MICROBIOLOGICAL METHODS

Clospore: A Liquid Medium for Producing High Titers of Semi-Purified Spores of *Clostridium difficile*

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Clostridium difficile **continues to cause infections in healthcare and other settings. Its spores survive well indoors and require sporicidal chemicals for infection control. However, proper testing of disinfectants is impeded due to difficulties in obtaining viable spores of high enough quality and titers to meet current regulations for sporicidal claims. A new liquid medium (Clospore) has been developed, based on a systematic review of the compositions of 20 other available media.** ***C. difficile*** **spores grown in the new medium and treated with a mixture of lysozyme and trypsin yielded final suspensions with >10^9 CFU/mL of viable spores, with a purity of >91% as tested by spore-staining and phase-contrast microscopy. The spores showed a biological decay rate of about 0.1 $\log_{10}$/month when dried on metal disks and stored indoors (air temperature 23 ± 2 C; relative humidity 52.76 ± 15.08%). Heating the purified spore suspensions to 70 C for 10 min to inactivate any vegetative cells showed no spore activation or inactivation. The spores could be stored for at least 14 months either refrigerated (4 C) or frozen (–20 or –80 C) in 50% (v/v) ethanol with virtually no loss in viability. The resistance of the enzyme-treated spores to three levels of sodium hypochlorite (1000, 3000, and 5000 ppm), using a standardized quantitative carrier test, was almost identical to that of the spores concentrated by centrifugation alone. The described procedure has been successfully applied to four standard (ATCC) and six clinical strains of** ***C. difficile*****.**

Clostridium difficile is now a major cause of acute and potentially fatal gastroenteritis (1, 2), having become more toxigenic (3) and resistant to standard therapies (4, 5). The frequent episodes of watery diarrhea contaminate the environment extensively with persistent (6, 7), and microbicide-resistant (8) spores, which poses a significant challenge for decontamination of the patient's immediate surroundings (9). Until recently, the label claims of many environmental surface disinfectants against *C. difficile* were based mostly on tests using its vegetative form, which is easier to inactivate. Now, the U.S. Environmental Protection Agency (EPA) will accept such claims only when the testing is conducted using the spores (10).

Although high titers of *C. difficile* spores can be obtained using semi-solid media (11, 12), the process yields crops of variable quality for routine testing of sporicidal activity. Thus far, good sporulation in liquid media has been difficult and, again, yields titers not high enough to demonstrate acceptable levels of sporicidal activity (13). After a systematic comparison of the compositions of 20 available media, we describe here a liquid medium along with a semipurification process to produce high-titered suspensions of *C. difficile* spores for use in testing environmental surface disinfectants.

Materials and Methods

Strains and Culture Media

Table 1 lists the strains of *C. difficile* used and their sources. The commercial media tested (manufacturer and catalog number) for spore production were: Brain-Heart Infusion (BHI; Oxoid, Ottawa, ON, Canada; CM0225); Columbia Broth (CB; QueLab, Mississauga, ON, Canada; QB-39-1106); Reinforced Clostridial Medium (RCM; Difco-BD; 218081-11808-17); RCM (Oxoid; CM0149); Thioglycolate Medium (Oxoid; CM173); Fluid Thioglycolate Medium (Difco-BD; 0256-01); Shahidi-Ferguson Perfringens-Agar Base (Difco-BD; 281110-0811-17); Bolton Broth (Oxoid; CM0983); and Special Peptone Mix (SPM; Oxoid; LP0072). The media were prepared and used according to label directions. BHI with glucose and thioglycolate broth (14); Wilson Broth (15); Duncan and Strong broth (16); and Meyer and Tholozan broth (17) were prepared following the details in their respective published papers. Unless stated otherwise, all media were autoclave-sterilized for 20 min at 121 C and prereduced for about 17 h. All incubations were at 36 ± 1 C in an anaerobic chamber (Thermo-Fisher Scientific, Ottawa, ON, Canada; Model 1025) with a gas mix of 5% CO_2, 10% H_2, 85% N_2 (Linde Ltd, Edmonton, AB, Canada).

Received September 15, 2010. Accepted by AH November 10, 2010.
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Table 1. Details on *Clostridium difficile* strains used on this study

No.	Strain and source	Characteristics
1	ATCC 700057	$TcdA^-/TcdB^-$
2	Clinical isolate from The Ottawa Hospital General Campus (TOHGC)	Toxigenic
3	ATCC 43594	Serotype A; $TcdA^+/TcdB^+$
4	ATCC 43598	Serotype F; Ribotype 001; $TcdA^-/TcdB^+$
5	Strain D24 (D. Tremblay, Université Laval, Québec City, QC)	Toxigenic
6	ATCC 9689	$TcdA^+/TcdB^+$
7	(V. Loo, McGill University, Montreal, QC)	PFGE:NAP1; Ribotype 027; $TcdA^+/TcdB^+$
8	(V. Loo, McGill University, Montreal, QC)	PFGE:NAP2; $TcdA^+/TcdB^+$ Clindamycin-resistant
9	(V. Loo, McGill University, Montreal, QC)	MutIc pulsovar C Toxigenic
10	(V. Loo, McGill University, Montreal, QC)	MutIc pulsovar E Toxigenic

Enzymes

Lysozyme (20 100 IU/mg) was obtained from Pharmacia LKB (Uppsala, Sweden). Trypsin Type IV (15 900 IU/mg) was from Sigma Aldrich (Oakville, ON, Canada). The yeast extract was from Difco-BD (Mississauga, ON, Canada). The rest of the chemicals were analytical grade from Sigma Aldrich or Fisher Scientific (Ottawa, ON, Canada).

Working Stocks of Spore Suspensions

All tested strains were first grown on fastidious anaerobic agar (FAA), and one isolated colony was separately picked for each and suspended in 10 mL CB. After overnight incubation, 50 L of the suspension was separately spread onto 10 culture plates (100 mm diameter) containing CB with 5% defibrinated sheep blood (QueLab, Montreal, QC, Canada) and 1.5% agar. These plates were held in an anaerobic chamber for 7 days at 36 ± 1 C, and then for a further 15 days under the same conditions but at 23 ± 2 C for spore maturation. The plates were then harvested by scraping and resuspending the growth on each in 200 mL sterile double-distilled water (ddH_2O). The suspension was washed three times in ddH_2O by centrifugation at 10 000 *g* for 10 min at 4 C, followed by resuspension of the pellet in 2 mL ddH_2O and heating at 70 C for 10 min to inactivate any remaining vegetative cells before storage at 3 ± 1 C. These spore suspensions were used as the starting inocula for all subsequent experiments.

Screening and Selection of Liquid Culture Media

The initial screening of different liquid media was performed as follows: An isolated colony was picked from an FAA plate incubated for 48 h, transferred to a sterile 15 mL plastic tube (Sarstedt, Montreal, QC, Canada) with 5 mL CB, and incubated for 36–48 h. A 10 L inoculum was then added to a 15 mL plastic tube (Sarstedt) containing 5 mL of the liquid medium under test (Table 2). The tubes were incubated for 72 h, and the levels of sporulation in them were compared.

Scale-up of Clospore, the New Liquid Medium

Based on the results of the initial screening, one liquid medium was selected for scale-up. An isolated colony was picked from an FAA plate that had been incubated for 48 h, transferred to a sterile 15 mL plastic tube (Sarstedt) with 5 mL CB, and incubated for 48 h. From this culture, 50 L was then inoculated into 20 mL CB in a 50 mL plastic tube (Sarstedt), incubated for 24 h, and the entire volume added to a 1 L Nalgene heavy-duty polypropylene bottle (Fisher Scientific) containing 500 mL of the test medium for 72 h of incubation.

Spore Purification

The procedure of Grecz et al. (18) was used with some modifications. The spore suspensions in Clospore were centrifuged at 10 000 *g* for 10 min in a Beckman Avanti (Beckman Coulter, Mississauga, ON, Canada; Model J25) centrifuge, and the pellets were washed three times each with 35 mL ddH_2O. The final pellet was transferred to a preweighed centrifuge tube. Each 1 g or less of wet-weight of the pellet was resuspended in 10 mL 0.1 mole/L sterile sodium phosphate buffer (pH 7.0), and vortexed. The tube received another 25 mL of the same buffer but containing 20 g lysozyme and 15 g trypsin/mg wet pellet; the enzyme-containing buffer was first filtered through a membrane (0.2 m pore diameter; 25 mm diameter; Millipore, Billerica, MA). The cell suspension was then sonicated (20 kHz) for 10 min in a Branson 1200 Ultrasonic water bath (Branson Ultrasonic Corp., Danbury, CT) and incubated in a water bath at 45 C for 6 h. Every 2 h, the suspension was sonicated for 10 min. At the end of incubation, the suspension was left overnight at 4 C, centrifuged and washed three times with the same volume (35 mL) ddH_2O, resuspended in 10–30 mL ddH_2O, and stored at 4 C. The spore suspensions were examined by phase-contract microscopy and spore-staining. The purity of the spore suspensions was calculated by counting the total number of spores, and the value was divided by the number of vegetative cells and spores together; this value was multiplied by 100.

Table 2. Arrangement in a 2^4 factorial experiment to examine additions to the SPM base for production of *C. difficile* spores (ATCC 43598)

Factors tested, g/L	Treatments																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16		
Special peptone mix (SPM)	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23		
Yeast extract	—	5	—	—	—	5	5	5	—	—	—	5	5	—	5	5		
L-cysteine HCl	—	—	1	—	—	1	—	—	1	1	—	1	1	1	—	1		
Glucose	—	—	—	5	—	—	5	—	5	—	5	5	—	5	5	5		
NaCl	—	—	—	—	5	—	—	5	—	5	5	—	5	5	5	5		
No. spores/mL	3.86×10^6	7.18×10^6	3.34×10^6	2.22×10^6	4.68×10^6	6.38×10^6	2.62×10^5	3.29×10^6	2.65×10^6	4.35×10^5	2.35×10^6	3.15×10^5	3.40×10^5	1.94×10^6	1.75×10^5	2.88×10^6	2.62×10^5	1.34×10^5
± SD	3.89×10^5	1.06×10^5	1.77×10^5	4.88×10^5	4.31×10^4	5.66×10^5	6.79×10^5	3.96×10^4	2.83×10^5	4.74×10^4	3.04×10^5	0.00×10^4	5.90×10^5	9.90×10^5	2.62×10^3	1.34×10^5	1.34×10^4	1.34×10^4

Spore Counting

Sporulation was followed by examining separate glass slides prepared with 10 L each of the test spore suspension. Ten random fields were selected and photographed in a phase-contrast and epifluorescence microscope (Axioskop 2 Plus, Carl Zeiss Ltd; Toronto, ON, Canada) with Lens Plan-NEOFLUAR. The images were processed with the Software Image-Pro Plus version 5.1, (Media Cybernetic Inc., Bethesda, MD) to count the total number of spores as highly refractile particles.

Recovery Medium for Viable Spores

The agar medium to recover viable spores was BHIYT-Ly prepared as follows: 3.7% BHI, 0.5% yeast extract (Y), 0.1% L-cysteine, 0.1% sodium taurocholate (T), and 1.5% agar. This medium was autoclaved for 20 min at 121 C and cooled to about 55 C before receiving a filter-sterilized solution of lysozyme (Ly) to give a final concentration of 10 mg/L in the medium. The numbers of viable spores were recorded as CFU/mL (8). Membrane filtration (8) was used to capture the spores in all samples to be assayed, and the filters were placed on plates of BHIYT-Ly agar.

Chlorine Measurements

Concentration of free chlorine used in the disinfection experiments were measured using a Hach colorimeter (DR820 Hach, Loveland, CO).

Sensitivity to Chlorine as a Disinfectant

To determine if the enzyme treatment for spore purification could in any way influence their susceptibility to disinfectants, the spores of *C. difficile* (ATCC 43598) were divided into two portions; one was subjected to differential centrifugation and the other to enzyme treatment. The spores in the two preparations were then tested in parallel against three levels (1000, 3000, and 5000 ppm) of available chlorine using a quantitative carrier test procedure as previously described (8).

Statistical Analyses

Mathematical calculations and statistical analysis used the Excel software (Microsoft Corp., Mississauga, ON, Canada); XLSTAT v. 7.5.2, Addinsoft (New York, NY); OriginPro 8 SRO (OriginLab Corp., Northampton, MA), and GraphPad Prisma, V5, GraphPad Software, Inc. (La Jolla, CA). The exact method of statistical analysis and the types of data analyzed are detailed in individual sections below.

Results

Formulation of Clospore, a New Liquid Sporulation Medium

Of the 20 available liquid media, 13 were subjected to laboratory screening with 10 different strains of *C. difficile.* There was either no sporulation or barely detectable sporulation in most, except the one with SPM made from digests of meat, plant proteins, and yeast cells, which gave the best spore yield (10^6 viable spores/mL). While Wilson Broth

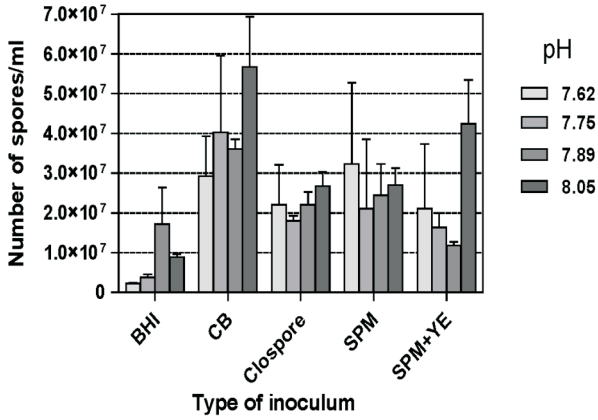


Figure 1. Inoculum selection and its interaction with the pH of Clospore. Five different media, each at four different pH values, were used to select the best combination to yield the highest sporulation levels in Clospore. The values are the average of two separate experiments. BHI = Brain Heart Infusion; CB = Columbia Broth; SPM = Special Peptone Mix; YE = Yeast Extract.

also gave good sporulation, the cultures were quite heterogeneous, with a high proportion of vegetative cells. Based on these results, SPM was selected as the protein concentrate and the base for the further formulation of Clospore. To enhance the sporulation levels obtained with SPM, a 2^4 factorial design was used to assess the role of four chemicals most frequently found in the media analyzed in this study. The components tested were yeast extract, glucose, L-cysteine, and the inorganic salt NaCl. When a formulation contained yeast extract, glucose or NaCl, the concentration was 5 g/L; when L-cysteine was used it was added as 1 g/L. The SPM base (23 g/L) was used as a control. The results of these experiments are given in Table 2.

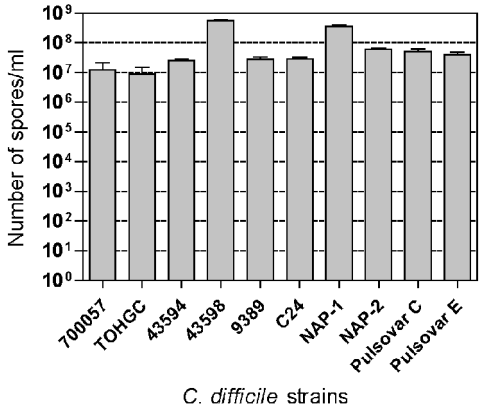


Figure 2. Sporulation levels of 10 *C. difficile* strains in optimized Clospore. One 48 h colony of *C. difficile* from FAA plate was inoculated into 5 mL of prereduced CB and incubated for 18 h. Then 50 L of the preinoculum was added to 20 mL of an 18 h prereduced CB medium and incubated for 17 h. The 20 mL cultures were inoculated in 500 mL Clospore and incubated for 3 days. The spores were enumerated as described. *See* Table 1 for abbreviations.

Sporulation levels increased by about two-fold (7.18 10^6 ± 1.06 10^5 CFU/mL), as compared with the basal SPM (3.86 10^6 ± 3.89 10^5 CFU/mL), only when yeast extract was included. A one-way analysis of variance (ANOVA) demonstrated significant differences ($P < 0.0001$) between the formulated media, whereas a Tukey's test with a confidence level of 95% showed the best combination to be SPM + yeast extract. A 5 g/L amount of glucose in the medium inhibited sporulation; this effect was confirmed using glucose levels of 0–35 mM. NaCl was also inhibitory but less so than glucose. L-cysteine showed no discernible effect (Table 2).

The salt composition was selected by including potassium salts instead of sodium salts to keep a lower Na^+/K^+ ratio as was reported for *C. perfringens* (17), as well as the inclusion of Ca^{2+} and Mg^{2+} salts and an inorganic ammonium source. The amended liquid media were then formulated as follows: SPM and yeast extract; K_2HPO_4 (2.6 g/L), KH_2PO_4 (0.3 g/L); K_2CO_3 (2.61 g/L), $MgSO_4$ (0.12 g/L); $CaCl_2{\cdot}2H_2O$ (0.08 g/L); and $(NH4)_2SO_4$ (0.3 g/L) as the salts. To test these combinations we conducted another 2^4 factorial experiment keeping the SPM (23 g/L), yeast extract (5 g/L), $MgSO_4$, $CaCl_2{\cdot}2H_2O$, and $(NH4)_2SO_4$ concentrations constant (data not shown).

A one-way ANOVA ($P < 0.0005$) and a Tukey's media comparison analysis at a confidence level of 95% gave the best medium with the composition: SPM (23 g/L); yeast extract (5 g/L); KH_2PO_4 (0.3 g/L; K_2CO_3 (2.61 g/L); $MgSO_4$ (0.12 g/L); $CaCl_2{\cdot}2H_2O$ (0.08 g/L); and $(NH4)_2SO_4$ (0.3 g/L); pH 7.8. Final sporulation levels in all of these media combinations were higher than 10^6 CFU/mL. The presence of K_2HPO_4 was inhibitory, so it was eliminated from the medium. The best formulated medium gave sporulation values in the order of 5 10^7 to 8 10^7 CFU/mL with two ATCC strains (43598 and 43594), and two clinical isolates (NAP-1 and NAP-2).

Selection of the Inoculum and pH for Clospore

After the liquid medium was formulated with significant levels of sporulation, the focus was on refining the inoculum composition and its interaction with the pH of Clospore.

Table 3. Concentrations of ingredients in the optimized liquid medium (Clospore)

Component	g/L
SPM	10
Yeast extract	10
$(NH_4)_2SO_4$	0.6
$MgSO_4{\cdot}7H_2O$	0.12
$CaCl_2{\cdot}2H_2O$	0.08
K_2CO_3	3.48
KH_2PO_4	2.6
pH	7. 9 ± 0.1

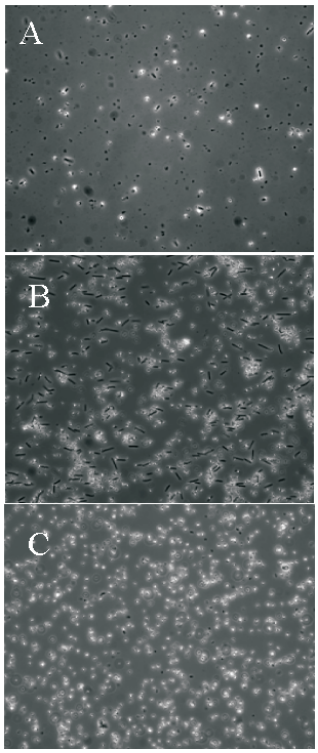


Figure 3. The appearance of spore suspensions before and after enzyme treatment as seen under phase-contrast microscopy. The bright spots are spores, and the dark spots are the vegetative cells or cell debris. Magnification X400. (A) The culture sedimented from 1 L of growth medium; (B) the pellet washed in water and concentrated; (C) the suspension after enzymatic treatment and washing in water.

Spores produced in five different media were inoculated into Clospore at four different pH levels. One 48 h colony of *C. difficile* (ATCC 43598) from an FAA plate was inoculated into 5 mL of each medium and incubated for 18 h. Then, 50 L of each inoculum variant was added to sporulation media adjusted at four different pH values, from 7.6 to 8.05, with 0.5 M KOH solution, and incubated for 72 h. Each set of conditions was run in triplicate. Spores were counted at the end of incubation in the sporulation broth and their titers were compared using a two-way ANOVA and Tukey's media comparison analysis. The two-way ANOVA showed a significant difference among inocula from different media ($P < 0.0001$), but the differences among the pH levels used were not significant ($P > 0.049$). However, a Tukey's media comparison analysis showed with 95% confidence that the best medium-pH interaction was CB, pH 8.05, followed by SPM+YE, pH 8.05 (Figure 1). Because the use of CB for inoculum production gave more stable results than the other media tested, it was selected as the medium of choice for producing the spore inoculum for Clospore.

Scale-up and Optimization of Clospore

Initially, increasing the volume of the selected liquid medium from 5 to 500 mL under the same conditions gave erratic and generally lower spore yields. This may have been due to an uneven gas exchange and an increase in the medium's pH by nearly one unit upon autoclaving.

A pH study with the scaled-up medium corroborated the previous finding that the best pH value for it was between 7.8 and 8. However, because the sporulation levels were still erratic, it was necessary to perform a further optimization of Clospore. To do this, we used a modification of the Rosenbrock's optimization method (19, 20). The seven components of the medium were used as variables to optimize ($n = 7$) with $n + 1$ combinations per trial. In total, we used eight combinations with two replicates per assay. The best combination in each trial was used as the starting point for the next. The step-size used was 50% of the initial component concentration. Only six attempts were necessary to reach nearly constant and maximal spore counts of 1.6 10^8/mL ± 2.6 10^7/mL with ATCC 43598. The final and optimal composition of the candidate liquid medium is presented in Table 3. The 10 strains used in the study were all grown in the medium, with final spore concentrations of 10^7–10^8 spores/mL (Figure 2).

Purification of the Spore Suspension and Recovery

Purification method.—Three different protocols were tried to obtain final spore suspensions as free as possible from cell debris: differential centrifugation in water, aqueous polymer

Table 4. Enzyme purification of spore crops of three different strains of *C. difficile*[a]

	Before enzyme treatment (culture volume, 1 L)			After enzyme treatment (35 mL)			
Strain	Sporulation, %	Total No. spores[b] 10^{11}	Sediment wet weight, g	Sediment wet weight, g	Total No. spores[b] 10^{10}	Spore recovery, %	Final spore purity, %[c]
ATCC 43598	70.34 ± 20.15	1.22 ± 0.46	0.610 ± 0.38	0.453 ± 0.11	4.37 ± 0.31	35.82 ± 6.81	95 ± 4.0
ATCC 700057	65.19 ± 10.24	0.53 ± 0.07	0.682 ± 0.23	0.41 ± 0.17	2.05 ± 0.22	38.68 ± 4.42	93 ± 2.0
NAP-1	75.65 ± 18.23	1.25 ± 0.38	0.583 ± 0.36	0.442 ± 0.29	5.12 ± 0.27	40.96 ± 5.34	96 ± 7.0

[a] Mean of data from nine separate experiments for ATCC 43598, five for ATCC 700057, and five for NAP-1.

[b] Total number of spores was determined by counting under a phase-contract microscope.

[c] Purity of spore suspensions was calculated by counting total number of spores and the value divided by the number of vegetative cells and spores together; this value was multiplied by 100.

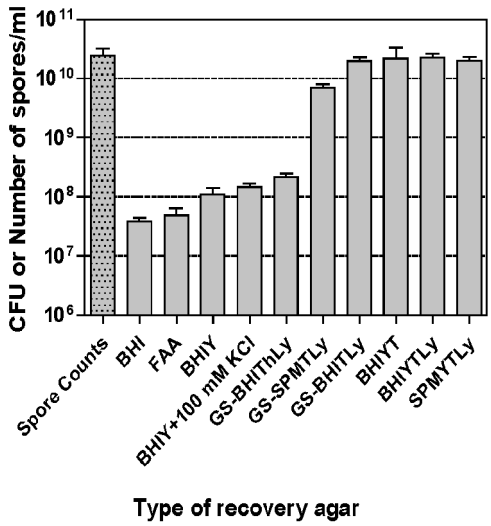
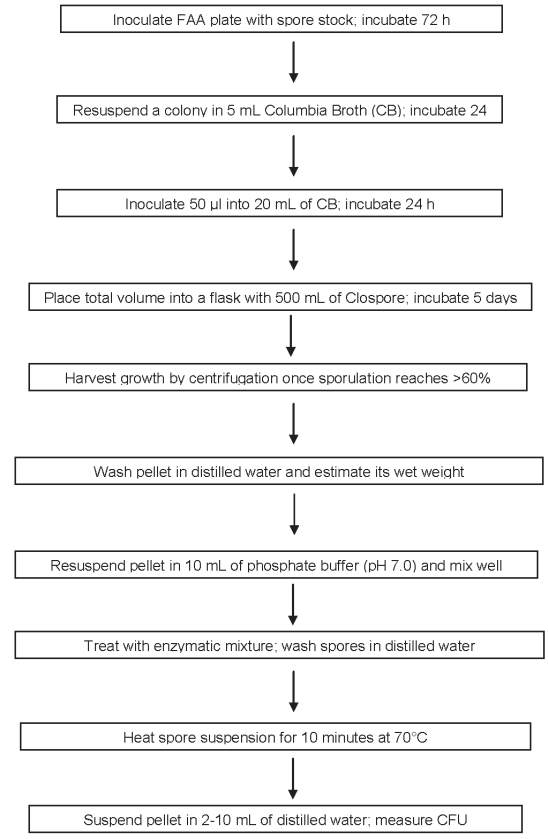


Figure 4. Comparison of various agar media for recovery of *C. difficile* spores. The agar media were modified by inclusion or substitution of 0.1% sodium taurocholate (T), sodium thioglycholate (Th), yeast extract (Y), lysozyme (Ly), and KCl. An enzyme-purified spore suspension of *C. difficile* (ATCC 43598) with 2.40 10^{10} spores/mL was serially diluted and filtered through 0.2 m membrane filters. The membranes were incubated at 36 ± 1 C on plates of each agar medium. CFU were counted at 48 h and 4 days. The values are the average of two replicates per agar medium in two separate experiments.



Flowchart 1. Main steps in the preparation of *C. difficile* spore suspensions.

two-phase systems (21), and enzymatic treatment (18). The enzymatic method, using a combination of lysozyme and trypsin, yielded suspensions with 91–99% purity (as examined microscopically; Figure 3) with the strains ATCC 43598, ATCC 700057, and NAP-1 (Table 4), as well as with all other strains.

Recovery and enumeration of viable spores.—The most common agar medium for recovering *C. difficile* from stool samples is Cycloserine-Cefoxitin-Fructose Agar with or without egg yolk (22, 23) or with taurocholate (15). Such a medium may not be needed when working with a pure culture of *C. difficile* or for enumerating survivors after a disinfection test. We have often used glucose-soluable starch-BHI (GS-BHI) agar with thioglycolate (8) as the preferred recovery medium. The comparison of BHI or FAA with GS-BHI agar, BHI supplemented agar plus taurocholate (11), and the incorporation or substitution of Ly and SPM to these media clearly demonstrated that only the inclusion of 0.1% taurocholate to the medium increased spore recovery to 95% (Figure 4). Use of BHI or SPM, as well as the addition of Ly, had no significant effect on spore recovery, and all differences in recovery observed could be attributed to taurocholate.

Flowchart 1 summarizes the main steps in the preparation of the spore suspensions.

Stability of spore suspensions.—The spores were suspended in ddH_2O or 50% (v/v) aqueous ethanol and tested for their viability when stored at 4 ± 1C, –20 ± 1C, and –80 ± 1 C. The comparison of the regression lines (Figure 5) using an *F* test did not show any significant differences in viability over a period of 14 months in either the aqueous or alcohol suspension separately for water (P = 0.75) and for 50% ethanol P = 0.98 (Figure 5). However, there was a significant difference (P = 0.046) between the liquids used. The average linear equation obtained for water was y = 8.82 – 0.04*t, and for 50% aqueous ethanol, y = 8.77 – 0.011*t. The slope for water was three-fold higher than that for 50% ethanol. These results suggest that the purified spores can be stored in water at 4 C for 2 years or longer, and in 50% aqueous ethanol for about 5 years without any significant loss in viability.

Survival of spores dried on metal disks and stored indoors.—To test the stability of the spores when they are dried and stored under ambient conditions (air temperature, 23 ± 2 C; relative humidity 52.76 ± 15.08), an enzyme-purified spore suspension of *C. difficile* (ATCC 43598) was mixed with the soil load (8). Each of several disks (1 cm diameter) of brushed stainless steel (8) received 10 L of the spore suspension and the inoculum dried for 1 h in a laminar flow cabinet. The disks were then placed on a laboratory shelf in a covered sterile glass Petri plate. Every 2 months, two disks were removed and eluted as described earlier (8), and the eluates were assayed for viable spores with BHIYT-Ly recovery agar. As shown in Figure 6, the rate of loss in viability of the spores grown in Clospore, purified

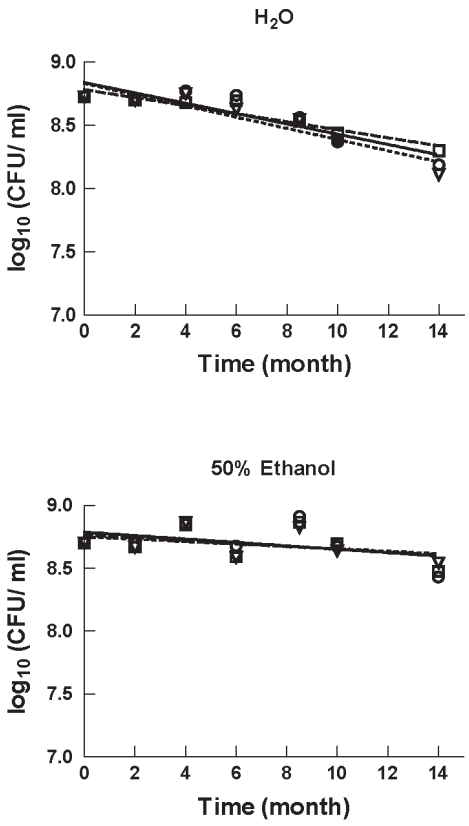


Figure 5. Viability of *C. difficile* (ATCC 43598) spores stored in ddH_2O or 50% aqueous ethanol at +4, –20, and –80 C. There was no significant difference in spore survival at the three temperatures when spores were stored in ddH_2O (P = 0.75); the same was noted for the spores stored in 50% ethanol (P = 0.98). However, there was a significant difference between spore survival in ddH_2O and 50% ethanol (P = 0.046). The linear equations are for water: y = 8.82 – 0.039*t and for 50% ethanol y = 8.77 – 0.011*t.

enzymatically and held under ambient conditions, was about 0.1 $\log_{10}$/month.

Heat-treatment of the spores.—The inherent heat-resistance of bacterial spores can be used, if necessary, to rid the suspensions of vegetative cells by heating. Such heating may also contribute to spore activation and germination. This was tested using our enzyme-purified spores by heating 1 mL aliquots of their aqueous suspensions in duplicate in 2 mL capped cryovials at different temperatures; no loss in volume was observed. At the end of 10 min of heating, the vials were immediately immersed in ice for 5 min, vortexed, and sonicated for 10 min; viable spores were then enumerated. As shown in Figure 7, there was no significant activation or inactivation up to 80 C. Above that temperature, there was a significant and rapid decrease in spore viability.

Sensitivity to disinfectants.—As can be seen in Figure 8, the resistance/susceptibility profile of the spores from the two preparations was almost identical (= 0.05, P > 0.55). These results indicate that the enzyme treatment of the spores did not compromise their resistance to chlorine as a prototypical environmental surface disinfectant.

Discussion

In spite of the mounting significance of *C. difficile* as a pathogen, obtaining high levels of good quality and viable spores of this anaerobe on a consistent basis has remained difficult. This has impeded the evaluation of environmental surface and medical device disinfectants for meaningful label claims of sporicidal activity. When our initial attempts to increase the sporulation levels in several commercial media, frequently used to grow fastidious anaerobes, were unsuccessful with seven different strains of *C. difficile*, we undertook this systematic investigation to develop a liquid medium to obtain consistently higher yields of good quality spores of standard and clinical strains of *C. difficile*.

A review of the composition of more than 20 media for culturing *C. difficile* and other anaerobic spore-formers identified several common features. While peptones came from diverse protein sources, sodium salts were present in nearly all the media (24), with the protein extract:sodium ratio as high as 100. Potassium salts and phosphate ions were frequently absent in many of these media. With this knowledge, our further work focused on developing a new liquid medium for high yields of viable spores of several strains. Most of this work, however, concentrated on one particular strain (ATCC 43598), which is among those recommended by the U.S. EPA for testing disinfectants against the spores of *C. difficile* (10).

Simple addition of yeast extract to SPM produced a two-fold increase in spore levels. Subsequent incorporation of phosphate and carbonate as potassium salts increased sporulation levels by more than one $\log_{10}$ to levels close to 10^8 CFU/mL. In contrast, the presence of glucose, NaCl, and K_2HPO_4 reduced sporulation. The composition of the medium for seeding our liquid sporulation medium also proved to be crucial, and the best results were obtained when CB was used

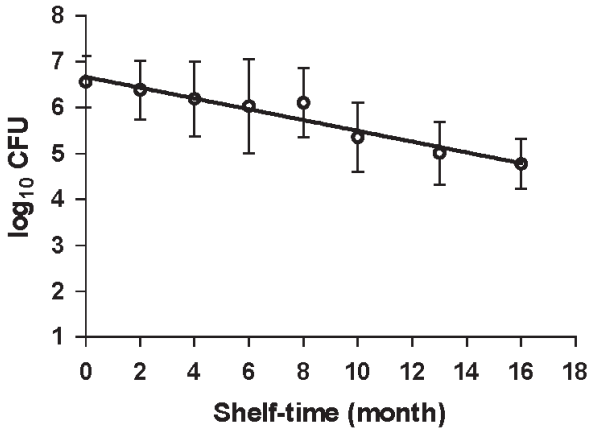


Figure 6. Survival of *C. difficile* (ATCC 43598) spores dried on stainless steel disks kept indoors. A 10 L volume of a >10^6 CFU/mL spore suspension (95% purity) in soil load was placed on metal disks and held indoors (air temperature 23 ± 2 C; RH 52.76 ± 15.08). At each sampling time, two disks were eluted and the surviving spores enumerated on BHIYT-Ly agar.

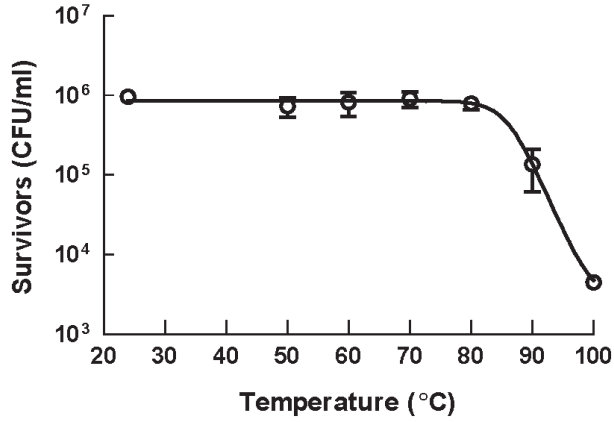


Figure 7. Effect of heating on *C. difficile* spore viability. Aliquots of 1 mL an enzyme-purified spore suspension of *C. difficile* (ATCC 43598), with about 10^6 spores/mL, were dispensed into 2 mL sterile cryovials. Capped vials were then heated in a water bath in duplicate for 10 min at different temperatures. The values are the average of two separate experiments with two different crops.

for the purpose. Our initial scale-up experiments yielded erratic results, possibly due to uneven gas exchange in the larger volumes and local variations in pH. Changes in K_2CO_3 concentration reaffirmed that the best pH was in the range of 7.8–8.0 (Figure 2).

Optimization of Clospore changed the concentrations of some components by lowering the SPM and increasing the quantities of yeast extract and changing the ratio K_2CO_3/KH_2PO_4 to give a final pH of 7.9 ± 0.1. Yeast extract appears to have one or more components that contribute to *C. difficile* sporulation.

The optimized Clospore gave spore yields of 60–85% in 3–5 days of incubation, although the spore suspension retained some vegetative cells, fore-spores, and cell debris. A quantitative and consistent spore recovery from a spore suspension is an important factor to satisfactorily evaluate the

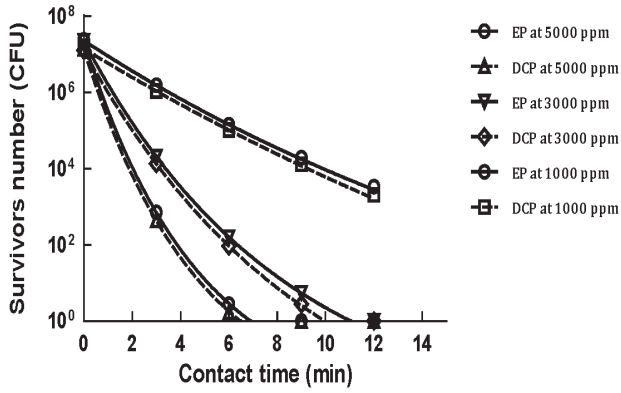


Figure 8. Comparative sodium hypochlorite (chlorine bleach) resistance of *C. difficile* (ATCC 43598) spores purified by enzymatic treatment (EP) and differential centrifugation (DCP). The free chlorine concentrations used were 1000, 3000, and 5000 ppm.

sporulation levels in pure cultures as well as for use in disinfection experiments. We found that sample concentration by differential centrifugation gave unacceptably low spore yields. Purification using the aqueous polymer two-phase system with polyethylene glycol 4000 (21) was effective, but too laborious. By contrast, purification with lytic enzymes and intermittent sonication gave us purity levels of 95–99%.

C. difficile spore germination and recovery from stools or pure cultures has been a recurrent topic in the scientific literature due to the difficulty to recover all the dormant spores in a sample. This difficulty appears to be related to the necessary presence of germinant and co-germinant factors in the recovery media. Our results clearly demonstrate that inclusion of only 0.1% sodium taurocholate to the media increases spore recovery by >90%, which is in agreement with other reports (25–27). The inclusion of yeast extract in the medium seems necessary for almost the total recovery of dormant spores. Incorporation of KCl into the media did not enhance spore recovery in our study. The level of germination generated by KCl is insufficient to counter the effect of taurocholate (11), and prolonged contact with KCl may inhibit spore outgrowth. Stability to desiccation and heat-resistance confirms that the spores do not suffer any significant change in resistance as a result of the purification process reported here. Nor does it alter the disinfectant resistance of the spores as shown with testing against various levels of free chlorine (Figure 8). Further work is needed to demonstrate that this observation applies to other sporicidal chemicals as well.

It is noteworthy here that neither Clospore nor the semi-solid spore recovery medium BHIYT-Ly contain any blood, thus eliminating potential problems of cost, availability, and variations in quality of such an additive. The spore suspensions from Clospore can be stored in ddH_2O or 50% aqueous ethanol at 4 ± 1 C for at least 14 months without any significant drop in viability (Figure 6), although for long-term conservation, suspension in 50% aqueous ethanol is better.

In summary, the newly developed liquid medium along with the purification procedure described can routinely produce *C. difficile* spores with viability titers of ≥10^9 CFU/mL and a purity level of >95. The procedure has been successfully applied to several standard and clinical strains. Our laboratory has been routinely using such spores to study their biology as well as to develop safer and better disinfectants for environmental decontamination.

Acknowledgments

We thank Michelle Alfa (St. Boniface Hospital, Winnipeg, MB, Canada); Frank Chan (Children's Hospital of Eastern Ontario, Ottawa, ON); Marc Desjardin (The Ottawa Hospital, Ottawa, ON); Vivian Loo (McGill University, Montreal, QC); and Denis Tremblay (Université Laval, Québec City, QC) for supplying *C. difficile* strains.

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