

EVALUATION OF METHYLXANTHINES AND RELATED
COMPOUNDS TO ENHANCE *CLOSTRIDIUM PERFRINGENS*
SPORULATION USING A MODIFIED DUNCAN AND
STRONG MEDIUM¹

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ABSTRACT

Methylxanthines and related compounds were added to Duncan-Strong (DS) medium in an effort to improve spore yields and enterotoxin production in three strains of Clostridium perfringens. Supplementation with 50–200 µg of caffeine/ml (0.26–1.03 mM) increased heat-resistant spore yields for strain NCTC 8238 from 4.32 to 6.03 log₁₀ spores/ml after 24 h of incubation. With strains NCTC 8239 and NCTC 10240, 50–200 µg/ml caffeine (0.26–1.03 mM) or theophylline (0.28–1.11 mM) were equally effective, and increased spore yields from 4.18–4.89 to 6.00–7.13 log₁₀ spores/ml. When sporulation and enterotoxin production were evaluated in DS medium containing either starch or raffinose and supplemented with 100 µg/ml caffeine, heat-resistant spores were first detected at 6 h, and enterotoxin at 3 h of incubation. Raffinose not only yielded higher spore numbers than starch, but also shortened the time to toxin detection and/or increased toxin production by two out of three strains. Strain NCTC 8238 produced enterotoxin (2 ng/ml) with raffinose within 3 h, while strain NCTC 10240 produced 2 ng/ml enterotoxin with starch and higher levels (4 ng/ml) with raffinose at 3 h. In contrast, strain NCTC 8239 produced enterotoxin (2 ng/ml) at 3 h only with starch.

¹Reference to a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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INTRODUCTION

Clostridium perfringens is an important cause of food poisoning all over the world including the United States (CDC 1985; Dische and Esek 1957; Stringer *et al.* 1980). Outbreaks primarily involve meat and meat products, and illness occurs due to consumption of food contaminated with large numbers of vegetative cells (Hauschild *et al.* 1970). The ingested cells sporulate in the intestine and release a heat-labile enterotoxin known as *C. perfringens* enterotoxin (CPE), which is responsible for typical symptoms (Duncan 1975; Stark and Duncan 1971). Many strains do not sporulate and produce enterotoxin or respond poorly in sporulation media (Duncan *et al.* 1972; Ting and Fung 1972; Harmon and Kautter 1986a). Thus, the routine microbiological examination of cultures implicated in food poisoning caused by *C. perfringens* is made more difficult when sufficient sporulation and enterotoxin production is not obtained.

Several studies have shown that CPE production is directly related to sporulation (Duncan *et al.* 1972; Tsai *et al.* 1974; Uemura *et al.* 1973). Therefore, the ideal sporulation medium for detection of enterotoxigenic strains should support spore formation in high yields. Several media (Duncan and Strong 1968; Kim *et al.* 1967; Labbe and Rey 1979; Sacks and Thompson 1975; Taniguti 1968; Tortora 1984) have been developed for *C. perfringens* sporulation. The most widely used is Duncan and Strong medium (Duncan and Strong 1968) without or with minor modifications (Harmon and Kautter 1986a; Labbe and Rey 1979).

Sacks and Thompson (1977) reported that the addition of purine analogs such as the methylxanthines to DS medium greatly promoted *C. perfringens* spore yields. Carbohydrates have also been shown to influence spore yield (Labbe *et al.* 1976). Labbe and Rey (1979) reported enhanced sporulation by some *C. perfringens* strains when starch was replaced with raffinose in DS medium. In the present study, we screened a number of methylxanthines and related compounds in modified DS medium containing starch or raffinose to quantify spore yield, enterotoxin production, and earliest time to enterotoxin detection.

MATERIALS AND METHODS

Test Organisms

Three strains of *Clostridium perfringens* NCTC 8238 (Hobbs serotype 2), NCTC 8239 (Hobbs serotype 3), and NCTC 10240 (Hobbs serotype 13) obtained from American Type Culture Collection were used in the study. The organisms were maintained at 4C in cooked-meat medium (Difco Laboratories, Detroit, MI) and used through the course of the study as stock cultures.

Chemicals

The methylxanthine and related compounds used included caffeine, theophylline, theobromine, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, 8-methylxanthine, 1,7-dimethylxanthine, 3-isobutyl-1-methylxanthine, xanthine, and hypoxanthine. All compounds were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). They were stored according to the supplier's instructions and were used as received.

Sporulation Medium

Duncan and Strong (DS) sporulation media (Duncan and Strong 1968) was prepared with either 0.4% starch (original formulation) or replacing starch with 0.4% raffinose (Labbe and Rey 1979) and supplemented with 50–200 $\mu\text{g/ml}$ of each of the methylxanthines or related compounds. These compounds were added prior to autoclaving. The compounds were tested at different concentrations to determine the optimum level yielding highest spore numbers. The pH of all the modified DS medium formulations was raised to 7.8 ± 0.1 by adding filter-sterilized 0.66 M sodium carbonate to autoclaved DS medium to enhance sporulation, as described by Harmon and Kautter (1986a).

Inoculation Procedure

From the spore stock cultures, 0.1 ml was transferred into 10 ml of fresh fluid thioglycollate (FTG) medium (Difco Laboratories, Detroit, MI), heat shocked at 75C for 20 min and then incubated for 18 h at 37C. A 1.0 ml portion from this culture was transferred to 10 ml of freshly steamed FTG medium and incubated for 4 h at 37C. This medium supports only vegetative growth. The culture was added at 1% concentration to DS medium and incubated for 24 h. No provisions were made to obtain anaerobic conditions due to the presence of sodium thioglycollate in the sporulation medium.

Incubation and Sampling

After inoculation, all DS cultures were incubated at 37C for 24 h to determine the formulation yielding the highest number of heat-resistant spores. In experiments to determine earliest time for spore formation and enterotoxin detection, sampling was done at 3 h intervals up to 24 h.

Enumeration of Heat-Resistant Spores

Tryptose-sulfite-cycloserine (TSC) agar without egg yolk enrichment (Hauschild and Hilsheimer 1974) was used for enumerative spiral plating (Spiral Systems Model D plating instrument; Cincinnati, OH). The TSC agar was freshly prepared prior to each experiment to avoid hydrogen peroxide production (Harmon and Kautter 1976). A 5 ml portion of each sporulating culture was heated at 75C for 20 min to kill vegetative cells and activate heat-resistant spores. Serial dilutions of heat-shocked samples were made in 0.1% peptone water (w/v) before duplicate plating of each dilution on TSC agar. The TSC agar plates were overlaid with an additional 10 ml of TSC agar and allowed to solidify before placing into anaerobic jars. Viable spore counts were made after 48 h of incubation at 37C in a GasPak system (Baltimore Biological Laboratory, Cockeysville, MD).

Quantification of Enterotoxin Formation

Enterotoxin concentration in the culture supernatant fluids was determined by the commercially available reversed passive latex-agglutination (RPLA) test kit (Oxoid, Columbia, MD), which can detect a minimum level of 2 ng/ml of sample. The RPLA test for CPE detection has been reported to be sensitive, specific, rapid and above all, does not require special equipment or test reagents (Berry *et al.* 1986; Harmon and Kautter 1986b). The remaining portion of the 20 ml DS culture was centrifuged at $900 \times g$ for 20 min at 4C to remove the cells. Enterotoxin assays were carried out in 96-well (V-wells) microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA). Twenty-five μ l of test sample was serially diluted in 0.15 M phosphate buffered saline containing 0.5% (w/v) bovine serum albumin. Then, 25 μ l of sensitized (anti-enterotoxin) latex or 25 μ l of unsensitized latex suspension (control) was mixed. The controls were used to test for nonspecific agglutination reaction. The test results were read after overnight incubation at room temperature. The endpoint at which latex agglutination could be clearly read without nonspecific agglutination was equivalent to an enterotoxin concentration of 2 ng/ml (the sensitivity of the test in detecting enterotoxin).

Phase Contrast Microscopy

Aliquots (2 ml) were taken from the sporulation media and centrifuged for 2 min in an Eppendorf Centrifuge (model 5413, Hamburg, Germany). Supernate fluid was decanted and the cell pellet resuspended in 400 μ l mixture of glycerol (J.T. Baker Chem. Co., Phillipsburg, N.J.) and 2% agarose (Bio-Rad Laboratories, Richmond, CA) heated to 50C. The mixture was mixed on highest setting using a Lab-Line Super Mixer II (Melrose Park, IL) to ensure total disper-

sion of cells in the pellet. A 10 μ l portion of the suspension was transferred to a clean glass microscope slide warmed to 50C and then covered with a clean glass coverslip. Vegetative cells and spores were observed and photographed using phase contrast at 1000 \times magnification (Olympus BH-2, Tokyo, Japan).

Transmission Electron Microscopy

A 900 μ l sample from the sporulation media was fixed with 100 μ l of a 10% solution of glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) and incubated at 4C for one hour. The cell pellet was washed two times with 0.1 M sodium cacodylate, pH 7.3 (Electron Microscopy Sciences, Ft. Washington, PA). A 2% osmium tetroxide in 0.1 M sodium cacodylate was added to the washed cell pellet for postfixing. The cell pellet was washed three times with 0.1 M sodium cacodylate, pH 7.3 and then dehydrated sequentially with 50, 70, 90 and 100% ethanol, and finally 100% propylene oxide. The dehydrated cell pellet was embedded in Polybed-812 epoxy resin (Electron Microscopy Sciences, Ft. Washington, PA). Thin sections were cut with a diamond knife and placed on Formvar-coated copper 200-mesh grids, and stained with uranyl acetate and lead citrate. Thin sections were observed and photographed using a transmission electron microscope (Model CM-12, Philips Electronics, Eindhoven, Netherlands).

Statistical Analysis

All data were analyzed by analysis of variance and orthogonal contrasts using SAS (SAS 1989). The independent variables were strains, compounds, concentration and replication. The dependent variable was spore yield. Bonferroni mean separation test was used to determine significant differences ($p < 0.05$) among means (Miller 1981).

RESULTS AND DISCUSSION

The relative numbers of heat resistant spores (those that survived 75C for 20 min) of the 3 strains of *C. perfringens* at 24 h in DS medium in the presence of methylxanthines and related compounds at concentrations ranging from 50 to 200 μ g/ml are shown in Table 1; the effect of concentration levels was not significant ($p < 0.05$).

For strain NCTC 8238, the addition of caffeine, theophylline, theobromine, xanthine or hypoxanthine gave similar spore yields, i.e., they were not significantly different ($p < 0.05$) from one another (Table 1). Caffeine significantly increased the spore yield by 51 fold compared to control DS medium. Phase-contrast

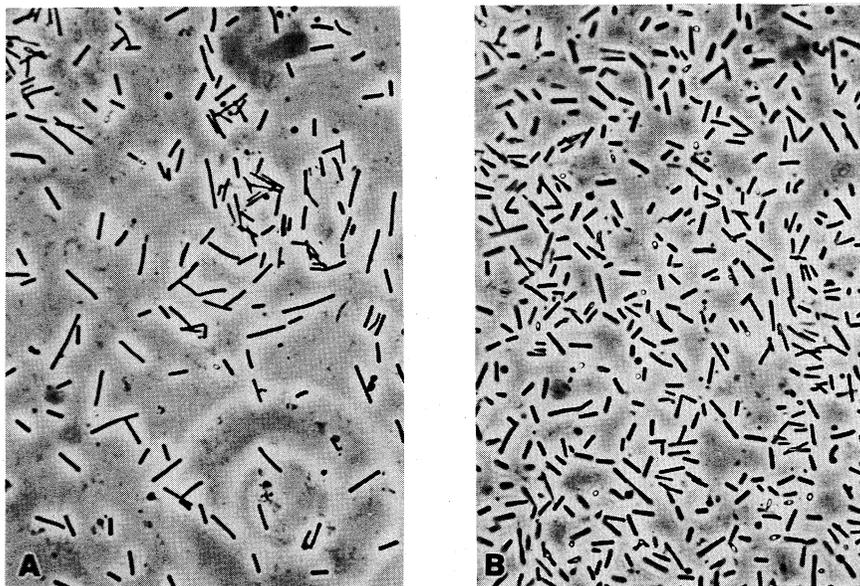


FIG. 1. PHASE-CONTRAST MICROSCOPY OF *CLOSTRIDIUM PERFRINGENS* STRAIN NCTC 8238 AT 24 h
(A) DS medium (control), and (B) DS medium containing 0.51 mM caffeine.

spore numbers increased ($P < 0.05$) from $4.18 \log_{10}/\text{ml}$ (control) to $6.00 \log_{10}/\text{ml}$ (caffeine) and $5.73 \log_{10}/\text{ml}$ (theophylline). Spore yields for this strain in the presence of caffeine, theophylline, theobromine, 1-methylxanthine and 1,7-dimethylxanthine were not significantly different ($p < 0.05$) from one another (Table 1).

For all 3 strains, mean spore yield was highest ($6.38 \log_{10}/\text{ml}$) in the presence of caffeine, followed by theophylline ($5.67 \log_{10}/\text{ml}$) and theobromine ($5.34 \log_{10}/\text{ml}$). The mean spore count in control DS medium was $4.46 \log_{10}/\text{ml}$. Therefore, caffeine increased spore yield by 83-fold, followed by theophylline (16-fold) and theobromine (8-fold). Our results indicate that all three methyl groups of caffeine (1,3,7-trimethylxanthine) are required to increase *C. perfringens* sporulation.

Preliminary experiments confirm and support findings reported by Labbe and Rey (1979) that vigorously growing culture in freshly prepared FTG medium was a prerequisite for high spore yields in DS medium. Poor growth in FTG resulted in poor sporulation response in DS medium. Glucose is known to be a sporulation repressor (Tsuboi and Yanagishima 1973). To avoid transfer of unutilized residual glucose from FTG medium to DS medium, the level of inoculation was 1% instead of the 10% used originally by Duncan and Strong (1968).

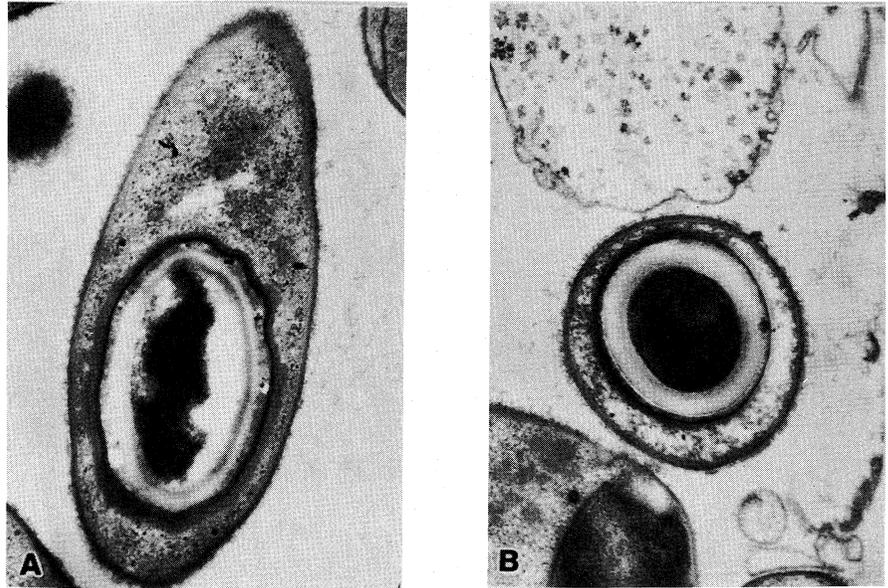


FIG. 2. TRANSMISSION ELECTRON MICROGRAPH OF THIN SECTIONS OF *CLOSTRIDIUM PERFRINGENS* NCTC 8238 AT 24 h CULTURED IN DS MEDIUM CONTAINING 0.51 mM CAFFEINE

(A) cell showing spore within sporangia, (B) free mature spore.

When sporulation and enterotoxin formation were followed in DS medium containing either starch or 0.4% raffinose, as suggested by Labbe and Rey (1979), and supplemented with 0.51 mM caffeine, heat-resistant spores could not be detected at 3 h of incubation with any of the three strains (Fig. 3-5). A direct correlation between heat-resistant spores and enterotoxin titers was observed (Fig. 3, 4 and 5). Spore counts per microscopic field were less than one at 3 h for strains NCTC 8238 (Fig. 3) and NCTC 8239 (Fig. 4) and were less than one for up to 9 h for strain NCTC 10240 (Fig. 5). For strain NCTC 8238, spore yield increased from 5.13 \log_{10}/ml at 6 h to 6.92 \log_{10}/ml at 24 h with starch and from 5.96 \log_{10}/ml to 7.34 \log_{10}/ml with raffinose in DS medium (Fig. 3). Spore counts per microscopic field increased from 1.5 at 6 h to 26 at 24 h with starch and 1.7 to 31 with raffinose. A corresponding increase in enterotoxin concentration was detected. Enterotoxin (0.3 \log_{10} ng/ml could be detected at 3 h only in the presence of raffinose for strain NCTC 8238 (Fig. 3). Presence of starch in DS medium was more effective in enhancing the spore yield for strain NCTC 8239 (Fig. 4). The spore yield ranged from 5.30 \log_{10}/ml at 6 h to 7.75 \log_{10}/ml at 24 h with starch and from 4.96 \log_{10}/ml to 6.97 \log_{10}/ml with raffinose. Spore counts per microscopic

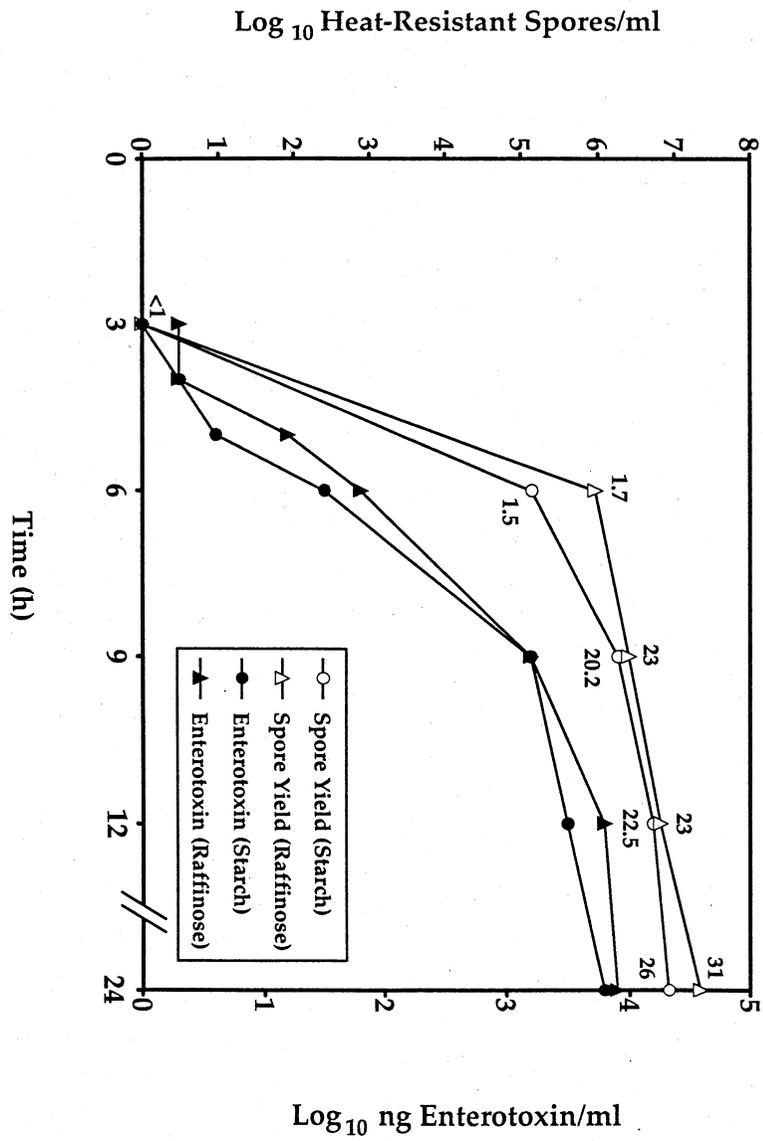


FIG. 3. THE TIME COURSE OF SPORULATION AND ENTEROTOXIN DETECTION BY *CLOSTRIDIUM PERFRINGENS* NTCC 8238. Values adjacent to symbols for heat-resistant spores are the spore count per microscopic field (average of 5 fields), as determined by phase-contrast microscopy.

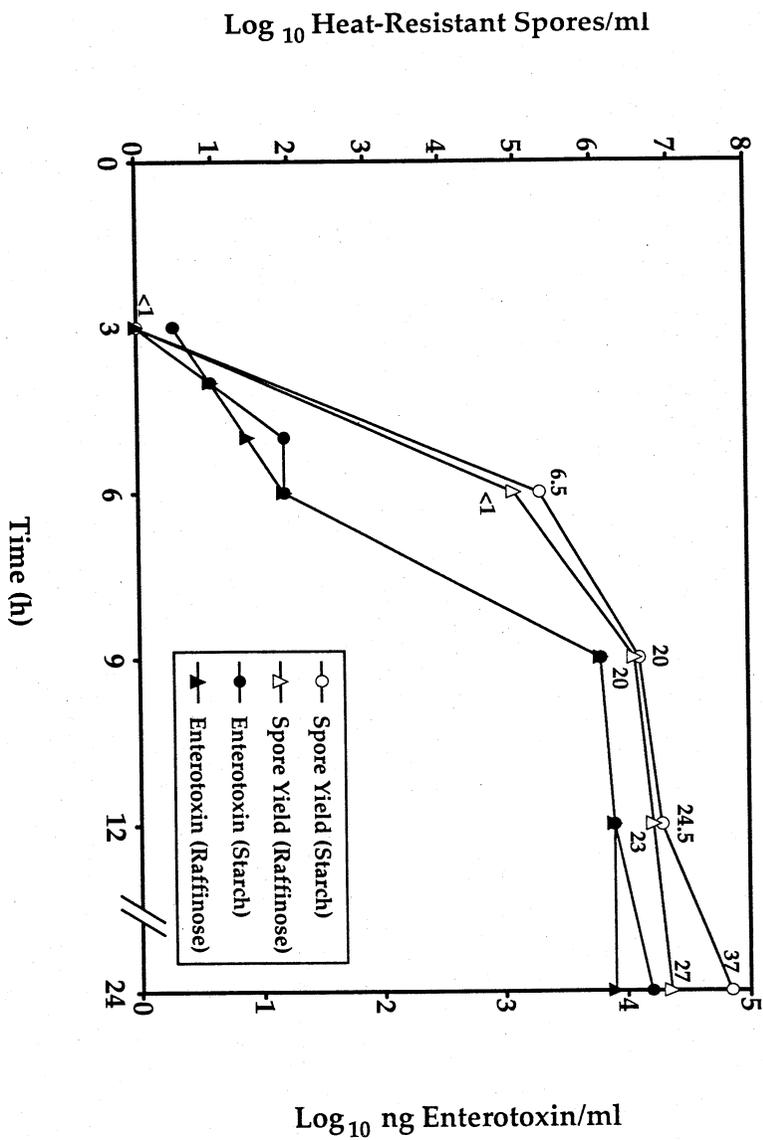
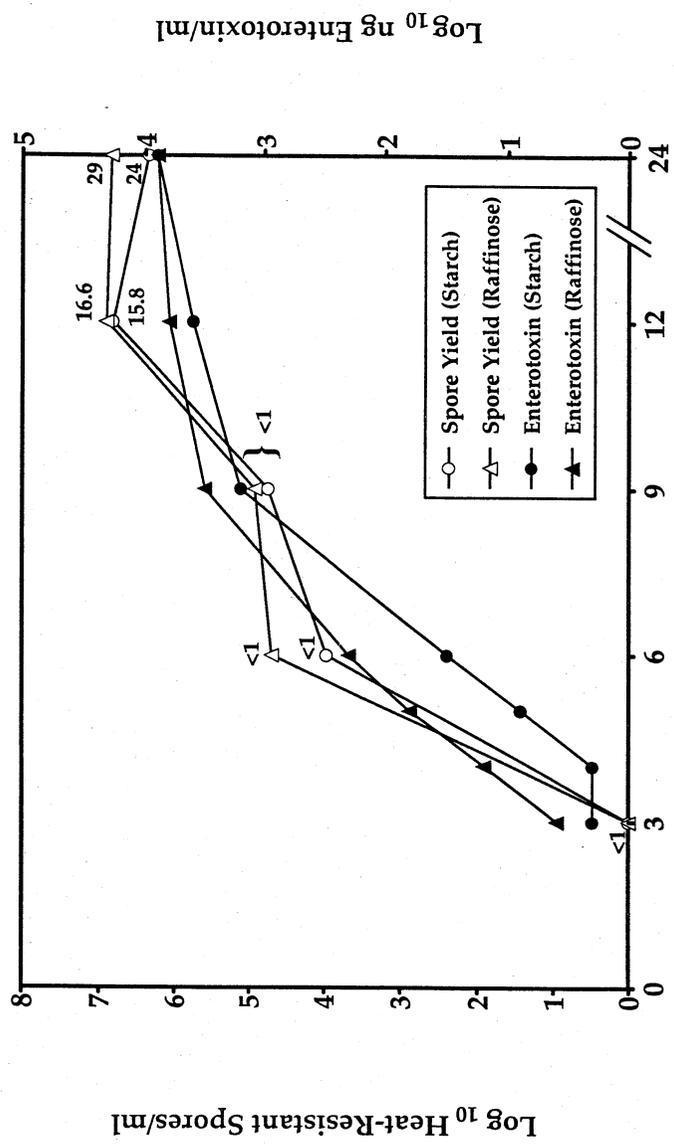


FIG. 4. THE TIME COURSE OF SPORULATION AND ENTEROTOXIN DETECTION BY *CLOSTRIDIUM PERFRINGENS* NTCC 8239
 Values adjacent to symbols for heat-resistant spores are the spore count per microscopic field (average of 5 fields), as determined by phase-contrast microscopy.



Time (h)

FIG. 5. THE TIME COURSE OF SPORULATION AND ENTEROTOXIN DETECTION BY *CLOSTRIDIUM PERFRINGENS* NTCC 10240

Values adjacent to symbols for heat-resistant spores are the spore count per microscopic field (average of 5 fields), as determined by phase-contrast microscopy.

field were 37 with starch and 27 with raffinose at 24 h. Strain NCTC 8239 produced detectable levels of enterotoxin ($0.3 \log_{10}$ ng/ml) at 3 h only in the presence of starch. Enterotoxin levels were higher for starch ($1.2 \log_{10}$ ng/ml) than raffinose ($0.9 \log_{10}$ ng/ml) at 5 h and at 24 h ($4.2 \log_{10}$ ng/ml, starch; $3.9 \log_{10}$ ng/ml, raffinose). Traditionally, strain NCTC 8239 has been used for enterotoxin production (Craven and Blankenship 1982; Hauschild 1970; Nilo 1976; Uemura *et al.* 1975). Our results confirm that NCTC 8239 is an ideal strain for enterotoxin formation with starch in DS medium. As with strain NCTC 8238 (Fig. 3), strain NCTC 10240 (Fig. 5) sporulated better in the presence of raffinose. Especially noteworthy were the spore counts per microscopic field for strain NCTC 10240. Spore counts per microscopic field were < 1 even when heat-resistant spores were as high as $4.93 \log_{10}/\text{ml}$ at 9 h with raffinose in DS medium (Fig. 5). These data indicate that spore counts cannot be used to approximate the heat resistant spore levels $< 5 \log_{10}/\text{ml}$. Spore yield increased from $3.97 \log_{10}/\text{ml}$ at 6 h to $6.36 \log_{10}/\text{ml}$ at 24 h with starch and $4.70 \log_{10}/\text{ml}$ to $6.85 \log_{10}/\text{ml}$ with raffinose. A parallel increase in enterotoxin levels was detected except at 24 h when levels were $3.9 \log_{10}$ ng/ml with starch or raffinose (Fig. 5).

In the present study, sporulation and enterotoxin production varied considerably among strains and depended upon the carbohydrate used. Such variation among strains is a well-known characteristic of this organism. Labbe and Rey (1979) suggested that the rate of extracellular hydrolysis of raffinose (as compared to starch) may contribute to the higher spore yield and enterotoxin production by some strains of *C. perfringens*. Lack of readily metabolizable carbohydrate in the culture medium may induce sporulation. Sacks and Thompson (1977) indicated that the influence of carbohydrate energy appeared to be a strain-related factor.

Methylxanthine compounds are structurally similar to purines and have been shown to retard bacterial growth (Raj *et al.* 1965). Labbe (1989) reported that methylxanthines stimulate spore formation, perhaps by affecting nucleic acid metabolism. Several purine derivatives have been shown to partially inhibit purine nucleotide synthesis and increase sporulation in *B. subtilis* (Freese *et al.* 1978; Heinze *et al.* 1978). Tsuboi and Yanagishima (1973) reported that methylxanthines inhibit phosphodiesterases and may result in an increased accumulation of 3',5'-monophosphoric acid. Papaverine, an inhibitor of phosphodiesterase, induced sporulation in *C. perfringens* strains FD-1 and PS52 (Sacks 1982). In contrast, Labbe and Nolan (1981) reported that papaverine did not stimulate spore formation of *C. perfringens* strain NCTC 8679. However, it should be noted that compounds such as 3',5'-monophosphoric acid are not found in spore forming bacteria (Setlow 1973).

Among the various biological effects attributed to methylxanthines, compounds other than caffeine complex with metals ions (Tu and Friederich 1968), a property that may stimulate spore production. In the present study, however, caf-

feine was found to be most efficacious in enhancing spore formation. Caffeine has been reported to have a variety of biological effects in various systems, including inhibition of cAMP phosphodiesterase and blocking of nucleoside receptors, alteration of DNA polymerase I (Balachandran and Srinivason 1982) and RNA polymerase I (Shields *et al.* 1981) activity. In *C. perfringens* FD-1, induction of arginine deiminase was markedly increased by caffeine in the presence of dextrin but not in the presence of maltose or maltotriose (Sacks 1985). The author suggested that caffeine may be adversely affecting polysaccharide utilization. Tortora *et al.* (1982) reported that caffeine, but not theophylline, uncoupled the regulation of glycolysis and gluconeogenesis in *Saccharomyces cerevisiae*. Specifically, caffeine appeared to inhibit the carbon catabolite inactivation of fructose-1,6-diphosphatase, resulting in depressed glycolytic activity and altered energy change. Theophylline was shown to inhibit L-alanine induced germination of *B. cereus* spores (Cassone and Simonetti 1971). These effects might have resulted in increased spore yield in the present study.

The addition of caffeine and replacement of starch with raffinose to DS medium increased sporulation and enterotoxin formation by two out of three *C. perfringens* strains. While additional *C. perfringens* strains need to be evaluated to assess the universality of the observed responses, the results of the current study indicate that these additives can be used to enhance identification of enterotoxigenic *C. perfringens* strains.

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