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Enhancement of Enterotoxin Production by Carbon Dioxide in *Vibrio cholerae*

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We found that *Vibrio cholerae* 569B produced much more cholera enterotoxin in the presence of added carbon dioxide than in its absence. An atmosphere of 10% carbon dioxide was optimal for maximal enterotoxin production.

We have recently developed an animal model for experimental cholera by using germfree suckling mice (5). In this model *Vibrio cholerae* 569B, classical Inaba, which is a high producer of cholera enterotoxin (CT) in vitro, did not cause disease, whereas *V. cholerae* V86, El Tor Inaba, which is a poor producer, caused disease in both germfree and conventional suckling mice. The mortality was lower in conventional mice as compared with germfree mice. These findings suggested that CT production in vivo and in vitro may be different and that the microenvironment in the intestines of animals may be an important factor for CT production by *V. cholerae*. During experiments designed to define conditions leading to high CT production, we found that carbon dioxide enhanced CT production by *V. cholerae* in vitro.

The organism used in this study was *V. cholerae* 569B, classical Inaba. The origin of this strain has been previously described (2). Ten microliters (1×10^6 to 5×10^6 CFU) of 24-h cultures in heart infusion broth (Difco Laboratories, Detroit, Mich.) were added to 5 ml of Syncase medium (4) in plastic tubes (17 by 100 mm, 2001 tube; Falcon Plastics, Oxnard, Calif.). The cultures were incubated for 7 or 24 h at 37°C under various cultural conditions. Bacterial cell growth was monitored by measuring the optical density of the culture at 640 nm with a spectrophotometer (Shimazu Bausch and Lomb, Tokyo, Japan). The cells were removed by centrifugation for 2 min at $4,700 \times g$ in a microcentrifuge (Micro-Centrifuge model 59A; Fisher Scientific, Co., Pittsburgh, Pa.), and the supernatant was filtered through a 0.2-μm membrane filter (Minisart NML; Sartorius Filters, Inc.). CT antigen in the filtrates was measured by reversed passive latex agglutination (6) by using latex particles sensitized with rabbit antibodies to CT and serial twofold dilution of filtrates. Titrations were carried out by using a VET-RPLA kit (Denka Seiken, Tokyo, Japan). Toxin titer was expressed as $\log_2$ of the highest dilution showing agglutination.

We first compared CT production and growth of strain 569B under various growth conditions. The results of a representative experiment are shown in Table 1. A stationary culture grown in an atmosphere of 5% CO_2 in air yielded 32 times as much CT as that grown in air. The anaerobic stationary culture grown in an atmosphere of 10% CO_2–80% N_2–10% H_2 in a glove box, and the aerobic shaking culture frequently used in toxin studies both yielded 16 times as much CT as did the aerobic stationary culture. Although in both the stationary culture with 5% CO_2 and the anaerobic culture bacterial cell growth was less than that in the aerobic shaking culture, CT production was greater. These results confirmed the finding of Fernandes and Smith (3) that anaerobic conditions with an atmosphere of 5% CO_2–88% N_2–7% H_2 at 37°C resulted in CT production by *V. cholerae* 569B at a high toxin-to-cell ratio. Our findings suggest that the high CT production under anaerobic conditions is due to the carbon dioxide content, since 5% CO_2 in air enhanced CT production to almost the same level as that achieved under anaerobic conditions.

TABLE 1. Comparison of CT production and growth of *V. cholerae* 569B in various cultural conditions

Cultural conditions	CT production and cell growth at h of incubation[a]			
	7		24	
	Toxin titer	OD	Toxin titer	OD
5% CO_2 in air, stationary	6	0.102	9	0.285
Anaerobic, stationary	7	0.133	8	0.285
Aerobic, stationary	2	0.050	4	0.340
Aerobic, shaking	5	0.250	8	1.10

[a] The toxin titer is expressed as $\log_2$ of the highest dilution showing a positive reaction. The optical density (OD) was measured at 640 nm.

We next examined the effect of CO_2 concentration on CT production and cell growth (Table 2). Carbon dioxide markedly enhanced CT production in spite of a slight suppression of cell growth. A CO_2 concentration of 10% yielded maximal CT production. Carbon dioxide also stimulated toxin production by *V. cholerae* V86, El Tor Inaba (CT), and enterotoxigenic *Escherichia coli* H10407 (heat-labile enterotoxin), although the enhancement of toxin production by these strains was less than that exhibited by strain 569B (data not shown).

A half-century ago Burnet found that carbon dioxide increased hemolysin production by *Staphylococcus aureus* (1). The effect of carbon dioxide was quantitative, and 20% CO_2 enhanced hemolysin production 40-fold. He hypothesized that the effect of CO_2 in increasing the yield of staphylococcal hemolysin was due to an increase in intracellular acidity. The mechanism by which carbon dioxide enhances CT production by *V. cholerae* 569B is unknown. CT production by *V. cholerae* in the presence of CO_2 has not been reported except for studies on CT production under anaerobic conditions (3). Thus, this study is the first demonstration that carbon dioxide alone enhances CT production by *V. cholerae*.

In conclusion, *V. cholerae* 569B produces a high level of

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TABLE 2. Effect of CO_2 concentration on CT production and growth of *V. cholerae* 569B

Atmospheric CO_2 concentration (%)	CT production and cell growth at 24 h of incubation[a]	
	Toxin titer	OD
0	5	0.311
5	10	0.290
10	12	0.270
20	10	0.260

[a] The toxin titer is expressed as $\log_2$ of the highest dilution showing a positive reaction. The optical density (OD) was measured at 640 nm.

CT in stationary cultures grown in an atmosphere of 5 to 10% CO_2 in air at 37°C. This cultural method may be useful for studies of the mechanism of CT production by *V. cholerae*.

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