Selective Medium for Isolation of *Clostridium butyricum* from Human Feces

M. R. POPOFF

Service des Anaérobies, Institut Pasteur, 75724 Paris Cedex 15, France

Received 8 February 1984/Accepted 4 June 1984

A selective medium, *Clostridium butyricum* isolation medium (BIM), is described for the isolation of *C. butyricum* from human feces. The BIM is a synthetic minimal medium and contains trimethoprim (16 µg/ml), D-cycloserine (10 µg/ml), and polymyxin B sulfate (20 µg/ml) as selective inhibitory agents. Qualitative tests indicated that *C. butyricum* and other butyric acid-producing clostridia grew on BIM, *Clostridium sphenoides* and *Bacillus cereus* produced small colonies, and other clostridia and other obligate anaerobic or facultatively anaerobic bacteria were inhibited. Quantitative recovery of *C. butyricum* from cultures or seeded fecal samples was comparable with BIM and with complex medium, but the quantitative recovery of the other butyric acid-producing clostridia tested (*C. beijerinckii*, *C. acetobutylicum*) was lower with BIM than with complex medium. The BIM should aid the rapid isolation of *C. butyricum* from fecal samples and should be useful for bacteriological investigation of neonatal necrotizing enterocolitis.

Clostridium butyricum has been incriminated in some cases of neonatal necrotizing enterocolitis (8, 10, 15, 19). This organism was present at high levels in the feces of these infants. However, *C. butyricum* was also recovered from stools of normal newborns. Neonatal necrotizing enterocolitis has multifactorial origin, and bacteria, especially clostridia, seem to play a central role in the initiation of intestinal lesions.

Qualitative and quantitative bacteriological examinations of fecal specimens are important methods for neonatal necrotizing enterocolitis etiological investigation. *C. butyricum* isolation is usually done by employing nonselective media designed for the recovery of clostridia. In a previous investigation of experimental infection in guinea pigs with *C. butyricum*, I recognized the need to use a selective medium for quantitative evaluation of this organism in intestinal content (manuscript in preparation). On the basis of the minimal medium for *C. butyricum* described by Cummins and Johnson (2), I have developed a selective medium for the isolation of *C. butyricum* from feces.

MATERIALS AND METHODS

Bacteria. I used 255 strains of bacteria: 34 strains of *C. butyricum*, 10 strains of *Clostridium beijerinckii*, 126 strains (39 species) of other clostridia, 24 strains (14 species) of non-spore-forming anaerobes, and 55 strains (52 species) of various facultatively anaerobic bacteria. *C. butyricum* and *C. beijerinckii* strains were identified by the method previously described (13). For each species of *Clostridium*, the type strain listed in the Approved Lists (17) and obtained from the American Type Culture Collection was included. The other strains of clostridia and of non-spore-forming anaerobes were identified in our laboratory according to the manual of Holdeman et al. (7). The facultatively anaerobic bacteria were obtained from various reference laboratories at the Pasteur Institute.

Isolation medium for *C. butyricum*. The basal medium used by Cummins and Johnson (2) for determining the nutritional requirements of *C. butyricum* was employed with some

modifications of the buffer solution. Several antimicrobial agents in various concentrations were tested. The better results, allowing growth of *C. butyricum* and inhibition of other fecal bacteria, were obtained with trimethoprim, D-cycloserine, and polymyxin B sulfate (all from Sigma Chemical Co.). The concentration of each antimicrobial agent was defined in a preliminary experiment (see Table 1). The *C. butyricum* isolation medium (BIM) had the following composition: concentrated mineral salts solution, 100 ml; phosphate solution, 100 ml; iron sulfate (0.1% aqueous solution), 10 ml; biotin (0.002% aqueous solution), 0.25 ml; cysteine-hydrochloride, 0.5 g; glucose, 10 g; agar, 15 g; trimethoprim, 16 mg; cycloserine, 10 mg; polymyxin B sulfate, 20 mg; distilled water, 790 ml. The pH was adjusted to pH 7.7, and the medium was autoclaved at 110°C for 20 min.

The concentrated mineral salts solution contained: NaCl, 9 g; CaCl₂, 200 mg; MgCl₂ · 6H₂O, 200 mg; MnCl₂ · 4H₂O, 100 mg; CoCl₂ · 6H₂O, 10 mg; distilled water, 1,000 ml. The phosphate solution contained: KH₂PO₄, 7 g; K₂HPO₄, 7 g; distilled water, 100 ml. The antimicrobial agents were added to the medium after it was autoclaved and cooled to 50°C in a water bath. The stock solutions of D-cycloserine (0.25%) and polymyxin B sulfate (0.25%) were made in distilled water. The trimethoprim stock solution (0.1%) was made at 55°C distilled water acidified with 0.05 N HCl as described by Dezfulian et al. (3). Sixteen milliliters of trimethoprim, 4 ml of the cycloserine, and 8 ml of the polymyxin B sulfate solution were added to 1 liter of medium.

Fluid basal medium (FBM), prepared as BIM without agar, glucose, and antimicrobial agents, was used for fecal specimen dilutions. In some experiments, we used a basal medium (BM) that was BIM without antimicrobial agents.

Evaluation of BIM. The growth of various bacteria was studied on BIM and on a complex meat-yeast extract medium (VL) (1). Overnight cultures in Trypticase (BBL Microbiology Systems)-yeast extract-glucose broth (16) were diluted 1:10 in FBM and streaked on BIM and VL plates. The plates were incubated for 48 h at 37°C in an anaerobic glove box flushed with H₂-CO₂, 95:5 (vol/vol). For the quantitative recovery of *C. butyricum* strains on BIM and VL media, dilutions in FBM were made in an anaerobic chamber (La

Calhène) containing N₂-CO₂-H₂, 80:15:5 (vol/vol); then 0.1 ml of 10³, 10⁴, and 10⁵ dilutions was spread over each plate. The colonies were counted after plate incubation for 48 h at 37°C in an anaerobic chamber. *C. butyricum* spores, obtained in D medium (9), were enumerated on BIM and VL plates after heat treatment of the culture at 80°C for 10 min.

BIM was also tested for *C. butyricum* recovery from seeded fecal specimens. A 1-g human fecal sample was homogenized in 3 ml of FBM. Then 1 volume of a 1:10 dilution of the overnight *C. butyricum* cell suspension in Trypticase-yeast extract-glucose broth was mixed with 4 volumes of fecal suspension and 0.1 ml of 10¹, 10², and 10³ dilutions in FBM was spread over the surface of BIM plates. All manipulations were done in an anaerobic chamber. The colonies were counted after 48 h of incubation at 37°C. Some colonies of each plate were examined for their microscopic appearances and tested for sugar fermentation. The number of *C. butyricum* colonies thus recovered from a fecal sample on BIM was compared with the number of viable bacteria added to the fecal sample, as counted on VL plates.

RESULTS

Concentrations of antimicrobial agents in BIM. The quantitative recovery of *C. butyricum* on VL medium and on BM with various concentrations of trimethoprim, cycloserine, and polymyxin B is shown in Table 1. Based on these data and data from several similar experiments, I decided to use 16 µg of trimethoprim, 10 µg of cycloserine, and 20 µg of B polymyxin per ml of medium. This preparation was designated BIM.

The *C. butyricum* colonies on BIM had more irregular margins and were slightly smaller (1.5 to 3 mm in diameter) than colonies grown on VL medium.

Qualitative tests. The growth of 34 strains of *C. butyricum*, 10 strains of *C. beijerinckii*, 126 strains of other clostridia, 24 strains of non-spore-forming anaerobes, and 55 strains of various facultatively anaerobic bacteria on BIM was compared with that obtained on VL medium (Table 2). In addition to the 34 strains of *C. butyricum* and 10 strains of *C. beijerinckii* tested, all strains of other species of butyric acid-producing clostridia (BAPC) grew on BIM. The colonial appearance of most of these species was indistinguishable. Among the other clostridia tested, only *C. sphenoides* produced very small colonies on BIM. Among the 24 strains of non-spore-forming obligately anaerobic bacteria and the 55 strains of facultatively anaerobic bacteria tested, only *Bacillus cereus* grew on BIM. The *B. cereus* colonies on BIM were regularly circular and much smaller than the *C. butyricum* colonies on the same medium.

Quantitative tests. The quantitative recovery of two *C. butyricum* reference strains, seven *C. butyricum* isolates, three *C. beijerinckii* strains, and one *Clostridium acetobutylicum* strain was determined on VL, BM, and BIM (Table 3). Small differences between the number of colonies on the three media appeared for *C. butyricum* reference strains or isolates, and the *C. beijerinckii* and *C. acetobutylicum* recovery on BIM was low compared with that obtained on VL medium.

Recovery of *C. butyricum* from seeded fecal samples. Eight strains of *C. butyricum*, one strain of *C. beijerinckii*, and 12 fecal human samples were tested for recovery of *C. butyricum* or *C. beijerinckii* from seeded feces. The numbers of colonies recovered on BIM from seeded fecal samples usually were 10 to 100% of the number obtained on VL medium for *C. butyricum* strains, but not for the *C. beijerinckii* strain (Table 4). The recovery rate of *C. beijerinckii*

TABLE 1. Quantitative recovery of *C. butyricum* (CB 1002) on VL medium and BM containing various concentrations of trimethoprim (TMP), cycloserine (Cycl), and polymyxin B (Pol B)

Medium	Concn (µg/ml) of:			No. of organisms recovered per ml of culture	No. of spores recovered per ml of culture
	TMP	Cycl	Pol B		
VL	0	0	0	1.75 × 10 ⁶	4.7 × 10 ⁷
BM ^a	0	0	0	1.39 × 10 ⁶	4.6 × 10 ⁷
	16	10	10	1.26 × 10 ⁶	2.7 × 10 ⁷
	16	10	20	1.15 × 10 ⁶	2.8 × 10 ⁷
	16	10	30	0.70 × 10 ⁶	ND ^b
	24	10	10	0.88 × 10 ⁶	ND
	24	10	20	0.51 × 10 ⁶	ND
	16	20	10	0.98 × 10 ⁶	ND

^a BM is a synthetic minimal medium without antimicrobial agents (see the text).

^b ND, Not done.

from seeded feces was 1% of the cells present in the inoculum.

The BIM inhibited the growth of the fecal flora. Only a few colonies that were smaller than 0.5 mm were present on plates inoculated with 1:10 dilutions of fecal samples.

TABLE 2. Growth^a of various species of bacteria of BIM

Species	No. of strains tested	No. of strains growing on:	
		BIM	VL medium
<i>C. butyricum</i>	34	34	34
<i>C. beijerinckii</i>	10	10	10
<i>C. acetobutylicum</i>	21	21	21
<i>C. "butylicum"</i>	5	5	5
<i>C. pasteurianum</i>	1	1	1
<i>C. tyrobutyricum</i>	1	1	1
<i>C. "saccharoacetoperbutylacetum"</i>	2	2	2
<i>C. sphenoides</i>	4	2 ^b	4
<i>B. cereus</i>	6	5 ^b	6

^a The strains that grew on VL control plates but not on BIM (number of tested strains) were: *Clostridium absonum* (1), *C. aminovalericum* (1), *C. barkeri* (1), *C. bifementans* (2), *C. botulinum* A (1), *C. botulinum* D (1), *C. botulinum* E (1), *C. cadaveris* (1), *C. carnis* (1), *C. clostriforme* (2), *C. cochlearium* (1), *C. difficile* (11), *C. glycolicum* (2), *C. ghoni* (2), *C. histolyticum* (1), *C. innocuum* (4), *C. limosum* (1), *C. lituseburens* (1), *C. malenominatum* (2), *C. manganotii* (2), *C. perenne* (2), *C. perfringens* (14), *C. putrificum* (1), *C. ramosum* (1), *C. rectum* (1), *C. sardiniensis* (2), *C. septicum* (3), *C. sordellii* (13), *C. sporogenes* (2), *C. sporosphaeroides* (1), *C. subterminale* (2), *C. tetani* (2), *C. tertium* (4); *Bacteroides bivius* (1), *B. fragilis* (4), *B. melaninogenicus* (1), *B. ureolyticus* (1); *Fusobacterium nucleatum* (1), *F. necrophorum* (3), *F. varium* (1); *Peptococcus magnus* (1), *Peptostreptococcus anaerobius* (1), *Peptococcus anaerobius* (1); *Propionibacterium acnes* (3); *Eubacterium lentum* (1); *Bifidobacterium* species (5); *Citrobacter* species (1); *Escherichia coli* (3); *Edwardsiella tarda* (1); *Enterobacter aerogenes* (1), *E. agglomerans* (1), *E. cloacae* (1), *E. hafniae* (1); *Klebsiella pneumoniae* (1), *K. oxytoca* (1), *K. ozaenae* (1), *K. rhinoscleromatis* (1); *Levinea amalonatica* (1), *L. malonatica* (1); *Proteus mirabilis* (1), *P. morgani* (1), *P. rettgeri* (1), *P. vulgaris* (1); *Providencia alcalifaciens* (1), *P. stuartii* (1); *Salmonella choleraesuis* subsp. *choleraesuis* serovar *oraniensis* (1), *S. cholerae-suis* subsp. *cholerae-suis* serovar *brandenburg* (1), *S. cholerae-suis* subsp. *cholerae-suis* serovar *typhimurium* (1); *Serratia liquefaciens* (1), *S. marcescens* (1); *Bacillus cereus* subsp. *mycoides* (1), *B. coagulans* (1), *B. firmus* (1), *B. licheniformis* (1), *B. macerans* (1), *B. polymyxa* (1), *B. subtilis* (1); *Lactobacillus acidophilus* (1), *L. casei* subsp. *casei* (1), *L. casei* subsp. *rhamnosus* (1), *L. delbrueckii* subsp. *delbrueckii* (1), *L. fermentum* (1), *L. jensenii* (1), *L. delbrueckii* subsp. *lactis* (1), *L. plantarum* (1), *L. salivarius* (1), *L. viridescens* (1); *Streptococcus faecalis* (1), *S. faecalis* subsp. *liquefaciens* (1), *S. faecalis* subsp. *zymogenes* (1), *S. faecium* (1), *S. salivarius* (1), *S. pyogenes* (1); *Staphylococcus aureus* (2), *S. epidermidis* (1), *S. haemolyticus* (1), *S. hominis* (1), *S. saprophyticus* (1).

^b Small colonies.

TABLE 3. Recovery of *C. butyricum* and other BAPC on VL, BM, and BIM

Strain	No. of organisms recovered per ml of culture on:		
	VL	BM	BIM
<i>C. butyricum</i>			
VPI 3266	6.6×10^6	2.0×10^6	1.0×10^6
VPI 2969	7.9×10^6	1.3×10^6	2.1×10^6
153-2 ^a	11.3×10^7	8.3×10^7	8.0×10^7
181-2 ^a	8.7×10^6	3.0×10^6	2.0×10^6
147-1 ^a	5.5×10^7	4.9×10^7	3.1×10^7
144-1 ^a	1.5×10^7	3.1×10^6	5.0×10^6
174-3 ^a	3.6×10^7	1.6×10^7	9.2×10^6
116-1 ^a	0.9×10^8	1.3×10^8	1.1×10^8
<i>C. beijerinckii</i>			
VPI 5481	1.7×10^8	5.0×10^6	10^5
VPI 2681	1.7×10^8	1.2×10^8	10^5
CNRZ 534	2.9×10^6	1.4×10^6	10^5
<i>C. acetobutylicum</i>			
AB 23	19.0×10^6	5.0×10^6	1.10^5

^a These *C. butyricum* isolates were isolated from newborn feces (P. Raibaud, Centre National de Recherches Zootechniques, Jouy-en-Josas, France).

DISCUSSION

The methods routinely used for isolation of *C. butyricum* from fecal samples are those described for most clostridia and include spore selection techniques by heat or alcohol treatment and the use of blood agar or egg yolk agar with neomycin and cycloserine (18) or with kanamycin (20). But these methods allow the growth of a wide variety of clostridia and other bacteria from fecal flora.

Cummins and Johnson (2) reported that *C. butyricum* but not *C. beijerinckii* strains grew on a basal glucose-mineral salts medium supplemented with biotin. Our observations showed that (i) both *C. butyricum* and *C. beijerinckii* strains grew on fluid BM, (ii) some *C. beijerinckii* strains produced colonies smaller than *C. butyricum*, and (iii) the quantitative recovery of *C. beijerinckii* strains on BIM was less efficient than that of *C. butyricum* strains (Tables 3 and 4). The use of

TABLE 4. Recovery of *C. butyricum* from seeded fecal specimens on BIM

Fecal specimen no.	<i>C. butyricum</i> strain	No. of colonies recovered from fecal specimen on BIM ^a	Size (CFU/ml) of the <i>C. butyricum</i> inoculum used to prepare seeded stools ^b
1	VPI 3266	8.0×10^3	6.2×10^3
2	VPI 2417	1.0×10^5	2.8×10^6
3	VPI 2969	8.0×10^4	6.2×10^4
4	VPI 2681 ^c	2.0×10^4	2.0×10^6
5	CB 174-3	5.9×10^3	11.3×10^3
6	CB 87-2	3.0×10^4	4.5×10^5
7	CB 116-1	2.3×10^4	3.2×10^4
8	CB 181-2	2.1×10^4	2.7×10^4
9	CB 1002	1.0×10^4	3.0×10^4
10	CB 1002	8.0×10^4	3.0×10^4
11	CB 1002	3.2×10^3	3.0×10^4
12	CB 1002	8.0×10^3	3.0×10^4

^a All colonies were considered to be *C. butyricum*, since unseeded specimens did not yield any colonies and all colonies tested produced the same microscopic appearance and the same pattern of sugar fermentation as *C. butyricum*.

^b The *C. butyricum* cell suspension used for seed fecal specimens was diluted in FBM as a fecal specimen and inoculated on VL plates.

^c *C. beijerinckii*.

the BM allowed the growth of some BAPC, principally *C. butyricum*, and inhibited the growth of the proteolytic clostridia and other clostridia with more complex nutritional requirements.

The incorporation of cycloserine, trimethoprim, and polymyxin B in BM improved the selectivity of this medium. The cycloserine had previously been used for the isolation of *Clostridium perfringens* (6) and *Clostridium difficile* (21); the cycloserine-cefoxitin combination had been used for the isolation of *C. difficile* (4); the cycloserine-trimethoprim-sulfamethoxazole combination had been used for the isolation and enumeration of clostridia in human feces (14); and polymyxin associated with oleandomycin and sulfadiazin had been used for *C. perfringens* quantitation in foods (5, 6). Although many *C. butyricum* isolates from human sources produce a β -lactamase (11, 12), β -lactam antibiotics would not be satisfactory for the isolation of those *C. butyricum* strains that do not produce a β -lactamase.

With BIM, some of the BAPC could be recovered from fecal specimens, whereas the majority of other fecal microorganisms were inhibited. Among the other bacteria, *B. cereus* strains and some *C. sphenoides* strains grew on BIM. The colonies of these organisms were circular and small and could be distinguished easily from BAPC colonies. No obvious differences were observed among the colonies of the different BAPC. However, *C. butyricum* is the main BAPC isolated from human feces (13) and is quantitatively recovered on BIM. If the number of *C. butyricum* organisms is low in fecal samples, fluid BIM could be used as enrichment medium before inoculation of BIM plates.

Our data also indicate that BIM, with some modifications, can be used for the isolation of some other BAPC, such as *C. acetobutylicum*. The recovery of BAPC from soil specimens was not attempted.

ACKNOWLEDGMENTS

We thank J. Morin for technical assistance and M. Sebald for helpful advice and criticism during the course of this work.

LITERATURE CITED

- Beerens, H. 1954. Amélioration des techniques d'étude et d'identification des bactéries anaérobies. Ann. Inst. Pasteur de Lille 6:36-52.
- Cummins, C. S., and J. L. Johnson. 1971. Taxonomy of the clostridia: wall composition and DNA homologies in *Clostridium butyricum* and other butyric acid-producing clostridia. J. Gen. Microbiol. 67:33-46.
- Dezfulian, M., L. M. McCroskey, C. L. Hatheway, and V. R. Dowell, Jr. 1981. Selective medium for isolation of *Clostridium botulinum* from human feces. J. Clin. Microbiol. 13:526-531.
- George, W. L., V. L. Sutter, D. Citron, and S. M. Finegold. 1979. Selective and differential medium for isolation of *Clostridium difficile*. J. Clin. Microbiol. 9:214-219.
- Handford, P. M., and J. J. Cavett. 1973. A medium for the detection and enumeration of *Clostridium perfringens* (welchii) in foods. J. Sci. Food. Agr. 24:487.
- Hauschild, A. H. W., and R. Hilsheimer. 1974. Enumeration of food-borne *Clostridium perfringens* in egg yolk-free tryptose-sulfite-cycloserine agar. Appl. Microbiol. 27:521-526.
- Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.). 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Va.
- Howard, F. M., J. M. Bradley, D. M. Flynn, P. Noone, and M. Szawatkowski. 1977. Outbreak of necrotizing enterocolitis caused by *Clostridium butyricum*. Lancet ii:1099-1102.
- Labbe, R. G. 1981. Enterotoxin formation by *Clostridium perfringens* type A in a defined medium. Appl. Environ. Microbiol. 41:315-317.
- Laverdiere, M., A. Robert, R. Chicoine, D. Salet, and R.

- Rosenfeld.** 1978. Clostridia in necrotizing enterocolitis. *Lancet* **ii**:377.
11. **Magot, M.** 1981. Résistance aux pénicillines dues à la production d'une bêta-lactamase chez des *Clostridium* du groupe *butyricum*. *C.R. Acad. Sci.* **292**:285-287.
 12. **Magot, M.** 1981. Some properties of the *Clostridium butyricum* group B-lactamase. *J. Gen. Microbiol.* **127**:113-119.
 13. **Magot, M., J. P. Carlier, and M. R. Popoff.** 1983. Identification of *Clostridium butyricum* and *Clostridium beijerinckii* by gas-liquid chromatography and sugar fermentation: correlation with DNA homologies and electrophoretic patterns. *J. Gen. Microbiol.* **129**:2837-2845.
 14. **Mevisen-Verhage, E. A. E., N. M. De Vos, W. C. M. Harmsen-Van Amerongen, and J. H. Marcelis.** 1982. A selective medium for the detection and enumeration of clostridia in human feces. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **48**:205-206.
 15. **Popoff, M. R., and M. Sebald.** 1981. Mise en évidence chez *Clostridium butyricum* d'un facteur thermostable responsable du pouvoir pathogène expérimental. *C.R. Acad. Sci.* **292**:763-766.
 16. **Sebald, M., and R. N. Costilow.** 1975. Minimal growth requirements for *Clostridium perfringens* and isolation of auxotrophic mutants. *Appl. Microbiol.* **29**:1-6.
 17. **Skerman, V. B. D., V. Mc Gowan, and P. H. A. Sneath.** 1980. Approved lists of bacterial names. *Int. J. Syst. Bacteriol.* **30**:225-420.
 18. **Smith, M. F., S. P. Borriello, G. S. Clayden, and M. W. Casewell.** 1980. Clinical and bacteriological findings in necrotizing enterocolitis: a controlled study. *J. Infection* **2**:23-31.
 19. **Sturm, R., J. L. Staneck, L. R. Stauffer, and W. W. Nebleh.** 1980. Neonatal necrotizing enterocolitis associated with penicillin resistant, toxigenic *Clostridium butyricum*. *Pediatrics* **66**:928-931.
 20. **Westra-Meijer, C. M. M., J. E. Degrener, G. Dzoljic-Danilovic, M. F. Michel, and J. W. Mettau.** 1983. Quantitative study of the aerobic and anaerobic faecal flora in neonatal necrotizing enterocolitis. *Arch. Dis. Child.* **58**:523-528.
 21. **Willey, S. H., and J. G. Bartlett.** 1979. Cultures for *Clostridium difficile* in stools containing a cytotoxin neutralized by *Clostridium sordellii* antitoxin. *J. Clin. Microbiol.* **10**:880-884.