37°C. Single colony was inoculated into tryptic soy broth (TSB, Difco) with 1% NaCl and incubated static at 37°C for 6 hrs (absorbance at 600 nm about 1). One milliliter of this culture was inoculated into 100 ml of casamino acid yeast extract medium (CAYE)[6] and cultured overnight at 37°C. The CAYE culture was used to inoculate (1 ml/100 ml medium) CAYE - lincomycin medium (CAYE-L)[6] and incubated at 30°C for the production of enterotoxin.

Crude toxin was prepared from culture supernatant by ammoniun sulfate precipitation (50 % saturation), dialyzed in TEAN buffer for 24-48 hrs[4] and subsequently in distilled water for 2 hrs. The toxin was lyophilized and stored at 4 °C.

II. *Determination of toxin titer*

Serial dilutions of culture filtrates or crude toxins were prepared. Twenty five μl aliquot from each dilution was assayed for the presence of cholera-like toxin by RPLA kit for CT toxin following the procedures of the supplier (Oxoid Co., Hampshire, England). The titer of toxin was determined as the reciprocal of the highest dilution that gave positive agglutination.

III. *Protein analysis*

The protein concentration was determined with the Coomassie blue dye-binding protein assay kit (Bio-Rad Lab., Richmond, California).

IV. *Agar gel double diffusion test*

Cholera toxin and CT-like enterotoxin preparations were adjusted to have similar titer (about 10 μg/ml of CT) by the RPLA. An agar gel (2%) double diffusion test was performed on microscopic slides[4]. After the precipitin lines developed, agar plates were washed with phosphate-buffer saline, dried and stained with Coomassie brilliant blue.

V. *Determination of cytotonic activity by Chinese Hamster Ovary cell*

The cultivation of Chinese Hamster Ovary (CHO) cells and assay of cytotonic activity were previously described[12]. One hundred μl of 10-fold dilution of toxin preparation was added to 900 μl cell culture (about 10^4 cells/ml) in each

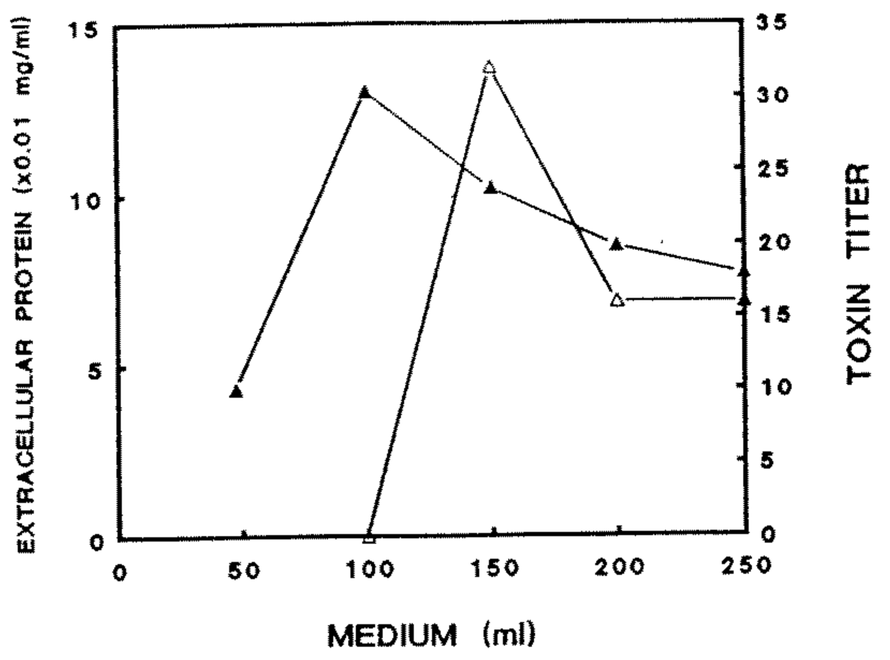

Figure 1. Production of CT-like enterotoxin by non-01 *V. cholerae* in different volumes of CAYE-L medium in 500-ml Erlenmeyer flask. The culture was incubated static at 30°C.
▲, extracellular protein; △; toxin titer.

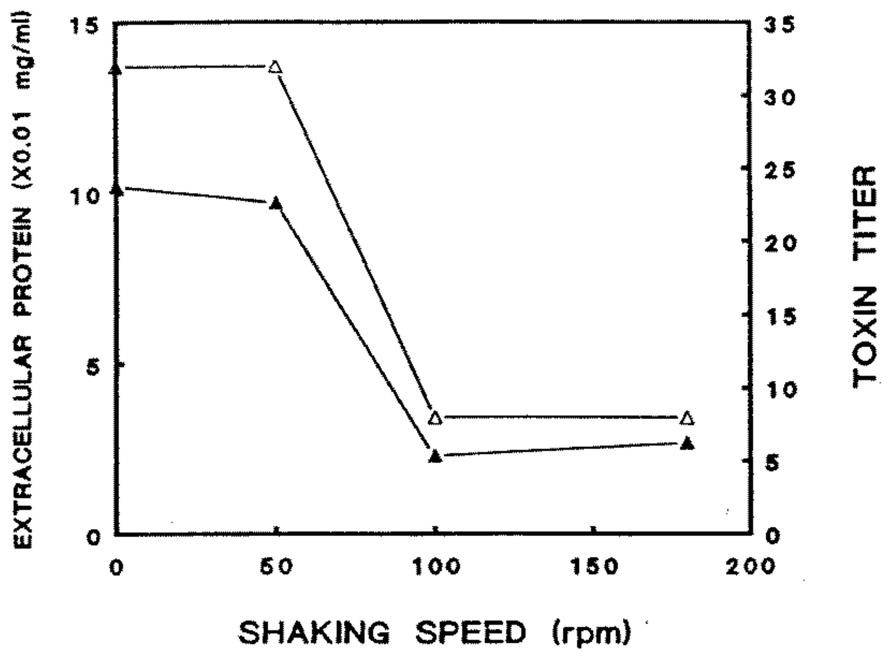

Figure 2. Production of CT-like enterotoxin by non-01 *V. cholerae* in CAYE-L medium incubated at 30 °C and different shaking speeds. ▲, extracellular protein; △, toxin titer.

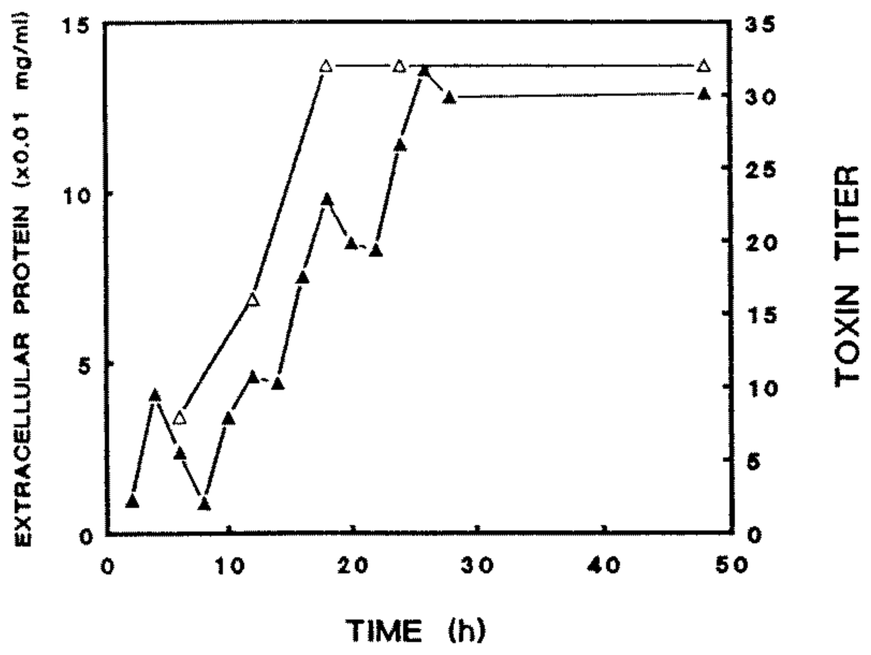

Figure 3. Time course for the production of CT-like enterotoxin by non-01 *V. cholerae* in CAYE-L medium incubated static at 30°C ▲, extracellular protein; △, toxin titer.

well of a 24-well microplate and incubated for 18-24 hrs. The percentage of elongated cells of three microscopic fields was determined (at least 500 cells observed). Means of three replicate experiments were determined. Purified CT (List Lab., Campbell, California) was used as a positive control.

RESULTS

I. *Production of cholera toxin-like enterotoxin*

Production of CT-like enterotoxin by this non-01 *V. cholerae* was optimum at 30°C with a limited amount of aeration. A different volume of CAYE-L in a 500-ml Erlenmeyer flask was inoculated and incubated static 30°C for 24 hrs. The toxin titer was highest at 150 ml medium (Fig. 1) Optimum ratio of surface area/volume of medium for toxin production was 0.6 ㎠/ml. Flasks containing 150 ml medium were cultured at various shaking speed. Optimum toxin production was found in static culture or at low speed, 50 rpm (Fig. 2). The time course for toxin production was shown in Fig. 3. Maximum activity was found at 16 hrs and remained unchanged through the rest of the culture period.

For large production of toxin, 300 ml CAYE-L in 2,000 ml Erlenmeyer flask (surface/volume ratio of 0.6 ㎠/ml) was inoculated and incubated static at 30°C for 24 hrs.

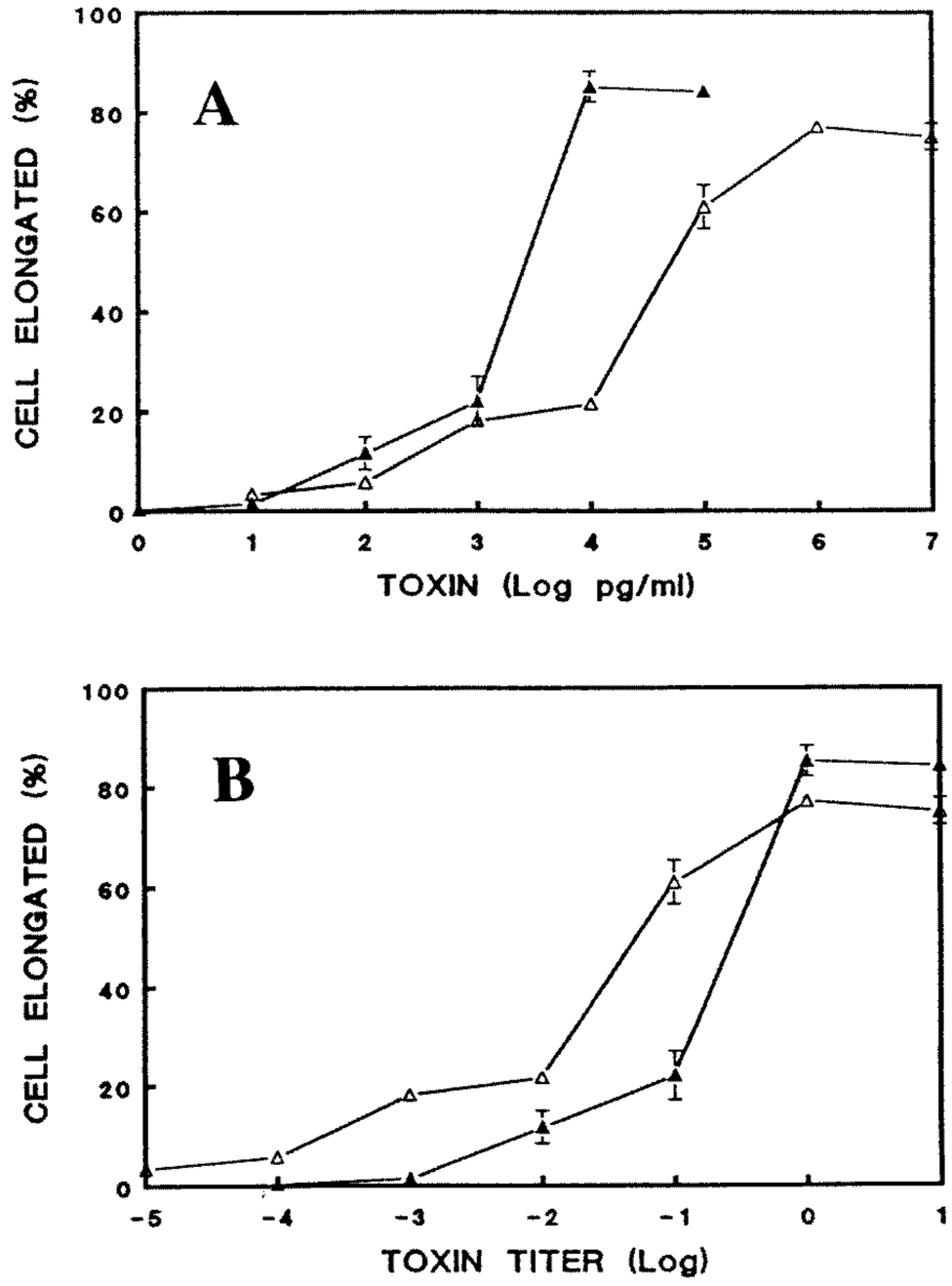

Figure 4. Cytotonic activity of CT-like enterotoxin produced by non-01 *V. cholerae* assayed by Chinese hamster overy cell. Cytotonic activity of CT was compared. A, based on protein quanity; B, based on toxin titer. △, Ct-like enterotoxin; ▲, CT.

II. *Immunological characteristics and bioactivity of cholera toxin-like enterotoxin*

The CT-like enterotoxin produced by this non-01 *V. cholerae* showed similar cytotonic activity like CT. Cytotonic activity of CT and this CT-like enterotoxin based on protein concentration was shown in Fig. 4A. The crude toxin preparation should contain other proteins, so another experiment was performed based on similar toxin titer (determined by RPLA). The CT-like enterotoxin showed higher activity than CT (Fig. 4B).

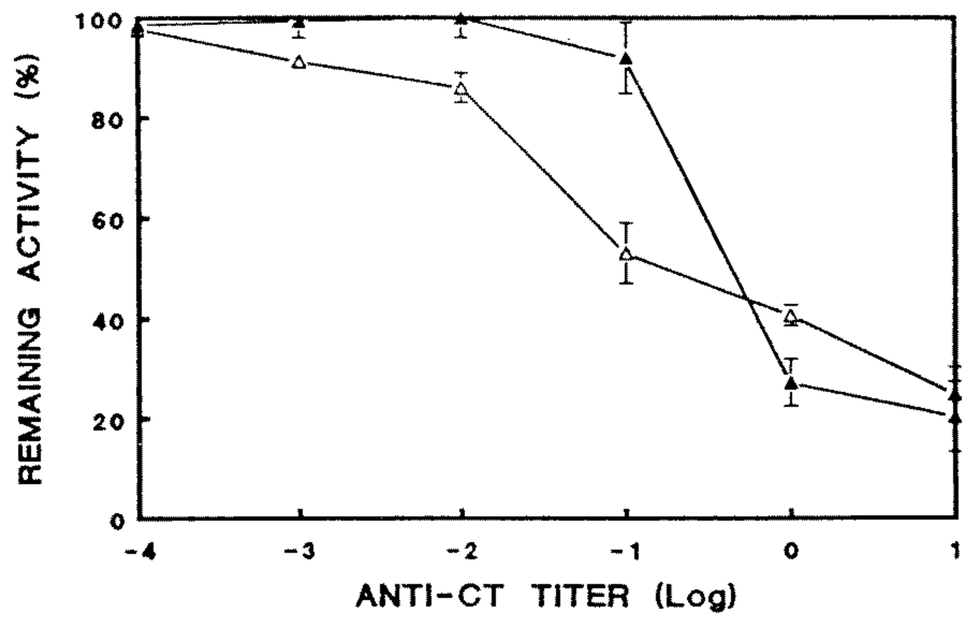

Figure 5. Neutralization of the cytotonic activity of CT and CT-like enterotoxin by anti-CT antiserum. Toxins (titer 1) were mixed with different quantities (titer) of anti-CT antiserum for 2 h and cytotonic activity was assayed by CHO cell. △, CT-like enterotoxin; ▲, CT.

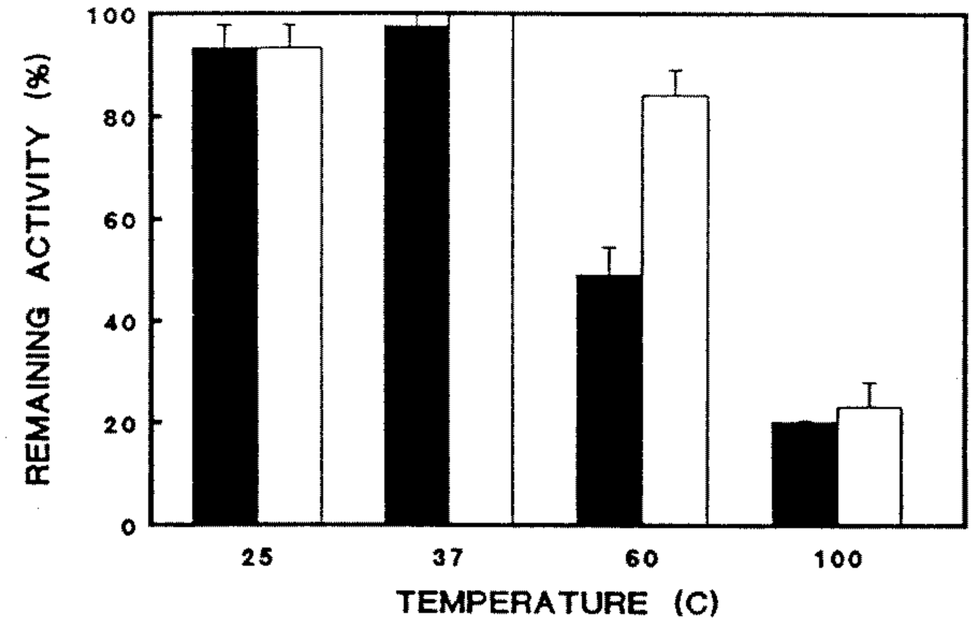

Figure 6. Thermostability of CT-like enterotoxin produced by non-01 *V. cholerae*. The toxin was heated at different temperatures for 10 min and cooled at room temperature (□) or on ice (■).

In a double diffusion test, a precipitin line was found in between CT and anti-CT antiserum , but not in between CT-like enterotoxin and anti-CT antiserum (data not shown).

Neutralization of toxin was performed by mixing the toxin (titer 1) with various 10-fold dilutions of anti-cholera toxin antiserum (List Lab.) and incubated at room temperature for 2 h. Cytotonic activity of the neutralized toxin was determined. Result showed that activity of both the CT and this CT-like enterotoxin was neutralized by incubating with anti-CT antiserum (Fig. 5).

III. *Thermostability of cholera toxin-like enterotoxin*

The toxin preparations were treated at 37°, 60° or 100°C for 10 min and cooled immediately at room temperature (25°C) or on ice. Activity remaining was determined by CHO assay (Fig. 6). The CT-like enterotoxin was denatured permanently after heating at 100°C for 10 min. This toxin was also inactivated by heating at 60°C for 10 min, but activity resumed after being incubated at room temperature.

DISCUSSION

The enterotoxin produced by this local non -01 *V. cholerae* was similar to CT. It was detected by the RPLA kit for CT and its activity was neutralized by anti-CT antiserum. It is also a heat-labile toxin. This strain also hybridized to the nucleic acid probe for CT [13]. However, by using similar toxin quantity (titer) in double gel diffusion assay, it failed to produce lines of identity with anti-CT antiserum. So, this toxin was similar but not identical to CT.

We compared the production of toxin by this non-01 *V. cholerae* in TSB, Syncase medium [14], CAYE and CAYE-L medium [6] and CAYE -L was the best one (unpublished data). Production of toxin was optimum at 30°C with limited oxygen tension achieved by static culture (surface/volume raito of 0.6 ㎠/ml) or low-speed shaking. Aerobic shaking flask culture was usually used for the large production of CT by 01 *V. cholerae*[15], nevertheless, optimum dissolved oxygen tensions for the production of CT-like toxins by non-01 *V. cholerae* were different in many studies. Craig et al.[4] also reported that toxin production by environmental strains of non-01 *V. cholerae* was optimum in static cul-

ture with optimun surface/volume ratio of 2.0 cm^2/ml. However, in another report reciprocal shaking culture with vigorous aeration were optimum for toxin prodcution by *V. mimicus,* 01 (Classical and El Tor) and non-01 *V. cholerae* in CAYE-L medium[6].

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REFERENCES

1. Joseph, S.W., Colwell, R. R. and K J.B. 1983. *Vibrio parahaemolyticus* and related halophilic vibrios. CRC Crit. Rev. Microbiol. 10 : 77-123.
2. Morris, J. G. and Black, R. E. 1985. Cholera and other vibrioses in the United States. N. Eng. J. Med. 312 : 343-350.
3. Kay, B. A., Sack, R. B., Spira, W. M., Guerra, H. E., Guerrero, C.E., Chaparro, E., Yi, A. E., Salazar-Lindo, E., Chea, E., Wachsmuth, I. K. and Davis, B. R. 1984. *Vibrio cholerae* non-01 isolated from five people with diarrhoea in Lima. Lancet. 1 : 218.
4. Craig, J. P., Yamamoto, K., Takeda, Y. and Miwatani, T. 1981. Production of cholera-like enterotoxin by a *Vibrio cholerae* non-01 strain isolated from the environment. Infect. Immun. 34 : 90-97.
5. Spira, W. M. and Fedorka-Cray, P.J. 1984. Purification of enterotoxins from *Vibrio mimicus* that appear to be identical to cholera toxin. Infect. Immun. 45 : 679-684.
6. Spira, W. M. and Fedorka-Cray, P. J. 1983. Production of cholera toxin-like toxin by *Vibrio mimicus* and non-01 *Vibrio cholerae:* batch culture conditions for optimum yields and isolation of hypertoxigenic lincomycin-resistant mutants. Infect. Immun. 42 : 501-509.
7. Iwanaga, M., Yamamoto, K., Higa, N., Ichinose, Y., Nakasone, N. and Tanabe, M. 1986. Culture conditions for stimulating cholera toxin production by *Vibrio cholerae* 01 El Tor. Microbiol. Immunol. 30 : 1075-1083.
8. Yamamoto, K., Takeda, Y., Mwatani, T. and Craig, J. P. 1983. Evidence that a non-01 *Vibrio cholerae* produces enterotoxin that is similar but not identical to cholera enterotoxin. Infect. Immun. 41 : 896-901.
9. Arita, M., Takeda, T., Honda, T. and Miwatani, T. 1986. Purification and characterization of *Vibrio cholerae* non-01 heat-stable enterotoxin. Infect. Immun. 52 : 45-49.
10. Yoh, M., Honda, T. and Miwatani, T. 1986. Purification and partial characterization of a non-01 *Vibrio cholerae* hemolysin that cross-reacts with thermostable direct hemolysin of *Vibrio parahaemolyticus*. Infect. Immun. 52 : 319-322.
11. O'Brien, A. D., Chen, M. E., Holmes, R. K., Kaper, J. and Levine, M. M. 1984. Environmental and human isolates of *Vibrio cholerae* and *Vibrio parahaemolyticus* produce a *Shigella dysenteriae* 1 (Shiga)-like cytotoxin. Lancet, 1 : 77-78.
12. Wong, H. C., Ting, S. H. and Shieh, W. R. 1992. Incidence of toxigenic vibrios in foods available in Taiwan. J. Appl. Bacteriol. 73 : 197-202.
13. Wong, H. C., Shieh, W. R. and Lee, Y. S. 1993. Toxigenic characterization of vibrios isolated in foods available in Taiwan. J. Food Prot. In press.
14. Finkelstein, R. A., Atthasampunna, P., Chulasamaya, M. and Charunmethee, P. 1966. Pathogenesis of experimental cholera : biologic activities of purified procholeragen A. J. Immunol. 96:440-449.
15. Iwanaga, M. and Kuyyakanond, T. 1987. Large production of cholera toxin by *Vibrio cholerae* 01 in yeast extract peptone water. J. Clin. Microbiol. 25 : 2314-2316.

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本土分離之Non-01霍亂弧菌類霍亂毒素之生產與特性

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摘　要

Non-01之霍亂弧菌能產生類霍亂毒素、耐熱腸毒素、類耐熱溶血素等毒素，引起腸炎。我們從台灣地區水產食物中分離到一株能產生類霍亂毒素的non-01菌株，本篇報導該毒素的生產、細胞毒性與耐熱性。最適合毒素生產的培養條件爲：casamino acid yeast extract lincomycin培養基、30°C、低震盪速率或靜止培養。該毒素不耐熱，在100°C加熱十分鐘後便喪失活性。在Chinese Hamster Ovary細胞測驗中顯示，該毒素的腸毒活性與霍亂毒素類似，其活性同時可被抗霍亂毒素抗血清所中和；在double diffusion試驗中該毒素卻不能與抗霍亂毒素抗血清形成作用線，因此該毒素應與霍亂毒素類似但不完全相同。