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Improved Media for Sporulation and Enterotoxin Production by *Clostridium perfringens*

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ABSTRACT

Sporulation of 24 strains of *Clostridium perfringens* isolated from stools of food poisoning patients and normal controls was improved by adding sodium carbonate to Duncan-Strong (DS) sporulation medium and replacing starch with raffinose in Taniguti's AEA medium. Viable counts of heat-tolerant spores (75°C for 20 min) were 2 to 186 times greater in modified AEA medium and 2 to 169 times greater in modified DS than in DS medium. Reversed passive latex agglutination assays revealed a corresponding increase in enterotoxin titers in supernatant fluids of the 12 enterotoxigenic strains grown in modified AEA medium and in modified DS medium.

Because *Clostridium perfringens* sporulates poorly in ordinary culture media such as cooked meat, a large number of media have been developed specifically for the sporulation of this microorganism (*1-3,7,9-14*). However, none of these proposed media is entirely satisfactory for culturing all strains. In a recent comparison of the performance of a new medium proposed by Tortora (*14*) and the AEA medium of Taniguti with two media that have been widely recommended in the United States, i.e., Ellner's (*3*) and Duncan-Strong (DS) media (*2*), we found that strains that failed to utilize starch sporulated poorly in all four media. Those that rapidly utilized starch often produced large numbers of vegetative cells but few spores, presumably because of the overproduction of acid end-products which killed the vegetative cells before sporulation could occur. This effect was most pronounced with DS medium, which has an initial pH only slightly above neutral (pH 7.2 to 7.3) (*2*). The best results were obtained with the AEA medium of Taniguti (*12*); however, sporulation of many food poisoning strains was still poor. Therefore, we modified AEA medium by replacing starch with raffinose (*8*), and DS medium by adjusting the initial pH of the medium from 7.3 to 7.8 with about 0.2 ml of sodium carbonate (*11*). In the present study, we cultured 24 *C. perfringens* strains isolated from stools and two strains isolated from outbreak foods in the modified media and compared their sporulation with that produced in DS medium. The relative number of spores in each medium was determined by examining stained smears of the sporulating cultures and by making viable counts after heating a portion of each culture at 75°C for 20 min.

The effects of adding certain components to the modified AEA medium, as recommended by Taniguti (*11*), on the development of heat-tolerant spores and the correlation between spore counts and enterotoxin titers in culture supernatant fluids were also determined.

MATERIALS AND METHODS

Cultures

Fecal isolates were obtained by homogenizing approximately 1 g of stool with 9 ml of 0.1% peptone in screw cap tubes with a Vortex mixer and heating the suspension for 20 min at 75°C before plating on Trypticase soy-sheep blood (TSB) agar. The inoculated plates were incubated anaerobically at 37°C, as described previously (*6*). Stock cultures were prepared by transferring typical colonies from TSB to cooked meat medium (CMM; Difco Laboratories, Detroit, MI). After incubation for 18 h at 35°C, the cultures were held at room temperature for 24 h. Strains FD-97 through FD-118, isolated from normal stools, were of the normal hemolytic biotype (on TSB) and were negative for enterotoxin, as reported earlier (*6*). Strains FD-1000 through FD-1049 were isolated from stools of patients who had experienced diarrhea during one of eight different *C. perfringens* food poisoning outbreaks. The characteristics of the causative strains were described by Harmon et al. (*6*). Stock cultures of the control strains (FD-1 and NCTC 8239) were prepared by resuspending lyophilized DS spore broth cultures in distilled water and transferring 0.2 ml of the suspension to freshly steamed CMM. The CMM control cultures were incubated and stored in the same manner as the fecal isolates.

Media

Duncan-Strong (DS) medium. DS medium, prepared according to the directions of Duncan and Strong (*2*), was modified by adding a sufficient amount of filter-sterilized 0.66 M sodium carbonate to sterilized DS medium to increase the pH from 7.3 to 7.8 ± 0.1.

AE base. Each liter of AE base consisted of 10 g polypeptone (BBL, Cockeysville, MD), 10 g yeast extract (BBL), 4.36 g dibasic sodium phosphate, 0.25 g monobasic potassium phosphate, 1.5 g ammonium acetate (Sigma Chemical Co., St.

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Louis, MO) and 0.2 g heptahydrate magnesium sulfate. Ingredients were dissolved in distilled water and, if necessary, the pH was adjusted to 7.5±0.1 with 2 M sodium carbonate. Volumes of 15 ml were dispensed into 20×150-mm screw cap tubes and sterilized by autoclaving for 15 min at 121°C. To prepare the final medium, 0.6 ml of separately sterilized 10% raffinose and 0.2 ml each of filter-sterilized 0.66 M sodium carbonate and 0.32% cobalt chloride ($CoCl_2 \cdot 6H_2O$) were added to each 15 ml of base. Just before use, the medium was steamed for 10 min; it was then cooled to <50°C, and 0.2 ml of filter-sterilized 1.5% sodium ascorbate (prepared daily) was added to each 15 ml. In some experiments, 0.2 ml of a solution containing 125 μg thiamine hydrochloride/ml was also added to each 15 ml. Each ingredient was added singly or in combination to AE base plus raffinose so that the effect of specific ingredients on sporulation could be determined.

Inoculation sequence

The inoculation sequence was similar to that used by Duncan and Strong (*2*), except that only one 4-h subculture was made in fluid thioglycollate medium and a 5% rather than a 10% inoculum was used to minimize the potentially adverse effect of adding too much glucose to the sporulation medium along with the inoculum (*8*).

Incubation

Cultures in modified AE medium were incubated for 24 h at 37°C in an anaerobic jar (Oxoid USA, Columbia, MD) (*11*). Cultures in DS and modified DS media were incubated in an air incubator at 37°C, as recommended by Duncan and Strong (*2*), except that 15-ml rather than 125-ml amounts were used.

Sampling

After 24 h of incubation, 5 ml of each sporulating culture was removed; a 1-ml portion was suspended in 9 ml of 0.1% peptone and heated for 20 min at 75°C before plating on tryptose-sulfite-cycloserine (TSC) agar (*6*). Dilutions were made in 0.1% peptone and the TSC agar plates were incubated at 37°C in an anaerobic jar (Oxoid). Viable counts were made after 20 to 24 h of incubation. The remainder of the test portion was centrifuged at 25,000×*g*, and the supernatant fluid was collected and tested for enterotoxin.

To determine the correlation between microscopic and viable counts, 0.05 ml of each culture was spread evenly onto a 2-cm^2 area of a microscope slide and air dried. The smears were fixed by flooding the slide with methanol and staining for 1 min with crystal violet (Difco). The number of mature spores in each microscopic field was determined and recorded as the ratio of spores to vegetative cells. At least five microscopic fields were examined and the spore count per ml was calculated.

Enterotoxin production

To establish the correlation between spore counts and enterotoxin levels, cell-free supernatant fluids were tested for enterotoxin by diluting them in phosphate-buffered saline plus 0.5% (wt/vol) bovine serum albumin and assaying for enterotoxin by reversed passive latex agglutination (*5*). Quantities were estimated by comparing the test results with those obtained with enterotoxin standards of a known concentration.

RESULTS AND DISCUSSION

Microscopic vs. viable counts

The relationship between direct microscopic counts made with stained smears and the corresponding viable counts of heat-tolerant spores in cultures of 16 different *C. perfringens* strains is shown in Table 1. These data indicate that although the microscopic count can be used to estimate spore populations in cultures that contain large numbers of spores (>5×10^6/ml), it is inappropriate when fewer spores are present. This technique was suitable for modified AEA because it produced large numbers of spores and contained fewer residual vegetative cells. In some instances, uneven distribution of spores in cultures, particularly those grown in DS media, also prevented accurate estimation of the spore populations by microscopic examination. These results indicated that viable counts provide an objective basis for comparison with different sporulation media (*2*). In subsequent experiments, therefore, we used microscopic counts only as a screening technique before testing for enterotoxin.

Effect of medium ingredients

Except for the combination of sodium ascorbate and cobalt chloride, differences in the numbers of heat-tolerant spores produced were negligible when certain ingredients were added to modified AE base (Table 2). However, the number of spores that could be seen by microscopic examination often increased when one or more of these ingredients was added. The final combination (variation 6) recommended by Taniguti (*11*) was the most effective. Although the actual increase in heat-tolerant spores was small, the ratio of spores to intact vegetative cells was high. This would explain the apparent increase in the number of spores reported by Taniguti, who judged the effectiveness of his medium by microscopic examination (*11,12*). The medium described in variation 6 was used in all subsequent experiments because it gave a better yield of mature spores, and the microscopic count was a simple, quick way to determine whether a culture had sporulated enough to permit valid testing for enterotoxin. When thiamine hydrochloride was added, many strains produced large atypical elongated spores that were unstable during storage of the culture at 4°C and appeared to be less heat tolerant than those produced in modified AEA or DS medium. This ingredient was omitted from the modified AEA medium used for general culturing.

Sporulation of food poisoning strains

The relative numbers of heat-tolerant spores (those that survived for 20 min at 75°C) and ratio of spores produced in modified AEA and DS media by enterotoxigenic strains compared with cultures grown in standard DS medium are shown in Table 3. In most instances, the viable spore counts of cultures grown in modified AEA were much greater than those of cultures grown in DS and modified DS media. The difference in sporulation response was also reflected in the larger amounts of enterotoxin found in supernatant fluids from cultures in the media (data not shown). The finding of Labbe and Rey (*8*), that sporulation was greatly influenced by the vigor of the fluid thioglycollate cultures used to inoculate the

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TABLE 1. *Correlation between direct microscopic counts and viable counts of* C. perfringens *spores in three different sporulation media*[a].

Strain source	Microscopic counts[b] DS	Modified DS	Modified AEA	Spores count/ml[c] (× 10^6) DS	Modified DS	Modified AEA
Outbreak stools[d]						
FD-1000	<1	<1	11	0.3	0.06	52
FD-1001	25	7	30	16	5.4	44
FD-1002	1.5	4	21	1.7	0.3	16
FD-1003	5	1	17	2	0.2	4
FD-1005	<1	<1	6	0.2	0.04	2
FD-1033	<1	2	23	0.7	0.8	18
FD-1035	<1	1.8	35	<0.001	0.1	32
Normal stools[e]						
FD-98	2	25	9	0.07	38	11
FD-99	<1	<1	0.5	0.62	0.08	0.5
FD-100	3.5	<1	2	0.15	0.06	0.8
FD-101	<1	4	5	0.3	5	15
FD-102	<1	<1	2	0.004	0.05	2
FD-103	<1	13	7.5	0.02	9	5
FD-104	1.7	<1	6	0.06	0.03	2
FD-108	1.3	1.8	5	0.7	2	3

[a]Media compared: Duncan-Strong (DS) broth; DS broth modified by adjusting to pH 7.8 with sterile sodium carbonate; and AEA broth modified by substituting raffinose for starch.

[b]Count per microscopic field (average of five fields). Each field represented ca. 5×10^{-5} ml of culture.

[c]On tryptose-sulfite-cycloserine agar after heating culture for 20 min at 75°C.

[d]Isolated from feces of food poisoning patients.

[e]Isolated from stools of normal healthy adults.

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TABLE 2. *Effect of various medium components on the production of heat-tolerant spores by* C. perfringens *in modified AEA spore broth*[a].

Strain source	Spore count[b]/ml (× 10^6) with variations[c] 1 None (pH 7.4)	2 + Sodium carbonate	3 + Sodium ascorbate	4 + Cobalt chloride	5 + Thiamine-HCl	6 + Cobalt and ascorbate
Outbreak stools						
FD-1000	30	22	28	27	29	59
FD-1022	0.05	0.8	0.96	0.11	0.01	0.13
FD-1009	1.6	2.9	4.8	3.9	6.3	23
FD-1010	0.3	12	15	16	21	46
FD-1008	48	22	61	18	51	41
FD-1036	9.5	12.5	10.5	15	17	7.8
FD-1001	99	63	55	40	50	90
Normal stools						
FD-113	7	46	11	4	33	50
FD-114	14	60	30	10	18	15
FD-117	0.05	0.8	1.0	1.1	0.12	2.4
FD-118	0.13	1.52	1.1	2.3	0.9	3.7
Control strains[d]						
FD-1	35	106	113	97	102	108
NCTC 8239	0.75	43	40	42	29	22

[a]AEA broth was modified by adding 0.6 ml of 10% sterile raffinose to each 15 ml of freshly steamed AE base without starch (pH 7.4).

[b]On tryptose-sulfite-cycloserine agar after heating culture for 20 min at 75°C.

[c]Variations: 0.2 ml of the following ingredients was added to each 15 ml of AE base: (1) none, pH 7.4; (2) 0.66 M sodium carbonate; (3) 1.5% sodium ascorbate; (4) cobalt chloride (3.2 mg Co^{++}/ml); (5) 125 μg thiamine-HCl/ml; (6) combination of 2, 3 and 4. All media except variation 1 were adjusted to pH 7.8 with sodium carbonate after sterilization.

[d]Isolated from foods implicated in food poisoning.

TABLE 3. *Relative numbers of spores produced by food poisoning strains of* C. perfringens *in three different sporulation media*[a].

Strain source	Spore count/ml[b] ($\times 10^6$)			Spore ratio[c]	
	DS	Modified DS	Modified AEA	Modified DS	Modified AEA
Outbreak strains[d]					
FD-1000	0.013	0.04	3.8	3.0	127
FD-1001	13	6.3	54	0.48	4
FD-1002	0.11	0.21	1.1	2.0	10
FD-1003	0.28	0.66	52	2.3	186
FD-1004	0.28	0.45	15	1.6	53
FD-1005	1.6	2.8	17	1.8	11
FD-1007	2.5	2.1	6.4	0.85	3
FD-1035	0.11	0.5	2.0	4.5	18
FD-1049	0.7	0.8	18	1.1	19
FD-1036	3.4	3.7	7.3	1.1	2
FD-1023	5	ND[e]	24	ND	5
FD-1024	0.01	0.01	30	1	3000
Control strains[f]					
FD-1	6.7	45	115	7	17
NCTC 8239	60[g]	20	1.7	0.33	0.03

[a]Media compared: Duncan-Strong (DS) broth; DS broth modified by adjusting base to pH 7.8 with sterile sodium carbonate; and AEA broth modified by substituting raffinose for starch.
[b]On tryptose-sulfite-cycloserine agar after heating culture for 20 min at 75°C.
[c]Compared with spore count obtained with DS broth culture.
[d]Isolated from feces of food poisoning patients.
[e]ND, no data available.
[f]Maintained by culturing in sporulation broths.
[g]Strain acclimated by repeated subculture in DS broth.

sporulation media, was confirmed. Cultures that grew slowly in fluid thioglycollate medium responded poorly in sporulation media, presumably because residual glucose in the inoculum encouraged vegetative growth and suppressed sporulation (*8*). This effect was more pronounced with the nonhemolytic food poisoning strains grown in DS media than in those grown in modified AEA medium. The most satisfactory results were obtained by culturing the food poisoning isolates only once in CMM and following the procedures recommended by Duncan and Strong (2) for heat shocking and culturing. Culturing in buffered tryptose-peptone-glucose-yeast extract broth (*4*) before testing adversely affected the sporulation response of many cultures (data not shown), as did the use of culture media that were more than 1 wk old (*8*).

Sporulation of normal stool isolates

The sporulation response obtained in the three media with 12 enterotoxin-negative strains isolated from normal stools is shown in Table 4. Although strains of this biotype have occasionally been implicated in food poisoning, the strains tested did not produce significant amounts of enterotoxin in the sporulation media studied (data not shown).

Sporulation of isolates from normal stools (strains FD-97 through FD-118) was less predictable than that from stools of food poisoning patients. Better sporulation was obtained with a few normal stool isolates in DS and modified DS media than with modified AEA medium; however, spore counts were usually greater with cultures grown in modified AEA medium (Table 4). Hence, although modified AEA medium did not yield the highest counts in every instance, it did so with 22 of the 24 strains studied. Strains FD-1 and NCTC 8239, which have been maintained as laboratory cultures for many years, responded very erratically in different trials (Tables 3 and 4). This suggests that such cultures are unsuitable for determining the likely response of freshly isolated *C. perfringens* in a particular sporulation medium because they may be adapted to specific culturing conditions.

Enterotoxin production

The correlation between viable spore counts in different media and the enterotoxin titers found in culture supernatant fluids with 12 food poisoning strains, as measured by reversed passive latex agglutination, is shown in Table 5. Enterotoxin titers were 10 to >500 times higher in culture supernatant fluids from modified AEA medium than from the other two media for all but two strains. This result could be attributed to the larger proportion of sporulating cells in cultures grown in modified AEA medium and possibly to actual increases in the amounts of enterotoxin produced when starch was replaced by raffinose (*8*). Strain NCTC 8239 sporulated better in DS and modified DS media than in modified AEA (Tables 3 and 4), apparently as a result of repeated culturing in DS medium. Similar results were obtained with the enterotoxin-negative FD-1 strain, which we isolated from roast beef implicated in food poisoning, but

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TABLE 4. *Relative numbers of spores produced by enterotoxin-negative* C. perfringens *strains isolated from normal stools in three different sporulation media*[a].

Isolate No.	Spore count/ml[b] (× 10⁶) DS	Modified DS	Modified AEA	Spore ratio[c] Modified DS	Modified AEA
FD-97	2	0.45	1	0.23	0.50
FD-98	0.61	390	110	639	180
FD-99	6.2	0.08	4.7	0.01	0.76
FD-101	5	0.55	8.6	0.1	2
FD-103	2.1	45	150	21	75
FD-104	0.01	0.04	1	4	100
FD-105	1	94	5	94	5
FD-111	0.03	0.61	13	20	433
FD-113	3.0	1.3	10	0.43	3
FD-114	7.0	15	7	2	1
FD-117	0.1	0.25	3	3	30
FD-118	0.21	1.8	15	9	71
Control strains[d]					
FD-1	12	2.2	32	0.18	2.7
NCTC 8239	41[e]	129[e]	48	3.1	1.2

[a]Media compared: Duncan-Strong (DS) broth; DS broth modified by adjusting base to pH 7.8 with sterile sodium carbonate; and AEA broth modified by substituting raffinose for starch.
[b]On tryptose-sulfite-cycloserine agar after heating culture for 20 min at 75°C.
[c]Compared with spore count obtained with DS broth culture.
[d]Maintained by culturing in sporulation broths.
[e]Strain adapted to culturing in DS broth.

TABLE 5. *Correlation between spore counts and enterotoxin levels produced by eight* C. perfringens *strains in three different sporulation media*[a].

Strain[b]	Spore count/ml[c] (× 10⁶) DS	Modified DS	Modified AE	Enterotoxin titer[d] (μg/ml) DS	Modified DS	Modified AE
FD-1000	0.4	0.06	23	0.3	ND[e]	25
FD-1001	16.3	5.4	44	25	25	25
FD-1002	2.0	0.2	4	1.3	0.12	12.5
FD-1003	0.17	0.3	17	0.6	0.25	25
FD-1005	0.1	0.05	2	0.01	0.01	25
FD.1006	0.3	0.1	1.0	0.006	0.007	1.25
FD-1004	4.4	0.5	5	1.25	0.10	25
FD-1033	0.5	0.8	18	0.05	0.10	25
FD-1035	0.01	0.01	32	0.01	0.125	25
FD-1049	0.01	0.01	0.5	0.01	0.1	1.25
FD-1021	0.01	0.01	0.02	12.5	25	50
FD-1023	0.4	19	45	0.01	12.5	12.5

[a]Media compared: Duncan-Strong (DS) broth; DS broth modified by adjusting base to pH 7.8 with sterile sodium carbonate; and AEA broth modified by substituting raffinose for starch.
[b]Isolated from feces of food poisoning patients (eight different outbreaks).
[c]On tryptose-sulfite-cycloserine agar after heating culture for 20 min at 75°C.
[d]After 24 h of incubation at 37°C. Enterotoxin was quantitated by reversed passive latex agglutination.
[e]ND, no data available.

which resembles the hemolytic heat-sensitive strains usually found in normal stools. Strains of this type usually sporulate abundantly in Ellner's medium but respond poorly in DS medium, probably because of the higher initial pH of Ellner's (pH 7.8 vs. 7.3) and the greater buffering capacity. This advantage has been noted by several workers (*3,9,11,14*) and may explain, at least in part, the better overall results obtained with modified DS medium; however, enterotoxin levels were always higher when modified AEA medium was used for culturing enterotoxigenic strains (Table 5).

CONCLUSIONS

Modification improved the performance of AEA and DS media in promoting sporulation and enterotoxin pro-

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duction by *C. perfringens*. Modified AEA with raffinose was most effective for both purposes. Although it did not always give superior results, modified AEA was the most effective of the three media studied in promoting sporulation of a majority of the strains.

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