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Effects of Microcosm Salinity and Organic Substrate Concentration on Production of *Vibrio cholerae* Enterotoxin

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The effects of aquatic processes on production of cholera toxin by *Vibrio cholerae* were studied with seawater microcosms. Several salinity and organic nutrient concentrations were employed. At 10 g of organic nutrient per liter of seawater, toxin production increased as the salinity was increased. At lower organic nutrient concentrations, toxin production was markedly enhanced when the salinity was 20 and 25‰. Toxin concentration increased with salinity, independent of cell concentration and toxin stability. From the results obtained in this study, it is concluded that physical and chemical parameters of the aquatic environment affect not only the physiological state of *V. cholerae*, but also its potential pathogenicity.

The incidence of cholera, and other enteropathogenic diseases, ranges from sporadic outbreaks to epidemics that characteristically occur in seasonal cycles (1, 3, 11, 12). Indeed, the cyclical nature of the occurrence of disease caused by *Vibrio cholerae* can be influenced by many variables, including the habits of human individuals and a great variety of environmental factors. The former has been well studied in classical epidemiology, especially in the examination of the direct transmission of diarrheal disease. The latter, however, is of special interest, from the perspective of the microbiologist, because the physiological state of the organism, *V. cholerae*, can be directly affected by temperature, salinity, nutrient concentration, and other environmental variables. If these also affect potential pathogenicity (for example, production of enterotoxin), then the environment can, indeed, influence the incidence of the disease in a very direct way (2).

In the present study, the effect of salinity and organic nutrient concentration on production of enterotoxin by *V. cholerae* was studied from the perspective of gaining information about the physiological and potentially pathogenic state of *V. cholerae* in the natural environment and, therefore, how it may be transmitted to humans from aquatic sources.

MATERIALS AND METHODS

Bacteria. Toxigenic isolates of *V. cholerae* were used in all experiments, including classical biotype strains 569B, CA401, ATCC 14035, and HV113; University of Maryland isolates 4507 and 4510; International Center for Diarrheal Disease Research, Bangladesh, isolates 599655, 599687, 599716, and 599717; and International Center for Diarrheal Disease Research, Bangladesh, isolate, El Tor biotype VCELOG52. Stock cultures were maintained in liquid nitrogen, and bench cultures were maintained on peptone agar, which comprised 1% peptone, 1% NaCl, and 1.5% agar (pH 7.4).

Bacterial inoculum. Bacteria were transferred from peptone agar to 100 ml of CAYEG broth (3% Casamino Acids [Difco Laboratories, Detroit, Mich.], 0.3% yeast extract, 0.2% glucose, 0.05% KH_2PO_4 [pH 8.4]) (15) and were incubated for approximately 15 h at 37°C. A 15-h culture (1 ml) was added to 100 ml of freshly prepared CAYEG broth and incubated at 37°C to mid-to-late logarithmic growth phase, after which cells were washed twice in phosphate-buffered saline (PBS) at 1,500 × *g* for 10 min. Cells were resuspended in PBS at approximately 10^8 CFU/ml.

Microcosms. Microcosms consisted of 50 ml of defined seawater medium, previously described by Singleton et al. (18). Concentrations of components in 32‰ salinity seawater were as follows (in grams per liter): NaCl, 23.926; $MgCl_2 \cdot 6H_2O$, 10.83; Na_2SO_4, 4.004; $CaCl_2$, 1.147; KCl, 0.677, $NaHCO_3$, 0.196; KBr, 0.098; H_3BO_3, 0.026; NaF, 0.003; $SrCl_2$, 0.024. Each microcosm contained 48.5 ml of seawater, 0.1 ml of bacteria in PBS, and 1 ml of organic nutrient (CYG) in H_2O. CYG consisted of 30 g of Casamino Acids, 3 g of yeast extract, and 2 g of glucose per liter. The resulting microcosms contained approximately 10^6 CFU/ml. Organic nutrient concentration in individual microcosms was adjusted to 10.0, 1.0, 0.1, 0.01, or 0.001 g/liter. Control microcosms did not receive bacteria. In some experiments, individual salt components were deleted from microcosms. Microcosms were incubated at 37°C for 15 h with rotary agitation at 100 rpm. Microcosm salinity was measured with a salinometer (American Optical Corp., Buffalo, N.Y.). In some experiments, microcosms were prepared with seawater collected from the open ocean at Hogsty Atoll in the Bahamas. Microcosms of various salinities were prepared with natural and artificial seawater diluted with sterile, distilled, deionized water. Toxin production was also measured in CAYEG and syncase broth (5), incubated at 37°C for 15 h at 150 rpm.

ELISA for toxin. Cholera toxin (CT) was detected by using a modified enzyme-linked immunosorbent assay (ELISA; 8). Polystyrene (Costar, Cambridge, Mass.) or polyvinyl chloride (Falcon, no. 3912; Becton Dickinson and Co., Oxnard, Calif.) 96-well microtiter plates were coated with 0.1 ml of anti-CT serum (S12 rabbit serum Peterson; National Institutes of Health, Bethesda, Md.) diluted 1:10,000 with 0.1 M carbonate buffer, pH 9.6. Broth and microcosm samples were dialyzed three times against 100 volumes of PBS at 6°C. A sample (0.1 ml) was added to coated plates and incubated for 1 h at room temperature. Bound CT was detected with anti-CT immunoglobulin G monoclonal antibody 227-65, prepared by Elaine Remmers (14), and affinity-purified horseradish peroxidase-conjugated goat anti-mouse immu-

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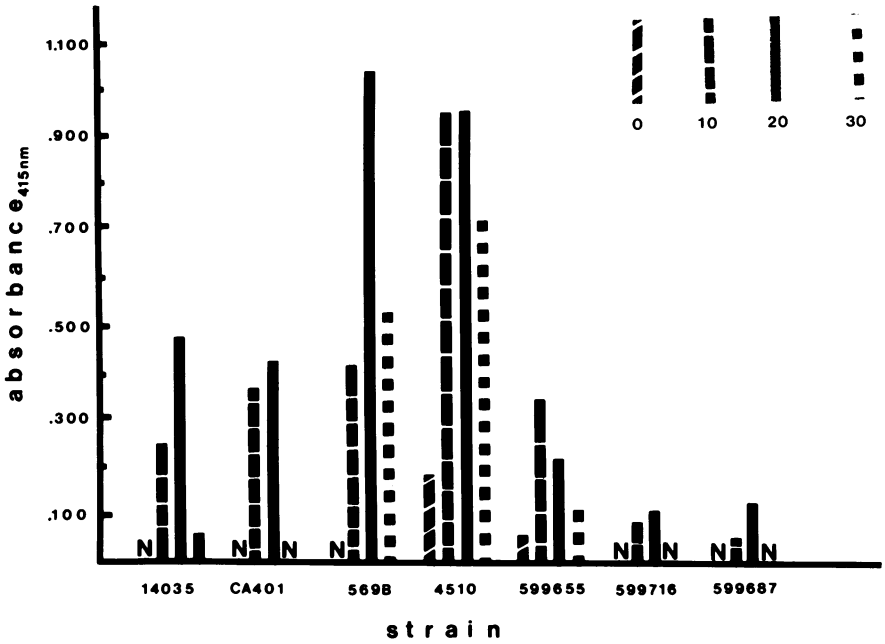

FIG. 1. Effect of salinity on CT production by *V. cholerae* strains. Conditions for incubation: 37°C, 100 rpm, 15 h, 1 g of CYG per liter of seawater. Toxin measured by CT ELISA (A_{415}). Absorbance units were corrected for control microcosms receiving PBS, not bacteria. Salinities tested were 0, 10, 20, and 30‰ as indicated. N, Not detected.

noglobulin G (Cooper Biomedical, Inc., West Chester, Pa.). The developing substrate employed was 2,2'-azino-bis(3-ethylbenz)thiazolinesulfonic acid (A 1888; Sigma Chemical Co., St. Louis, Mo.) in 0.05 M citric acid, pH 4.0. Negative controls consisted of plate wells receiving uninoculated microcosms or broths. Purified CT used for positive controls, and in some microcosms, was purchased from Sigma. CT was stored at 5°C at 1 mg of protein per ml in buffer composed of 0.05 M Tris, 0.001 M disodium EDTA, 0.003 M NaN_3, and 0.2 M NaCl.

Y-1 adrenal cell assay. The Y-1 cell assay was used to measure the biological activity of CT (10).

RESULTS

Effect of salinity and organic nutrient concentration on CT production. CT production increased when *V. cholerae* strains were grown in 10 to 20‰ seawater and 1 g of CYG per liter (Fig. 1). This effect was not related to CFU concentration at 10, 20, and 30‰, although CT levels were associated with CFU concentration of strain 599687 (Table 1). When salinity was increased in 5‰ increments, 20 to 25‰ was optimal for strain 569B (Fig. 2). In contrast, when the concentration of CYG was increased (e.g., from 1 to 10 g/liter), the enhanced production of CT associated with salinity was reduced (Fig. 2). The highest levels of CT production were at 30‰. CT was not detected in microcosms when the organic nutrient concentration was less than 0.1 g/liter (data not shown).

Stability of CT in microcosms. Toxin was added to 10 ml of sterile seawater (0, 5, 10, 15, 20, 25, and 30‰) (final concentration, 1 μg of CT per ml) to determine whether CT was stable at various concentrations of salt. Results of ELISA and Y-1 cell assays confirmed that biological and antigenic properties of CT were stable under conditions of the experiments employed in the present study (data not

TABLE 1. Effect of salinity and organic nutrient on cell concentration of *V. cholerae* strains

Strain	CFU/ml (10^7)[a]						
	Salinity (‰)				Medium		
	0	10	20	30	Seawater[b]	Syncase	CAYEG
14035	1.2×10^{-4}	175	95	192	205	330	42
CA401	3.0×10^{-4}	128	125	160	45	67	70
599655	1.8×10^{-3}	5.3	3.5	7.1	71	325	73
599716	5.3×10^{-4}	8.0	16	8.1	70	325	265
599687	3.5×10^{-4}	7.0	140	7.5	59	275	315
588717	4.5×10^{-4}	15	3.9	4.8	NT[c]	NT	NT
VCELOG52	$<5.0 \times 10^{-5}$	4.0	3.5	7.4	NT	NT	NT
4510	1.5×10^{-2}	53	53	45	60	255	40
569B	9.4×10^{-4}	72	76	100	46	405	6.6

[a] 0, 10, 20, and 30‰ salinity microcosms (1 g of CYG per liter) were incubated at 37°C and 100 rpm for 15 h; seawater, syncase, and CAYEG media were incubated at 37°C and 150 rpm for 15 h.

[b] Seawater, − 20‰, 10 g of CYG per liter.

[c] NT, Not tested.

shown). Extended measurements of CT over a 6-month period yielded results showing that CT was remarkably stable at salinities of 10 to 30‰. Very high concentrations of salt (25 to 30‰) provided greatest stability.

Effect of components of defined seawater medium on CT production. The effects of individual salt components on CT production were tested by deleting individual salts of 20‰ (1 g of CYG per liter) microcosm medium. CT was not detected in microcosms when sodium bicarbonate or potassium chloride were individually deleted (Table 2). Elimination of NaCl, $MgCl_2 \cdot 6H_2O$, Na_2SO_4, $CaCl_2$, or $SrCl_2$ did not significantly reduce CT production. However, a small reduction in CT ($P < 0.05$) was observed when KBr, H_3BO_3, or NaF was deleted.

CT production in seawater, CAYEG, and syncase media. Of 10 strains of *V. cholerae*, 7 produced more CT in defined seawater medium (20‰, 10 g of CYG per liter) than in CAYEG (35 g of nutrient per liter) or syncase (15 g of nutrient per liter) (Fig. 3). The enhanced production of CT in seawater was not related to CFU concentration (Table 1), particularly since the CFU concentration in syncase broth was higher than that in seawater or CAYEG.

DISCUSSION

Many reports have described properties and mechanisms of pathogenicity of bacteria and have provided an understanding of disease processes in general. However, little is known about environmental factors affecting the physiological state of enteropathogenic bacteria and, inferentially, how they control pathogenicity of these bacteria.

Experiments reported in the present study were done to elucidate the effect of marine and estuarine environments on the physiology of *V. cholerae* by monitoring the production of a factor associated with pathogenicity. Since estuarine and brackish waters have been identified as a source of *V. cholerae*, factors of these waters may affect the viability and physiology of *V. cholerae* and, thereby, influence the transmission of cholera (3).

Singleton et al. (17, 18) have shown that the viability of *V. cholerae* is enhanced in waters of 25 to 35‰ salinity, when organic nutrient concentrations (e.g., tryptone) are between 0 and 0.5 g/liter. At nutrient concentrations greater than 0.5 g/liter, salinity dependency was found to be reduced. Miller et al. (13) confirmed the findings of Singleton et al. (17, 18),

TABLE 2. Effect of seawater medium components on production of CT

Salt component deleted from seawater medium	CT ELISA[a] (A_{415})
None	0.118
NaCl	0.118
$MgCl_2 \cdot 6H_2O$	0.126
Na_2SO_4	0.125
$CaCl_2$	0.101
KCl	0.000[b]
$NaHCO_3$	0.000
KBr	0.083
H_3BO_3	0.079
NaF	0.046
$SrCl_2$	0.096

[a] Absorbance values were corrected for microtiter plate wells receiving uninoculated microcosms.

[b] CT levels in microcosms with KCl, $NaHCO_3$, KBr, H_3BO_3, NaF, or $SrCl_2$ deleted were significantly lower than those in complete medium.

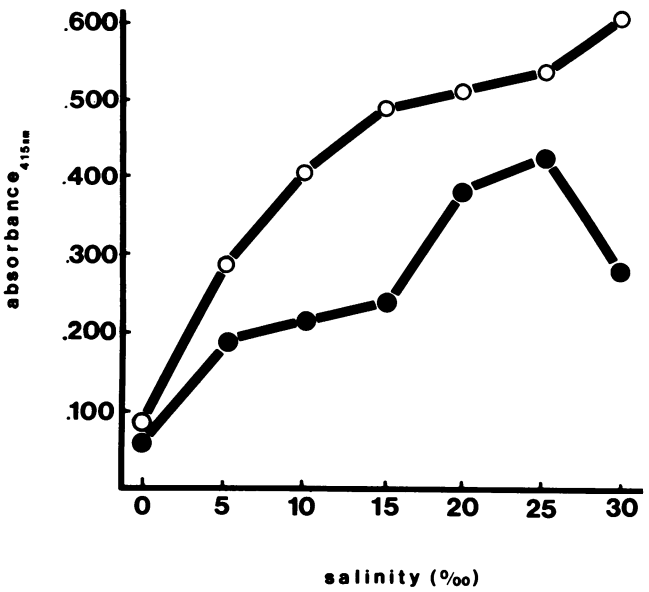

FIG. 2. Effect of salinity and organic nutrient concentration on production of cholera toxin by *V. cholerae* 569B. Symbols: ○, 10 g of CYG per liter; ●, 1 g of CYG per liter.

reporting that the optimum salt concentration for survival of toxigenic *V. cholerae* is 20‰ and that salinities within the range of 25 to 30‰ to support survival of the organism.

The findings reported here also demonstrate a salinity dependency of *V. cholerae*, measured at a nutrient concentration of 0.1 to 1.0 g/liter, nearly twofold greater than that reported by Singleton et al. (18). It is probable that there is a greater sensitivity of direct methods in measuring viability, which allows detection of effects of nutrient at lower concentrations than is possible with ELISA.

The results of the present study show that at high concentrations of organic nutrient (e.g., 10 g of substrate per liter) CT production increases with salinity in the range of 0 to 30‰. However, when organic nutrient is less than 1 g/liter, CT production is markedly enhanced at salinities of 20 to 25‰. The salinity effect is concluded to be directly related to the rate of CT production into the extracellular environment and not to the concentration of culturable cells or stability of CT in various salinities.

Sodium bicarbonate and potassium chloride were essential salts for CT production at 20‰. The former finding is similar to that of Iwanaga and Yamamoto (7), who showed that CT is not detected in their modified CT medium when $NaHCO_3$ is absent. Importantly, seawater medium, in general, supported higher CT production than did CAYEG or syncase medium, even though the organic concentration was 1.5- to 3.5-fold less. The fact that seawater medium enhances CT production and survival of *V. cholerae* (17) further supports studies which show that *V. cholerae* is autochthonous to estuarine environments (2, 3).

Singh and McFeters (16) describe the injurious effects of copper on production of heat-stable toxin by *Escherichia coli* strains. They report that injured strains retain the capacity to produce toxin, yet are not detected on selective media. These authors describe the effects of artificial aquatic processes on injured enteropathogenic bacteria, although viability of the total cell population was not determined. It is highly probable that a large population of nonculturable cells

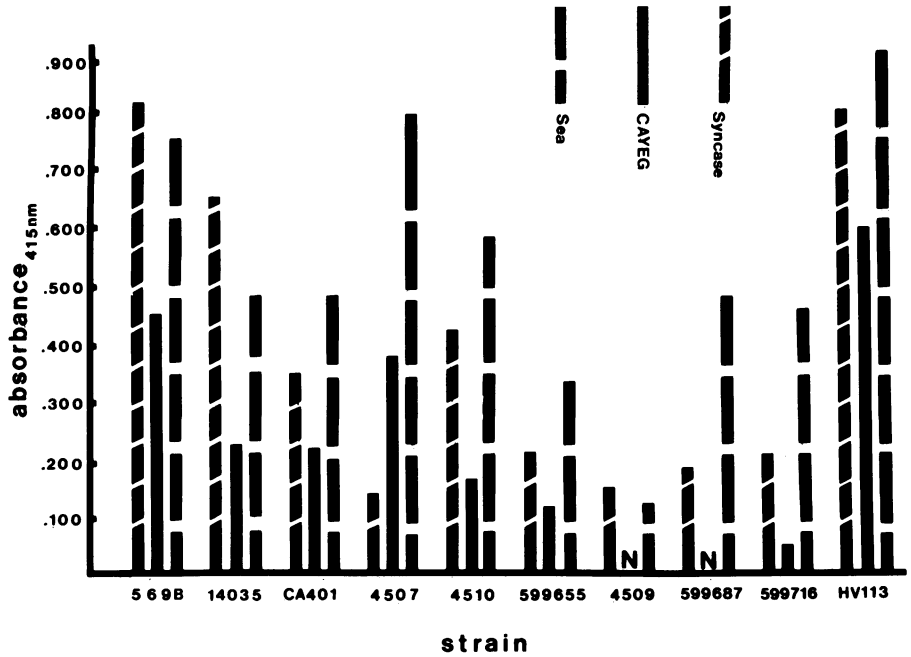

FIG. 3. Comparison of toxin production in defined seawater medium, CAYEG, and syncase broth. Absorbance values for CT ELISA were corrected for uninoculated control cultures. Seawater, 10 g of CYG per liter; N, not detected.

(2) was not detected by a differential test of growth on selective versus nonselective media, which could have been determined by direct measurement of viability (9). Colwell et al. (2) have shown that natural aquatic processes induce a nonculturable form of *V. cholerae*, which is not detected by standard agar and broth methods of culture, but which is viable by the direct microscopic technique of Kogure et al. (9).

Chemical and physical factors of the aquatic environment have a significant effect on the physiology of *V. cholerae*, reflected in the production of enterotoxin associated with clinical disease. We believe that the altered physiology of cells responding to physiochemical variations in the natural environment is reflected in enteropathogenic disease processes. Furthermore, the natural variation in environmental parameters of brackish water and estuarine ecosystems induce physiological and metabolic responses in the bacterial populations, whether temperature, salinity, cation concentration, anion concentration, nutrient concentration, variations in nutrient composition, or other natural variation associated with season, climate, or geography. Thus, the induced physiological or metabolic response (or both) of the microorganism will be reflected in those factors known to be associated with virulence and pathogenicity, such as production of enterotoxin. In certain climes and during specific seasons or meteorologic conditions, pathogenic microorganisms in the environment will be less or more virulent or pathogenic, an example of which is the classical and well-known bimodal distribution of cholera in certain cholera-endemic countries.

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LITERATURE CITED

1. **Black, R. E., K. H. Brown, S. Becker, A. R. M. A. Alim, and M. H. Merson.** 1982. Contamination of weanling foods and transmission of enterotoxigenic *Escherichia coli* diarrhoea in children in rural Bangladesh. Trans. R. Soc. Trop. Med. Hyg. **76**:259–264.
2. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3**:817–820.
3. **Colwell, R. R., R. J. Seidler, J. Kaper, S. W. Joseph, S. Garges, H. Lockman, D. Maneval, H. B. Bradford, N. Roberts, E. Remmers, I. Huq, and A. Huq.** 1981. Occurrence of *Vibrio cholerae* serotype 01 in Maryland and Louisiana estuaries. Appl. Environ. Microbiol. **41**:555–558.
4. **Finkelstein, R. A.** 1973. Cholera. Crit. Rev. Microbiol. **2**:553–623.
5. **Finkelstein, R. A., P. Atthasampunna, M. Chulasamaya, and P. Charunmethee.** 1966. Pathogenesis of experimental cholera: biological activities of purified procholeragen A. J. Immunol. **96**:440–449.
6. **Glass, R. I., S. Becker, M. I. Huq, B. J. Stoll, M. U. Khan, M. H. Merson, J. V. Lee, and R. E. Black.** 1982. Endemic cholera in rural Bangladesh, 1966–1980. Am. J. Epidemiol. **116**:959–970.
7. **Iwanaga, M., and K. Yamamoto.** 1985. New medium for the production of cholera toxin by *Vibrio cholerae* 01 biotype El Tor. J. Clin. Microbiol. **22**:405–408.
8. **Ketyi, I., and A. S. Pacsa.** 1980. Estimation of *Vibrio cholerae* and *Escherichia coli* heat-labile enterotoxin by enzyme-linked immunosorbent assay (ELISA). Acta Microbiol. Acad. Sci. Hung. **27**:89–97.
9. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **25**:414–420.
10. **Maneval, D. R., R. R. Colwell, S. W. Joseph, R. Grays, and S. T.**

Donta. 1980. A tissue culture method for the detection of bacterial enterotoxins. J. Tissue Culture Methods **3**:85–90.
11. **Martin, A. R., W. H. Mosley, B. S. Binapani, A. Shamsa, and I. Huq.** 1969. Epidemiological analysis of endemic cholera in urban East Pakistan 1964–1966. Am. J. Epidemiol. **89**:572–582.
12. **McCormack, W. M., W. H. Mosley, M. Fahimmuddin, and A. S. Benenson.** 1969. Endemic cholera in East Pakistan. Am. J. Epidemiol. **89**:393–404.
13. **Miller, C. J., B. Drasar, and R. G. Feachem.** 1984. Response of toxigenic *Vibrio cholerae* 01 to physio-chemical stresses in aquatic environments. J. Hyg. **93**:475–495.
14. **Remmers, E. F., R. R. Colwell, and R. A. Goldsby.** 1982. Production and characterization of monoclonal antibodies to cholera toxin. Infect. Immun. **37**:70–76.
15. **Sack, D. A., S. Huda, P. K. B. Neogi, R. R. Daniel, and W. M. Spira.** 1980. Microtiter ganglioside enzyme-linked immunosorbent assay for Vibrio and *Escherichia coli* heat-labile enterotoxins and antitoxin. J. Clin. Microbiol. **11**:35–40.
16. **Singh, A., and G. A. McFeters.** 1986. Recovery, growth, and production of heat-stable enterotoxin by *Escherichia coli* after copper-induced injury. Appl. Environ. Microbiol. **51**:738–742.
17. **Singleton, F. L., R. Attwell, S. Jangi, and R. R. Colwell.** 1982. Effects of temperature and salinity on *Vibrio cholerae* growth. Appl. Environ. Microbiol. **44**:1047–1058.
18. **Singleton, F. L., R. A. Attwell, M. S. Jangi, and R. R. Colwell.** 1982. Influence of salinity and organic nutrient concentration on survival and growth of *Vibrio cholerae* in aquatic microcosms. Appl. Environ. Microbiol. **43**:1080–1085.