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Nutritional Aspects of Cytotoxin Production by *Clostridium difficile*

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Arginine was the only amino acid used by *Clostridium difficile* that permitted cytotoxin synthesis in a peptone-based medium. Synthesis of cytotoxin was delayed when glucose was used as the substrate. Addition of rifampin or puromycin to cultures prior to release of cytotoxin inhibited the release of cytotoxin, suggesting that a protein essential for cytotoxin release is synthesized after cytotoxin is synthesized.

Clostridium difficile is the major causative agent of pseudomembranous colitis in humans and in animals (4, 11, 19). *C. difficile* and the diseases that it causes are associated with oral antibiotic, especially clindamycin, therapy for treating anaerobic bacterial diseases (1, 3, 10). The pathology includes diarrhea and severe inflammation of the colonic mucosa with elaboration of pseudomembranes (11). Pseudomembranous colitis may result from nosocomial infections after the organism is released into the environment and spread through hospital personnel and facilities (19). The production of two toxins by *C. difficile* was reported in 1981 (4, 17, 18). Toxin A, an enterotoxin, is primarily responsible for the symptoms associated with pseudomembranous colitis, including extensive tissue damage and fluid response. Toxin B is responsible for the potent cytotoxic effect; this toxin has a molecular weight of 269,696, as determined from the nucleotide sequence (2), and it is usually detected by the tissue culture assay (5, 6).

Toxin production depends on the bacterial strain used and on the growth medium used (7, 12–14). Most cell-associated toxin B is found during the exponential phase of growth; the extracellular levels increase from 36 to 72 h (9). However, in one previous study workers observed a decline in cell-associated toxin B content during the exponential phase (14), and in another study workers found the maximum amount of free toxin between 24 and 48 h after inoculation (3). During a study of the regulation of cytotoxin synthesis, we found that initiation of cytotoxin synthesis and the time of cytotoxin release differed when *C. difficile* was cultured in Trypticase-yeast extract (TY) medium containing arginine or glucose as a substrate. When arginine and glucose were used, the highest titer of toxin observed (10^6 at 72 h) was obtained. Addition of rifampin or puromycin prior to toxin release inhibited cytotoxin release.

MATERIALS AND METHODS

Bacterial strain. *C. difficile* VPI 10463 (Tox^+) was obtained from the culture collection of the Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg.

Turbidity and pH measurements. Stock cultures were grown in chopped meat medium (Difco) and were used to inoculate medium containing Trypticase (BBL) and yeast extract (Scott Laboratories) (8) with or without added substrate. Trypticase-vitamin (TV) medium containing a vitamin solution in place of yeast extract (8) was also used. The cultures were grown to the early stationary phase and were used to inoculate the same types of media. Before and after incubation, the pH of each medium was measured with a Corning model 7 pH meter. Optical density was measured at 540 nm with a Bausch & Lomb Spectronic 20 spectrophotometer.

Glucose and arginine determinations. Arginine (15) and glucose (16) assays were performed by using aliquots of culture supernatants (centrifuged at 12,000 × *g* for 20 min at 4°C) to determine residual levels of these substrates.

Toxin preparation. Cultures were centrifuged at 12,000 × *g* for 20 min. Free cytotoxin was collected by passing the supernatants through a 25-mm membrane filter (pore diameter, 0.45 μm; type HAWP cellulose mixed esters; Millipore). Cell-associated cytotoxin was prepared by resuspending cell pellets in 0.05 M Tris-HCl buffer (pH 7), and the cells were broken by two passages through a French pressure cell at 15,000 lb/in^2. The crude cell extract was centrifuged at 12,000 × *g* for 20 min, and the supernatant was passed through a 25-mm membrane filter (pore diameter, 0.45 μm; type HAWP; Millipore).

Cytotoxicity assay. Chinese hamster lung cells (cell line CHL V79) were diluted to a concentration of approximately 2.5×10^4 cells per ml in HAM F12 medium containing L-glutamine (GIBCO Laboratories) and 10% fetal calf serum (Sigma Chemical Co.). The diluted cells were added to the wells of 24-well tissue culture plates (1 ml per well), and the plates were incubated for 24 h at 37°C in a CO_2 incubator. Tenfold serial dilutions (0.1 ml per well) of the toxin samples were added to the cell monolayers. The plates were incubated for an additional 24 h and then examined for cell rounding. The toxin titer was the reciprocal of the dilution that resulted in 50% cell rounding (17).

Protein and RNA synthesis inhibitors. Rifampin (dissolved in dimethyl sulfoxide) and puromycin dihydrochloride (dissolved in sterile water) were added to produce final concentrations of 10 and 150 μg/ml, respectively, in cultures at various times during growth to determine their effects on the cytotoxin titer. Rifampin and puromycin were purchased from U.S. Biochemicals, Cleveland, Ohio.

RESULTS AND DISCUSSION

Previously, we observed repression of cytotoxin synthesis by elevated levels (>0.2%) of glucose in the medium (13). To alleviate this effect, we grew *C. difficile* in TY medium containing individual amino acids as energy sources. Table 1

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TABLE 1. Effects of yeast extract and amino acids on growth of and cytotoxin production by *C. difficile* VPI 10463

Medium	Substrate	Optical density at 540 nm	Cytotoxin titer at 72 h[a]
TY	Alanine (50 mM)	1.2	ND[b]
	Isoleucine (50 mM)	0.76[c]	ND
	Leucine (50 mM)	0.76[c]	ND
	Serine (50 mM)	1.1	ND
	Threonine (50 mM)	0.97	ND
	Valine (50 mM)	0.64[c]	ND
	None	0.85	10^3
	Arginine (50 mM)	1.5	10^6
	Glucose (0.2%)	1.5	10^4
	Glucose (1%)	1.5	10^6
TV	None	0.46	ND
	Alanine (50 mM)	0.29	ND
	Arginine (50 mM)	0.87	10^4
	Serine (50 mM)	0.47	ND
	Threonine (50 mM)	0.73	ND
	Glucose (1%)	0.56	ND

[a] Titer resulting in rounding of 50% of hamster CHL V79 cells.
[b] ND, no cytotoxic activity detected.
[c] Growth and cytotoxin production were inhibited.

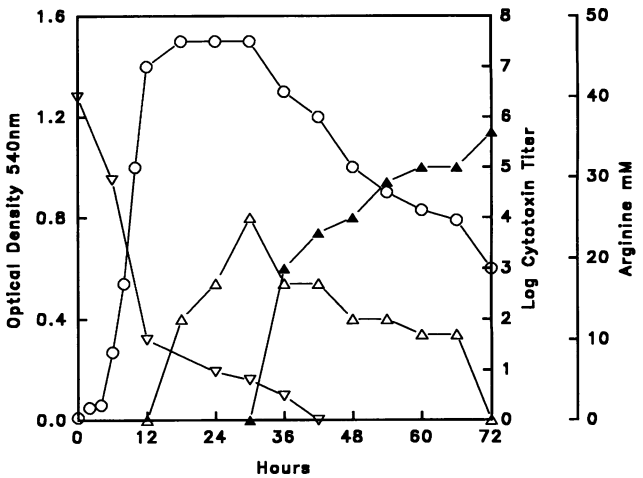

FIG. 1. Growth of *C. difficile* VPI 10463 and arginine utilization in TY broth containing 1% arginine and detection of cytotoxin in cell lysates and free cytotoxin in culture supernatants. Symbols: ○, optical density at 540 nm; ▽, arginine concentration; △, cell-associated cytotoxin titer; ▲, free cytotoxin titer.

shows the effects of different substrates on growth of and cytotoxin titers produced by *C. difficile* grown in TY media and TV media. No cytotoxin was detected in the supernatants of cultures containing substrate levels (50 mM) of the amino acids alanine, serine, leucine, isoleucine, threonine, and valine. Only addition of substrate levels of arginine resulted in any significant cytotoxin titer. A cytotoxin titer of 10^3 was obtained when *C. difficile* was grown in TY medium. Addition of 0.2% glucose to TY medium produced a 100-fold increase in the cytotoxin titer compared with the titer obtained with TY medium, and an additional 100-fold increase in cytotoxin titer was observed when 1% glucose was added (Table 1). No spores were detected by microscopic examination of TY-glucose or TY-arginine medium cultures.

To determine whether the presence of yeast extract affected cytotoxin production when amino acids were added as substrates, TV medium was used. The effect of adding yeast extract as a substrate for growth of *C. difficile* was determined by comparing TY medium cultures and TV medium cultures. There was almost twice as much growth when yeast extract was present (Table 1). Again, only cultures containing arginine-supplemented TV media produced cytotoxin. No cytotoxin was detected when *C. difficile* was grown in TV medium containing glucose. The fact that *C. difficile* did not produce cytotoxin when it was grown in TV media containing glucose but did produce cytotoxin when the medium contained arginine could be related to the immediate elevation of the pH by release of ammonia from arginine. *C. difficile* grew poorly and produced no detectable cytotoxin when it was grown in TV media that did not contain added substrate. Defined minimal medium containing glucose was also used for growth of *C. difficile*; however, growth was not very good, and very little cytotoxic activity was detected. Similar results for growth and cytotoxin synthesis by *C. difficile* were observed by Haslam et al. (7) when they used a defined medium.

The TY-arginine medium culture reached maximum growth (optical density at 540 nm, 1.5) in less than 24 h, and no residual arginine was detected after 36 h (Fig. 1). Figure 2 shows the growth of and utilization of glucose by *C. difficile* cultured in TY medium containing 0.2% glucose. With medium containing 0.2% glucose, the optical density reached 1.5 at 10 h, and only low levels of residual glucose were detected after 36 h. Figure 3 shows that the TY medium culture supplemented with 1% glucose reached an optical density of 1.5 at 18 h and had a residual glucose level of 22 mM at 18 h.

Substrates that permitted cytotoxin production were used to determine free (supernatant) and cell-associated cytotoxin levels. Bacteria grown in TY media (without additional substrates) produced cytotoxin intracellularly within 18 h, and the cytotoxin level increased to a maximum cell-associated titer of 10^2 at 24 h. No cell-associated toxin was detected after 30 h of incubation. Free cytotoxin was not detected until 42 h, and its titer increased slightly to 10^3 at 72 h in TY medium cultures. Addition of 0.2% glucose resulted

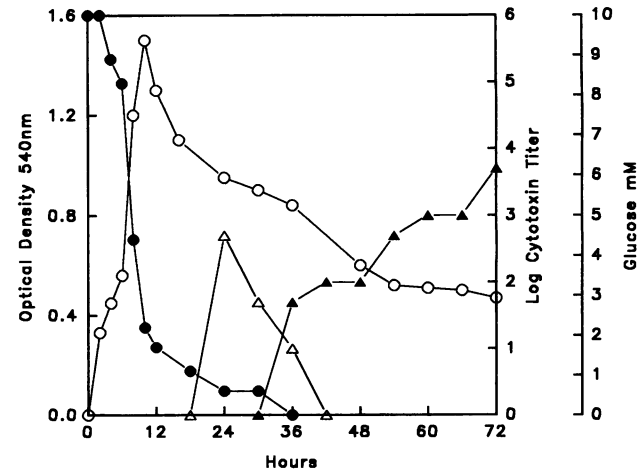

FIG. 2. Growth of *C. difficile* VPI 10463 and glucose utilization in TY broth containing 0.2% glucose. Production of cytotoxin was assayed in cell lysates and culture supernatants. Symbols: ○, optical density at 540 nm; ▼, glucose concentration; △, cell-associated cytotoxin titer; ▲, free cytotoxin titer.

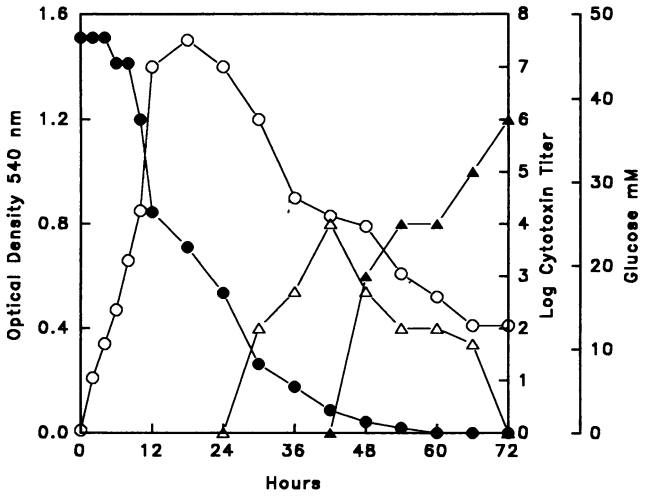

FIG. 3. Growth of *C. difficile* VPI 10463 and glucose utilization in TY broth containing 1% glucose. Production of cytotoxin was assayed in cell lysates and culture supernatants. Symbols: ○, optical density at 540 nm; ●, glucose concentration; △, cell-associated cytotoxin titer; ▲, free cytotoxin titer.

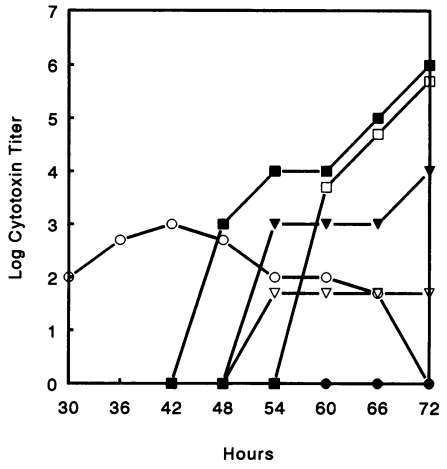

FIG. 4. Effect of addition of rifampin on production and release of cytotoxin by *C. difficile* in TY media containing 1% glucose. Symbols: ○, cell-associated cytotoxin titer; ●, free cytotoxin titer when rifampin was added at 36 h; ▽, free cytotoxin titer when rifampin was added at 42 h; ▼, free cytotoxin titer when rifampin was added at 48 h; □, free cytotoxin titer when rifampin was added at 54 h; ■, free cytotoxin titer when no rifampin was added.

in a cell-associated cytotoxin titer of nearly 10^3 at 24 h. Extracellular cytotoxin appeared at 36 h, and its level increased to a titer of 10^4 by 72 h (Fig. 2). When the carbohydrate level was increased to 1%, cell-associated cytotoxin appeared later (>24 h), but reached a titer of 10^4 at 42 h. Free cytotoxin did not appear until 48 h and increased to a titer of 10^6 at 72 h (Fig. 3). At both levels of carbohydrate, free cytotoxin was not observed until the glucose concentration was almost depleted (<2 mM). Furthermore, when *C. difficile* was grown in media containing glucose as a substrate, autolysis occurred immediately after the maximum optical density occurred, yet 24 to 36 h elapsed before cytotoxin was detected in the supernatant (Fig. 2 and 3). This finding and the fact that the optical densities had to decrease to almost one-half of their maximum values before measurable cytotoxin was released make it doubtful that the cytotoxin was released only during autolysis of the bacteria.

When arginine was used as a substrate, cell-associated cytotoxin appeared at 18 h, and the level of this cytotoxin peaked at 30 h and slowly declined until 72 h, after which no toxin was detected (Fig. 1). Free cytotoxin was observed initially at 36 h at a titer of 10^3, and its level increased to a titer of more than 10^5 by 72 h. No residual arginine was detected in the medium after 36 h. Some differences in cytotoxin titers and times of cytotoxin synthesis in previous reports (1, 7, 9, 12, 14) on the production of cytotoxin by *C. difficile* can be explained by strain variation or the use of different media for growth and toxin production. In this study, a more controlled assay was performed by using TY medium containing glucose or arginine. As in previous studies, extracellular cytotoxin was found during the death and decline phase of growth. This study showed that cell-associated cytotoxin was detected in the late exponential phase of growth when *C. difficile* was grown in TY media containing arginine or in the death and decline phase in media containing glucose and that the cytotoxin was detected after most of the substrate was used. We suggest that cytotoxin is synthesized during the stationary phase of growth and is released by autolysis or is secreted by some other mechanism during the death and decline phase.

The transcriptional inhibitor rifampin and the translational inhibitor puromycin were added to cultures to determine whether cytotoxin was synthesized continuously during the decline phase of growth. Figure 4 shows that when *C. difficile* was grown in TY medium containing 1% glucose, addition of rifampin prior to 42 h prevented cytotoxin release, although cell-associated cytotoxin was already present. When rifampin was added at 42 h, the amount of cytotoxin released was slight. The cytotoxin titer was 100-fold less than the titer in the control when rifampin was added at 48 h. There was only a slight decrease in the final titer when rifampin was added at 54 h. These data indicate that a new mRNA was synthesized between 36 and 48 h, allowing synthesis of a protein that is required for the release of the cytotoxin. The more than 10-fold difference in the observed titer caused by the addition of rifampin at 48 h and the fact that the total cytotoxin content increased 100-fold from 48 h to 72 h (Fig. 3) suggest that cytotoxin was actively synthesized between 48 and 72 h. Generally similar results were obtained with TY media to which 0.2% glucose or arginine was added.

Puromycin (150 μg/ml) was added to *C. difficile* grown in TY medium containing 1% glucose or arginine, and the extracellular cytotoxin content was determined at 72 h (Table 2). No cytotoxin was released from *C. difficile* cultures grown in TY medium containing 1% glucose when puromycin was added before 36 h. Addition of puromycin at 36 h resulted in the release of a small amount of cytotoxin. Subsequently, there was a 10-fold increase in the amount of cytotoxin released for each 6 h of delay before the addition of puromycin. This corroborates the rifampin data which indicated that cytotoxin was synthesized at 48 h and later.

Inhibition of cytotoxin release by rifampin or puromycin after initiation of cytotoxin synthesis (i.e., the presence of cell-associated cytotoxin) leads us to suggest that late synthesis of either a secretory protein or an autolytic enzyme is required for the release of cytotoxin from the bacteria.

TABLE 2. Effect of puromycin on production of cytotoxin by *C. difficile* grown in TY medium containing 1% glucose or 1% arginine

Time (h) that puromycin was added to the culture	Extracellular cytotoxic activity (titer) at 72 h	
	TY-arginine medium	TY-glucose medium
30	10^2	ND[a]
36	10^3	10^2
42	NA[b]	10^3
48	10^4	10^4
54	10^5	10^5
No puromycin added	10^6	10^6

[a] ND, no cytotoxin detected.
[b] NA, not assayed.

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