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Clostridium perfringens

I. Sporulation in a Biphasic Glucose-Ion-Exchange Resin Medium

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A biphasic culture medium suitable for cultivation and sporulation of *Clostridium perfringens*, *C. botulinum*, and *C. sporogenes* was devised. The medium designed for use in a disposable, compartmented, plastic film container contained peptones, yeast extract, minerals, an anion exchange resin, and glucose in 4% agar as the solid phase and $(NH_4)_2SO_4$ and 0.1% agar as the liquid phase. With the biphasic system, it was not necessary to use active cultures as inocula. Growth was at least equal to that obtained in conventional media, and spore production of 9 out of 12 strains of *C. perfringens* equalled or usually exceeded that of conventional media.

The sporulation of *Clostridium perfringens* has been a relatively involved procedure incorporating a number of serial transfers, several incubation periods, and various media used in sequence. It has been difficult to attain consistently high spore yields with such methods. This study was undertaken to develop a simplified method wherein a single medium would support proliferation and sporulation of this organism in one incubation period and offer as good or better spore production levels as methods currently in use.

C. perfringens can readily be cultured in a simple medium with glucose. However, glucose repressed spore production (4, 6, 14, 18), and, instead, a heavy vegetative crop followed by autolysis occurred. Of the several broth media reported for the sporulation of *C. perfringens*, all, with the exception of that of Duncan and Strong (5), which incorporated starch, contain a nitrogen source without carbohydrate. All of these media require a heavy, active vegetative inoculum from a separate carbohydrate-enriched growth medium to induce sporulation.

In the present study, the biphasic cultural technique reviewed by Schultz and Gerhardt (17) and described for the sporulation of *C. botulinum* (1, 3, 16) was adopted for use with *C. perfringens*. A new medium containing glucose was compounded to support both vegetative proliferation and sporulation in a disposable, compartmented, plastic film container.

For comparative purposes, 12 strains of *C. perfringens* were sporulated in the proposed biphasic system and in accordance with the methods of Hall (9) and Duncan and Strong (5), whose media had been selected as the most promising of 14 conventional media screened in our laboratory. The proposed system was also evaluated for the production of spores of *C. botulinum* and *C. sporogenes*.

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MATERIALS AND METHODS

Test organisms. Twelve strains of *C. perfringens* incriminated in food poisoning outbreaks were obtained from C. H. Duncan, University of Wisconsin, Madison (NCTC-8238, NCTC-8798, NCTC-9851, NCTC-10240, FD-1, FD-2, FD-5, FD-7, T-65, 68900, 77455, and 80535); three strains of *C. botulinum* (62A; 9B; and Langeland F, which was initially acquired through the courtesy of N. Walls, Georgia Institute of Technology, Atlanta) and one strain of *C. sporogenes* (PA 3679-H) were selected from our own collection. Stock cultures were prepared in Cooked Meat Medium (Difco) supplemented with 0.1% glucose in screw-cap tubes (20 by 150 mm) and after incubation at 37 C for 48 hr were stored at 2-5 C.

Inoculum preparation. To prepare the *C. perfringens* inocula for the conventional media (5, 9), 1 ml of the stock suspension was subcultured at 37 C after the method of Halvorson (10) in 25 ml of freshly autoclaved and cooled Fluid Thioglycollate Medium (BBL) for 16 hr, followed by two subsequent (2.5 ml) transfers (each incubated 4 hr at 37 C) into 22.5 ml of steamed (10 min) and cooled Fluid Thioglycollate Medium. The entire final culture (25 ml) was placed in 225 ml of the above sporulation broths.

The biphasic systems were inoculated with 1 ml of the Cooked Meat Medium stock.

Sporulation media. Both conventional *C. perfringens* media (5, 9) were dispensed (225 ml) into 500-ml flasks equipped with vented stoppers and serum-stoppered side arms. A Trypticase-peptone medium (1) was used to sporulate strains 62A and 9B of *C. botu-*

linum. These media were sterilized at 121 C for 15 min, cooled rapidly, and used immediately.

The liquid phase of the proposed biphasic medium consisted of (w/v) 2.0% $(NH_4)_2SO_4$ and 0.1% agar (Fisher Scientific Co.) in distilled water. The solid phase contained 0.5% Soy peptone (Sheffield), 0.5% N-Z-Amine NAK (Sheffield), 0.5% Edamine-S (Sheffield), 0.5% Trypticase (BBL), 0.5% yeast extract (BBL), 0.05% glucose, 0.01% $MnSO_4$, 0.01% $MgSO_4$, 0.01% thiamine-HCl, 4.0% agar (Fisher), and 0.2% AG-1-X-4 (chloride form, 200–400 mesh) anion exchange resin (Bio-Rad Laboratories). Both phases were adjusted to *p*H 7.5 with 5 N KOH.

The system used 30 ml of the liquid phase and 100 ml of the solid phase in a disposable plastic container. All plastic containers were refrigerated at 2 to 5 C to hasten solidification of the agar phase and were used within 3 hr after autoclaving.

Enumeration media. *C. perfringens* spores were counted in the following medium: 1.5% Trypticase (BBL), 1.0% yeast extract (BBL), 0.05% sodium sulfite, 0.05% ferric citrate, 0.1% glucose, and 1.5% agar (Fisher Scientific Co.) adjusted to *p*H 7.2 with 5 N KOH. *C. botulinum* and *C. sporogenes* spore counts were made in a medium containing 5.0% Thiotone (BBL), 0.5% yeast extract (BBL), 0.5% Trypticase (BBL), 0.05% sodium thioglycolate, 0.01% glucose, and 0.75% agar (Fisher Scientific Co.) adjusted to *p*H 7.2 with 5 N KOH. Also included in each tube with the latter medium was 0.3 ml of filter-sterilized 5% $NaHCO_3$ aqueous solution (0.12% final concentration).

Plastic film culture container. All biphasic sporulation experiments were carried out in a disposable, compartmented, plastic film container modified from an earlier noncompartmented culture bag designed by D. Berkowitz (*personal communication*). Commercially available food-grade polyolefin bags (CEDANCO, Framingham, Mass.) were fabricated as shown in Fig. 1 with a heat-sealing device. Seal lengths were controlled by inserting a sheet of Teflon-impregnated glass cloth (Sentinel, Hyannis, Mass.) between the layers of film where lamination was not desired. The central pocket (A) contained the liquid phase of the system, the lower compartment (B) contained the solid nutrient phase, and a gauze-covered cotton plug (C) permitted aseptic inoculation and venting of gases. Containers measuring 23 by 30 cm were used for solid phases up to 100 ml, with a ratio of one part liquid to 3.3 parts solid phase.

Containers were suspended from a special support rack (Fig. 1) with two parallel rods (R) which ran through prepunched holes (F) in the container. Dehydrated agar for the solid phase was placed into compartment (B) followed by a solution of the nutrients and salts to insure that no agar remained in the access channel to cause adherence of the container walls. A weight distribution rod (D) was placed through fold (E), and the container was autoclaved and cooled rapidly at 2 to 5 C. The weight distribution rod prevented the heat-softened plastic film from tearing loose from the support rack.

After solidification of the agar, the rod (D) was removed, the inoculum was pipetted through the opening (C), and the container was heat-sealed at (J). The entire container was removed from the support rack and tipped on its side so that the liquid phase would run down onto the solid phase, washing any adhering inoculum from the walls.

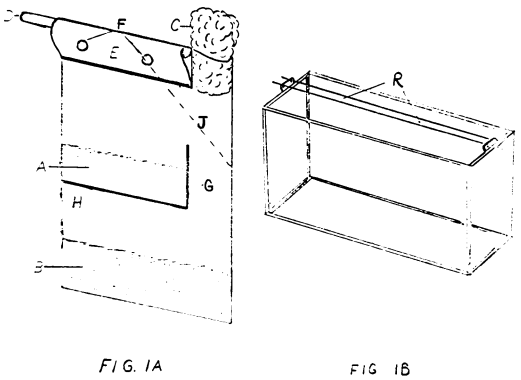

FIG. 1A FIG 1B

FIG. 1. *(a) Schematic drawing of a disposable compartmentalized plastic film container for the biphasic production of anaerobic spores. (A) liquid phase compartment, (B) solid phase compartment, (C) cotton plug, (D) metal rod, (E) upper fold, (F) holes for support rack rods, (G) harvest area, (H) sampling point, and (J) heat seal line after inoculation. (b) Support rack for film container with support rods (R).*

The above container was enclosed in a disposable nonautoclavable gas barrier bag (film no. 48, 3M Company, Minneapolis, Minn.) which was heat-sealed to leave a 5-cm opening in one corner. The air surrounding the inner bag was displaced by gassing for 1 min with prepurified nitrogen, and the barrier bag was completely sealed. Apparently, the exchange of gases through the walls of the inner container was sufficient to enable the rapid growth of the clostridia involved in the study, whereas no growth occurred without the barrier bag.

Incubation and sampling. Both conventional and biphasic systems were incubated at 37 C, with the biphasic containers resting upright on the incubator shelves without any support.

The flask cultures were sampled periodically by suspending contents and withdrawing a sample with a sterile syringe through the serum-stoppered side arm previously wiped with 70% ethanol.

Periodic samplings were also made through the film containers with a sterile 2-ml syringe equipped with a 25-gauge hypodermic needle. Greatest protection from contamination was obtained by tilting the container away from the access channel (G) and removing a sample by aseptic insertion of the needle through both bags at H (Fig. 1). Samplings were made every 4 hr beginning at 16 hr of incubation and were examined in wet mount under phase contrast misroscopy for the appearance of refractile spores.

Harvesting. After microscopic spore counts reached a maximum, the cultures were harvested. The technique for harvesting the biphasic cultures was to remove the outer barrier bag and aseptically cut the access channel at (G) for decanting the sample. Each

sample was washed three times by centrifuging and suspension in sterile Sorensen's phosphate buffer (*p*H 7.2). The washed pellet was suspended to the original culture volume (31 ml for the biphasic and 250 ml for the conventional cultures) in buffer and placed in screw-capped containers at 2 to 5 C. *C. perfringens* was heated at 65 C for 30 min, and the other organisms were heated at 80 C for 10 min to inactivate vegetative cells and heat-shock the spores.

Enumeration. Cells which survived the above heat treatment and formed macrocolonies in the enumeration media were regarded as spores. Spores were heat-shocked a second time if more than 24 hr had elapsed since harvesting.

C. perfringens spore counts were made on duplicate 1.0-ml portions per sample by using three plates per dilution. Incubation was at 37 C for 24 hr under an atmosphere of 90% nitrogen–10% CO_2 (exhausted three times) in an anaerobic container modified from a model 2Z-368 commercial pressure spray paint tank (Fig. 2; Dayton Electric Mfg. Co., Chicago, Ill.) which held 160 plates.

C. botulinum and *C. sporogenes* spore counts were made on duplicate 1.0-ml portions per sample with three tubes (11 by 203 mm) per dilution. Each tube used 13 ml of medium, with incubation at 30 C for 48 hr.

RESULTS

Comparison of C. perfringens sporulation media. Table 1 presents the comparative spore yield data for 12 strains of *C. perfringens* with identical lots of enumeration medium. Strains 77455 and 80535 were the most difficult to sporulate under all conditions tested. With the conventional techniques, the Duncan-Strong medium (5) produced at least fivefold higher spore yields than Hall's broth (9) with four strains (NCTC 8238, NCTC 8798, FD-1, and FD-7), gave equal sporulation with six strains (NCTC 9851, NCTC 10240, FD-2, FD-5, T-65, and 77455), and somewhat lower spore concentrations with 68900 and 80535 (Table 2). Compared with the Duncan-Strong medium, the biphasic system was superior by 10-fold or more with five strains (NCTC 8238, NCTC 9851, FD-5, T-65, and 68900), was approximately equal with four others (FD-1, FD-2, FD-7, and 80535), and was inferior with three strains (NCTC 8798, NCTC 10240, and 77455). When compared with the Hall medium, the biphasic technique was superior by 10-fold or more with six strains (NCTC 8238, NCTC 8798, NCTC 9851, FD-5, FD-7, and T-65), about equal with four strains (FD-1, FD-2, 68900, and 77455), and somewhat lower with two strains (NCTC 10240 and 80535).

Incubation periods ranged between 20 and 30 hr, depending on strain, and, when incubated beyond optimal time, the spores germinated (became phase dark, stained with methylene blue, and did not tolerate heating at 65 C). As reported by Despaul (4), after the initial spore production peak the cultures reverted to vegetative cells and began to lyse.

Effect of modification of the biphasic system medium on sporulation. Table 3 indicates the importance of maintaining the proposed biphasic system for the maximum sporulation of *C. perfringens*. When glucose was excluded, very poor growth resulted and no spores were recovered after heating (65 C for 30 min). Strains FD-1, FD-2, and FD-7 produced spores at reduced levels in the biphasic system without resin but the remaining nine strains produced none, although heavy vegetative growth occurred with all strains.

To determine whether the biphasic interface was essential, plain agar was omitted from the solid phase of the medium. Excellent vegetative growth was achieved with all strains, but four strains did not sporulate at all and the remaining eight formed markedly reduced spore numbers. The addition of agar to the liquid phase proved beneficial in establishing a more rapid vegetative

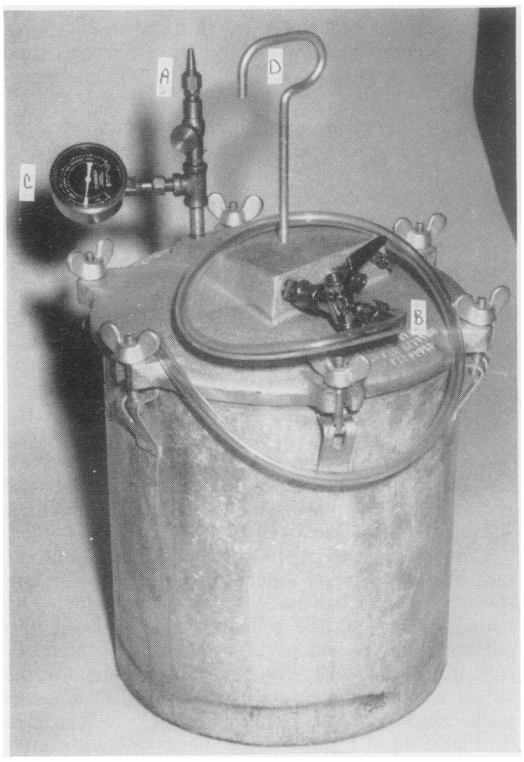

FIG. 2. *Painter's pressure tank modified to serve as an anaerobic container for 160 plates. (A) connection to vacuum source, (B) connection line to gas cylinder, (C) pressure/vacuum gauge, and (D) carrying handle.*

TABLE 1. *Comparative spore concentrations of Clostridium perfringens produced by the conventional and the biphasic methods*

Strain	Duncan-Strong medium[a]		Hall medium[a]		Biphasic culture medium[a]		
	1	2	1	2	1	2	3
NCTC 8238	6.5	5.3	1.7	0.8	630.0	760.0	1,900.0
NCTC 8798	46.0	31.0	1.0	1.1	6.2	6.0	3.7
NCTC 9851	0.41	0.20	0.066	0.5	4.7	3.9	5.3
NCTC 10240	7.6	5.4	0.64	2.7	0.68	0.24	0.26
FD-1	37.0	27.0	3.6	6.3	53.0	34.0	35.0
FD-2	6.3	6.0	4.7	7.1	30.0	26.0	29.0
FD-5	0.79	0.10	0.65	0.62	7.0	25.0	19.0
FD-7	9.8	18.0	2.0	2.3	20.0	44.0	23.0
T-65	0.37	2.0	0.15	0.70	5.7	21.0	18.0
68900	1.4	0.58	3.2	—[b]	36.0	27.0	18.0
77455	0.27	0.55	0.12	0.40	0.42	0.13	0.14
80535	0.31	0.31	0.98	0.40	0.31	0.21	0.20

[a] Counts given $\times 10^5$ spores/ml.
[b] Sample lost.

TABLE 2. *Ratios comparing the conventional and the biphasic methods for sporulation of Clostridium perfringens*[a]

Strain	DS/H[b]	BP/DS[c]	BP/H[d]
NCTC 8238	6.3	186.4	1,117.0
NCTC 8798	35.0	0.42	14.5
NCTC 9851	1.1	15.3	16.4
NCTC 10240	3.8	0.06	0.23
FD-1	6.4	1.3	8.2
FD-2	1.14	4.6	4.7
FD-5	0.7	37.8	26.6
FD-7	6.4	3.1	19.5
T-65	2.8	12.5	34.9
68900	0.3	27.0	8.4
77455	1.6	0.6	0.9
80535	0.45	0.8	0.3

[a] Based on averages of data from Table 1.
[b] Duncan-Strong/Hall ratio of sporulation.
[c] Biphasic/Duncan-Strong ratio of sporulation.
[d] Biphasic/Hall ratio of sporulation.

growth, but its omission did not reduce the final spore concentration levels. In fact, strains NCTC 9851 and NCTC 10240 showed a 10-fold increase over the unmodified biphasic control.

Effect of modification of the biphasic system environment on sporulation. Table 3 also demonstrates the importance of maintaining the physical environment in the container system. When normal biphasic systems were incubated on a reciprocating shaker (0.5 cycle/sec) which kept the liquid phase in gentle motion, heavy vegetative growth levels were obtained but sporulation was depressed in 10 of 12 strains. Incorporation of 10% CO_2 in the nitrogen atmosphere in the barrier envelope produced profuse vegetative proliferation with cell levels up to 10^{11} per ml with most strains, followed by a transition to autolysis without detectable sporulation. Without the barrier envelope, there was no growth by any strain.

Sporulation of other clostridia. In three separate experiments with the biphasic system, *C. botulinum* types A, B, and F spore concentrations reached 10^7 or more spores per ml with total production levels of 10^9 spores (Table 4). The A and B *C. botulinum* strains were also sporulated by the conventional method of Anellis and Rowley (1). Approximately equal spore concentrations were attained by the two procedures, although total spore yield was consistently higher in the larger broth volume (250 ml) than in the biphasic system (31 ml). *C. sporogenes* PA 3679-H produced 10^8 spores per ml, or a total production of 10^9 spores in the biphasic system.

Maximum sporogenesis of these clostridia occurred in 60 hr at 37 C. Except for the type F botulinal strain, which produced only 30% sporulation, the other clostridia attained 70 to 90% sporulation by the biphasic procedure. Mice injected with *C. botulinum* types A and B culture filtrates obtained by both procedures exhibited typical botulinal symptoms and were protected by their respective antisera.

DISCUSSION

The proposed system has several advantages over conventional sporulation methods. Serial transfers of cultures and several media are not required. Incubation time is considerably re-

TABLE 3. *Effect of modifying cultural conditions on Clostridium perfringens spore yields in the biphasic system*[a]

Strain	Unmodified biphasic system[b]	Modifications of biphasic system						
		Deletions from solid phase[c]			Agar deleted in liquid phase[d]	Agitation (0.5 cycle/sec)	10% CO_2-90% N_2	Incubated without gas barrier bag
		Glucose	Resin	Agar				
NCTC 8238	11,000.0	ZC[e]	ZC	0.22	14,000.0	100.0	ZC	NG[f]
NCTC 8798	160.0	ZC	ZC	3.3	180.0	3.3	ZC	NG
NCTC 9851	46.0	ZC	ZC	0.036	640.0	4.1	ZC	NG
NCTC 10240	3.9	ZC	ZC	ZC	42.0	0.3	ZC	NG
FD-1	410.0	ZC	85.0	53.0	440.0	0.2	ZC	NG
FD-2	280.0	ZC	26.0	0.0029	120.0	0.1	ZC	NG
FD-5	170.0	ZC	ZC	ZC	63.0	0.2	ZC	NG
FD-7	430.0	ZC	22.0	ZC	280.0	0.2	ZC	NG
T-65	150.0	ZC	ZC	ZC	670.0	0.2	ZC	NG
68900	270.0	ZC	ZC	0.29	120.0	0.03	ZC	NG
77455	2.3	ZC	ZC	0.18	6.0	6.7	ZC	NG
80535	2.4	ZC	ZC	0.16	2.0	28.0	ZC	NG

[a] Counts given as 10^4 spores/ml.
[b] Average counts derived from Table 1.
[c] Biphasic medium was modified by deletion of a single ingredient. Omission of agar was from the solid phase.
[d] Single experiment; solid phase unmodified.
[e] Zero colonies on plates inoculated with undiluted, heat shocked (65 C for 30 min) samples.
[f] No growth.

TABLE 4. *Comparative spore concentrations*[a] *of Clostridium botulinum and Clostridium sporogenes produced by the conventional and the biphasic methods*

Organism	Strain	Type	Expt no.	Method[b]	
				Biphasic, 10^7 spores/ml	Conventional, 10^5 spores/ml
C. botulinum	62A	A	1	6.0	2.8
			2	5.2	11.0
			3	3.0	2.1
C. botulinum	9B	B	1	4.5	1.0
			2	4.3	2.3
			3	4.9	1.7
C botulinum	Langeland F	F	1	1.9	
			2	3.0	
			3	2.6	
C. sporogenes	PA 3679-H		1	13.0	
			2	12.0	
			3	10.0	

[a] Average of duplicate samples per experiment with three tubes per dilution after heating at 80 C for 10 min. Tubes incubated for 48 hr at 30 C.
[b] Counts given at 10^7 spores/ml.

duced and spore concentrations are as good or substantially better than with conventional methods. Additionally, the plastic film containers are easily fabricated, inexpensive, and relatively immune to breakage. Storage and washing problems are eliminated since they are disposable. Moreover, compartmentalization eliminates the handling of several containers.

The improvements in the system are dependent on at least three factors: (i) the ability to use glucose in the sporulation medium for supporting heavy vegetative proliferation, (ii) the use of an anion-exchange resin to facilitate sporogenesis, and (iii) the static biphasic interface.

Without glucose, we were unable to propagate large numbers of vegetative cells and had to resort to preparation of large vegetative inocula in a separate growth medium as is done with the conventional procedures. At best, only marginal success was attained in using such externally prepared inocula in this manner. The depressive effect of glucose (and perhaps other factors) on sporulation in the biphasic system was overcome by using a resin in the medium.

Prompted by reports of antisporulants either in the medium or from cultural by-products (5, 11, 15), resin was selected as a possible sequestering agent because of its ease in handling, stability at autoclave temperatures, and commercial availability in purified form. Used previously by Malin and Finn (13) as an aid to anaerobiosis, it is not known whether the resin is actually a sequestering agent in our application or whether it functions in another capacity. They reported that their resin permitted prolific growth but they did not indicate whether it

encouraged sporogenesis. Since the resin beads were incorporated in the agar block, it was virtually impossible to retrieve them for post-cultural chemical analysis without heating or eluting with agents which would disrupt components adsorbed onto the resin. A major portion of the turbidity in actively growing cultures was concentrated near the lower part of the agar block where the resin tended to settle, whereas the agar was in the liquid state, although some settling of organisms was also expected due to gravity.

Combinations of the glucose and resin without the complete biphasic system did not elicit sporulation. They could be placed into the broth medium or into the liquid phase of the proposed system without generating the desired results. It is not known whether the two substances interact directly or only through intermediates during heating and culturing, or whether the resin is simply a sequestering agent for entities created during the utilization of glucose by the organism. A preliminary attempt to improve the conventional media by adding resin or plastic film, or both, to the broth failed to increase spore yields.

In any case, the biphasic interface appears to play an important role in controlling the interaction of these two materials. Our results imply a balanced exchange of materials at the interface which can easily be unbalanced by setting the liquid phase in motion. A second interface formed by the liquid and plastic film container apparently produces a second gradient where gaseous exchange can take place. Evidently, there is sufficient inflow of nitrogen to create reduced conditions for anaerobic growth and there is a pronounced outflow of culture odor into the surrounding gas envelope formed by the barrier bag.

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