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Stimulation of *Clostridium perfringens* Enterotoxin Formation by Caffeine and Theobromine

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In the presence of 100 μg of caffeine per ml or 200 μg of theobromine per ml, sporulation of *Clostridium perfringens* NCTC 8679 rose from less than 1 to 80 or 85%. Enterotoxin concentration increased from undetectable levels to 450 μg/mg of cell extract protein. Heat-resistant spore levels increased from less than 1,000 to between 1×10^7 and 2×10^7/ml. These effects were partially reversible by the addition of adenosine or thymidine. In the case of NCTC 8238, caffeine and theobromine caused a three- to fourfold increase in the precentages of cells possessing refractile spores and a similar increase in enterotoxin concentration. Heat-resistant spore levels, however, were unaffected. Inosine was ineffective in promoting sporulation in NCTC 8679.

Clostridium perfringens is a leading cause of human food poisoning. The responsible strains which have been isolated belong to type A (9), although other types have been shown to produce enterotoxin (22). McDonel and co-workers have described the biological activity of the enterotoxin (9-14) which is only produced during sporulation by enterotoxin-positive strains (5).

Sacks and Thompson reported that certain methylxanthines, including caffeine, 3-isobutyl-1-methylxanthine, and theophylline, increase spore yields of *C. perfringens* (16-18). These compounds are structurally similar to purines. In fact, Lopez et al. (8) have shown that sporulation in *Bacillus subtilis* can be induced by using inhibitors of purine nucleotide synthesis or by purine deprivation in auxotrophs. Sekar et al. (20) recently demonstrated that inosine reverses the inhibition of sporulation caused by *m*-aminobenzeneboronic acid and suggested that inosine levels may increase specifically during sporulation.

To our knowledge, there have been no reports linking caffeine with bacterial toxin formation. In the present study, we demonstrate the ability of caffeine and theobromine (which possesses one less methyl group) to promote sporulation and enterotoxin formation by *C. perfringens*.

MATERIALS AND METHODS

Cultures. Cultures of *C. perfringens* NCTC 8238 (Hobbs serotype 2) and NCTC 8679 (Hobbs serotype 6) were maintained at 4°C in cooked-meat medium (Difco Laboratories, Detroit, Mich.).

Sporulation medium. Vegetative cells were grown in fluid thioglycolate medium (BBL Microbiology Systems, Cockeysville, Md.), washed, and inoculated into D medium (18) as previously described (6), except in the case of NCTC 8679, the combination of solutions 2 and 3 was not mixed with solution 1 after its addition to solution 1 (except as noted below).

Induction of sporulation. The various compounds tested for their ability to induce sporulation were added before autoclaving. In the case of caffeine, the medium containing caffeine was sonified briefly to disperse clumps before autoclaving. Nucleosides and guanine were dissolved in 0.25 N HCl (thymine in 0.25 N NaOH) and added at the time of inoculation of D medium. In these experiments, the medium was mixed at the time of inoculation, and the bottles containing the medium were placed in Gas-Pak jars (BBL Microbiology Systems).

Sporulation and enterotoxin formation. Heat-resistant spore levels (HRSL), percent sporulation, and turbidity were determined at 10 h of growth as previously reported (6, 7). In these strains, free spores appear in D medium at about 12 h. Cultures in D medium were harvested at 10 h by centrifugation at 10,000 × *g* for 20 min. Enterotoxin concentration in cell extracts was determined by electroimmunodiffusion as previously described (7). Samples were assayed in duplicate. All experiments were done in duplicate.

Reagents. Reagents were obtained from Sigma Chemical Co., St. Louis, Mo., except for 1-methylxanthine, 3-methylxanthine, and 9-methylxanthine, which were obtained from ICN Nutritional Biochemicals, Cleveland, Ohio. Decoynine was a gift from G. Whitfield, The Upjohn Co., Kalamazoo, Mich.

RESULTS

Effects of caffeine and theobromine on sporulation and toxin formation. We investigated the effects of caffeine and theobromine

on sporulation and enterotoxin formation with two strains of *C. perfringens*. In the case of NCTC 8679, caffeine produced a dose-dependent response in the number of heat-resistant spores per milliliter (Fig. 1). These numbers increased from 2×10^2/ml in the presence of 10 μg of caffeine per ml to 1.8×10^7/ml at a caffeine concentration of 100 μg/ml. Similarly, the percentage of the population which possessed refractile or partially refractile spores increased from less than 1 to 87%, and enterotoxin concentration rose from undetectable levels to 450 μg/mg. Increasing the caffeine concentration to 200 μg/ml only moderately increased the enterotoxin level and had essentially no effect on spore formation.

When theobromine replaced caffeine in D medium, a similar dramatic increase in sporulation and enterotoxin formation occurred (Fig. 2). However, percent sporulation and enterotoxin concentration were slightly lower, even at a theobromine concentration of 200 μg/ml. HRSL were similar to those obtained in the presence of caffeine. Since their molecular weights are similar, comparisons between theobromine (molecular weight, 180) and caffeine (molecular weight, 194) based on weight are justified.

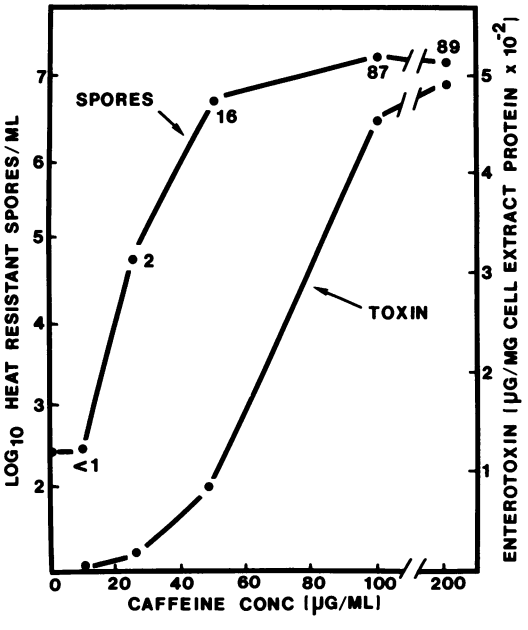

FIG. 1. *Sporulation and enterotoxin formation by C. perfringens NCTC 8679 in the presence of caffeine. Enterotoxin concentration is expressed as micrograms of enterotoxin per milligram of cell extract protein. Numbers adjacent to symbols are the percentages of the population possessing refractile or partially refractile spores.*

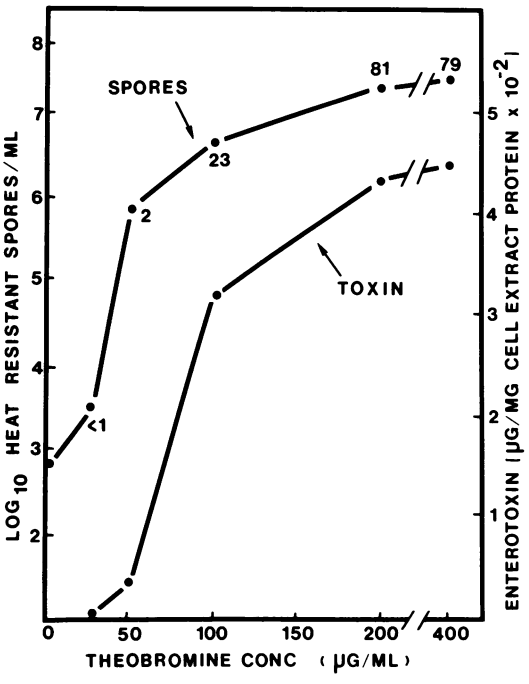

FIG. 2. *Sporulation and enterotoxin formation by C. perfringens NCTC 8679 in the presence of theobromine. Details as in the legend to Fig. 1.*

Next, we wished to determine the effect of caffeine and theobromine on another strain, NCTC 8238. These two compounds were also effective in this case (Table 1). Again, as little as 50 μg of either caffeine or theobromine per ml increased enterotoxin levels and percentages of refractile spores. A 200-μg/ml level of caffeine or theobromine was no more effective than 50 μg/ml. Interestingly, both compounds failed to increase HRSL above that of the control. Caffeine and theobromine partially inhibited growth as reflected in decreased Klett readings (Table 1).

Since caffeine and theobromine are both purine analogs, we checked the effects of a number of purine and pyrimidine analogs on growth and sporulation of NCTC 8679 (Table 2). Concentrations selected were those which resulted in an inhibition of growth similar to that caused by caffeine up to a maximum of 400 μg/ml (200 μg/ml in the cases of 1-, 3-, and 9-methylxanthine). All analogs inhibited growth to some extent, but none stimulated sporulation as well as did caffeine or theobromine. Of those listed in Table 2, theophylline was most effective. Decoynine, an inhibitor of guanosine monophosphate synthetase, has been reported to stimulate sporulation in *B. subtilis* (15). It was only moderately effective in this study; a concentration of 5 μg/ml resulted in 2% sporulation and an HRSL of 1.1

TABLE 1. *Effect of caffeine and theobromine on sporulation and enterotoxin formation by C. perfringens NCTC 8238*

Addition	Concn (μg/ml)	Klett units	% Refractile[a]	Heat-resistant spores/ml[b]	Enterotoxin concn[b,c]
None		145	22	$1.3 \times 10^7 \pm 0.2$	142 ± 23
Caffeine	50 (0.25)[d]	98	81	$1.1 \times 10^7 \pm 0.1$	318 ± 7
	200 (1.0)	83	80	$8.9 \times 10^6 \pm 1.1$	392 ± 34
Theobromine	50 (0.27)	86	73	$8.9 \times 10^6 \pm 0.1$	485 ± 83
	200 (1.1)	89	75	$1.2 \times 10^7 \pm 0.3$	416 ± 67

[a] Percentage of the population which possessed refractile or partially refractile spores.
[b] Numbers are means ± average deviation.
[c] Micrograms of enterotoxin per milligram of cell extract protein.
[d] Millimolar concentration given in parentheses.

TABLE 2. *Effect of purine and pyrimidine analogs on growth and sporulation of C. perfringens NCTC 8679*

Addition	Concn (μg/ml)	Klett units	% Refractile[a]	Heat-resistant spores/ml[b]
None		260	<1	$6.0 \times 10^2 \pm 0.8$
Fluorouracil	1.0 (0.007)[c]	222	<1	<30
	5.0 (0.035)	140	<1	<30
8-Azadiaminopurine	1.0 (0.004)	251	<1	$1.3 \times 10^3 \pm 0.5$
	5.0 (0.02)	219	<1	$1.0 \times 10^3 \pm 0.6$
	25.0 (0.10)	170	<1	$7.7 \times 10^1 \pm 0.5$
2-Amino-6-mercaptopurine	1.0 (0.005)	188	<1	$3.1 \times 10^3 \pm 3.0$
6-Mercaptopurine	0.5 (0.003)	243	<1	$1.7 \pm 10^3 \pm 0.6$
	1.0 (0.006)	224	<1	$5.6 \times 10^1 \pm 0.5$
	10.0 (0.06)	100	<1	<30
6-Methylaminopurine	100 (0.67)	246	<1	$4.7 \times 10^4 \pm 3.5$
	400 (2.68)	223	2	$1.2 \times 10^5 \pm 0.9$
6-Azathymine	100 (0.78)	215	1	$1.0 \times 10^6 \pm 0.9$
	400 (3.15)	213	2	$2.0 \times 10^6 \pm 1.8$
Papaverine	100 (0.29)	228	<1	ND[d]
	200 (0.58)	105	<1	ND[d]
6-Azauracil	200 (1.76)	230	<1	$2.6 \times 10^3 \pm 0.5$
	400 (3.54)	215	<1	$8.7 \times 10^3 \pm 0.5$
Theophylline	100 (0.55)	196	16	$9.0 \times 10^6 \pm 0.7$
	400 (2.22)	200	30	$1.6 \times 10^7 \pm 0.3$
1-Methylxanthine	200 (1.19)	234	<1	$2.8 \times 10^4 \pm 2.6$
3-Methylxanthine	200 (1.19)	256	<1	$1.1 \times 10^3 \pm 0.9$
9-Methylxanthine	200 (1.19)	208	8	$1.3 \times 10^5 \pm 0.6$
Decoynine	1 (0.004)	255	<1	$1.7 \times 10^2 \pm 0.8$
	5 (0.018)	236	2	$1.1 \times 10^5 \pm 0.1$
	10 (0.036)	64	<1	$2.3 \times 10^3 \pm 0.3$
Caffeine	100 (0.52)	160	87	$1.8 \times 10^7 \pm 0.1$
Theobromine	200 (0.48)	150	81	$2.2 \times 10^7 \pm 0.1$

[a,b,c] As in Table 1.
[d] ND, Not determined.

$\times 10^5$/ml. Other compounds found to be ineffective in inhibiting growth or promoting sporulation were N^6-methyladenosine and inosine (data not shown). 6-Azauracil and inosine caused a 2- to 3-log increase in HRSL (3) or a fivefold increase in percent sporulation (20) in *B. subtilis*, whereas papaverine and inosine have been reported to stimulate sporulation in *C. perfringens* (16; L. Sacks, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I128, p. 105). At a concentration of 200 μg/ml, 1-, 3-, and 9-methylxanthine were not as effective as was caffeine, theobromine, or theophylline in promoting sporulation.

In an attempt to determine the mode of action of caffeine, we added, at the time of inoculation, excess (3.0 mM) nucleosides to D medium containing 0.3 mM caffeine (58 μg/ml). All nucleosides tested reduced enterotoxin concentration (Table 3). Adenosine and thymidine caused an especially large decrease in caffeine-stimulated HRSL, percent sporulation, and enterotoxin level. This was also reflected by an increase in turbidity. However, adding guanosine and thymidine did not simply increase the vegetative cell population and thereby decrease the percentage of sporulating cells and enterotoxin concentration in cell extracts (due to increased levels of protein in cell extracts). These nucleosides also caused a nearly 2-log reduction in HRSL. Furthermore, enterotoxin levels, expressed as micrograms per milliliter of cell extract, averaged 655 μg/ml in the presence of caffeine alone and 66 and 60 μg/ml in the presence of caffeine plus thymidine or adenosine, respectively (data not shown). By comparison, guanosine, uridine, and cytosine reduced enterotoxin concentrations to 335, 460, and 401 μg/ml, respectively. Thymidine and adenosine, therefore, depressed HRSL and total enterotoxin formation by cells growing in the presence of caffeine. Thymine and adenine were ineffective in this regard.

TABLE 3. *Effect of nucleosides on caffeine-induced sporulation of C. perfringens NCTC 8679*

Addition[a]	Klett units	% Refractile[b]	Heat-resistant spores/ml[c]	Enterotoxin concn[c,d]
Caffeine	175	17	$1.3 \times 10^7 \pm 0.2$	94 ± 3
Caffeine + uridine	188	16	$1.1 \times 10^7 \pm 0.1$	67 ± 1
Caffeine + cytosine	169	8	$5.8 \times 10^6 \pm 0.4$	57 ± 2
Caffeine + guanosine	210	17	$9.2 \times 10^6 \pm 1.4$	44 ± 4
Caffeine + thymidine	216	1	$2.4 \times 10^5 \pm 0.8$	8 ± 1
Caffeine + adenosine	200	2	$2.4 \times 10^5 \pm 0.6$	7 ± 1

[a] Caffeine added at a concentration of 0.3 mM; nucleosides added at 3.0 mM. No enterotoxin was detected in the absence of caffeine or in the presence of individual nucleosides alone.
[b,c,d] As in Table 1.

DISCUSSION

An explanation(s) for the mechanism by which caffeine and theobromine stimulate sporulation must center on their effects on sporulation, since it has been well established that sporulation is required for enterotoxin formation (2, 7). The effects of these two compounds on sporulation and toxin formation support this concept. Both were unusually effective in promoting sporulation. The degree (percentage of sporulating cells) of sporulation obtained here in the presence of caffeine is among the highest obtained with this organism. Only one other strain, NCTC 10239, has been reported to produce such a high percentage of mature, refractile spores in a defined medium (6). However, HRSL higher than those obtained here have been reported for this organism in both defined and complex media (1, 6, 16). In the case of complex media, Sacks and Thompson (17) showed that with *C. perfringens* PS52, a concentration of 200 μg of caffeine per ml increased HRSL by 2 to 3 logs. Theophylline produced similar results when the same strain was grown in a defined medium (18). In these experiments, no attempt was made to measure enterotoxin. In the present study, caffeine and theobromine increased HRSL to even a greater extent, i.e., 4 to 5 logs above the caffeine-free medium. Corresponding dramatic increases occurred in enterotoxin levels. However, the response of *C. perfringens* to caffeine and theobromine is strain dependent. Some strains (NCTC 8239, ATCC 3624, FD1, and 2498) failed to increase their sporulation levels in the presence of these compounds. Other strains (NCTC 8449 and NCTC 1090) showed only a modest increase (data not shown).

Although caffeine and theobromine stimulated toxin formation by NCTC 8238, no corresponding increase in HRSL was noted. This apparent anomaly may be explained by the increased percentages of sporulating cells and the concomitant decrease in turbidity when cells of this strain were grown in the presence of these compounds. The net effect, therefore, was fewer cells, but better sporulation.

Caffeine, theobromine, and theophylline are methylxanthines, a class of compounds with one or more methyl groups at various positions on the purine ring. Attempts to determine whether a methyl group at a specific site on the ring was responsible for the stimulatory effect of methylxanthines were unsuccessful. A single methyl group at position 1, 3, or 9 on the purine ring was not as effective as caffeine (methyl groups at positions 1, 3, and 7), theobromine (methyl groups at positions 3 and 7) or theophylline

(methyl groups at positions 1 and 3). It is possible that two methyl groups, one at position 3, are necessary for the stimulatory effect of these compounds.

Methylxanthines are known to inhibit phosphodiesterases, possibly resulting in an accumulation of adenosine 3',5'-monophosphoric acid (23). However, the latter compound seems to be absent in sporulating bacteria (21). In addition, in the present study, papaverine, also an inhibitor of phosphodiesterase, was ineffective in promoting sporulation.

Freese et al. (3) reported that the partial reduction in the synthesis of purine nucleotides increases the HRSL of *B. subtilis* by 2 to 3 logs. This was demonstrated by the use of mutants or inhibitors of nucleotide synthesis. They also reported that caffeine and theophylline are ineffective in promoting sporulation of *B. subtilis*. However, in this study, methylxanthines were more effective than purine or pyrimidine analogs in stimulating sporulation.

Caffeine is known to interfere with deoxyribonucleic acid repair mechanisms (4); yet the partial reversal of caffeine-induced sporulation and enterotoxin formation by exogenous nucleosides suggest that in the case of *C. perfringens*, caffeine may induce sporulation and, hence, enterotoxin formation by affecting nucleotide synthesis, e.g., by inhibiting a nucleoside phosphorylase (which catalyzes the reaction of the free base to its nucleoside) or a phosphoribosyltransferase (which catalyzes the reaction of the free base to its nucleotide). However, in the case of *Escherichia coli*, thymidine kinase was the only enzyme of those tested which was inhibited by caffeine (19). The ability of inhibitors (hadacidin and decoynine) of adenosine monophosphate and guanosine monophosphate synthesis to promote sporulation in *B. subtilis* (3, 15) also suggests that an alteration in nucleotide synthesis may promote sporulation. Decoynine was moderately effective in this study. We are currently attempting to determine the specific enzymes whose activity may be affected by caffeine.

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