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Enrichment, Isolation, and Cultural Characteristics of Marine Strains of *Clostridium botulinum* Type C

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Terrestrial strains of *Clostridium botulinum* type C, designated 468 and 571, were used to screen various media for growth and sporulation at 30 C. Of the various formulations tested, only egg meat medium fortified with 1% additions of yeast extract, ammonium sulfate, and glucose (FEM medium) gave good growth and satisfactory sporulation. FEM medium was used to recover four marine type C isolates from inshore sediments collected along the Atlantic, the Gulf of Mexico, and the Pacific coasts of the United States. The isolation techniques involved repeated transfer of cultures showing type C toxin in FEM medium and purification by a deep tube method. The medium used for purification was beef infusion-agar supplemented with 0.14% sodium bicarbonate and 0.1% L-cysteine hydrochloride. L-Cysteine was adopted in preference to sodium thioglycolate, because some lots of the latter were definitely inhibitory for growth. The addition of bicarbonate markedly increased viable spore counts of both the marine and terrestrial strains. Various cultural and biochemical characteristics of the marine and the terrestrial strains were compared. With the exception of some variations in their fermentation patterns, both groups showed similar characteristics. Of 23 fermentable compounds tested, the terrestrial strains attacked only glucose and mannose. The marine strains fermented glucose, mannose, galactose, and ribose actively; dextrin, inositol, maltose, and melibiose were weakly fermented.

Clostridium botulinum type C is usually associated with animal botulism. Large outbreaks among various aquatic wild birds, fur-bearing animals, sheep, horses, and cattle are known (16). In contrast, outbreaks involving humans are extremely rare. A human outbreak occurred in California in 1950 involving four persons with one death. The food responsible is unknown, but type C toxin reportedly was present in the stomach contents of the deceased (17). Prévot et al. (20) described a human type C outbreak in France, and Matveev et al. (15) cited the occurrence of two cases in Russia.

Indifference to type C botulism by food microbiologists is, of course, due to the rarity of foodborne outbreaks in humans. As a result, little information is available concerning the possible low-temperature growth of this organism; yet, there are seemingly valid reasons to suggest that strains from marine environments might possess such ability. All known strains of *C. botulinum* type C are reported to be nonproteolytic, so the organism is similar in that regard to those botulinum types capable of low-temperature growth. Only type E and nonproteolytic types B and F are known to possess psychrophilic characteristics (8, 9, 22). It is also known from extensive ecological surveys that type C is present in marine environments with these types (3, 7, 25-27). Therefore, it is conceivable that marine type C strains might also exhibit low-temperature growth ability.

This paper describes the isolation of four type C strains from marine sediments and certain cultural and biochemical characteristics of these strains. Studies concerning the minimal temperature for growth and other growth characteristics of the marine strains will be presented later.

MATERIALS AND METHODS

Origin of terrestrial strains. Strains 468 and 571 were obtained from the National Canners Association Laboratory at Berkeley, Calif. They were originally isolated by A. R. Prévot from animals reportedly dead from type C botulism.

Sources of marine sediments. Various marine sedi-

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ments containing *C. botulinum* type C were acquired from B. Q. Ward and M. W. Eklund. In general, the sediments consisted either of sand, a mixture of sand and fine gravel, or mud. Sediment numbered 613 was collected at New Dorp Lane, Staten Island, N.Y., sediment 616 at Yacht Club, Edgewood, Md., and sediment 345 at Lagund Madre, Corpus Christi, Tex. (25, 26). Sediment 10 was collected between Lake Washington and Puget Sound at a depth of 6 to 9 meters (Eklund, *personal communication*).

Sporulation media. Before attempting to recover type C cultures from various marine sediments, some exploratory work was conducted to learn more about the sporulation requirements of the terrestrial strains 468 and 571. These strains were selected because they were known to be extremely difficult to sporulate. It was considered that the probability of recovering and purifying marine type C strains from the sediments would be considerably improved if a suitable sporulation medium was used. It was hoped that a medium permitting sporulation of the terrestrial strains would also sporulate marine strains.

The composition of the noncommerical media is given below on the basis of grams per liter. Beef infusion (BI) broth consisted of 1,000 ml of filtrate of beef infusion (454 g of lean beef/liter), 5 g of NaCl, and 10 g of peptone (Difco). The medium was adjusted to *p*H 7.4 before sterilization. Liver infusion (LI) broth consisted of 1,000 ml of liver infusion (454 g of liver/liter), 10 g of tryptone (Difco), 1 g of soluble starch, and 1 g of K_2HPO_4; before sterilization, the medium was adjusted to *p*H 7.2. Cardella's medium was formulated according to his recommendations (2). Jensen's medium (13) was slightly modified; it consisted of 30 g of Lactalysate (BBL), 20 g of yeast extract (Difco), 10 g of glucose, and 3.5 g of sodium citrate. Eklund's cooked meat formulation has been described (8). Trypticase-peptone-glucose (TPG) broth was prepared according to Schmidt, Nank, and Lechowich (23). A medium resembling TPG, but incorporating additions of yeast extract (Difco) and ammonium sulfate (TPAY-GT), was prepared according to Roberts (21). The commercially prepared media were rehydrated according to each suppliers recommendations. Fortified egg meat medium, hereafter abbreviated FEM, comprised Egg Meat Medium (Difco) with 1% additions each of yeast extract (Difco), ammonium sulfate, and glucose. All of the media described above were dispensed in 20-ml quantities in screw-cap tubes (20 by 150 mm) and autoclaved at 121 C for 15 min. Sodium thioglycolate (20%) sterilized at 121 C for 10 min was added aseptically to each medium not containing discrete meat particles; the final concentration equaled 0.1%. All of the media tested were in the *p*H range of 7.0 to 7.2 after autoclaving. Actively growing cultures of strains 468 and 571 in Egg Meat Medium (Difco) were used to inoculate the various media. Growth and sporulation at 30 C were assessed daily by means of phase-contrast microscopy.

Isolation of marine strains. Duplicate sets of tubes of FEM medium, with three or five tubes per set, were inoculated with about 1 g of sediment per tube. One set was preheated at 71 C for 15 min; the other set was not preheated. The preheating treatment used was arbitrarily selected, because it showed no destructive effect for spores of the terrestrial strains. Assays for type C toxin were made after 3 days of incubation at 30 C.

Undiluted enrichment cultures invariably showed nonspecific reactions when assayed for botulinum toxin, as marked by convulsion and death of the mice within 5 to 10 min after intraperitoneal injection. Such deaths were avoided by diluting the cultures 10-fold; gelatin-phosphate buffer was used as the diluent (6). The presence of type C toxin was confirmed by the toxin-antitoxin neutralization test with the use of white mice. Botulinum antitoxins including monovalent sera for types A through F (Communicable Disease Center, Atlanta, Ga.) were employed. Cultures showing type C toxin were titrated to determine the minimal lethal dose (MLD) levels of toxin present. Those cultures showing the highest titers were transferred and subjected to repeated enrichment in FEM medium.

For purification, a deep-tube method was used. Tubes about 12.5 cm long were prepared from glass tubing (outer diameter, 12 mm). The tubes were loosely rubber-stoppered at each end and autoclaved at 121 C for 15 min. A preliminary viable count was made on each enrichment culture exhibiting a comparatively high toxin titer. The count was based on preheating for 15 min at 71 C. By use of this count, a series of 40 to 50 stoppered tubes were inoculated with a preheated dilution to give one or two colonies per tube. The recovery medium consisted of BI containing 1.5% Difco Noble Agar (BIA). Filter-sterilized sodium bicarbonate and L-cysteine hydrochloride were added aseptically to give respective concentrations of 0.14 and 0.10%. L-Cysteine hydrochloride was prepared as a 20% solution and sterilized (121 C for 10 min); both heat-treated and membrane-filtered solutions gave comparable counts. Sterile 1 N sodium hydroxide was added to compensate for the acidity of the L-cysteine hydrochloride. Each inoculated tube was poured with about 6 ml of the medium. Colonies developing after 24 to 48 hr at 30 C were picked from the agar and inoculated into FEM medium. The tubes were incubated for about 72 hr and assayed for type C toxin. To insure the purity of each type C isolate, a second purification step was employed as described above. Type-specific antitoxin and microscopic examination were used to confirm the purity of each marine strain.

Spore production. FEM medium was used for sporulation of the marine and the terrestrial strains. The medium was prepared in 135-ml quantities in screw-cap bottles (200-ml capacity) and sterilized by autoclaving. Sterile L-cysteine was incorporated into the medium just prior to inoculation to give a final concentration of 0.1%; the medium was adjusted to *p*H 7.0. Four or six bottles were inoculated in preparing each spore suspension. The inoculum consisted of 1 ml of a recently sporulated culture per bottle. Incubation was at 30 C. Sporulation was followed by phase-contrast microscopy. At peak sporulation (2 or 3 days of incubation, depending upon the strain), the spores were recovered. After mixing by inversion, the

liquid was decanted through a loosely packed, sterilized cheese-cloth filter in an 8.9-cm Büchner funnel, the spores being collected in a sterilized screw-cap flask (1-liter Erlenmeyer). With aseptic techniques used throughout, the suspension was poured into 250-ml screw-cap centrifuge bottles. The spores were sedimented at 9,500 rev/min for 30 min with a Servall model SS-4 centrifuge. After decanting, each spore sediment was resuspended in distilled water to approximately one-tenth its original volume. Finally, the suspension was pipetted into a bottle containing glass beads and stored at about 1 C.

Standardization of spore suspensions. Each suspension was standardized with BIA. The medium was prepared in 150-ml quantities in 8-oz (ca. 230-ml) prescription bottles. Immediately before use, sterile L-cysteine hydrochloride and sodium bicarbonate were added. A deep-tube counting method with five replicates per dilution was used. Dilutions were made to give 10 to 20 colonies/tube (16 by 125 mm screw-cap). After inoculation, each tube was poured with about 13 ml of BIA. When the medium had solidified, a cover layer of the same medium was poured (about 6 to 8 mm in thickness), and the tubes were incubated at 30 C. Colony counts were usually made after 24 to 30 hr, since longer incubation times failed to give higher counts and occasionally gas production caused splitting of the agar.

Cultural and biochemical tests. Gelatin hydrolysis was determined with BI broth containing 12% gelatin (Difco). BI broth with small strips of coagulated egg white and FEM were used to determine the ability of type C to digest more complex proteins. Catalase, indole, and nitrite tests were conducted according to Collins (4). Lecithinase and lipase activities were assessed by use of egg yolk medium (14) as modified by Dowell and Hawkins (5). Petri dishes containing the medium were streaked and incubated in a Brewer jar charged with a mixture of 90% hydrogen and 10% carbon dioxide gases. After incubation for 5 days at 30 C, isolated colonies were examined for zones of precipitation (lecithinase) and so-called pearly layers (lipase). The base medium for the fermentation tests was Difco Thioglycollate Medium without Glucose or Indicator (5). Noble Agar was added to the base formulation at a 1.5% concentration; Andrade's indicator (Harleco) also was added prior to sterilization of the medium at 121 C for 15 min. Each fermentable compound selected was prepared as a 10% stock solution and membrane-sterilized. Screw-cap tubes (16 by 125 mm) were inoculated with 0.1 ml of suspension or 10^6 viable spores per tube. Each tube was poured with about 13 ml of medium containing the requisite fermentation compound at a final concentration of 0.5%. The tubes were incubated at 30 C and examined for a change in indicator and gas production at various times up to 1 month.

RESULTS

Among the media screened for sporulation of the two terrestrial strains (Table 1), only those formulations containing discrete meat particles permitted any noticeable sporulation. Use of

TABLE 1. *Various media screened for growth and sporulation of strains 468 and 571 with incubation at 30 C*

Medium[a]	Growth	Sporulation[b]
Beef infusion	Good	None
Liver infusion	Good	None
Cardella's medium	Excellent	None
Jensen's medium	Excellent	None
Eklund's medium	Excellent	2%
Cooked Meat (Difco)	Excellent	5%
Cooked Meat (Oxoid)	Excellent	2%
RCM (Oxoid)	Poor	None
TPG	Poor	None
TPAY-GT	Good	None
Egg Meat (Difco)	Excellent	5%
FEM	Excellent	80%

[a] The composition of the noncommercial media is given in Materials and Methods.

[b] Estimated microscopically by phase-contrast microscopy. Sporulation was followed daily during 1 week of incubation. Both strains gave similar results.

TABLE 2. *Effect of glucose, yeast extract, and ammonium sulfate on the sporulation of strain 468 in FEM*

Additive[a]	Concn added (%)	Refractile spores[b]/ml
Glucose	0.1	7×10^6
	0.5	63×10^6
	1.0	69×10^6
	1.5	40×10^6
Yeast extract	0.5	67×10^6
	1.0	65×10^6
	1.5	60×10^6
$(NH_4)_2SO_4$	0.5	27×10^6
	1.0	50×10^6
	2.0	30×10^6

[a] When one of the additives was varied, the other two additives were held at a 1% level.

[b] Refractile spore counts were made with a Petroff-Hausser chamber after 3 days of incubation at 30 C.

FEM medium permitted excellent growth and approximately 80% sporulation. Peak sporulation in FEM was seen after about 72 hr as judged by refractile spore counts made with a Petroff-Hausser chamber. Longer incubation produced significant germination and a loss in spore yield.

Strain 468 was used to determine the effect of various concentrations of yeast extract, ammonium sulfate, and glucose on sporulation (Table 2). Yeast extract, within the levels used, had little effect on spore yield, although its deletion practically eliminated sporulation. Ammonium sulfate

at a 1% concentration gave the highest yield of the three levels tested. Glucose present at 0.5 and 1.0% levels gave similar yields, but higher or lower amounts reduced sporulation. Considering these results, it was decided that the medium would be used as it was originally formulated.

FEM was employed to recover type C cultures from the four marine sediments as described.

TABLE 3. *Effect of preheating on the recovery of mixed type C cultures from marine sediments in FEM with incubation at 30 C*

Sediment	Fraction of samples showing toxin	
	Unheated	Preheated[a]
613	2/5	4/5
616	0/8	1/8
345	4/8	7/8
10	2/5[b]	4/5
Totals	8/26	16/26

[a] At 71 C for 15 min.
[b] Both samples contained a combination of types C and E botulinum toxins.

TABLE 4. *Effect of serial transfers of mixed enrichment cultures on the increase in type C toxin titers*

Sediment	Transfer[a]	MLD of toxin/ml	
		Unheated	Preheated[b]
613	1		10,000
	2	100,000	20,000
	3	500,000	500,000[c]
616	1		50,000
	2	200,000	1,000,000[c]
	3	500,000	1,000,000
345	1		10,000
	2	2,000	50,000
	3	2,000	50,000
	4	50,000	50,000
	5	50,000	50,000[c]
10	1		1,000
	2		5,000
	3		20,000
	4		50,000
	5		50,000
	6		200,000[c,d]

[a] Initial enrichment is designated one.
[b] At 71 C for 15 min with the exception noted below.
[c] Denotes transfers used to obtain pure cultures.
[d] Based on a preheating at 82 C.

TABLE 5. *Yield and viability of spores of C. botulinum type C produced in FEM*

Strain	Refractile count/ml		Viability expressed as % of refractile count	
	Culture	Stock	Unheated	Preheated[a]
468	65×10^6	120×10^6	88	71
571	90×10^6	200×10^6	95	100
6812	85×10^6	510×10^6	26	41
6813	168×10^6	780×10^6	100	86
6814	—[b]	840×10^6	83	86
6816	70×10^6	330×10^6	79	55

[a] At 71 C for 15 min.
[b] Result not determined.

More enrichments containing type C toxin were obtained with a moderate preheating than without preheating (Table 3). Preheating appeared to eliminate all of the microorganisms except the sporeforming bacteria.

Repeated efforts to isolate type C directly from the initial enrichment cultures showing type C toxin were unsuccessful, so successive transfers in FEM were tried to increase the number of type C cells or spores present to facilitate isolations. With the exception of sediment 10, transfers were made both with and without preheating. As shown in Table 4, it was possible to increase the levels of type C toxin by repeated transfer and hence presumably the number of type C cells both with and without preheating.

Four marine type C strains were isolated in pure culture by use of the successive transfer technique and a deep-tube purification method. Strain 6812 was isolated from sediment 613, strain 6813 from sediment 616, strain 6814 from sediment 345, and 6816 from sediment 10. Three strains (6812, 6813, and 6814) were isolated with a 71 C preheating treatment. Strain 6816 was isolated with preheating at 82 C; repeated attempts to isolate type C from the concomitant sporeformers by use of the lower preheating temperature were unsuccessful.

Spore suspensions of the marine and terrestrial strains were produced in FEM medium. The yield and the viability of spores of each strain are summarized in Table 5. All strains showed good yields and viabilities. BIA gave good recoveries based on comparisons of refractile to viable-count data.

The effect of Na_2HPO_4 on viable counts should be mentioned. The BIA used in our laboratory normally contains 0.5% Na_2HPO_4. In most cases, the addition of phosphate seemed to reduce

colony size and to produce diffused colonies. Although there were some differences between strains, in general, added phosphate reduced the viable counts. Since phosphate showed no beneficial effect, it was omitted from the medium.

Table 6 shows the effect of various preheating temperatures on the viable counts of spores of the six strains. At 71 C and lower, there was no major reduction in counts. In fact, spores of 6812 showed somewhat higher counts up to 77 C than without preheating, suggesting a possible heat-activation effect.

Earlier it was stated that the reducing compound sodium thioglycolate was used in the initial sporulation studies on 468 and 571. With the production lot of thioglycolate used in these studies, no growth-inhibitory effects were observed. However, a second lot of thioglycolate from a newly opened bottle caused marked reduction in viable counts. The counts with this lot of thioglycolate and the comparative counts with L-cysteine hydrochloride are shown in Table 7. With the exception of data for spores of strain 6816, the presence of thioglycolate at a concentration of 0.1% gave very low counts. Lower concentrations permitted increased counts in most instances, but not as high as those obtained with L-cysteine. Two additional lots of thioglycolate from newly opened bottles produced similar inhibitory effects. On the other hand, various lots of L-cysteine tested failed to show any inhibitory response. The optimal concentration of L-cysteine was considered to be 0.1%.

The incorporation of 0.14% sodium bicarbonate into the BIA medium caused a pronounced increase in viable counts (Table 8). The addition of bicarbonate permitted maximal counts at 30 C after 24 to 30 hr, but counts at 24 hr without bicarbonate were appreciably lower. In two cases,

TABLE 6. *Effect of preheating on the viability of spores of the terrestrial and marine type C strains*

Preheat temp (C)[a]	Viable count/ml[b]					
	Nonmarine		Marine strains			
	468	571	6812	6813	6814	6816
None	105×10^6	190×10^6	130×10^6	800×10^6	700×10^6	140×10^6
60	88×10^6	190×10^6	200×10^6	780×10^6	780×10^6	95×10^6
66	86×10^6	210×10^6	170×10^6	760×10^6	760×10^6	110×10^6
71	85×10^6	200×10^6	210×10^6	680×10^6	720×10^6	100×10^6
77	90×10^6	180×10^6	260×10^6	580×10^6	700×10^6	60×10^6
82	56×10^6	180×10^6	120×10^6	160×10^6	160×10^6	35×10^6
88	32×10^6	34×10^6	50×10^6	2×10^6	2×10^6	13×10^6

[a] Preheat time = 15 min at temperature shown.

[b] Deep-tube colony counts based on recovery in BIA containing 0.14% $NaHCO_3$ and 0.1% L-cysteine hydrochloride. Each count represents the average of a five-tube replicate set at 30 C after 24 to 30 hr of incubation.

TABLE 7. *Effect of one production lot of sodium thioglycolate and one lot of L-cysteine hydrochloride on the viable counts of spores of type C*[a]

Compound	Concn (%)	Unheated viable count/ml					
		468	571	6812	6813	6814	6816
Thioglycolate	0	28×10^6	90×10^6	38×10^6	66×10^6	180×10^6	25×10^6
	0.02	75×10^6	90×10^6	100×10^6	720×10^6	460×10^6	139×10^6
	0.05	$<10 \times 10^6$	$<10 \times 10^6$	120×10^6	520×10^6	380×10^6	126×10^6
	0.10	$<10 \times 10^6$	$<10 \times 10^6$	20×10^6	64×10^6	160×10^6	122×10^6
L-Cysteine	0.02	91×10^6	42×10^6	150×10^6	740×10^6	620×10^6	82×10^6
	0.05	97×10^6	100×10^6	140×10^6	870×10^6	660×10^6	104×10^6
	0.10	110×10^6	180×10^6	150×10^6	760×10^6	880×10^6	135×10^6
	0.15	100×10^6	150×10^6	100×10^6	870×10^6	870×10^6	139×10^6

[a] See footnote *b*, Table 6. Figures represent the maximal counts obtained during 1 month of incubation.

TABLE 8. *Effect of sodium bicarbonate on the viable counts of spores of C. botulinum type C*[a]

Suspension[b]	Viable count/ml			
	No $NaHCO_3$		$NaHCO_3$ added	
	24 hr	30 days	24 hr	30 days
468	60×10^5	50×10^5	103×10^5	90×10^5
571	3×10^5	8×10^5	97×10^5	86×10^5
6812	1×10^5	34×10^5	93×10^5	81×10^5
6813	44×10^5	81×10^5	102×10^5	89×10^5
6814	22×10^5	103×10^5	115×10^5	105×10^5
6816	18×10^5	25×10^5	103×10^5	73×10^5

[a] See footnote *b*, Table 6.

[b] Suspensions were prepared to contain 10^7 spores/ml based on preheating at 71 C for 15 min; viable counts shown are based on this preheating.

TABLE 9. *Comparative characteristics of marine and terrestrial strains of C. botulinum type C*

Characteristic	Terrestrial		Marine			
	468	571	6812	6813	6814	6816
Toxicity	+	+	+	+	+	+
Digestion						
Gelatin	+	+	+	+	+	+
Coagulated egg white	−	−	−	−	−	−
Meat	−	−	−	−	−	−
Catalase	−	−	−	−	−	−
Motility	−	−	−	−	−	−
Indole	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−
Lecithinase	+	+	+	+	+	+
Lipase	+	+	+	+	+	+
Fermentable compound[a]						
Dextrin	−	−	(AG)	(AG)	(AG)	(AG)
Galactose	−	−	AG	AG	AG	AG
Galactose	−	−	AG	AG	AG	AG
Glucose	AG	AG	AG	AG	AG	AG
Inositol	−	−	(AG)	(AG)	(AG)	(AG)
Maltose	−	−	(AG)	(AG)	(AG)	(AG)
Mannose	AG	AG	AG	AG	AG	AG
Melibiose	−	−	(AG)	(AG)	(AG)	(AG)
Ribose	−	−	AG	AG	AG	AG

[a] AG = acid and gas within 48 hr; − means sugar not fermented; reaction in parentheses means weak and delayed fermentation. Adonitol, arabinose, dulcitol, fructose, glycerol, inulin, lactose, mannitol, raffinose, salcin, sorbitol, starch, sucrose, trehalose, and xylose were not fermented during 1 month of incubation at 30 C.

longer incubation seemed to offset partially the stimulatory effect of bicarbonate.

Table 9 shows the comparative cultural and biochemical characteristics of the marine and terrestrial strains. Only moderate variations in their fermentation patterns distinguished the two groups. Microscopically, the marine and the terrestrial strains were very similar. Vegetative cells grown in FEM medium at 20 hr ranged from 1.0 to 1.2 μm in width and 4 to 7 μm in length. Spores were cylindrical-shaped, ranging from 0.6 to 1.0 by 1.2 to 1.4 μm. Motility was absent in hanging-drop preparations. Both the marine and the terrestrial strains showed positive lecithinase and lipase reactions. The marine isolates gave zones of precipitation measuring about 3 mm from the edge of the colonies. On the other hand, the terrestrial strains produced somewhat narrower

zones. The strong lecithinase reactions observed were attributed to the longer incubation time (5 days) at 30 C than is commonly used.

DISCUSSION

The wide geographical distribution of *C. botulinum* type C in marine environments emphasizes the need for a comprehensive study to determine its possible capacity to grow at low temperatures. Type C growth within the refrigeration temperature zone would have considerable relevance to the public health safety of certain perishable foods. Of particular concern is the conceivable application of low doses of ionizing radiation to extend the refrigerated storage life of various seafoods. This possibility is being explored in various laboratories under grants from the U.S. Atomic Energy Commission. If type C can grow at low temperatures, it might involve a health hazard situation comparable to that discussed for *C. botulinum* type E (22, 23).

The success in isolating *C. botulinum* type C from sediments representing various coastal areas of the United States is attributed to the use of FEM medium for enrichment and for serial transfers. Preheating at 71 C for 15 min also favored the recovery of more initial enrichment cultures showing type C toxin than corresponding unheated cultures. This preheating treatment is also advised for each serial transfer step in attempted isolations. In some cases, when isolations are difficult, somewhat higher preheating treatments may be used advantageously.

Growth inhibition of some species of clostridia by the reducing compound sodium thioglycolate is well known (10, 11, 24), so the fact that some production lots of thioglycolate are quite inhibitory for type C growth is not particularly surprising. Our results only emphasize the caution that must be taken when using thioglycolate for growing anaerobes. Where the incorporation of a supplemental reducing system is desired, L-cysteine hydrochloride is advised for growing *C. botulinum* type C.

The stimulatory effect of sodium bicarbonate, or presumably carbon dioxide, on spore germination and the growth of various clostridia has frequently been cited, since the first reports of Olsen and Scott (18) and Wynne and Foster (28). The beneficial effect of bicarbonate for type C growth appears to be rather universal for both marine and terrestrial strains. The addition of bicarbonate to the medium for viable counts is definitely recommended.

Considering the cultural and biochemical characteristics of the marine and terrestrial strains reported here, both groups appear to be closely related. Only moderate differences in their fermentation patterns appear to separate the marine from the terrestrial strains, but the significance of such differences seems questionable, since widely variable patterns have been reported in the literature (1, 12, 19).

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