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New Medium for the Production of Cholera Toxin by *Vibrio cholerae* O1 Biotype El Tor

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A new medium that stimulates in vitro production of cholera toxin by *Vibrio cholerae* O1 El Tor (El Tor vibrios) was developed. The medium contains 0.5% NaCl, 0.3% $NaHCO_3$, 0.4% yeast extract, and 1.5% Bacto-Peptone. El Tor vibrios were cultured in a stationary test tube at 37°C for 20 h. The culture supernatant was assayed for cholera toxin by a reversed passive latex agglutination method. Most vibrios grown in this medium produced 10 to 20 times more toxin than in traditional syncase medium. The number of live vibrios at the end of culture was about 10^8/ml in the new medium (AKI medium) and about 10^{10}/ml in the syncase medium. As a result, each individual organism in the new medium should have produced as much as 1,000 times more toxin than in syncase medium. Sodium bicarbonate was found to be the most important factor in toxin production by El Tor vibrios in the new medium. We recommend this new medium because of its high yield of cholera toxin and its technical simplicity.

Many efforts have been made to increase in vitro production of cholera toxin (CT) by *Vibrio cholerae* O1. As a result of these efforts, syncase medium was developed in 1966 (3), TCY medium was developed in 1969 (8), CAYE medium was developed in 1970 (7), and yeast extract peptone water was developed. These media have been used with aerobic conditions at 25 to 30°C. Investigation with a classical biotype of *V. cholerae* O1 (classial vibrios), especially the high-toxin-producing strain 569B, resulted in these optimum culture conditions. However, the present cholera pandemic is caused by El Tor vibrios. The data in our laboratory revealed that 20 to 30% of El Tor vibrios of human origin did not produce a detectable amount of CT under the above conditions. However, they have produced a large amount of CT in the infected intestine (6, 9). These findings suggest that the above media or culture conditions or both are not necessarily optimum for El Tor vibrios. The temperature in the intestine is higher (37°C), and oxygen tension is most likely much less than in well-aerated cultures. Actually, a *V. cholerae* 569B produces a substantial amount of CT even at 37°C in syncase medium if it is anaerobically cultured (2). We tried to make a medium with a composition similar to that of cholera stool and to culture El Tor vibrios at 37°C in poorly aerobic, stationary conditions. This paper describes the process of developing a new medium for stimulating in vitro CT production by El Tor vibrios and includes comparative studies on CT production in this new medium and in syncase medium.

MATERIALS AND METHODS

Bacterial strains. One hundred thirteen strains of El Tor vibrios, isolated in Kenya, the Philippines, and Taiwan after 1980, were used. El Tor vibrios isolated in Kenya originated from healthy carriers, those isolated in the Philippines were from cholera patients, and those isolated in Taiwan were from the environment.

Media. A tentative medium, in which the main electrolyte composition resembles that of cholera stool, was made. This tentative medium contained 0.5% NaCl, 0.15% KCl, 0.4% $NaHCO_3$, 0.4% yeast extract (Difco Laboratories, Detroit, Mich.), and 1.0% peptide. To prepare the medium, the ingredients except $NaHCO_3$ were dissolved in a half volume of distilled water and autoclaved. $NaHCO_3$ (0.8%) was sterilized by filtration. Equal volumes (5 ml each) of both sterilized ingredients were then gently mixed in the test tube. Final concentrations of Na^+, K^+, Cl^-, and HCO_3^- were 134, 19, 106, and 47 meq/liter, respectively. The concentration of each ingredient in this tentative medium was varied to get the optimum concentration for CT production. When one ingredient was being varied, the concentrations of the others were kept constant as indicated.

Culture conditions. To achieve a constant gas condition, test tubes (diameter, 15 mm; height, 150 mm) with 10 ml of medium were usually used (test tube culture). In comparative studies, organisms were cultured in media with a large surface-to-volume ratio (flask culture, 10 ml of medium in a 100-ml Erlenmyer flask) in the resting state or with reciprocal shaking.

Assay of CT. A reversed passive latex agglutination test (RPLA) was used to titrate CT. Culture fluids were centrifuged at 14,000 × *g* for 20 min at 4°C. The supernatant was diluted twofold serially in 0.025-ml volumes in microdilution

TABLE 1. CT production with various concentrations of yeast extract[a]

Yeast extract (%)	CT production (ng/ml) by:	
	A66	82p6
0.00	7.8	3.9
0.05	15.6	15.6
0.10	15.6	15.6
0.20	31.3	31.3
0.40	31.3	62.5
0.60	62.5	62.5
0.80	62.5	62.5
1.00	62.5	62.5

[a] Strains were cultured in media containing 0.5% NaCl, 0.15% KCl, 0.4% $NaHCO_3$, 1.5% Bacto-Peptone, and yeast extract as indicated at 37°C for 20 h in stationary test tubes.

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TABLE 2. CT production at various concentrations of sodium bicarbonate[a]

$NaHCO_3$ (%)	CT production (ng/ml) by:			
	A66	82p6	A239	B16
0.00	UD	UD	UD	UD
0.05	2.0	15.6	7.8	31.3
0.10	7.8	31.3	31.3	125
0.20	15.6	62.5	62.5	500
0.40	15.6	62.5	62.5	500
0.70	7.8	62.5	62.5	250
1.00	3.9	7.8	31.3	31.3
1.50	UD	7.8	15.6	7.8
2.00	UD	UD	7.8	UD
2.50	UD	UD	UD	UD

[a] Strains were cultured in media containing 0.5% NaCl, 0.15% KCl, 0.4% yeast extract, 1.5% Bacto-Peptone, and $NaHCO_3$ as indicated. Strains A66, A239, and B16 were isolated in Kenya, and strain 82p6 was isolated in the Philippines. UD, Undetectable.

plates with latex diluent (10 mM sodium phosphate buffer, 0.15 M NaCl, 1% bovine serum albumin, 0.05% polyvinylpyrrolidone, 0.1% NaN_3 [pH 7.0]). An equal volume of a 0.025% suspension of sensitized latex was added to each well. Microdilution plates were shaken thoroughly and then allowed to stand at room temperature. The agglutination was judged visually after 18 h. The lowest concentration of CT detectable by this method was 1 to 2 ng/ml. The purified CT used for the control titration was obtained from Sanko Junyaku Co., Tokyo, Japan.

Sensitization of latex with anti-CT antibody for reversed passive latex agglutination. Specific anti-CT antibody was purified by affinity column chromatography from rabbit anti-CT serum as described previously (5). Forty milliliters of purified antibody (10 μg/ml) in 10 mM phosphate-buffered saline (pH 7.0) was mixed with an equal volume of 0.25% latex (SDL59, Takeda Pharmaceutical Co., Osaka, Japan) suspension in the same buffer in a 500-ml Erlenmyer flask. The mixture was shaken gently for 1 h at room temperature. The latex–anti-CT conjugate was washed with latex diluent three times by centrifugation at 10,000 × *g* for 10 min and kept at 4°C in 0.5% suspension.

RESULTS

Development of a new medium. Three kinds of peptide were examined in the tentative medium. When Bacto-Peptone was used, strain A66 produced 125 ng of CT per ml, whereas 31.3 and 7.8 ng/ml were produced with Casamino Acids (Difco) and tryptone, respectively, in test tube culture at 37°C for 20 h. Although slight elevation of CT was seen at 1 to 2% Bacto-Peptone, its concentration had a minimal effect on the production of CT. A decrease or increase of NaCl or KCl concentration also had no significant influence.

On the contrary, yeast extract was an important component for toxin production (Table 1). CT production was much lower when yeast extract was removed from the medium. CT production increased when it was added up to 0.4 to 0.6%.

In the tentative medium, sodium bicarbonate was an essential component for enhancement of CT production. When it was removed, there was no detectable CT in the culture fluid, but 0.1 to 0.7% sodium bicarbonate markedly stimulated CT production (Table 2). A concentration over 1%, however, suppressed the effect. The optimum concentration of sodium bicarbonate was in the range of 0.2 to 0.4%. The pH of the tentative medium was about 7.6 with a minimum variation. When sodium bicarbonate was removed and the pH was adjusted back to 7.6 by sodium hydroxide, CT production was not stimulated.

From the above data, the composition of the medium for CT production in this study was established as follows: Bacto-Peptone, 1.5%; yeast extract, 0.4%; sodium bicarbonate, 0.3%; and sodium chloride, 0.5%. This medium had a pH of 7.5 ± 0.1 without further adjustment, and it was designated AKI medium.

Culture conditions for AKI medium. AKI medium produced more CT than syncase medium in several strains of El Tor vibrios (Table 3). Regarding the temperature, 37°C was better than 30°C for CT production, although its effect was not marked. A shallow culture or a shake culture in a flask suppressed CT production in five of six strains examined but rather enhanced CT production in one culture. This indicates that aeration in AKI medium is harmful for CT production in most El Tor vibrios. The number of vibrios at the end of culture was about 10^8/ml in AKI medium with test tube culture, but it was about 10^{10}/ml in syncase medium with shaking. CT concentration reached a maximum in 6 h and kept this level until 24 h (Table 4). Therefore, for AKI medium, stationary culture at 37°C for 20 h in test tubes was used in the following studies.

Comparison of AKI and syncase media. Figure 1 shows the frequency distribution of El Tor vibrios differentiated by the concentration of CT produced in AKI and syncase media. A peak frequency of CT concentration was at 62.5 ng/ml in AKI medium and 3.9 ng/ml in syncase medium. In AKI medium, 82 strains produced more than 30 ng of CT per ml, but in syncase medium, only 4 strains produced more than 30

TABLE 3. CT production under various conditions

Strain	CT production in[a]:						
	AKI at 37°C			AKI at 30°C		Syncase at 30°C	
	Stationary test tube	Stationary flask	Shaken flask	Stationary test tube	Stationary flask	Stationary flask	Shaken flask
82p6	31.3 (2.6×10^8)	15.6	3.9	31.3	15.6	2.0	UD (1.0×10^{10})
82p7	62.5 (2.8×10^8)	62.5	125	62.5	62.5	7.8	7.8 (9.5×10^9)
B16	250 (1.4×10^8)	62.5	15.6	62.5	62.5	7.8	15.6 (1.2×10^{10})
C128	62.5 (1.1×10^8)	31.3	7.8	62.5	62.5	2.0	3.9 (1.2×10^{10})
A66	125 (9.0×10^7)	UD	2.0	7.8	2.0	UD	7.8 (9.1×10^9)
H5	500 (1.7×10^8)	125	7.8	125	125	2.0	3.9 (7.0×10^9)

[a] Incubations were performed for 20 h in AKI and for 24 h in syncase medium as described in the text. Numbers in parentheses denote live vibrios per milliliter at the end of culture. UD, Undetectable (less than 2 ng/ml).

ng/ml. In syncase medium, 85 strains produced less than 4 ng of CT per ml, but only 12 did so in AKI medium. In syncase medium, 21 strains of 113 did not produce a detectable amount of CT. When these 21 strains were cultured in AKI medium, however, 13 strains produced 3.9 to 125 ng of CT per ml.

DISCUSSION

In this communication, we developed a new method to achieve high CT production by El Tor vibrios by using a medium in which the ingredients imitate cholera stool characteristics. The method is superior to traditional ones for detection of CT production by El Tor vibrios because of high toxin production; also, it is a simple medium to prepare and does not require shaking.

In several studies (1, 2, 3, 7, 8, 10) it has been pointed out that aeration is necessary and that 30°C is a better temperature than 37°C for CT production, but this was not necessarily applicable to El Tor vibrios in AKI medium. In the process of developing this new medium, we found that sodium bicarbonate markedly stimulates CT production. The optimum concentration, 0.2 to 0.4%, was almost the same concentration as that in cholera stool. It is most likely that bicarbonate plays a critical role in stimulating CT production. This is not simply a pH effect, because the adjustment of pH by sodium hydroxide had no effect. Recently, it has been suggested (T. Shimamura, A. Watanabe, and S. Sasaki, Proc. 58th Annu. Meet. Jpn. Soc. Bacteriol. **40**:190, 1985) that bicarbonate has a stimulating effect on CT production; i.e., culture of *Vibrio cholerae* O1 in a CO_2 incubator resulted in high CT production. Although the mechanism is unknown, sodium bicarbonate also stimulates CT production by El Tor vibrios in the intestine.

Yeast extract, which also plays an important role, is a complex. Some elements may be key materials which should be clarified hereafter. Bacto-Peptone did not have a significant effect on stimulating CT production, nor did sodium chloride or potassium chloride. Thus, a medium consisting of only sodium bicarbonate and yeast extract may be enough to produce a substantial amount of CT.

Bacto-Peptone had a minimum effect on CT production, and when tryptone was substituted for Bacto-Peptone, CT production was very low. It has been suggested that tryptone may have some suppressive factor on CT production by El Tor vibrios.

In most strains, the CT concentration in AKI medium was about 10 to 20 times higher than in syncase medium, and the population of vibrios at the end of culture was 100 times less than in syncase medium. This means that each individual organism may produce as much as 1,000 times more CT in AKI medium than in syncase medium.

There is a question of whether the induced toxin in AKI medium is biologically active, because we used an immunological method for detection of the toxin. The possibility has not been eliminated that the production of only an inactive antigenic portion, e.g., choleragenoid (4), might have been stimulated instead of biologically active holotoxin.

We are modifying this culture method for further improvement so that we can purify enough CT produced by El Tor vibrios to permit analysis.

TABLE 4. Growth of vibrios and the production of CT[a]

Incubation (h)	Growth (CFU/ml) in:		CT production in:	
	AKI	Syncase	AKI	Syncase
0	1.4×10^4	1.6×10^4	NT	NT
3	1.0×10^5	1.0×10^5	NT	NT
4	2.0×10^6	1.8×10^5	2.0	UD
5	1.2×10^7	1.0×10^6	125	UD
6	2.0×10^8	6.0×10^6	250	UD
7	2.6×10^8	6.0×10^6	250	UD
8	1.2×10^8	2.0×10^7	250	UD
12	2.7×10^8	6.0×10^7	250	3.9
24	1.2×10^8	8.1×10^9	250	15.6

[a] El Tor vibrio strain B16 was cultured in AKI medium in stationary test tubes at 37°C and in syncase medium in flasks with shaking at 30°C. NT, Not tested; UD, undetectable (less than 2 ng/ml).

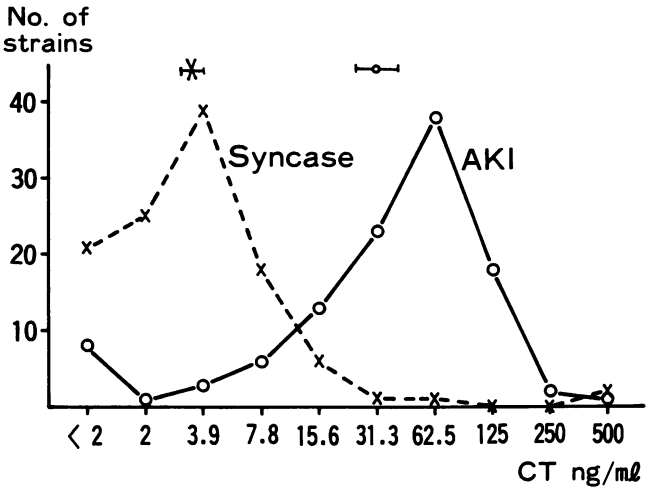

FIG. 1. Production of CT by various strains of El Tor vibrios in AKI and syncase media. A total of 113 strains were cultured in AKI medium (○) at 37°C for 20 h (test tube culture, stationary) and in syncase medium (×) at 30°C for 24 h (flask culture, with shaking). Horizontal bars above the graphs represent mean ± standard error values.

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