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Increased Spore Yields of *Clostridium perfringens* in the Presence of Methylxanthines

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The methylxanthines caffeine, theophylline, and isobutylmethylxanthine greatly increased spore yields of *Clostridium perfringens* strains FD-1, PS52, and PS49 when grown on Duncan-Strong medium or on a new casein-digest medium. Four other strains (KA3, and National Collection of Type Cultures strains 8798, 8238, and 10240) failed to show any significant increase when tested under similar conditions. The degree of sporulation increase was influenced by the carbohydrate energy source in some strains but not in others. Strain PS52 showed a large increase in spore yield when dextrin was the energy source but only a slight increase when raffinose served as the energy source. Strain FD-1 showed similar increases in spore yield with either dextrin or raffinose.

The difficulty commonly associated with inducing sporulation of *Clostridium perfringens* on laboratory media (22) has impeded studies on the spores of this important organism for many years. The most commonly employed sporulation medium for *C. perfringens* is probably that of Duncan and Strong (6). Although satisfactory spore yields (>10^7/ml) have been obtained on this medium with some strains, many strains sporulate only to a very limited extent on it. Ellner's medium (7) and casein-digest media (10) have also been used (3, 4, 8, 9, 15; H. Riemann, Ph.D. thesis, University of Copenhagen, Denmark, 1963), but none are consistently satisfactory (22).

Recently we reported that methylxanthines could greatly increase spore yields of strain PS49 in a defined medium and in a new casein-digest medium (14). In the present report, we show that strains PS52 and FD-1 also exhibited large increases in spore yield when methylxanthines were incorporated with the casein-digest medium. Strains PS49, PS52, and FD-1 also demonstrated very significant increases in spore yield when methylxanthines were added to Duncan-Strong (DS) medium. Four other strains showed no increase in spore yield when tested under similar conditions.

MATERIALS AND METHODS

Medium. The basal medium (CPS) contained Casitone (Difco) (6%), K_2HPO_4 (0.1%), ethylenediaminetetraacetic acid, ferric salt (0.02%) (11), and $MnCl_2$ (0.2 mM), and was adjusted to pH 7.0 with NaOH before autoclaving. An energy source (dextrin, starch, or raffinose) was added at the concentration level specified in the text. 3-*N*-(morpholino)-propane sulfonic acid was prepared separately as a 10% solution, adjusted to pH 7.6 with NaOH, autoclaved separately, and added aseptically to the fresh, sterile basal medium (1:9). Sodium thioglycolate (0.1%) was added to both solutions just before autoclaving.

DS medium was prepared according to the authors' formula (6); activated carbon was not employed.

Spore stocks. Stock cultures were prepared in CPS medium in 100-ml serum bottles with aluminum caps and incubated anaerobically in GasPak (Becton, Dickinson and Co.) jars at 35°C. The bottles contained 67 ml of CPS medium and were inoculated soon after autoclaving with 1 ml of fresh inoculum growing in fluid thioglycolate medium (Difco) or brain heart infusion (Difco) prepared as described below. Newly received strains were grown in cooked-meat medium (Difco) for 18 h at 35°C, and 1 ml of this culture was inoculated directly into the serum bottle as above. At 44 h, the serum bottles were removed from the GasPak jar, and a presterilized conventional sleeve-type rubber serum-bottle cap was used to stopper the serum bottle. Heat-resistant spores were counted (see below), and the bottle was stored at 5°C. It is desirable that spore stocks have as high a spore content as possible. Starch (0.3%), raffinose (0.7%), or no carbohydrate was added, depending on the strain. Most spore stocks have maintained a stable spore count for more than 2 years.

Inocula. When an inoculum was required, a 0.2-ml sample was withdrawn from the spore stock with a sterile syringe, transferred to 10 ml of sterile 0.1% peptone in a 16-mm tube, and heat shocked at 75°C for 20 min. One milliliter of this suspension was then transferred to 13 ml of freshly prepared fluid thioglycolate medium in a 16-mm culture tube and incubated at 42 to 43°C for 4 to 6 h until a Klett reading of 125 to 175 U was obtained. This culture was then used as the inoculum; 0.8 ml was added to 13 ml of freshly prepared CPS or DS medium. Our

basic aim was to obtain rapid germination of a large number of the spores in the stock suspension and permit their rapid growth in order to obtain a young, relatively dense, exponential-phase inoculum within the span of an 8-h day.

Culture conditions. Culture tubes (16 by 150 mm) were washed in conventional fashion, soaked in 6 M HCl, and rinsed six times in glass-distilled water. Anaerobiosis was achieved by using deep layers of fresh medium (13.8 ml in 16-mm tubes) and relatively heavy, rapidly growing inocula. Parallel cultures incubated in GasPak jars generally showed spore yields equivalent to those of tubes incubated in air (except for strain T-65). After inoculation, cultures were incubated at 35°C for up to 44 h. Spore counts were carried out at 20 and 44 h; Klett readings (no. 66 filter) and pH were determined at 20 h.

Spore enumeration. Spore counts were obtained by diluting samples of well-stirred cultures 1:10 with sterile 0.1% peptone, heating the samples at 75°C for 20 min, and "plating" them in oval tubes in SFP basal agar (Difco) (17) or SPS agar minus antibiotics (1). Colonies on SPS base medium generally formed excessive gas, whereas black pigment from colonies in SFP tended to diffuse excessively. The three strains showing marked sporulation responses (FD-1, PS49, PS52) formed well-defined black colonies without gas in SFP basal medium if tryptone (Difco) replaced tryptose. Many of the experiments reported here were enumerated in this medium. Unfortunately, a number of other strains failed to grow in this medium and were counted in one of the other media. Black colonies were counted after 20 h at 35°C. Lysozyme (2 μg/ml) (5) failed to increase counts of the strains used in this study.

Strains. Strains PS49, PS52, and KA3 were obtained from the Communicable Disease Center, Atlanta, Ga. Strains FD-1 and T-65 and NCTC strains 8798, 8238, and 10240 were obtained from S. M. Harmon, Food and Drug Administration, Washington, D.C.

Chemicals. Theophylline, lysozyme, and 3-*N*-(morpholino)-propane sulfonic acid were obtained from Calbiochem, La Jolla, Calif. Caffeine and 3-isobutyl-1-methylxanthine (IMX) were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. Ethylenediaminetetraacetic acid, ferric salt, was obtained from Eastman Kodak Co., Rochester, N.Y.

RESULTS

Figure 1 shows the influence of caffeine on spore yield of strain PS52 in CPS medium (0.3% starch). Very large increases in spore yield were observed when the caffeine concentration was in the range of 100 to 1,000 μg/ml. Incubation of tubes in GasPak jars gave results very similar to those obtained when tubes were incubated in air. The use of a relatively heavy exponential-phase inoculum and freshly autoclaved, deep tubes of culture medium seemed to obviate the need for strict anaerobiosis. With most strains, no major differences were observed between culture tubes incubated in air and in GasPak jars. Only strain T-65 showed greatly increased spore yields when grown under strictly anaerobic conditions.

Figure 2 shows the influence of methylxanthine concentration on the spore yield of strain PS52 in DS medium. Both caffeine and IMX induced large increases in spore yield and were highly effective for this strain in DS medium.

Figure 3 shows the effect of caffeine on strain FD-1 in DS medium. It is noteworthy that strain FD-1 apparently required higher levels of caffeine for maximum sporulation than did strains PS52 and PS49. Although no 44-h counts are shown in Fig. 3, several other experiments showed that 44-h counts showed the same pattern of increased spore yields in high caffeine concentration.

Figure 4 shows the influence of methylxan-

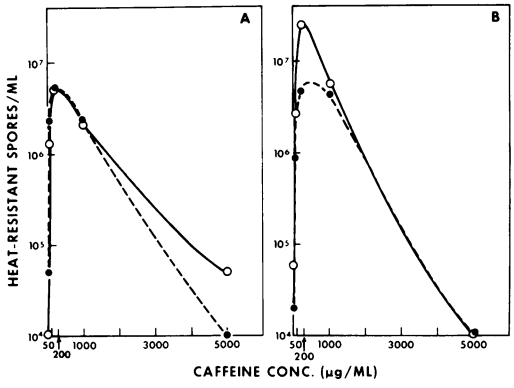

FIG. 1. *Influence of caffeine concentration on spore yield of C. perfringens PS52 in CPS medium (0.3% starch). (A) Tubes incubated in air. (B) Tubes incubated in GasPak jars. Symbols: ○, 20-h counts; ●, 44-h counts.*

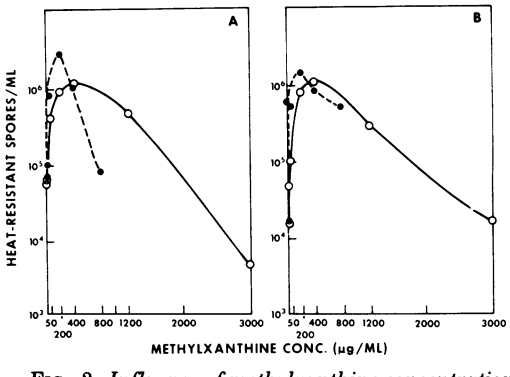

FIG. 2. *Influence of methylxanthine concentration on spore yields of C. perfringens PS52 in DS medium. (A) 20-h counts. (B) 44-h counts. Symbols: ○, caffeine added; ●, IMX added.*

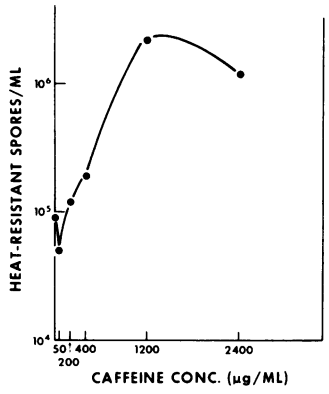

FIG. 3. *Influence of caffeine on spore yields of C. perfringens FD-1 in DS medium. All counts at 20 h.*

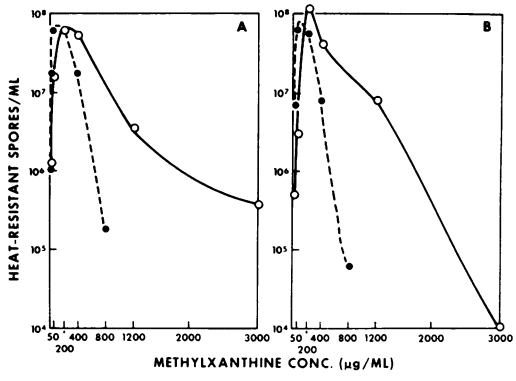

FIG. 4. *Influence of methylxanthine concentration on spore yield of C. perfringens PS49 in CPS medium (0.3% dextrin). (A) 20-h counts. (B) 44-h counts. Symbols: ○, caffeine added; ●, IMX added.*

thines on spore yields of strain PS49 in CPS medium (0.3% dextrin). Again, large increases were observed at appropriate concentrations. Control counts for strain PS49 were sometimes higher than those shown in this experiment, whereas cultures containing optimum levels of methylxanthine were consistently high; in such cases, the degree of increase in spore yield might be no more than threefold. The reason for the fluctuations in these control counts remains unclear.

Figure 5 shows the influence of methylxanthine concentration on spore counts of strain PS49 grown in DS medium. Addition of theophylline and isobutylmethylxanthine resulted in large increases in spore yield in the appropriate concentration range. Caffeine, in this case, was much less effective. Strain PS49 tended to clump in DS medium, but methylxanthine-containing tubes exhibited notably less clumping. This represented the only instance in which the effects of methylxanthines on spore yield might be attributed partly to an influence on the degree of dispersion of the spores.

Strain T-65 showed significant increases in spore yield on both DS and CPS (0.3% starch or dextrin) with caffeine (200 to 400 μg/ml) or IMX (50 μg/ml) after incubation in GasPak jars.

Previous work with strain PS49 in a defined medium indicated a possible influence of the carbohydrate energy source on the methylxanthine effect (14); larger spore increases were induced by methylxanthines in the presence of dextrin as compared with raffinose. Figure 6 shows the influence of theophylline on spore yields of FD-1, using 0.4% dextrin, 0.4% raffinose, and 0.7% raffinose as the energy sources in CPS medium. This strain showed equivalent

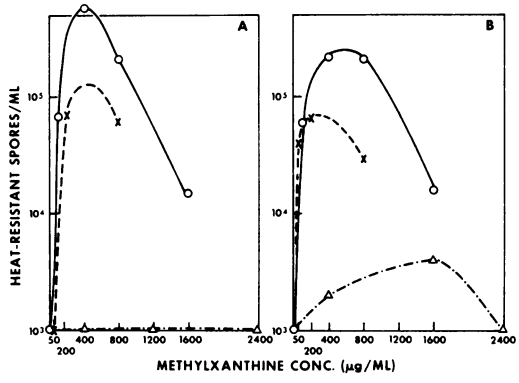

FIG. 5. *Influence of methylxanthine concentration on spore yield of C. perfringens strain PS49 in DS medium. (A) 20-h counts. (B) 44-h counts. Symbols: ○, theophylline added; ×, IMX added; △, caffeine added.*

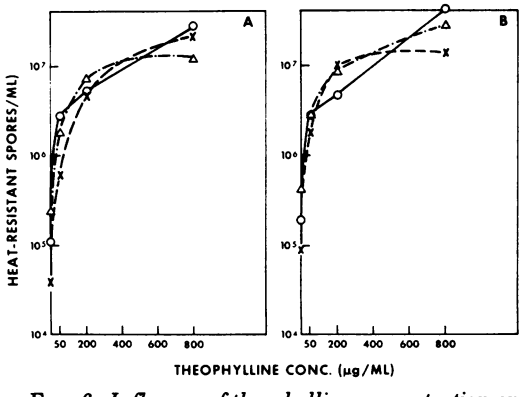

FIG. 6. *Influence of theophylline concentration on spore yield of C. perfringens strain FD-1 in CPS medium supplemented with different carbohydrate energy sources. (A) 20-h counts. (B) 44-h counts. Symbols: ○, 0.4% dextrin added; △, 0.7% raffinose added; ×, 0.4% raffinose added.*

spore increases in the presence of theophylline when either raffinose or dextrin served as the energy source. Raffinose concentration did not materially influence the result.

However, strain PS52 responded very differently to caffeine when raffinose, rather than dextrin, served as the energy source (Fig. 7). A much larger increase in spore yield occurred with caffeine when dextrin was the energy source. Other experiments showed similar effects with theophylline.

Strain KA3 and NCTC strains 8798, 8238, and 10240 showed no significant increases in spore yield when methylxanthines were added to CPS medium, regardless of the carbohydrate present.

Phase microscopy was frequently used to verify sporulation; in general, qualitative microscopic examinations showed a much larger fraction of sporulating cells at effective methylxanthine concentrations. Clumping was generally not evident. Only in the experiments with PS49 in DS medium was pronounced clumping observed in control tubes and reduced clumping in tubes containing active concentration of methylxanthines. Klett readings generally showed only slight differences between control and methylxanthine-treated tubes. High concentrations of methylxanthine were, in some cases, associated with markedly reduced Klett readings. Routine pH determinations were performed on 20-h cultures. In general, methylxanthine-containing cultures showed slightly higher pH levels; the difference was usually less than 0.3 pH unit.

DISCUSSION

The results of the present work show that the ability of methylxanthines to increase spore yields of *C. perfringens* is limited to certain strains. Strains PS52, PS49, and FD-1 showed very large increases in heat-resistant spores when methylxanthines were added to DS or casein-digest medium. Strain KA3 and NCTC strains 8798, 8238, and 10240 failed to show increased spore yields when tested similarly. The methylxanthine response appears to be strain related.

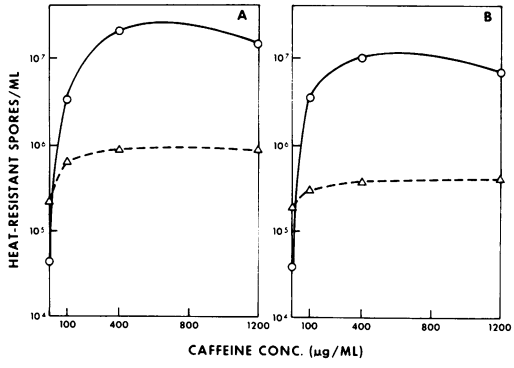

FIG. 7. *Influence of caffeine concentration on spore yield of C. perfringens strain PS52 in CPS medium with 0.4% starch and 0.7% raffinose. (A) 20-h counts. (B) 44-h counts. Symbols: ○, 0.4% starch added; △, 0.7% raffinose added.*

The influence of the carbohydrate energy source also appears to be a strain-related factor. Strain PS52 showed very large increases in spore yield when dextrin served as the energy source in CPS medium; when raffinose replaced dextrin, spore increases were much reduced. On the other hand, strain FD-1 exhibited large increases in spore yield with either carbohydrate in the presence of methylxanthines.

The mechanism involved in the induction of endospore formation is not yet understood, and few guidelines are available to those attempting to develop satisfactory sporulation media for organisms such as *C. perfringens*. In general, sporulation media contain all the nutrients required for rapid growth, but may lack a rapidly metabolizable carbohydrate and may contain excess manganese. Most of the substances found to increase sporulation of anaerobes have been common nutrients (glutamic acid [18], thiamine [10], etc.). We know of no instance in which spore yield is greatly increased by the addition of non-physiological chemicals such as the methylxanthines employed here. For this reason, further studies of the methylxanthine effect might have interest extending beyond the development of a satisfactory sporulation medium for *C. perfringens*.

Several possible mechanisms for the action of methylxanthines on *C. perfringens* exist. Interference with some aspect of nucleic acid metabolism is one possibility. Methylxanthines stimulate sporulation of *Saccharomyces cerevisiae* (19) and also increase ribonuclease activity (20); premeiotic deoxyribonucleic acid synthesis in yeast is known to utilize ribonucleic acid breakdown products preferentially. Methylxanthines are also known to inhibit phosphodiesterases, and this action could result in an accumulation of adenosine 3′,5′-cyclic monophosphoric acid (19). However, the apparent absence of 3′,5′-cyclic monophosphoric acid in sporulating bacteria (16) seemingly eliminates this possibility. The much larger effects of methylxanthines in the presence of dextrin as compared with raffinose observed with strains PS49 (14) and PS52 might be considered consistent with an influence on 3′,5′-cyclic monophosphoric acid concentration, but the present finding that strain FD-1 shows equally strong methylxanthine ef-

fects in the presence of either dextrin or raffinose greatly weakens this hypothesis. Methylxanthines are known to inhibit bacterial growth (13), but at somewhat higher concentration levels (1,250 to 5,000 μg/ml) than those shown to be active here.

The metal-chelating properties of methylxanthines (21) may play a role in increasing the spore counts. Inhibition of phosphodiesterase by chelating agents has been attributed to binding of the chelating agent to the iron in the enzyme (12). The present experiments demonstrating large effects on spore yield of PS49 in DS medium with theophylline and IMX but not with caffeine (Fig. 5) are consistent with this type of mechanism. Caffeine does not appear to be capable of metal chelation (21). However, in view of the large effects exhibited by caffeine in most experiments, the major effects of methylxanthines are not likely to be asociated with the metal-binding capacities of these compounds.

Another possibility emerges from the demonstrated ability of theophylline to inhibit germination under some conditions (2). The ability to inhibit "recycling" of spores could result in greatly increased spore yields for some clostridial strains. Although this possibility has not been conclusively eliminated, our data do not seem to support it.

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