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Michael T. Kazamias

Jay F. Sperry

*University of Rhode Island*

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Kazamias, M. T. & Sperry, J. F. (1995). Enhanced Fermentation of Mannitol and Release of Cytotoxin by *Clostridium difficile* in Alkaline Culture Media. *Applied and Environmental Microbiology*, 61(6), 2425-2427.

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## Enhanced Fermentation of Mannitol and Release of Cytotoxin by *Clostridium difficile* in Alkaline Culture Media

MICHAEL T. KAZAMIAS AND JAY F. SPERRY\*

Department of Biochemistry, Microbiology & Molecular Genetics,  
University of Rhode Island, Kingston, Rhode Island 02881

Received 29 September 1994/Accepted 4 April 1995

*Clostridium difficile* ATCC 43255 fermented less than 10% of the mannitol in a medium at pH 7; however, when the initial pH of the medium was adjusted to 8.5 or 9, about 80% of the mannitol was fermented. Cell extracts of *C. difficile* phosphorylated mannitol with phosphoenolpyruvate, not ATP, indicating a phosphoenolpyruvate phosphotransferase system transport phosphorylation of mannitol. The phosphorylation product was dehydrogenated by D-mannitol-1-phosphate:NAD oxidoreductase. Growth at an initial pH of 8.5 yielded cytotoxin titers of  $10^7$  to  $10^8$  in Trypticase-yeast extract-mannitol medium, with a titer of  $10^8$  as early as 13 h.

During the study of the regulation of cytotoxin synthesis and its release from *Clostridium difficile*, a delay in the synthesis and release of the cytotoxin was observed when glucose was present in the medium (13). Mannitol was substituted for glucose as an energy source to alleviate an apparent glucose repression of the synthesis and release of its cytotoxin. This caused more growth than that in the Trypticase-yeast extract (TY) medium (15); however, only 6% of the mannitol was metabolized (Fig. 1A). Residual mannitol was assayed by a periodate oxidation method (3) with mannitol as the standard.

*Bergey's Manual of Systematic Bacteriology* suggests that fermentation of mannitol is an important characteristic of the species *C. difficile*, and this is the best way to differentiate it from *Clostridium sporogenes* (2), one of the more-similar organisms. Therefore, we wanted to determine why the fermentation of mannitol by *C. difficile* was so limited. *C. difficile* ATCC 43255 (VPI 10463) was grown in TY medium (pH 7) containing 1% mannitol (TYM) under  $O_2$ -free  $CO_2$  at  $37^\circ C$  for 24 h. The cells were pelleted at  $10,000 \times g$ , washed once, and then resuspended in 0.05 M Tris-HCl buffer (pH 7) and were broken by passage through a French pressure cell twice at  $15,000 \text{ lb/in}^2$ . The lysate was centrifuged at  $8,000 \times g$  for 5 min at  $4^\circ C$  to pellet whole cells and large debris. The supernatant was assayed for mannitol:NAD oxidoreductase, with no activity being observed. When the supernatant was assayed for D-mannitol-1-phosphate:oxidoreductase (9), slight activity ( $0.0062 \mu\text{mol per min per mg of protein}$ ) was detected at pH 7. For most bacteria this enzyme usually has a pH optimum around 9 to 9.6 (6, 9-11). When the pH of the assay mixture was adjusted to 8.9, there was an increase in specific activity to 0.273. The difference in activity (44 times greater activity at pH 8.9) of this enzyme could explain why so little of the mannitol was fermented at pH 7.

No mannitol kinase activity was detected with ATP in a coupled enzyme assay (1 ml) containing mannitol ( $0.83 \mu\text{mol}$ ), Tris-HCl buffer (pH 8.9) ( $200 \mu\text{mol}$ ), NAD ( $0.5 \mu\text{mol}$ ), ATP (or phosphoenolpyruvate [PEP]) ( $1.25 \mu\text{mol}$ ), and bacterial cell extract ( $0.5$  to  $1 \text{ mg of protein per ml}$ ). The  $A_{340}$  was read. When we substituted PEP for ATP in the coupled enzyme assay at pH 8.9, a specific activity of  $0.047 \mu\text{mol per min per mg}$

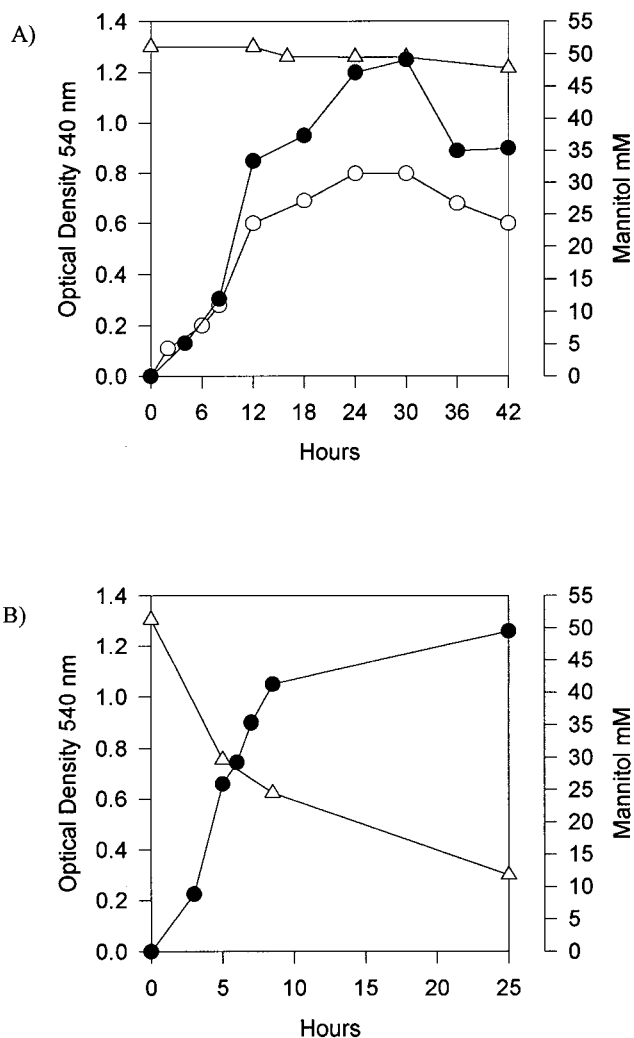


FIG. 1. (A) Growth of *C. difficile* ATCC 43255 in TY broth and TYM at an initial pH of 7. Symbols: ○, optical density in TY broth; ●, optical density in TYM; △, residual mannitol. (B) Growth of *C. difficile* ATCC 43255 in TYM at an initial pH of 8.5. Symbols: ●, optical density; △, residual mannitol.

\* Corresponding author. Mailing address: Department of Biochemistry, Microbiology & Molecular Genetics, University of Rhode Island, Kingston, RI 02881. Phone: (401) 792-5908. Fax: (401) 792-7148. Electronic mail address: AUN102@URIACC.URI.EDU.

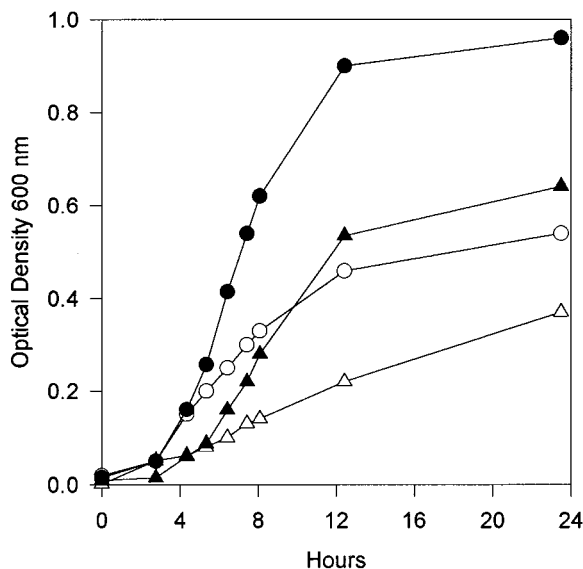


FIG. 2. Growth of *C. difficile* ATCC 43255 in Trypticase broth containing 0.1% or 0.25% yeast extract with or without 1% mannitol at an initial pH of 6.5 or 8.5. Symbols:  $\Delta$ , 0.1% yeast extract, pH 6.5;  $\blacktriangle$ , 0.1% yeast extract, pH 8.5;  $\circ$ , 0.25% yeast extract, pH 6.5, 1% mannitol;  $\bullet$ , 0.25% yeast extract, pH 8.5, 1% mannitol.

of protein was observed. This indicates that *C. difficile* uses a PEP-phosphotransferase (PTS) transport system for metabolism of mannitol. The PEP-PTS transport system for mannitol and fructose has been previously observed in clostridia (12).

To determine whether adjustment of the initial pH of the culture medium would allow for increased fermentation of mannitol, the pH of the culture medium was adjusted to 8.5 to 8.9 by the addition of potassium hydroxide (the pH of the medium was adjusted by adding 10 M KOH under  $O_2$ -free  $CO_2$ ). Hardman and Stadtman (4) have reported that other species of clostridia require a pH higher than 7 to ferment carbohydrates. When *C. difficile* was grown in TYM at pH 8.5 to 8.9, there was an increase in the rate of growth, the cell yield, and the amount of mannitol utilized (Fig. 1B).

We had previously noted that there was some fermentable substrate in the yeast extract, and so the growth of *C. difficile* was measured in TYM containing 0.25% or 0.1% yeast extract instead of the standard 1% yeast extract. Figure 2 shows that *C. difficile* grew more rapidly and produced higher cell yields in TYM at pH 8.5 (closed symbols) than at pH 6.5 (open symbols). The amount of yeast extract in the TYM affected the rate of growth slightly, but the cell yield was affected more dramatically. The products of fermentation were assayed (5) with a CAPCO model 700 gas chromatograph. Only acetic and isocaproic acids were observed at concentrations above those of the *C. difficile* TY culture control. However, 3.5  $\mu$ mol of *n*-butanol per ml and 0.5  $\mu$ mol of *n*-pentanol per ml were produced (data not shown). Nine strains of *C. difficile* and one strain of *C. sporogenes* were cultured in TYM (1% yeast extract) at pH 8.5. Only ATCC 43255 showed a sizable drop in pH (6.35), and ATCC 43255 was the only one that showed a real increase in optical density (optical density at 600 nm = 1.46). Fermentation products for strain 649 were very slight, with only traces (<0.1  $\mu$ mol per ml) of *n*-butanol and *n*-pentanol, indicating very slight fermentation of carbohydrate, possibly of mannitol or yeast extract. These results indicate that the fermentation of mannitol by strains of *C. difficile* might not be as common as has been reported (2).

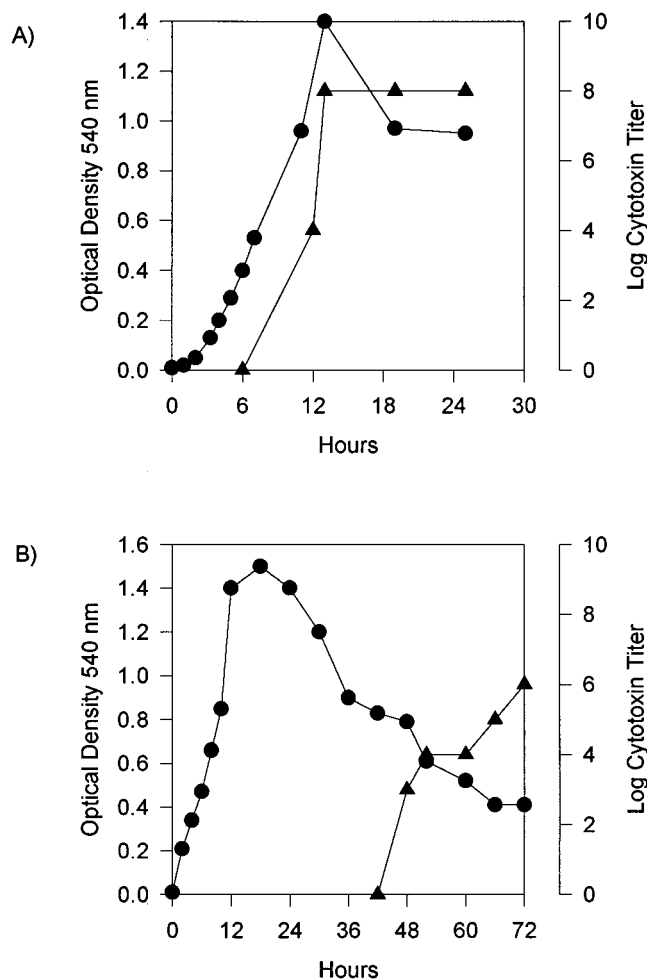


FIG. 3. (A) Cytotoxin production by *C. difficile* ATCC 43255 growing in TYM at an initial pH of 8.5. Symbols:  $\bullet$ , optical density;  $\blacktriangle$ , cytotoxin free in culture supernatant. (B) Cytotoxin production by *C. difficile* ATCC 43255 growing in TY broth containing 1% glucose at an initial pH of 7. Symbols:  $\bullet$ , optical density;  $\blacktriangle$ , cytotoxin free in culture supernatant. One cytotoxic unit causes rounding of 50% of cells.

Production of the cytotoxin by *C. difficile* was also affected by the higher initial pH of the growth medium. Cytotoxin was assayed with Chinese hamster lung (V79) cells grown in F12 medium containing 10% fetal calf serum. Twenty-four-well microtiter plates were used, and 1 ml of cells ( $10^4$ ) was added per well. After 24 h at 37°C, 0.1-ml aliquots of dilutions of samples were added in triplicate, and then the plates were incubated for another 24 h. The titer was the reciprocal of the dilution that gave 50% rounding of the lung cells. Figure 3A shows that by 12 h, during late exponential phase, the cytotoxin titer was already  $10^4$ , and it reached  $10^8$  by 13 h. This titer of cytotoxin was 10- to 100-fold higher than what we normally detect for *C. difficile* ATCC 43255 cultured in TY broth containing 1% glucose at an initial pH of 6.5 to 7 (Fig. 3B). Reports of *C. difficile* cytotoxin titers range from  $10^3$  50% cytotoxic units/ml to  $10^7$  100% cytotoxic units/ml, usually after 96 h of incubation (1, 7, 8, 14). The release of cytotoxin during the late logarithmic phase of growth (Fig. 3A) suggests that the cytotoxin may be released from the bacteria by a secretion mechanism rather than by autolysis.

This research was supported in part by Public Health Service Biomedical Research Support grant RR07086-15.

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