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Culture Medium for Selective Isolation and Enumeration of Gram-Negative Bacteria from Ground Meats†

F. J. CYZESKA, J. A. SEITER, S. N. MARKS, AND J. M. JAY*
Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202

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We developed a new medium, designated peptone bile amphotericin cycloheximide (PBAC) agar, which contains (per liter) 10 g of peptone, 300 mg of bile salts, 1 mg of amphotericin B, 1 g of cycloheximide, and 15 g of agar. When 21 samples of fresh ground beef were studied and plate count agar counts were used as references, we obtained a mean recovery of 28% of total counts with violet red bile agar overlay, whereas we obtained 48% recovery with PBAC agar. With 12 samples of frozen ground beef, recovery on violet red bile agar overlay was 29% of the recovery on plate count agar, whereas the corresponding value on PBAC agar was 45%. PBAC agar allowed the enumeration of 1.4 times as many gram-negative bacteria as violet red bile agar overlay. None of eight strains of gram-positive bacteria and none of eight strains of yeasts grew on PBAC agar. Of 158 colonies randomly selected from pour plates of eight fresh ground meat samples, 95% stained gram negative. In comparison, only 70% of 151 colonies selected from corresponding plate count agar plates were gram negative. The lack of background color, turbidity, and ease of use make PBAC agar easier to handle than other media used for gram-negative bacteria, such as violet red bile agar, violet red bile agar overlay, and crystal violet tetrazolium agar. In the preparation PBAC agar, all ingredients are autoclaved together except amphotericin B, which is filter sterilized and added before the plates are poured.

A large number of culture media have been developed for the growth and enumeration of specific groups of gram-negative bacteria. Media such as violet red bile agar (VRBA), MacConkey agar, eosin methylene blue agar, and Endo agar are well known and are widely used to isolate and enumerate gram-negative bacteria, such as coliforms. These media are made selective for gram-negative bacteria by ingredients such as bile salts, methylene blue, and others. Although these media were developed primarily to culture enteric bacteria, they have been used to isolate and enumerate other gram-negative bacteria as well.

Several investigators have reported the inhibition of metabolically injured cells by bile salts, especially in VRBA, and that this inhibition can be partially overcome by using an agar overlay technique (1, 3, 4). In this technique, samples are first plated onto nonselective media for 0 to 3 h to allow cell repair before being exposed to VRBA. After we used this "repair detection" method, it became clear that not all gram-negative bacteria that are found in refrigerated fresh ground meats develop on this medium. Although crystal violet tetrazolium agar was developed to enumerate gram-negative psychrotrophs, this medium also allows the growth of lactic acid bacteria. Since fresh ground meats may contain relatively high numbers of these organisms, we found that this medium is not a good selective medium for gram-negative bacteria in ground meats.

In our efforts to relate the amounts of endotoxin in fresh meats to actual numbers of viable gram-negative cells, we have been hampered by the lack of a culture medium that is not toxic toward the gram-negative flora of meats but yet is selective enough to inhibit gram-positive bacteria and fungi. Employing the principles that gram-negative bacteria do not require as rich a nutrient base as gram-positive bacteria and that gram-negative bacteria grow in the general absence of preformed B vitamins, we developed a simple medium for the selective isolation and enumeration of gram-negative bacteria, which in our hands is far superior to crystal violet tetrazolium agar and VRBA.

MATERIALS AND METHODS

Cultures. Gram-negative meat isolates were obtained from both fresh and spoiled meat samples. The gram-positive bacteria and some of the gram-negative

† Contribution 398 from the Department of Biological Sciences, College of Liberal Arts, Wayne State University.

bacteria used were standard stock culture strains. Although most of the yeast cultures were obtained from the American Type Culture Collection, some were isolated from fresh ground beef. All cultures were maintained on plate count agar (PCA) (Difco Laboratories, Detroit, Mich.). Strains to be tested were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) for 24 h at 30°C.

Media and plate counts. PCA was used as a control plating medium throughout this study. All plates were poured in duplicate and were incubated for 48 h at 30°C. Peptone bile amphotericin cycloheximide (PBAC) agar was compared with the VRBA overlay (VRBAO) method of Hartman et al. (1, 2), as well as with VRBA (BBL Microbiology Systems) pour plates. Briefly, in the VRBAO method petri dishes were poured first with about 15 ml of PCA, which was allowed to harden. Then, a sample was placed on each PCA surface, and freshly boiled VRBA was overlaid immediately. These plates were incubated for 48 h at 30°C.

PBAC agar adjusted to pH 6.7 contained the following ingredients (per liter): 10 g of peptone, 300 mg of bile salts no. 3, 1 mg of amphotericin B, 1 g of cycloheximide, and 15 g of agar. The antibiotics were prepared in stock solutions and stored in 2-ml portions at −20°C. Cycloheximide was dissolved in 95% ethanol and was added to the medium before autoclaving. Amphotericin B was dissolved in dimethyl sulfoxide (Fisher Scientific Co., Pittsburgh, Pa.), filter sterilized, and added to the medium after it was autoclaved and cooled to 45°C.

Food samples. To test the effectiveness of PBAC agar for recovery of gram-negative bacteria, we used fresh and frozen meats, as well as sterile skim milk. The ground meats were obtained from supermarkets in the metropolitan Detroit, Mich., area and were handled as previously described (2). Frozen meat samples were acquired in the same way, frozen in Nasco Whirl-Pak bags at −20°C, and stored for 1 to 8 months. Portions of frozen samples to be tested were removed from freezer bags which had been thawed in cold water. All meat samples were homogenized with a Stomacher 400 (Dynatech Laboratories, Inc., Alexandria, Va.) in plastic Stomacher bags for 2 min before plating.

Recovery of organisms from 10% sterile skim milk was demonstrated by inoculating individual tubes of skim milk with either gram-negative or gram-positive cultures. After 5 h of incubation, dilutions were made, and samples were plated and incubated as described above.

The percentages of gram-negative bacteria which developed on PBAC agar and PCA were determined. Eight fresh ground meat samples were plated onto PCA and PBAC agar and incubated as described above. Ten colonies were selected randomly from each of the duplicate countable plates. Each colony was suspended in 1 ml of nutrient broth and incubated for 24 h at 30°C. Sterile cotton swabs were then used to streak PCA plates and to make smears for staining. The PCA plates were examined for growth, and Gram stain preparations made for all colonies showing growth.

RESULTS

Preliminary work in which we used a range of bile salt concentrations in several basal media, including brain heart infusion broth, Casitone medium, and peptone medium, indicated the need to test the combination of 300 mg of bile salts no. 3 per liter plus 10 g of peptone per liter further. Since yeasts developed on this medium, we examined the inhibitory effects of amphotericin B and cycloheximide. Amphotericin B at a concentration of 1 μg/ml inhibited four of five yeast strains tested at 10^{-1} and 10^{-2} dilutions of 10^4 to 10^5 cells per ml in Trypticase soy broth. Cycloheximide inhibited three of five yeast strains tested when it was used at a concentration of 1,000 μg/ml. These antibiotics seemed to be active against different yeast strains, so we tried a combination of the two, using the concentrations described above. Of the eight yeast strains examined, none grew on this medium containing amphotericin B and cycloheximide when we tested 10^{-2} dilutions of broth cultures containing 10^5 to 10^6 cells per ml (Table 1). Thus, we developed a peptone bile salts medium containing both of these antibiotics (PBAC agar).

A total of 10 gram-negative cultures were plated onto PCA, VRBA, and PBAC agar after they were incubated for 5 h in skim milk. When PCA was used as a control to calculate the percent recoveries on VRBAO and PBAC agar, we obtained a mean recovery of 87% of the colonies that developed on PCA with VRBAO (range for the 10 cultures, 52 to 110%) (Table 2), whereas with PBAC agar there was an overall recovery of 95% of PCA controls (range, 84 to 110%).

TABLE 1. *Effectiveness of PBAC agar in inhibiting the growth of eight yeast strains*

Yeast	No./ml ($\times 10^3$) on:	
	PCA	PBAC agar
Candida guilliermondii ATCC 6260[a]	1,800	<0.1
Debaromyces cantarelli ATCC 24172[a]	1,200	<0.1
Rhodotorula rubra ATCC 9449	830	<0.1
Saccharomyces cerevisiae WSU 800	930	<0.1
Torulopsis candida ATCC 12790[b]	680	<0.1
Fresh beef isolate 001-4	1,600	<0.1
Fresh beef isolate 028-4	820	<0.1
Fresh beef isolate 1-100	650	<0.1

[a] Not inhibited when cycloheximide was the sole antibiotic.

[b] Not inhibited when amphotericin B was the sole antibiotic.

PBAC agar was tested for its ability to select against gram-positive bacteria. Table 3 shows that when PBAC agar was used, eight gram-positive strains were not recovered from spiked skim milk which had been incubated at 30°C for 5 h. Neither the antibiotics nor the milk reduced the selective properties of PBAC agar.

We tested the effectiveness of PBAC agar as a medium for enumerating gram-negative bacteria by performing plate counts on 21 fresh ground beef samples. The mean number of bacteria on VRBA was 2.0×10^6 cells per g, whereas the recoveries on VRBAO and PBAC agar were 3.0×10^6 and 4.1×10^6 cells per g, respectively (Table 4). Compared with counts on PCA, these values represented mean recovery rates of 17, 28, and 48% for VRBA, VRBAO, and PBAC agar, respectively. Thus, PBAC agar allowed recovery of 1.4 times as many gram-negative bacteria as VRBAO and 2.1 times as many as VRBA. The difference between VRBAO and PBAC agar was significant ($P < 0.05$).

TABLE 2. *Relative numbers of 10 gram-negative bacteria recovered on PCA, VRBA, and PBAC agar at 30°C for 48 h after culture in skim milk for 5 h at 30°C*

Bacterium	No./ml (×10³) on:[a]		
	PCA	VRBAO	PBAC agar
Pseudomonas fluorescens WSU 274	80	65 (81)[b]	67 (84)
Pseudomonas sp. WSU 2797	940	720 (77)	870 (93)
Pseudomonas sp. WSU 5	55	60 (100)	52 (95)
Pseudomonas fluorescens ATCC 17400	1,200	1,100 (89)	1,100 (91)
Pseudomonas sp. WSU 2796	1,200	900 (73)	1,300 (110)
Pseudomonas sp. WSU 2803	870	450 (52)	850 (98)
Pseudomonas sp. WSU 2808	1,200	1,100 (94)	1,100 (93)
Pseudomonas sp. WSU 2798	160	170 (110)	160 (99)
Pseudomonas geniculata ATCC 14150	1,100	1,100 (96)	1,100 (98)
Aeromonas salmonicida WSU 611	190	180 (96)	180 (95)

[a] The mean recoveries on PCA, VRBAO, and PBAC agar were 700×10^3, 580×10^3, and 680×10^3 bacteria per ml, respectively.

[b] The numbers in parentheses are percent recoveries. The mean percent recoveries on VRBAO and PBAC agar were 87 and 95%, respectively.

TABLE 3. *Inhibition of eight strains of gram-positive bacteria by PBAC agar after growth in skim milk at 30°C for 5 h*

Bacterium	No./ml (×10³) on:	
	PCA	PBAC agar
Bacillus megaterium WSU 63	2,500	<0.1
Bacillus subtilis WSU 61	140	<0.01
Brevibacterium taipei ATCC 13744	1,200	<1
Corynebacterium sp. WSU 280	650	<0.1
Lactobacillus sp.	260	<0.01
Micrococcus luteus WSU 82	29	<0.01
Staphylococcus aureus 196E	900	<0.1
Streptococcus sp.	420	<0.01

Compared with VRBA and VRBAO, PBAC agar was more efficient in recovering gram-negative cells from both frozen and fresh samples. The VRBAO method was developed to enumerate metabolically injured cells. We presumed that some of the bacteria in our frozen meats suffered freeze injury. Table 5 shows that when PBAC agar was used, the mean percent recovery of gram-negative bacteria from frozen meats was 45%, whereas with VRBA and VRBAO the mean recoveries were only 25 and 29%, respectively. Thus, PBAC agar allowed recovery of 1.3 times as many gram-negative bacteria as VRBAO and 1.4 times as many as VRBA. The difference in recovery on these two media was significant ($P < 0.05$). These results indicated that PBAC agar, which contained only one-fifth the concentration of bile salts that was in VRBA, was not as toxic to freeze-injured cells as the VRBAO.

With regard to the percentage of colonies that developed on the plating media and were gram negative, 95% of 158 colonies selected from PBAC agar stained negative, whereas only 70% of 151 colonies selected from PCA stained negative. Upon subculture onto MacConkey agar and incubation at 30°C for 24 h, 94% of the 158 colonies from PBAC agar grew, whereas only 94 (62%) of the 151 colonies from PCA grew.

DISCUSSION

PBAC agar was developed in response to the need for a medium which allows an accurate count of gram-negative bacteria in foods. Although there are a number of commercially available media for gram-negative bacteria, these media were developed for other purposes and are not entirely suitable for enumerating gram-negative bacteria in meats, especially when injured cells are present.

With regard to the rationale for PBAC agar, our findings indicate that 1% peptone provides enough nutrients for gram-negative bacteria to develop but not enough for gram-positive bacteria to develop during 48 h of incubation at 30°C. When PBAC agar plates were examined after 24 h, gram-negative colonies were well developed, although small in size. The additional

TABLE 4. *Comparison of VRBA, VRBAO, and PBAC agar for the enumeration of gram-negative bacteria in 21 ground meat samples, using PCA plate counts as controls*

Sample	Type[a]	No./g (×10³) on:[b]			
		PCA	VRBA	VRBAO	PBAC agar
172	GC	1,500	350 (23)[c]	860 (57)	1,100 (73)
173	GR	6,900	720 (10)	1,200 (17)	2,000 (30)
174	HB	8,400	1,500 (18)	4,600 (55)	5,900 (70)
175	GR	27,000	8,100 (30)	19,000 (70)	20,000 (74)
178	GR	21,000	9,700 (46)	12,000 (57)	16,000 (76)
179	GC	22,000	9,300 (42)	12,000 (54)	17,000 (77)
180	HB	1,000	30 (3)	39 (3.9)	91 (9.1)
182	HB	1,300	<1 (<0.08)	35 (2.7)	210 (16)
183	GC	1,200	46 (3.8)	120 (10)	620 (52)
185	HB	1,700	53 (3.1)	250 (15)	400 (82)
187	GR	2,500	200 (8)	570 (23)	1,200 (48)
188	GC	1,900	380 (20)	560 (29)	1,300 (68)
191	GC	450	43 (9.6)	63 (14)	100 (22)
192	HB	4,300	98 (2.3)	210 (4.9)	710 (17)
193	GR	5,100	190 (3.7)	310 (6.1)	640 (13)
194	HB	3,400	NT[d]	440 (12.9)	440 (13)
195	GC	3,500	NT	110 (3.1)	110 (3.1)
196	HB	44	6 (14)	12 (27)	13 (30)
197	HB	12,000	3,200 (27)	8,500 (71)	16,000 (130)
198	HB	2,200	380 (17)	690 (31)	2,000 (91)
199	GC	60	<0.1 (<1.7)	<0.1 (<1.7)	4 (6.7)

[a] GC, Ground chuck; GR, ground round; HB, hamburger meat.
[b] The mean recoveries on PCA, VRBA, VRBAO, and PBAC agar were 6,100 × 10³, <2,000 × 10³, 3,000 × 10³, and 4,100 × 10³ bacteria per g, respectively.
[c] The numbers in parentheses are percent recoveries. The mean percent recoveries on VRBA, VRBAO, and PBAC agar were 17, 28, and 48%, respectively.
[d] NT, Not tested.

TABLE 5. *Comparison of VRBA, VRBAO, and PBAC agar for the enumeration of gram-negative bacteria from 12 samples of frozen ground meat, using PCA counts as references*

Sample	Type[a]	No./g (×10³) on:[b]			
		PCA	VRBA	VRBAO	PBAC agar
057	HB	1,200	24 (2)[c]	100 (8.3)	410 (34)
086	HB	1,300	7.5 (0.58)	12 (0.92)	56 (4.3)
088	GC	3,100	55 (0.18)	17 (0.55)	30 (0.97)
089	GR	1,500	7.4 (0.49)	17 (1.1)	29 (1.9)
063	GC	650	5.7 (0.88)	10 (1.5)	13 (2)
099	HB	1,200	64 (5.3)	84 (7)	140 (12)
071	GC	77	8.1 (11)	9.2 (12)	15 (19)
101	GC	1,700	450 (26)	620 (36)	1,200 (71)
105	HB	2,100	1,400 (67)	1,800 (86)	2,700 (130)
106	GR	840	460 (55)	470 (56)	800 (95)
107	HB	13,000	8,800 (67)	8,900 (68)	11,000 (85)
139	GV	9,300	5,800 (62)	6,300 (68)	7,900 (85)

[a] HB, Hamburger meat; GC, ground chuck; GR, ground round.
[b] The mean recoveries on PCA, VRBA, VRBAO, and PBAC agar were 3,000 × 10³, 1,400 × 10³, 1,500 × 10³, and 2,000 × 10³ bacteria per g, respectively.
[c] The numbers in parentheses are percent recoveries. The mean percent recoveries on VRBA, VRBAO, and PGAC agar were 25, 29, and 45%, respectively.

24 h of incubation permitted the colonies to develop further so that counting was easier. After 48 h of incubation at 30°C, some pinpoint colonies of gram-positive bacteria began to develop, but these were few in number. Although peptone and Casitone both contain free amino acids, neither contains significant quantities of B vitamins. Preformed B vitamins are required by gram-positive bacteria, especially the lactic acid bacteria, which are fairly common in meats. The success of PBAC agar as a selective medium for gram-negative bacteria is due primarily to the faster growth rate of the gram-negative bacteria compared with gram-positive bacteria and

the fact that gram-negative bacteria can grow in the absence of preformed B vitamins. By employing a nutrient poor base, the bile salt concentration can be reduced to one-fifth of that in VRBA and MacConkey agar and still inhibit gram-positive bacteria. Gram-negative bacteria which are inhibited by the bile salt concentration in VRBA are able to grow in PBAC agar, even if they are freeze injured.

When 1% Casitone was used with bile salts, all four gram-positive strains tested grew, whereas these strains did not grow when 1% peptone was used with the same level of bile salts. Although the precise reason for this is not clear, it may have been due to the occurrence of larger quantities of B vitamins in the casein digest, which provided a more optimum nutritional base for the gram-positive bacteria.

With respect to PBAC agar and VRBAO, the former medium offers several advantages over the latter. PBAC agar recovered one-third more gram-negative bacteria from meat samples than previously reported methods. In addition, PBAC agar allows direct pour plating, whereas VRBAO requires that plating of samples be made onto prepoured nonselective media. The latter procedure often leads to uncountable plates because of spreaders and the development of colonies in groups so that enumeration is difficult. Because PBAC agar does not contain beef extract, this medium is quite clear and thus makes the enumeration of colonies easier. When low dilutions of meat homogenates are plated, the clarity of this medium is such that food particulates can be distinguished easily from bacterial colonies.

Overall, PBAC agar is an excellent medium for enumerating gram-negative bacteria in ground meats and other food samples and, perhaps, from water and other environmental sources as well.

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