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Production of Cholera Toxin-Like Toxin by *Vibrio mimicus* and Non-O1 *Vibrio cholerae*: Batch Culture Conditions for Optimum Yields and Isolation of Hypertoxigenic Lincomycin-Resistant Mutants

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Vibrio mimicus 61892, isolated in 1977 from a case of watery diarrhea in Bangladesh, produces an enterotoxin which possesses activity in Y-1 mouse adrenal cells and in rabbit ileal loops which is identical to the prototype cholera toxin (CT) produced by *Vibrio cholerae* 569B. The neutralization of the adrenal cell activity of 61892 toxin and 569B CT by homologous and heterologous antisera generates parallel titration curves which show complete neutralization in all cases. Paired titrations in the ganglioside GM1 enzyme-linked immunosorbent assay (using either CT or *Escherichia coli* heat-labile toxin antitoxin) of both toxins indicates that 61892 toxin is antigenically indistinguishable from 569B CT. The specific activity of the two toxins in the rabbit ileal loop is virtually identical. Batch culture production of CT-like toxin and CT by isolates of *V. mimicus* and different biotypes of *V. cholerae* was found to be highest in shake flask cultures of Casamino Acids-yeast extract broth grown at 27°C with vigorous aeration. Incorporation of lincomycin into the growth medium at a concentration of 50 μg/ml increased yields from wild-type strains. Dramatically higher yields were obtained when a spontaneous resistance mutant of strain 61892 was grown in the presence of 200 to 300 μg of lincomycin per ml. Under these conditions, yields of CT-like toxin were increased by 300- to 500-fold, and the highest yields reached more than 100 μg/ml after 44 h of culture. This is substantially higher than that reported in the literature for CT production by any strain of *V. cholerae*, including hypertoxigenic strain 569B.

A number of non-O1 *Vibrio cholerae* have been shown to produce an extracellular toxin that resembles the cholera toxin (CT) of *V. cholerae* biotypes *cholerae* and *eltor*. Zinnaka and Carpenter (26) and Ohashi et al. (15) have reported that strains isolated from outbreaks of watery diarrhea in the Sudan and the Philippines, respectively, produce extracellular heat-labile permeability factors which react with at least partial immunological identity with CT produced by O1 *V. cholerae*. Other investigators working with these organisms have reported permeability factors or factors which elicit fluid accumulation in ligated rabbit intestinal loops (1, 20), but the relationship of these to CT is unclear.

More recently, Craig et al. (4) and Yamamoto et al. (25) carefully documented the production of a CT-like toxin by a non-O1 *V. cholerae* isolated from the environment (surface water) in association with an outbreak of cholera in Louisiana in 1978 (2). Using the rabbit skin permeability assay and highly purified reagents, they demonstrated that the enterotoxin produced by this strain was indistinguishable in almost all respects, both biologically and antigenically, from the prototype CT of *V. cholerae* 569B. Similar CT-like toxin-producing non-O1 *V. cholerae* have also been implicated in cases of diarrhea occurring in the region of the Gulf Coast (11).

Kaper et al. (10), using a gene probe consisting of *Escherichia coli* heat-labile (LT) enterotoxin DNA (which cross-reacts with CT genes), demonstrated the presence of CT genes in two strains of non-O1 *V. cholerae*: S-21, one of the Sudan isolates from Zinnaka et al. (27, 28), and 61956, a strain isolated by Spira et al. (22) in Dacca, Bangladesh (later redesignated *Vibrio mimicus*; see below). This same probe was later used to identify CT genes in two additional isolates of non-O1 *V. cholerae* from clinical cases detected in follow-up studies on the Louisiana cholera outbreak (9).

TABLE 1. Source of isolates used in this study

Species and biotype	Strain	Source	Comments
V. mimicus	61892	W. M. Spira	Diarrhea; Bangladesh; 1977
	63616	W. M. Spira	Diarrhea; Bangladesh; 1977
V. cholerae non O1	S7	Y. Zinnaka	Diarrhea; Sudan; 1968
	Q40223	M. I. Huq	Diarrhea; Bangladesh; 1977
V. cholerae eltor	Stokes 1	R. B. Sack	Diarrhea; Australia; 1977 (16)
	U1399	R. Glass	Diarrhea; Bangladesh; 1981
V. cholerae cholerae	569B	R. B. Sack	Diarrhea; India
	Ogawa 395	R. B. Sack	Diarrhea; India
	T19766	R. Glass	Diarrhea; Bangladesh; 1980

Spira et al. (22) used a number of assays to identify toxigenic strains from clinical and environmental isolates from various places around the world, particularly Bangladesh (21). Culture filtrates of non-O1 *V. cholerae* strains were screened for permeability factor activity, ability to elicit fluid accumulation in ligated rabbit loops, ability to cause rounding of Y-1 adrenal cells (18), and elongation of Chinese hamster ovary (CHO) cells (8). Enterotoxins identified in this manner were confirmed by Y-1 adrenal cell neutralization tests with cholera antitoxin. Overall, they found that 24% of the clinical isolates and 7% of the environmental isolates produced a CT-like toxin. A cluster of sucrose nonfermenting strains isolated from cases of watery diarrhea in Dacca from November 1977 to March 1978, which were originally identified as non-O1 *V. cholerae*, have since been designated *V. mimicus*, a recently described species closely allied to *V. cholerae* (5).

In this paper, we report on the characteristics of *V. mimicus* enterotoxin (in crude preparation) which indicate that it is biologically and immunologically indistinguishable from CT. We also report on culture conditions for increasing the production of CT-like toxin by *V. mimicus* and non-O1 *V. cholerae* and on the isolation of a spontaneous lincomycin-resistant mutant which is hypertoxigenic when grown in the presence of this antibiotic.

MATERIALS AND METHODS

Bacterial strains and stock cultures. The strains used in this study are described in Table 1. All stocks were maintained as cultures frozen at −70°C in brain heart infusion broth containing 15% glycerol, and subcultures were prepared from frozen stocks by streaking a small amount of frozen suspension onto blood agar base (BAB) plates. Well-isolated colonies were picked after 24 h of incubation at 32°C to inoculum flasks. For lincomycin-resistant mutants, lincomycin was added to the stock medium and BAB plates at a concentration of 250 μg/ml.

Culture conditions. The medium used for toxin production was a modification of the Casamino Acids-yeast extract (CAYE) medium of Kusama and Craig (12). It contained Casamino Acids (Difco Laboratories), 3%; K_2HPO_4, 0.05%; glucose, 0.2%; and yeast extract dialysate, which we prepared by passing a 6% (wt/vol) solution of yeast extract (Difco) through an Amicon H1P10-8 hollow fiber (nominal molecular weight 10,000 exclusion). The dialysate was added to the medium in a ratio of 1:10. The final pH of the CAYE was 7.0. For resistance mutants, sterile lincomycin was added (Lincocin; Upjohn, Co.) to CAYE after the medium had been autoclaved and allowed to cool. This medium, designated CAYE-L, was routinely prepared in 500-ml Erlenmeyer flasks containing 25, 50, or 100 ml of CAYE.

Inoculum flasks were prepared by picking clones from BAB purity plates to 50-ml Erlenmeyer flasks containing 10 ml of CAYE or CAYE-L. These were incubated for 24 h in a 30°C reciprocal water bath shaker set at 240 oscillations per min. A 1% (vol/vol) inoculum from this culture was transferred to a fresh flask of CAYE or CAYE-L, and the second culture was incubated under the same conditions for 4 h. This 4-h culture was used to inoculate the production flasks at a proportion of 1% (vol/vol).

The rate of shaking and the temperature of incubation were varied as described below, and samples were collected aseptically from cultures at 8, 21, and 44 h after inoculation to determine pH, viable cell count, and CT concentration. Viable cell counts were determined by plating appropriate dilutions in 0.01 M phosphate-buffered saline (PBS) (pH 7.4) onto gelatin agar plates. The remaining culture was then centrifuged at 11,000 × *g* for 20 min, and the supernatant fluids were frozen at −20°C until they were assayed for toxin (usually within 72 h).

Reagents. Purified CT from strain 569B (Schwarz-Mann), 25.8 limit of blueing (Lb) per μg, was routinely used for a CT standard. Stocks of this material were prepared by rehydrating 1 mg of toxin in 1 ml of sterile distilled water and further diluting this to 100 μg/ml in PBS. Portions of this were stored at −70°C. Crude preparations of *E. coli* LT toxin were obtained by concentrating tryptic soy broth culture filtrates from *E. coli* H10407 grown at 37°C in shake flasks for 24 h. Concentrates, made 10× by ultrafiltration over an Amicon UM-20 membrane, were stored at −70°C until used.

The following antitoxins were used. (i) Cholera antitoxin produced by immunizing three New Zealand White rabbits with two doses of Schwarz-Mann CT. The first dose contained 20 μg of toxin in PBS and was given intramuscularly with Freund complete adjuvant;

the second contained 50 μg of toxin and was given subcutaneously on day 17 after the initial dose. Serum collected on day 31 was subsequently found to form a single precipitin line against either pure Schwarz-Mann CT or concentrated culture filtrates of strain 569B. (ii) Rabbit antiserum against crude concentrated filtrate from cultures of strain 61892 produced by the same protocol used for CT. The first and second doses of crude filtrate contained, respectively, 20 and 50 μg of CT-like toxin as determined by GM1 enzyme-linked immunosorbent assay (ELISA). (iii) Purified cholera antitoxin from the Swiss Serum Vaccine Institute, Berne, kindly provided by R. B. Sack. (iv) Burro antiserum against either crude CT (NIH lot 001) or crude *E. coli* LT, also provided by R. B. Sack.

GM1-ELISA procedure. CT was routinely assayed by using a modification of the microtiter ganglioside GM1-ELISA assay of Sack et al. (17). The reagents and procedures were as described previously, except that a peroxidase-conjugated goat anti-rabbit immunoglobulin G antiserum (Cappel Laboratories) was used in place of the alkaline phosphatase conjugate in the published procedure. The substrate employed was 2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid. In brief, our procedure involved coating the wells of polyvinyl microtiter plates with a solution of ganglioside GM1 (Supelco), 2 μg/ml in PBS, followed by successive additions of test preparations, rabbit anti-CT antiserum (diluted 1:4,000 in PBS, 0.1% Tween 20, 1% bovine serum albumin), and peroxidase-conjugated goat anti-rabbit immunoglobulin G antiserum (diluted 1:1,000 in PBS, Tween 20, bovine serum albumin). The substrate consisted of 0.004 M 2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid in 0.05 M sodium citrate buffer (pH 4.0) plus 0.0015 M H_2O_2. Plates were held for 60 min after the substrate was added, and color development was measured at 410 nm by using a fiber optic ELISA spectrophotometer (Dynatech). A titration of Schwarz-Mann CT (from 1.0 to 15 ng/ml) was included on every plate, and all test values were related to this standard curve.

Biological assays. Titrations of CT activity in rabbit ileal loops were done by a modification of the procedure of De and Chatterje (6). Serial dilutions of test preparations were prepared in normal saline containing 0.1% gelatin. Between 15 and 18 ligated intestinal loops were prepared in rabbits weighing 1.5 to 2.0 kg. Loops were injected in a partially randomized pattern with 1.0 ml each of PBS (negative control), a standard dose of purified CT (from 1.0 to 3,200 ng/ml), or a dilution of test preparation. The last loop at either end of the series was left blank. Animals were held for 20 h and sacrificed, and the fluid which had accumulated in each loop was measured. The results from rabbits with incorrect control loop responses were discarded. The length of each loop was also measured to the nearest 0.5 cm so that we could calculate a fluid accumulation ratio (volume to loop length in milliliters per centimeter) to correct partially for unavoidable variations in the lengths of different loops.

The Y-1 mouse adrenal cell assay was carried out with the microtiter plate modification exactly as described by Sack and Sack (18). The degree of rounding of cells (positive response) was subjectively graded by two independent observers on a scale from 0 (no rounding) to 4 (virtually all cells rounded).

Neutralization of toxins by homologous and heterologous antiserum was carried out by using the Y-1 adrenal cells. Serial twofold dilutions of antiserum (cholera antitoxin from the Swiss Serum and Vaccine Institute, initially diluted 1:894, and rabbit antiserum against *V. mimicus* 61892 culture filtrates, initially diluted 1:10) were incubated for 1 h at 37°C with either purified CT (Schwarz/Mann; 100 ng/ml) or concentrated culture filtrates of strain 61892 (diluted 1:10 to give an approximately equivalent Y-1 adrenal cell titer to that of the CT standard). The mixtures were transferred to microtiter wells containing Y-1 adrenal cells and held for 15 min before being washed off. The cells were then processed and observed in the routine manner.

Spontaneous lincomycin mutant of strain 61892. Spontaneous mutants to lincomycin resistant from *V. mimicus* 61892 were obtained by plating suspensions of 4-h CAYE shake flask cultures on BAB plates containing 100 μg of lincomycin per ml. Colonies growing out after 24 h at 32°C were picked to three successive purity plates containing the same concentration of lincomycin. Ten clones picked at this point showed virtually no difference in toxin production, so one was selected as a stock culture, 61892-1. This stock was later passaged through BAB containing 300 μg of lincomycin per ml to ensure that this strain was highly resistant to the antibiotic. The stock culture has since been maintained at −70°C without further passage.

RESULTS

Characteristics of *V. mimicus* CT-like toxin. Cell-free filtrates from cultures of strain 61892 were capable of eliciting a response in Y-1 adrenal cells that appeared to be identical to that elicited by pure CT. Virtually all cells were rounded, and there was no evidence of a cytotoxic effect like that observed with filtrates from many non-O1 *V. cholerae* and *V. mimicus* (14). Figure 1 demonstrates that this response can be completely inhibited by antiserum against 61892 filtrate and purified CT antitoxin. Similarly, the antiserum against 61892 filtrate appears to be as effective as CT antitoxin in abolishing the adrenal cell activity of purified CT. Although titers vary somewhat, all four titration curves appear to have almost the same slope, suggesting that the different antigen-antibody combinations may possess similar affinities.

We have further demonstrated the antigenic similarity of 61892 toxin to CT by comparative titrations of these two toxins and *E. coli* LT in the GM1 ELISA (Fig. 2). Preparations of the three toxins were standardized to equivalent titers in the Y-1 adrenal assay and then titrated in the GM1 ELISA assay by using either anti-CT or anti-LT antiserum. For CT and LT, the titration curve of the homologous antibody-antigen pair is at least fourfold more sensitive than that for the heterologous pair. When the 61892 toxin is titrated in this system, its titration curve parallels that of purified CT, suggesting that it is antigenically more similar to CT than to LT.

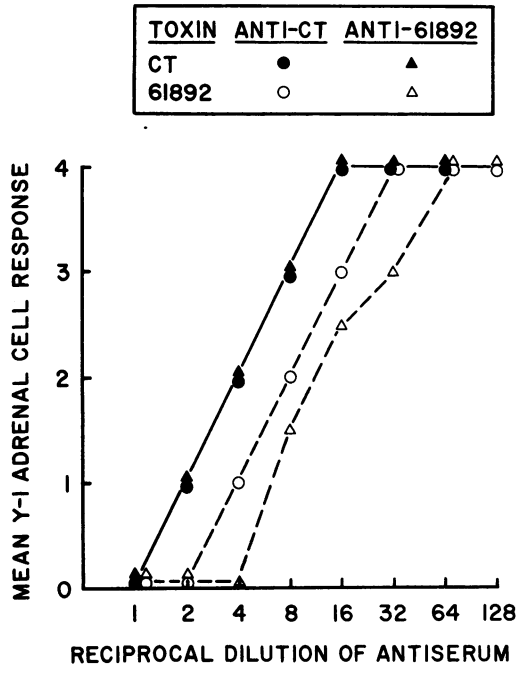

FIG. 1. Neutralization of the Y-1 adrenal cell activity of purified 569B CT and crude CT-like toxin from *V. mimicus* 61892 by serial dilutions of cholera antitoxin (Swiss Serum) or rabbit antiserum against concentrated filtrates from cultures of strain 61892. Adrenal cell activity is expressed as 0 (no cells rounded) to 4 (virtually all cells rounded). Values are the means of estimates made by two independent observers.

The CT-like toxin produced by strain 61892 is identical in specific bioactivity to that of 569B CT. Figure 3 presents the ileal loop titration of crude concentrated 61892 toxin compared to that of purified CT. Both toxin preparations have been standardized on the basis of ELISA antigen concentration. The specific bioactivity of 61892 toxin is, within the limits of this assay, virtually identical to that of CT. The amount of either toxin necessary to give a response of fluid accumulation = 1.00 (approximately a 50% response) is about 25 ng per loop.

Culture conditions for toxin production by *V. mimicus* and biotypes of *V. cholerae*. Our preliminary studies indicated that resting cultures did not support as high yields of CT-like toxin as did shake cultures (21). In this aspect, these strains were similar to *V. cholerae* biotype *cholerae* and unlike biotype *eltor* strains, which produced more CT in resting culture.

Further studies on shake flask conditions (C. B. Stephenson, P. J. Fedorka, P. H. Pettebone, and W. M. Spira, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B67, p. 29) revealed a significant relationship between biotype and incubation temperature which suggested that *V. mimicus* and non-O1 *V. cholerae* more closely resemble *V. cholerae* biotype *eltor* than biotype *cholerae*.

Table 2 shows overall results when cultures of *V. mimicus*, non-O1 *V. cholerae*, and *V. cholerae* biotype *cholerae* (classical) or biotype *eltor* were grown at different temperatures and shaking rates. All cultures were grown for 44 h in 50-ml Erlenmeyer flasks containing 10 ml of CAYE (1:5 medium to flask volume) at pH 7.0. Samples taken at 21 h had toxin concentrations which were the same as or lower than those collected at 40 h.

The data from this experiment were analyzed in a three-way analysis of variance without replication to examine the influence of and interactions between biotype, incubation temperature, and agitation rate. This analysis suggested that no significant difference existed between responses of *V. mimicus* and non-O1 *V. cholerae*, so the data from these two groups were pooled for the final analysis shown in Table 3.

Table 3 reveals highly significant differences

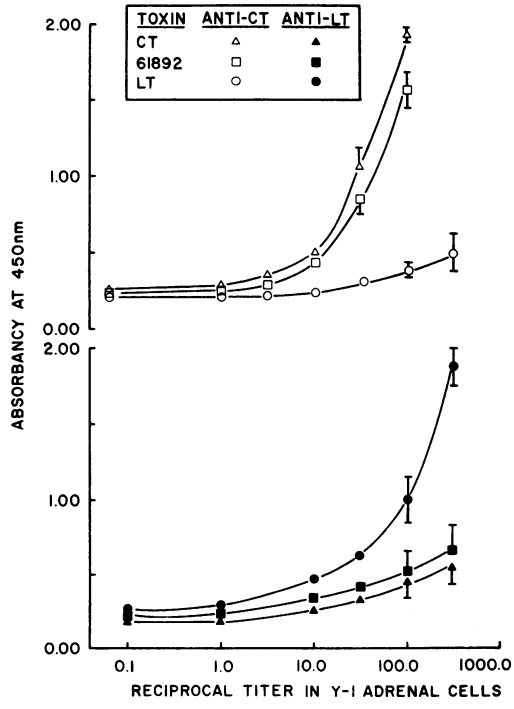

FIG. 2. Titration of 569B CT, 61892 CT-like toxin, and *E. coli* H10407 LT in the GM1 ELISA assay with burro CT antitoxin or LT antitoxin to overlay bound toxin. Equivalent concentrations of toxin were established between the purified 569B CT preparation and the crude preparations of 61892 CT-like toxin and LT by standardizing dilutions on the basis of bioactivity in the Y-1 adrenal cell assay. Values are the means of three trials ± 1 standard error.

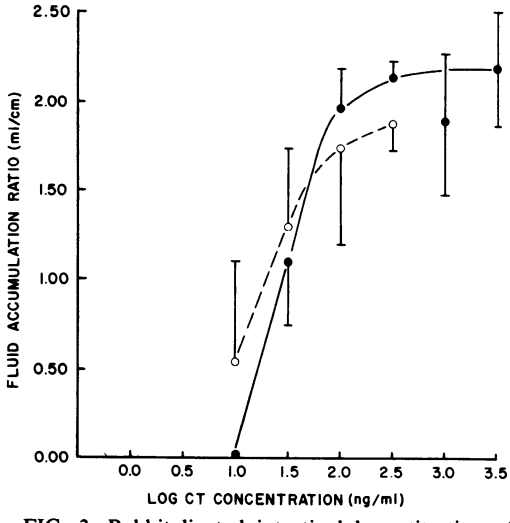

FIG. 3. Rabbit ligated intestinal loop titration of crude 61892 CT-like toxin (concentrated 10× in culture filtrates with an Amicon H1P10 hollow fiber). The toxin concentration is expressed in nanograms per milliliter of ELISA-based antigen. The loop response is expressed as a fluid accumulation ratio: volume of fluid in loop to length of loop. Values are the means of five trials ± 1 standard error. Symbols: ○, 569B CT; ●, 61892 CT-like toxin.

in toxin production between biotypes, between incubation temperatures, and between shaking rates. The interaction between biotype and incubation temperature is statistically significant, as is the interaction between temperature and shaking rate. The relationships underlying these interactions were further analyzed in Fig. 4.

A strong positive log-linear relationship between shaking speed and toxin production for the *eltor* and the non-O1-*V. mimicus* clusters was apparent in cultures incubated at 27°C. Highest yields were obtained in cultures shaken at 240 cycles/min. At 32°C, however, we found an apparent maximum when cultures were shaken at 150 cycles/min. This pattern of response distinguished these two clusters from the classical strains which did not exhibit a maximum at 150 cycles/min at 32°C, although a modest maximum was present at 37°C. The decrease in toxin production at 37°C was also proportionately more dramatic in classical strains than in the other clusters.

For all strains, we found a negative relationship between temperature and toxin production in the range examined. Our data suggest that an optimum may exist at a temperature at or below 27°C, coupled with higher agitation rates than we achieved. Control of temperatures below 30°C is difficult with our current shaker bath, so we settled on a standard temperature of 29 to 30°C for the remainder of this study. However, we extended the study of aeration effects by the simple expedient of reducing the volume of medium from a 1:5 (vol/vol) ratio to 1:10 and 1:20. When 2,000-ml Erlenmeyer flasks containing 400, 300, 200, or 100 ml of CAYE were used to grow strain 61892 at 29 to 30°C and 200 cycles/min for 44 h, toxin yields (± 1 standard error; $n = 4$) were 5 ± 1, 8 ± 2, 65 ± 11, and 105 ± 12 ng/ml, respectively. With optimum culture conditions, we were able to obtain maximum yields from *V. mimicus* 61892 of 250 to 300 ng/ml and from non-O1 *V. cholerae* Q40233 of 95 ng/ml. It is possible that higher rates of aeration may further improve toxin yields.

Effect of lincomycin on CT-like toxin production. We have found that all of our study strains are stimulated by lincomycin to produce higher yields of toxin, as reported by Levner et al. (13) for strain 569B and by Yamamoto et al. (24) for strains of non-O1 *V. cholerae*. Our study strains

TABLE 2. Toxin production by different biotypes of *V. cholerae* and *V. mimicus* grown in shake flask cultures at different temperatures and shaking rates[a]

Biotype cluster (no.)	Geometric mean toxin conc. (μg/ml)						
	Shaking rate			Incubation temp			Mean value for cluster
	50 rpm	150 rpm	240 rpm	27°C	32°C	37°C	
MIM-/NO1[b] (4)	0.005	0.041	0.038	0.022	0.029	0.015	0.019
eltor (2)	0.002	0.004	0.005	0.005	0.003	0.002	0.004
Classical (3)	0.057	0.350	0.680	1.100	0.440	0.029	0.240
Mean value for parameter	0.007	0.039	0.049	0.047	0.030	0.010	

[a] Cultures were grown in 50-ml Erlenmeyer flasks containing 10 ml of CAYE (pH 7.0) for 44 h. Nine cultures were grown for each strain used: one for each combination of temperature and shaking rate. Values for specific temperature or shaking rate represent geometric means calculated over all levels of the alternate parameter. Toxin concentration was measured as GM1 ELISA antigen.

[b] MIM/NO1 cluster contains two strains each of *V. mimicus* and non-O1 *V. cholerae*. Results were pooled after preliminary statistical analysis.

TABLE 3. Summary of three-way analysis of variance without replication

Source of variation	df	MS	F	P
Biotype cluster (C)	2	8.145	286.5	≤0.001
Shaking rate (S)	2	1.804	63.45	≤0.001
Incubation temp (T)	2	1.139	40.05	≤0.001
C × S	4	0.1017	3.576	0.059
C × T	4	0.4772	16.78	<0.001
S × T	4	0.2316	8.147	0.006
Error (C × S × T)	8	0.0284		

grown in the presence of 50 μg of lincomycin per ml yielded from 1.3- to 12.-fold more CT-like toxin than they did when grown without lincomycin. However, yields between batches were unpredictable, and our attempts to control growth conditions rigorously were inadequate to deal with this problem.

We produced resistance mutants of *V. mimicus* 61892 by multiple passage on BAB plates containing 100 and then on 300 μg of lincomycin per ml. The spontaneous mutant 61892-1 grew well in the presence of 300 μg of lincomycin per ml but still not as well as in its absence (Table 4). Nevertheless, we did not observe substantial inhibition of growth until the concentration exceeded 500 μg/ml.

With this mutant strain, we have obtained very impressive increases in CT-like toxin yield in the presence of lincomycin (also shown in Table 4). Under the conditions indicated, CT-like toxin accumulation was substantially higher at 44 h than at 24 h. The maximum yield obtained from a culture grown with 200 μg of lincomycin per ml was 105.6 μg/ml. Although this was the highest absolute yield, the specific productivity (expressed as femtograms of CT-like toxin per CFU) was slightly higher in the presence of 300 μg of lincomycin per ml.

We examined the kinetics of growth, pH change, and CT-like toxin accumulation with strain 61892-1 in a four-cell trial to study the interaction between lincomycin stimulation and aeration. We grew cultures with (CAYE-L) or without (CAYE) lincomycin (300 μg/ml) at medium to flask volume ratios of either 1:20 (25 ml to 500 ml) or 1:5 (100 ml to 500 ml). The results from the cultures with the 1:20 ratio are given in Fig. 5.

Although we inoculated each flask with an exponential-phase culture grown in CAYE or CAYE-L to match the growth flask condition, the cultures grown in CAYE-L demonstrated a profound lag phase lasting 4 h, in contrast to the almost immediate initiation of exponential growth in CAYE. The viable cell count in the CAYE-L culture actually decreased from 3×10^6 to 1×10^6 per ml between 0 and 4 h. During early exponential growth, however, both cultures demonstrated similar doubling times, ranging from 0.67 to 0.77 h. Maximum cell density in the CAYE-L culture was about half that achieved in CAYE. We observed a similar pattern in the cultures grown with a 1:5 ratio.

We found almost no detectable effect of medium to flask ratio on the kinetics of exponential- or transition-phase growth. Cultures in CAYE reached early stationary phase by 8 h postinoculation, whereas those in CAYE-L reached it at about 12 to 16 h. The decrease in pH during exponential growth followed by the consistent rise during transition and stationary phase was characteristic of all cultures. In CAYE-L cultures, this pattern was delayed to the extent that the lag phase was extended.

The medium to flask ratio did appear to have an influence on the viability of cells in stationary phase. Regardless of lincomycin, the viable cell concentration in the 1:20 ratio cultures began to decrease rapidly within 4 h after stationary phase had been established but was relatively stable in the 1:5 ratio cultures.

Lincomycin and medium to flask ratio inter-

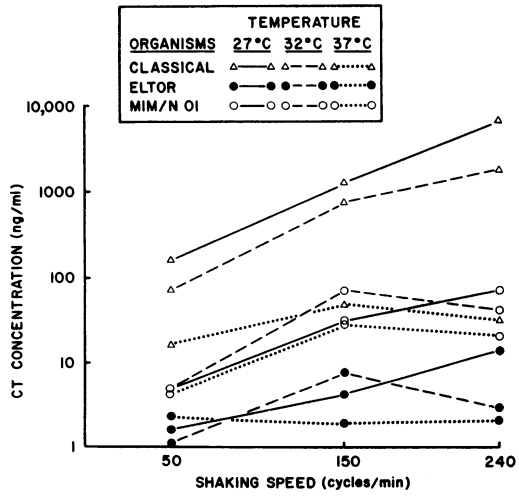

FIG. 4. CT-like toxin production by biotypes of *V. cholerae* and *V. mimicus* grown in shake flask cultures at different temperatures and rates of shaking. Cultures were grown in 50-ml flasks containing 10 ml of CAYE medium (pH 7.0) at 29 to 30°C for 44 h. Values are the geometric means of results from *V. cholerae* biotype *cholerae* (classical) (three strains), *V. cholerae* biotype *eltor* (two strains), and *V. mimicus* and non-O1 *V. cholerae* (two strains each). Nine cultures were grown for each strain used, one for each combination of temperature (27, 32, and 37°C) and shaking speed (50, 150, and 240 cycles/min).

acted in their effect(s) on CT-like toxin production in that toxin appeared more rapidly and peaked sooner in cultures with ratios of 1:20 compared to those with ratios of 1:5. CAYE cultures with 1:20 ratios produced higher yields (two to threefold) than CAYE cultures with 1:5 ratios, but the kinetics of production was similar.

The concentration of CT-like toxin decreased somewhat between 24 and 44 h in both CAYE cultures and in the CAYE-L culture grown with a 1:20 ratio. In contrast, the period of peak CT-like toxin concentration in cultures grown in CAYE-L with a 1:5 ratio lasted from 28 to at least 44 h.

DISCUSSION

The key points we present in this paper are that (i) toxigenic *V. mimicus* 61892 produces an extracellular toxin that appears to be biologically and antigenically indistinguishable from the prototype CT produced by the *V. cholerae* 569; (ii) production of CT-like toxin by *V. mimicus* and non-O1 *V. cholerae* responds to environmental conditions in some ways that resemble the response pattern of strains of *V. cholerae* biotype *eltor*; (iii) at least some *V. mimicus* and perhaps non-O1 *V. cholerae* appear to have the potential to be as toxigenic as any strains of *V. cholerae* biotype *cholerae* (classical); and (iv) lincomycin stimulation of spontaneous resistance mutants appears to be a simple and generally successful method for significantly increasing toxin yields from these organisms.

Our results with crude toxin from *V. mimicus* 61892 suggest a close relationship between it and 569B CT. Both toxins have the same specific activity in rabbit ileal loops. The Y-1 adrenal cell activities of 61892 and 569B CTs can be completely cross-neutralized by the heterologous antitoxin with parallel titration curves. The two toxins have virtually identical titration curves in the GM1 ELISA when assayed by either CT antitoxin or *E. coli* LT antitoxin. We find no demonstrable differences, thus far, between 61892 CT-like toxin and 569B CT.

Strain 61892 has also been examined by J. Kaper, University of Maryland Center for Vaccine Development, Baltimore, for the presence of CT gene with DNA probes. Another *V. mimicus* isolated at the same time as strain 61892, strain 61956, was previously reported to possess the toxin gene (10). Kaper has found that strain 61892 also possesses genes with homology for the probe (personal communication).

Although the CT-like toxin of strain 61892 appears to be indistinguishable from 569B CT, it will not be surprising if small differences do

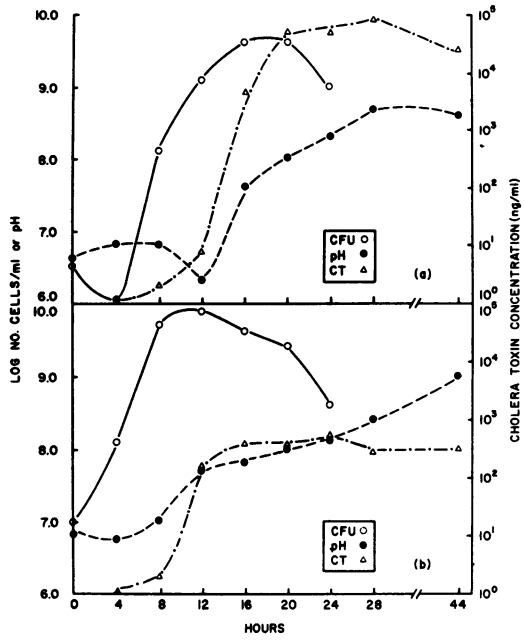

FIG. 5. Effect of lincomycin on the growth of and CT-like toxin production by *V. mimicus* 61892-1 (spontaneous lincomycin-resistant mutant) in shake flask cultures with a 1:20 medium to flask volume ratio. Cultures were grown in 500-ml Erlenmeyer flasks containing 25 ml of CAYE or CAYE-L and incubated at 29 to 30°C with shaking at 240 cycles/min. CT-like toxin concentration is expressed in nanograms per milliliter on the basis of titrations in the GM1 ELISA assay. (a) Growth in CAYE-L (containing 300 μg of lincomycin per ml). (b) Growth in CAYE (lincomycin-free medium).

TABLE 4. Growth and CT-like toxin production by *V. mimicus* spontaneous lincomycin-resistant mutant 61892-1 cultured in the presence of various concentrations of lincomycin[a]

Lincomycin conc. (μg/ml)	Cell density (21 h) (CFU/ml × 10^9)	Toxin conc. (μg/ml) at 8 h	21 h	44 h	Spec act at 44 h[b]
0	6.3	0.003	0.200	0.065	0.01
50	2.2	0.001	0.950	4.450	2.02
100	1.7	0.001	6.77	22.600	13.30
200	4.2	0.040	3.60	105.600	25.20
300	2.6	0.030	17.40	66.400	25.50
500	2.1	0.040	1.44	47.600	22.70
700	<0.1	ND[c]	0.300	0.700	>7.00
900	<0.1	ND	ND	0.030	>0.30

[a] Cultures were grown in CAYE shake flasks with a 1:5 medium:flask volume ratio at 30C and 240 cycles per min. CT-like toxin was assayed using the G_{M1}-ELISA assay.

[b] Specific activity is expressed as femtograms of CT-like toxin at 44 h per CFU at 21 h.

[c] ND, Not determined.

become apparent as the purified toxin is characterized. We have recently purified the CT-like toxin of strain 61892 and will report in a forthcoming article (W. M. Spira and P. J. Fedorka-Cray, submitted for publication) that slight differences have already been noted between its fine structure and that of 569B CT.

A number of published observations suggest that differences in fine structure exist between other CT-like toxins or CT produced by non-O1 *V. cholerae* or *V. cholerae eltor* and the prototype 569B CT, but that these are relatively small. Ohashi et al. (15) studied in detail a non-O1 *V. cholerae* (strain P4) isolated in the Philippines. This strain produced a toxin (expressed as PF in the rabbit skin assay of Craig [3]) whose activity could be neutralized by antitoxin against crude 569B holotoxin but not against the B subunit of 569B CT. They proposed that the two toxins differed by at least one antigenic site on the B subunit.

Zinnaka and Carpenter (26) and Zinnaka et al. (27, 28) purified a PF from another non-O1 *V. cholerae*, S-2 (isolated in the Sudan at the same time as strain S-7 used in the current work), which showed complete cross-neutralization with 569B CT but which failed to produce lines of identity with the 569B CT in Ouchterlony gel diffusion. They also concluded that the toxin with which they were working was related to but different from 569B CT.

Recently, Yamamoto et al. (25) purified a CT-like toxin from an environmental non-O1 *V. cholerae*, E8498, isolated in Louisiana in association with the 1978 outbreak of cholera (2, 4). They found this toxin to be completely neutralized by highly purified monospecific cholera antitoxin and to give a single line of identity with 569B CT in double gel diffusion. They could also show no quantitative difference in ganglioside GM1-binding activities between the two toxins in PF neutralization assays, although earlier studies with crude E8498 toxin (4) suggested that a difference did exist.

The evidence for variations within CTs produced by *V. cholerae* O1 consists of (i) a report by Finklestein et al. (7) that the CT of *eltor* strain 3083-2 Ogawa contained a B subunit which showed only partial immunological cross-reactivity with that of 569B CT; and (ii) the recent work of Saunders et al. (19), which demonstrated that *eltor* strain RJ1 CT and 569B CT were immunologically indistinguishable by double gel diffusion assay, but that both of these toxins were incompletely cross-reactive with *eltor* strain 3083-2 Ogawa CT.

Identification and characterization of fine structural differences between CTs and CT-like toxins may open the way to future studies concerning the relationship between the fine structure of these toxins and their biological and immunological properties. Such studies with unmodified toxins produced by wild-type strains are attractive because of their documented relationship to human diarrhea. Even when it becomes possible to produce fine-structure mutants with facility in the laboratory, these natural toxins will serve as an important benchmark.

This work will be aided by techniques to increase production of CT or CT-like toxins from strains that normally produce only small amounts in vitro. Our studies on toxin production in batch culture suggest that substantial increases in yield depend on careful control of dissolved oxygen tension. Such control has proven difficult in shake flask cultures, particularly with the reciprocal shakers we use. Differences in the position of flasks on the shaking platform and the size of the flask used (even when the medium to flask volume is kept constant) led to significant differences in the extent to which the medium was aerated and in toxin yields. Partially in response to this, we have recently developed a laboratory-scale recycling fermentor with 100% biomass feedback which appears to be a useful alternative to shake flask cultures for the production of CT and CT-like toxins for further purification (22a).

The facility with which spontaneous lincomycin-resistant mutants can be obtained and the enhanced yields which they make possible suggest that this may be a generally valuable approach. Yamamoto et al. (24) reported that they obtained maximum yields with a spontaneous resistance mutant of *V. cholereae* E8498 in the presence of 300 μg of lincomycin per ml. The yield (at 48 h) ws 1.5-fold that obtained in the presence of 100 μg/ml (538 versus 365 ng/ml). The difference was achieved even though growth in the 300 μg/ml medium was only 19% that in the lower concentration (38 versus 200 mg/ml [dry weight]). Thus, the specific productivity (nanograms of CT-like toxin per milligram of cell dry weight) was 14.1 in the presence of 300 μg of lincomycin per ml versus 1.8 in the presence of 100 μg/ml.

Our attempt to combine optimum growth conditions with lincomycin stimulation of a highly lincomycin-resistant mutant of *V. mimicus* 61892 has led to dramatic increases in CT-like toxin yield from this organism. The highest yield we obtained, 105 μg/ml, is better than the highest published yields of CT from hypertoxigenic strain 569B (40 μg/ml [23]). The purified CT-like toxin produced by this mutant appears to be biologically and antigenically indistinguishable from that produced by the parent strain or from 569B CT (W. M. Spira and P. J. Fedorka-Cray, submitted for publication). We are currently using the protocol described here to produce

CT-like toxin from a number of other strains and have already obtained two mutants which produce high concentrations of CT-like toxin under lincomycin stimulation (at least 50-fold increase over the parent strain). This will greatly simplify the production and purification of CT-like toxin from normally low to moderate producers.

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