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Monensin-Based Medium for Determination of Total Gram-Negative Bacteria and *Escherichia coli*†

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Plate count-monensin-KCl (PMK) agar, for enumeration of both gram-negative bacteria and *Escherichia coli*, is composed of (per liter) 23.5 g of plate count agar, 35 mg of monensin, 7.5 g of KCl, and 75 mg of 4-methylumbelliferyl-β-D-glucuronide (MUG). Monensin was added after the medium was sterilized. The diluent of choice for use with PMK agar was 0.1% peptone (pH 6.8); other diluents were unsatisfactory. Gram-negative bacteria (selected for by the ionophore monensin) can be used to judge the general quality or sanitary history of a commodity. *E. coli* (differentiated by its ability to hydrolyze the fluorogenic compound MUG) can be used to assess the safety of a commodity in regard to the possible presence of enteric pathogens. Pure-culture studies demonstrated that monensin completely inhibited gram-positive bacteria and had little or no effect on gram-negative bacteria. When gram-negative bacteria were injured by one of several methods, a few species (including *E. coli*) became sensitive to monensin; this sensitivity was completely reversed in most instances by the inclusion of KCl in the medium. When PMK agar was tested with food and environmental samples, 96% of 535 isolates were gram negative; approximately 68% of colonies from nonselective medium were gram negative. PMK agar was more selective than two other media against gram-positive bacteria and was less inhibitory for gram-negative bacteria. However, with water samples, KCl had an inhibitory effect on gram-negative bacteria, and it should therefore be deleted from monensin-containing medium for water analysis. MUG was useful in determining the general level of contamination by *E. coli* of raw hamburger, creekwater, and raw milk if certain precautions were taken; MUG could not be used to determine *E. coli* in frozen mixed vegetables because of the high level of false-positive reactions caused by flavobacteria.

The enumeration of all aerobic and facultatively anaerobic gram-negative bacteria in a sample may be desirable for several reasons. Because most psychrotrophic bacteria in foods are gram negative, an estimation of the number of gram-negative bacteria in some foods may be correlated with the keeping quality or shelf life of those products (2, 13, 27). Numbers of psychrotrophic bacteria in raw milk also correlate well with the sanitary conditions of the dairy farm from which the milk originates (16). In this regard, gram-negative bacteria may be used to judge the sanitary history of a raw commodity or to follow changes in the microbial flora during storage, transport, or processing. Coliforms are widely used for these purposes for historical reasons, but detection of the production of acid or gas from lactose by coliforms is beset with numerous problems (10, 11). The enumeration of gram-negative bacteria avoids many of these problems and is more sensitive because of the wider range of indicator-significant bacteria that can be detected.

Enumeration of gram-negative bacteria may also serve another purpose. Because gram-negative bacteria are generally more sensitive to heat than are gram-positive bacteria (4, 20), gram-negative bacteria may be used to assess the efficiency of pasteurization and other bactericidal heat processes. Thus, the presence of gram-negative bacteria in a thermally processed food signifies inadequate heat treatment or postpasteurization contamination (2, 4, 33, 41). Counts of gram-negative bacteria in foods, therefore, serve as indicators of how the food was handled (34).

Several media, such as crystal violet-triphenyl tetrazolium chloride (CV-TTC) agar (2) and peptone bile amphotericin cycloheximide (PBAC) agar (5), have been developed for the enumeration of gram-negative bacteria. Crystal violet-containing media, however, do not inhibit all gram-positive bacteria (5, 23, 37), and such media are inhibitory to some gram-negative bacteria (17, 33, 37). PBAC agar performed well, but some gram-positive bacteria grew on this medium after 48 h of incubation (5).

Although gram-negative bacterial counts on media such as CV-TTC and PBAC agars can be used to obtain an indication of how a food was treated, gram-negative counts cannot be used to evaluate the safety of a food in regard to the presence or absence of pathogenic or toxigenic microorganisms. For this purpose, an index organism must be used. Mossel (34) defines index organisms as organisms of fecal origin that may be used to signal the possible presence of bacteria hazardous to the health of the consumer. In the present study, we used *Escherichia coli* as an index organism.

Feng and Hartman (12) have developed rapid and reliable tests for *Escherichia coli* by incorporating the fluorogenic compound 4-methylumbelliferyl-β-D-glucuronide (MUG) in coliform media. Greater than 96% of *E. coli* isolates produced β-glucuronidase (GUD), an enzyme responsible for the hydrolysis of MUG (21, 29). Among the members of the families *Enterobacteriaceae* and *Vibrionaceae*, approximately 50% of *Shigella* spp. and a minority of *Salmonella* spp. and *Yersinia enterocolitica* were the only bacteria other than *E. coli* that produced GUD (21, 25, 29).

The development of a medium to enumerate both an indicator group (gram-negative bacteria) and an index organism (*E. coli*) would make possible the evaluation, with a single medium, of both the general bacteriological quality and the safety of a product (or the processes used to produce it). We report here the development of a medium that

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contains the ionophore monensin for the selection of gram-negative bacteria and MUG for the differentiation of *E. coli*.

MATERIALS AND METHODS

Bacterial strains. Control strains of *Yersinia* spp. were obtained from three sources: R. V. Lachica, Department of Nutrition and Food Science, University of Arizona, Tucson, Ariz.; N. J. Stern, Beltsville Agricultural Research Center, U.S. Department of Agriculture, Beltsville, Md.; L. A. Holcomb, State Hygienic Laboratory, University of Iowa, Iowa City, Iowa. *Pseudomonas alcaligenes* was also obtained from L. A. Holcomb. *Erwinia carotovora* subsp. *carotovora* and *Xanthomonas phaseoli* pathovar *sojensis* were obtained from E. J. Braun, Department of Plant Pathology, Seed and Weed Sciences, Iowa State University, Ames, Iowa. *Pseudomonas fragi* was obtained from T. G. Rehberger, Department of Food Technology, Iowa State University, Ames, Iowa. All other cultures were obtained from the culture collection of the Department of Microbiology, Iowa State University, or were isolated during this study.

Chemicals and media. Monensin (90 to 95% pure) and 3,3'-thiodipropionic acid (TDPA) were obtained from Sigma Chemical Co. (St. Louis, Mo.). MUG was obtained from Sigma and from Hach Chemical Co. (Ames, Iowa). Difco Plate Count Agar (PCA) was used.

Monensin was aseptically weighed, dissolved in 95% ethanol, and added to sterile, molten (47°C) medium to obtain the desired concentration; the final concentration of ethanol in the medium was ca. 1%. Upon addition of monensin to most media, a slight turbidity developed; this did not seem to affect the results.

PCA was chosen as the basal medium, and PCA containing 35 μg of monensin per ml will be referred to as PM agar. The final formulation of the medium, designated PMK agar, consisted of the following: 23.5 g of PCA, 38 mg of monensin preparation (90% pure, in 10 ml of 95% ethanol), 7.5 g of KCl (0.1 M), 75 mg of MUG, and 1,000 ml of distilled water. MUG was added before the medium was sterilized in an autoclave; it was omitted during injury and pure-culture studies. PBAC agar was prepared as described previously (5), except that stocks of cycloheximide were not frozen; rather, solutions were prepared as needed. CV-TTC agar was prepared as described previously (2). TDPA was dissolved in warm water, and the pH was adjusted to 7.0 before the other ingredients were added (31). Potassium phosphate buffer (0.1 M phosphate [pH 7.0]) was used as the diluent for some freeze-injury studies with *E. coli*. This was prepared by dissolving 5.3 g of KH_2PO_4 and 10.7 g of K_2HPO_4 in 1 liter of distilled water and adjusting the pH, if necessary, with KOH or HCl solutions. However, 0.1% Bacto-Peptone (pH 6.8; Difco) performed better with monensin-containing media, and this diluent was used in all other studies; several other diluents were tested and were unsatisfactory for use with PMK agar.

Injury studies. The activity of monensin against gram-positive and gram-negative species was tested by suspending overnight slants of the organism under study in 4.5 ml of 0.85% NaCl and using this suspension to inoculate appropriate media with and without monensin. The activity of monensin was also tested with injured gram-negative cells. Cultures were injured by freezing as follows: a 7-ml volume of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) was inoculated and incubated for 20 h at 35°C to obtain a population of cells in the stationary phase of growth. If cells in the exponential phase of growth were desired, the cultures in Trypticase soy broth were incubated until a moderate turbidity was observed (usually 2 to 3 h for *E. coli*). The cells were pelleted by centrifugation, the pellet was resuspended in 5 ml of chilled distilled water, and the cells were sedimented again. The resulting pellet was resuspended in 10 ml of chilled distilled water and frozen at −20°C. If desired, the suspension was sampled before freezing to obtain cells that had not been injured. After storage for 24 ± 1 h at −20°C, the suspension was thawed at room temperature with occasional shaking and diluted immediately. The diluent was chilled to 4°C to decrease temperature shock. Cells were plated in triplicate on PCA, PM, and PMK agars and sometimes on PBAC and CV-TTC agars.

E. coli K-12 was also injured with heat. The procedure of Maxcy (30) was used. The cell suspension was sampled after 0 and 12 min of heating, diluted in 0.1% peptone, and plated in triplicate on PCA and PBAC, PM, and PMK agars. A different procedure was used to injure other gram-negative bacteria with heat. A 7-ml volume of Trypticase soy broth was inoculated with the culture and incubated for 20 h at 35°C. The culture was then agitated well with a vortex mixer and was placed in a 55°C water bath. Samples were withdrawn after 0 and 10 min and plated on PCA and PBAC, PM, and PMK agars.

E. coli K-12 was also injured by exposure to NaCl by using the procedure of Maxcy (30). The cell suspension in 5% NaCl was sampled after 20, 60, and 120 min, diluted in 0.1% peptone, and plated on PCA and PBAC, PM, and PMK agars. Plates for all injury studies were incubated at 32°C and were counted after 24 h. The plates were reincubated for an additional 24 h and reexamined for the appearance of more colonies. This was repeated until few or no additional colonies were observed to arise. Final counts for most strains, however, were obtainable after 24 h.

Food and environmental samples. All samples were diluted in 0.1% peptone and surface plated in quadruplicate except on those media from which colonies were to be isolated; these were plated in sets of six or eight. Most samples were plated on PBAC agar, PCA with and without 0.1 M KCl, and PM and PMK agars. In addition, some samples were also plated on the latter four types of media with 1% TDPA. All plates were incubated at 32°C. With some samples, numbers of *E. coli*, as determined with PMK agar, were compared with those obtained by using the French most-probable-number method (38). This method relies on the production of indole and gas from lactose at 44.5°C.

Strains isolated for determination of Gram reaction or identification were picked at random; 56 colonies were usually isolated from each type of medium. Identifications of gram-negative bacteria were made with the Minitek Numerical Identification System (BBL Microbiology Systems). The procedures used were the same as those recommended by the manufacturer, with two exceptions. First, the inoculum broth was prepared by using the formula specified by Minitek except that it was supplemented with 0.1% yeast extract (8). The second modification was that Minitek kits inoculated with presumed nonfermenters were incubated at 32°C because the isolates were from food and environmental sources, not clinical material.

If the unknown was a nonfermenter, additional tests were made: disks of esculin, mannitol, and rhamnose were inoculated. Production of gelatinase, by using a tubed-medium method, and production of DNase, by using DNase test agar (Difco) with 0.01% toluidine blue O, were determined as described by MacFaddin (28). Flagellation of some isolates was determined by the staining method of Kodaka et al. (22). Motility was determined by using the motility stock culture

medium of Hugh (18) or by the hanging-drop technique. When the identification of an isolate by Minitek was dubious, the biochemical reactions were compared with published reactions of pseudomonads and other nonfermenters (14).

Because *P. fragi*, a frequent isolate from meats (40), was not included in the Minitek data base, a known strain of this organism was run through the Minitek system. The numerical code that was generated (corresponding to an atypical *Pseudomonas fluorescens*) was identical to the code number of a large number of strains isolated during this study from raw hamburger. Thus, these isolates were identified as *P. fragi*. Biovars of *P. fluorescens* were determined, and the identification of *P. fragi* was confirmed by using the biochemical reactions reported by Molin and Ternström (32) for 200 isolates from meat.

RESULTS

Selectivity and injury studies. Our findings agree with previous reports that monensin inhibits gram-positive bacteria but demonstrates little or no activity against gram-negative bacteria (15, 26). In the present study, media containing 10 μg of monensin per ml completely inhibited the seven gram-positive species tested: *Bacillus subtilis*, *Lactobacillus plantarum*, *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Streptococcus pyogenes*. No colonies were observed on plates of monensin-containing media of any of the dilutions plated (data not shown). Counts of 12 genera of gram-negative bacteria plated on media containing 35 or 50 μg of monensin per ml, however, were not significantly different from counts on media without monensin (data not shown).

Because naturally contaminated samples may contain injured indicator and index bacteria that are often inhibited by selective media on which they are supposed to be enumerated, cultures of gram-negative bacteria were injured by one of several different treatments, and their recovery with several media was evaluated. When *E. coli* K-12 was injured by freezing, it became sensitive to monensin (Table 1). Counts of freeze-injured *E. coli* K-12 averaged 90% lower on PM agar containing 35 μg of monensin per ml than on nonselective PCA without monensin. Five other strains of *E. coli* that were tested were sensitive to monensin after freezing, but two strains became sensitive only if cells were in the exponential phase of growth when frozen (data not shown).

A variety of substances reportedly help revive or decrease the rate of bacterial injury and death (31, 35, 39). The inclusion of $MgSO_4 \cdot 7H_2O$, $MgCl_2$, sucrose, mannose, maltose, aspartate, or proline in monensin-containing medium had either no or detrimental effects on the recovery of freeze-injured *E. coli* K-12 (data not shown). Likewise, diluents containing various concentrations of magnesium salts did not affect recovery of freeze-injured *E. coli*. However, the inclusion of 1% TDPA in monensin-containing medium completely reversed the inhibition by monensin of freeze-injured *E. coli* K-12; when a suspension of freeze-injured *E. coli* was plated on PCA, PM agar, and PM agar containing 1% TDPA, counts were 1.1×10^6, 7.7×10^5, and 1.5×10^6, respectively. However, as will be explained later, TDPA could not be used with monensin-containing media.

The addition of 0.1 M KCl to PM agar completely reversed the inhibition of freeze-injured *E. coli* K-12 (Table 1). In each of 11 replications, counts on medium with monensin and KCl (PMK agar) were actually higher than counts on nonselective PCA. The addition of KCl to PM agar also reversed the inhibition by monensin of all five of the other strains of injured *E. coli* (data not shown). Results of other experiments demonstrated that 0.1 M KCl was the minimum concentration necessary for full reversal of the inhibition by monensin and that concentrations above 0.1 M did not result in the recovery of significantly more cells (data not shown). The reversal by KCl of the inhibition of injured *E. coli* by monensin was dependent on the diluent used to dilute the injured cells (data not shown); the diluent of choice was 0.1% peptone. Inhibition of injured *E. coli* was also reversed by Li^+, Na^+, and Rb^+ ions (data not shown).

TABLE 1. Inhibition of freeze-injured *E. coli* K-12 by monensin and reversal of the inhibition by addition of 0.1 M KCl to the medium[a]

Trial	Count of cells growing on the following medium:		
	PCA	PM (% injured)	PMK (% increase over PCA)
1	9.7×10^7	8.7×10^5 (99)	1.3×10^8 (34)
2	8.0×10^6	6.5×10^5 (92)	1.9×10^7 (138)
3	5.1×10^7	5.1×10^6 (90)	1.0×10^8 (96)
4	4.1×10^7	2.4×10^6 (94)	8.1×10^7 (98)
5	2.9×10^7	6.4×10^6 (78)	5.2×10^7 (79)
6	2.7×10^7	8.8×10^5 (97)	6.1×10^7 (126)
7	2.3×10^7	1.2×10^6 (95)	2.5×10^7 (9)
8	1.1×10^8	7.6×10^7 (31)	1.4×10^8 (27)
9	4.1×10^7	2.4×10^7 (42)	9.4×10^7 (129)
10	1.5×10^8	3.6×10^7 (76)	2.3×10^8 (53)
11	2.0×10^8	1.5×10^7 (93)	2.8×10^8 (40)
Geometric mean	4.9×10^7	5.0×10^6 (90)	8.3×10^7 (69)

[a] Stationary-phase cells were frozen. Potassium phosphate diluent was used to plate cells.

A number of other gram-negative species were frozen and thawed before plating, but unlike *E. coli*, most species did not become sensitive to monensin (Table 2). With the species that were inhibited, the addition of KCl to PM agar usually reversed the inhibition. In contrast, more species were inhibited (to a greater degree) by PBAC and CV-TTC agars (Table 2).

E. coli K-12 also became sensitive to monensin (PM agar) when injured with heat, and this inhibition was reversed by the addition of KCl to the medium; heat-injured cells were also sensitive to PBAC agar. After exposure to 65°C for 12 min, cells were plated on PCA and PM, PMK, and PBAC agars, and the following counts were obtained (geometric means of two trials): 1.8×10^4, 1.2×10^4, 1.8×10^4, and 3.8×10^3, respectively. Six other gram-negative species were injured with heat and were plated on PCA and PM, PMK, and PBAC agars (Table 3). Five of the six species tested were not sensitive to monensin; counts on PMK agar were the same as or were higher than counts on nonselective PCA. Several of these same species, however, were inhibited to a significant degree by PBAC agar.

E. coli K-12 also became sensitive to monensin when it was injured by exposure to 5% NaCl, but this inhibition was overcome by the addition of 0.1 M KCl to PM agar. After 120 min of exposure to NaCl, the cells were plated on PCA and PM, PMK, and PBAC agars, and the following counts were obtained: 1.8×10^6, 8.1×10^5, 2.3×10^6, and 2.1×10^5, respectively. Results observed after 20 and 60 min of exposure also demonstrated equivalent recoveries on PCA and PMK agar and inhibition by PBAC agar (data not shown).

Food samples. The performance of PMK agar was also tested by plating food and environmental samples. The

TABLE 2. Recovery of selected gram-negative bacteria when frozen, thawed, and plated on five media

Bacteria	Recover of bacteria on the following medium:				
	PCA	PM	PMK	PBAC	CV-TTC
Acinetobacter calcoaceticus biovar *haemolyticus*[a]	1.9×10^7	1.7×10^7	2.4×10^7	7.6×10^6	NT[b]
Aeromonas hydrophila[c]	7.7×10^6	6.4×10^6	6.1×10^6	2.2×10^6	NT
Bordetella bronchiseptica before freezing[d]	8.4×10^8	9.7×10^8[e]	8.5×10^8[e]	NT	NT
Bordetella bronchiseptica after freezing[d]	1.3×10^8	6.5×10^7[e]	6.3×10^7[e]	NT	NT
Enterobacter agglomerans biogroup 6[f]	2.6×10^8	2.9×10^8	3.4×10^8	4.3×10^8[e]	2.6×10^8
Enterobacter gergoviae[f]	8.2×10^6	8.1×10^6	1.1×10^7	9.8×10^6	7.5×10^6
Erwinia carotovora subsp. *carotovora*	1.3×10^7	1.4×10^7	1.6×10^7	4.7×10^6	ND[g]
Escherichia coli K-12[h]	2.8×10^6	2.7×10^6	3.5×10^6	2.6×10^6	1.5×10^6
Escherichia coli K-12[i]	5.0×10^5	3.8×10^5	8.3×10^5	8.7×10^3	4.3×10^4
Escherichia coli KJ2F[h,j]	1.5×10^6	1.6×10^6	2.4×10^6	1.3×10^6	8.3×10^5
Flavobacterium indologenes	8.5×10^6	1.3×10^6	5.3×10^6	2.4×10^6	NT
Klebsiella oxytoca	5.8×10^8	5.5×10^8	5.3×10^8	NT	NT
Neisseria perflava before freezing[k]	1.0×10^8	2.7×10^7	7.2×10^7	3.6×10^7	NT
Neisseria perflava after freezing[k]	1.5×10^7	7.2×10^5	4.4×10^5	ND	NT
Providencia alcalifaciens	1.4×10^8	1.3×10^8	1.8×10^8	5.1×10^7[e]	NT
Pseudomonas aeruginosa	1.4×10^7	1.4×10^7	2.0×10^7	NT	NT
Pseudomonas alcaligenes	1.0×10^7	9.3×10^6	9.1×10^6	1.1×10^7	4.8×10^6
Pseudomonas fluorescens biovar I[l]	3.1×10^7	2.6×10^7	3.1×10^7[e]	3.2×10^7[e]	2.8×10^7
Pseudomonas fluorescens biovar III[l]	3.6×10^6	3.6×10^6	4.7×10^6	2.9×10^6	NT
Pseudomonas fragi[l]	1.6×10^7	2.2×10^7	2.1×10^7	1.6×10^7	NT
Salmonella arizonae	1.3×10^9	1.3×10^9	1.2×10^9	1.2×10^9[e]	NT
Salmonella typhimurium ATCC 14028	4.6×10^6	2.3×10^6	6.8×10^6	4.1×10^6	3.2×10^6
Serratia marcescens[f]	1.8×10^8	1.8×10^8	1.9×10^8	1.8×10^8	NT
Xanthomonas phaseoli pv. *sojensis*	8.2×10^6	8.1×10^6	1.1×10^7	9.8×10^6	7.5×10^6

[a] Isolated from frozen vegetables on violet red-bile-glucose (VRBG) agar.
[b] NT, Not tested.
[c] Isolated from water on VRBG agar.
[d] Brain heart infusion agar was substituted for PCA and counted at 48 h; colony sizes indicated that the cells may be sensitive to KCl.
[e] Colonies were substantially smaller than those on other media.
[f] Isolated from raw milk on PBAC agar.
[g] ND, Not determined. Medium was very inhibitory, and counts could not be determined.
[h] Addition of 0.05 M K_2SO_4 to PM agar also reversed inhibition; 3.2×10^6 cells of *Escherichia coli* K-12 and 1.9×10^6 cells of *Escherichia coli* KJ2F were recovered on PM agar plus K_2SO_4.
[i] Saline was used as diluent. Cells sampled before freezing were also sensitive to PBAC and CV-TTC agars.
[j] Isolated from water.
[k] Before freezing, PCA and PMK agars were counted at 24 h, and PM and PBAC agars were counted at 72 h; after freezing, PCA and PMK agars were counted at 48 h, PM agar was counted at 72 h, and colonies on PBAC agar were pinpoint and impossible to count.
[l] Isolated from raw hamburger on VRBG or PBAC agars.

results of preliminary experiments indicated that when raw hamburger was plated on PCA, PBAC, and PMK agars, a significant number of colonies appeared between 24 and 48 h of incubation. Therefore, 48 h was chosen as the incubation period for all subsequent experiments. An incubation temperature of 32°C was chosen because temperatures of 32°C or less yield higher total and coliform counts than temperatures of 35 or 37°C (36, 43). In addition, it was determined that monensin concentrations of less than 35 μg/ml were not selective enough (data not shown). Therefore, 35 μg/ml was the concentration used in all further studies.

The selectivity of PMK agar was very good with samples of raw hamburger. Three different lots were plated on PMK agar or PMK agar plus 1% TDPA (PMKT agar), and 98% of 324 strains examined were gram negative. Of 213 isolates from nonselective PCA or PCA plus 0.1 M KCl, 75% were gram negative. The results from one sample that was also plated on PBAC agar demonstrated that PMKT agar was more selective; 95% of 55 isolates from PMKT agar were gram negative, whereas 91% of the 55 isolates from PBAC agar were gram negative.

The inclusion of TDPA in PMK agar did not significantly affect the selectivity of that medium when hamburger was examined, but TDPA interfered with selectivity with other sample types. When frozen mixed vegetables (Table 4, sample H) or raw milk (Table 5) were plated on PMK and PMKT agars, substantial numbers of the isolates from PMKT agar were gram positive. However, when one creekwater sample was plated on PMK and PMKT agars, all isolates (53 from each of the media) were gram negative.

The data in Table 4 also demonstrate that the selectivity of PMK agar with frozen vegetables was good and that PBAC agar was not as selective as PMK agar. The results obtained with creekwater samples also demonstrated the better selectivity of PMK agar; all 108 isolates from PMK agar were gram negative, whereas only 87% of the 55 isolates from PBAC agar were gram negative. When soil was examined, all 40 colonies from PMK agar were gram negative; of 40 colonies from PBAC agar, 98% were gram negative.

The selectivity of PMK agar with raw milk was not as good as with the other sample types, but it was still more effective than PBAC agar (Table 5). Some of the yeasts isolated on PBAC agar were retested for resistance to amphotericin B and cycloheximide by streaking them on fresh PBAC agar prepared with freshly prepared stocks of the antibiotics; these isolates were confirmed to be resistant to these antibiotics at the concentrations used in PBAC agar. More yeasts grew on PMK agar than on PBAC agar, but significantly more gram-positive bacteria grew on PBAC agar than on PMK agar. Thus, gram-negative bacteria accounted for a greater proportion of colonies appearing on PMK agar than on PBAC agar.

Results of nine experiments with five sample types were combined to obtain an overall estimate of the selectivity of PMK agar; 96% of 535 isolates from PMK agar were gram negative. If results obtained with hamburger samples plated on PMKT agar are also included, 96% of 689 strains isolated from 10 samples by using PMK and PMKT agars were gram negative. By comparison, of 318 strains isolated on PBAC agar from six of the same nine samples, 88% were gram negative, and control plates of PCA showed that only 68% of 476 colonies examined were gram negative.

The preceding data indicate that PMK agar effectively suppresses the growth of gram-positive bacteria, but the data in Table 6 show that not all of the gram-negative bacteria that grew on nonselective PCA also grew on PMK and PBAC agars. PMK agar recovered approximately the same number of gram-negative bacteria from hamburger as did PCA, but the numbers of gram-negative bacteria recovered with PMK agar from the other sample types were substantially lower than numbers recovered with nonselective PCA. PBAC recovered about the same or fewer numbers of gram-negative bacteria than PMK agar.

Inclusion of KCl in both PCA and PM agar greatly increased the number of bacteria recovered from raw hamburger (Table 7). Inclusion of KCl in these two media resulted in slight to moderate increases in the numbers of bacteria recovered from frozen vegetables and raw milk, but with creekwater samples, the inclusion of KCl in either PCA or PMK agar reduced counts.

Tables 8, 9, 10, and 11 show the identities of isolates recovered from four sample types by using PMK and PBAC agars. These data will be discussed later.

MUG has been incorporated into several agar media at concentrations of 100 or 150 μg/ml to detect *E. coli* (1, 6, 12, 42). In the present study, however, visual examination of PMK agar containing various concentrations of MUG inoculated with *E. coli* demonstrated that a concentration of 75 μg/ml was sufficient for strong positive reactions. In addition, incorporation of KCl and monensin in PCA enhanced the fluorescence; most of this effect was produced by KCl.

We also observed that MUG cannot be incorporated into PBAC agar because pinpoint MUG-positive colonies were recovered from several sample types. These were gram-positive cocci, probably staphylococci or streptococci, which produce β-glucuronidase.

All of the MUG-positive colonies that appeared on PMK agar inoculated with five lots of raw hamburger were isolated and identified. Of the 38 strains isolated, 66% were *E. coli*, 26% were *Y. enterocolitica* sensu stricto biovar 1, and the rest were *Pseudomonas testosteroni*-like strains and *Flavobacterium meningosepticum*. All *Yersinia* isolates were from the same sample. If the *Yersinia* isolates are excluded, 90% of the MUG-positive isolates from hamburger were *E. coli*. The significance of MUG-positive *Yersinia* sp. will be considered below. The number of *E. coli* in one hamburger sample was estimated by both most-probable-number method and by the incorporation of MUG in PMK agar. The most-probable-number estimate was 17,000 *E. coli* per 100 g (95% confidence interval, 5,000 to 46,000). The estimate obtained with MUG in PMK agar was 100,000 ± 38,000 *E. coli* per 100 g.

MUG cannot be used in conjunction with PMK agar to determine the number of *E. coli* in frozen vegetables. Many MUG-positive colonies were observed; the majority of these were identified as *Pseudomonas paucimobilis*, *Flavobacterium thalpophilum*, and *Flavobacterium multivorum*. A significant number of MUG-positive isolates from four samples of creekwater were oxidase-positive strains (*P. paucimobilis*, *F. multivorum*, *P. testosteroni*-like strains, and others). However, all these organisms did not produce fluorescent zones until after 24 h of incubation. In contrast, all of the MUG-positive *E. coli* recovered from creekwater produced fluorescent zones within 24 h. Therefore, the level of *E. coli* in creekwater was determined by counting the MUG-positive colonies on PMK agar at 24 h of incubation. The number of *E. coli* cells in one water sample was 700/100 ml (95% confidence interval, 230 to 1,700) by a most-probable-

TABLE 3. Recovery of heat-injured gram-negative bacteria on four media

Bacterium	Recovery of bacteria on the following medium:			
	PCA	PM	PMK	PBAC
Acinetobacter calcoaceticus[a]	2.3×10^7	2.5×10^7	2.4×10^7	2.4×10^7
Aeromonas hydrophila[b]	1.0×10^6	9.7×10^5	1.2×10^6	4.8×10^{5c}
Enterobacter gergoviae[d]	1.1×10^6	1.4×10^6	1.1×10^6	1.2×10^6
Flavobacterium indologenes[d]	1.6×10^6	9.2×10^{5c}	1.2×10^{6c}	8.2×10^{5c}
Pseudomonas fragi[e]	7.7×10^8	7.6×10^8	8.1×10^8	6.9×10^8
Serratia marcescens[d,f]	5.6×10^7	3.8×10^{7c}	6.5×10^{7c}	1.0×10^7

[a] Biovar *haemolyticus*, isolated from frozen vegetables on VRBG agar.
[b] Isolated from water on VRBG agar.
[c] Colonies were substantially smaller on this medium.
[d] Isolated from raw milk on PBAC agar.
[e] Isolated from raw hamburger on PBAC agar.
[f] Counts were determined at 48 h.

TABLE 4. Percentages of gram-positive and gram-negative bacteria and yeasts isolated from frozen vegetables by using various media

Sample (medium)	Total cells/g	% Gram-positive	% Gram-negative	% Yeasts
H				
PMK (n = 50)[a]	1.1×10^3	2	96	2
PMKT (n = 44)	3.3×10^3	30	66	5
I				
PMK (n = 54)	2.2×10^3	0	100	0
PBAC (n = 56)	1.6×10^3	7	93	0

[a] n, Number of colonies examined.

TABLE 5. Percentages of gram-positive and gram-negative bacteria and yeasts isolated from raw milk by using various media

Sample (medium)	Total cells/ml	% Gram-positive	% Gram-negative	% Yeasts
P				
PCA (n = 56)[a]	6.2×10^3	57	43	0
PMK (n = 56)	1.8×10^3	2	93	5
PMKT (n = 54)	2.1×10^3	14	82	4
PBAC (n = 56)	1.7×10^3	9	89	2
Q				
PCA plus 1% TDPA (n = 55)	8.1×10^2	44	53	4
PMK (n = 56)	3.4×10^2	5	80	14
PBAC (n = 55)	2.6×10^2	18	76	6

[a] n, Number of colonies examined.

TABLE 6. Plate counts, percentages, and adjusted counts and percentages of gram-negative bacteria recovered from food and water samples on three media

Sample, type	PCA			PMK				PBAC			
	Count	% G−	G− count[a]	Count	% G−	G− count	% of PCA G−[b]	Count	% G−	G− count	% of PCA G−
B, hamburger	7.0×10^6	74	5.2×10^6	6.8×10^6	96	6.5×10^6	125	NT[d]			
D, hamburger	1.4×10^7	85	1.2×10^7	1.1×10^7	100	1.1×10^7	92	NT			
E, hamburger	7.0×10^6	60	4.2×10^6	3.8×10^6	95	3.6×10^6	86	3.9×10^6	91	3.6×10^6	86
L, water	1.6×10^5	87	1.4×10^5	6.5×10^4	100	6.5×10^4	46	NT			
P, milk	6.2×10^3	43	2.7×10^3	1.8×10^3	93	1.7×10^3	63	1.7×10^3	89	1.5×10^3	56
Q, milk	8.1×10^2	53	4.3×10^2	3.4×10^2	80	2.7×10^2	63	2.6×10^2	76	2.0×10^2	47
T, milk	1.1×10^4	85	9.4×10^3	1.0×10^4	98	9.8×10^3	104	NT			

[a] Gram-negative (G−) count = (total count) × (% gram-negative bacteria).
[b] % of PCA gram-negative bacteria = (G− count on selective medium)/(G− count on PCA) × 100.
[c] NT, Not tested.

number method and 350 ± 200/100 ml when PMK agar was used. With raw milk, all the MUG-positive strains from five samples were isolated and identified; 95% of them were *E. coli*.

DISCUSSION

Monensin imparted the desired selectivity when added to culture media. Pure cultures of gram-positive species were completely inhibited by monensin, but gram-negative species were not. Even when injured by one of several methods, most gram-negative species did not become sensitive to monensin. For those that did become sensitive, the addition of 0.1 M KCl to PM agar reversed the inhibition in most instances. The sample diluent was also an important factor; 0.1% peptone should be used in conjunction with monensin-containing media. In contrast, however, PBAC and CV-TTC agars were inhibitory to several freeze-injured gram-negative species. Likewise, several of the gram-negative species injured with heat were partially inhibited by PBAC agar (CV-TTC agar was not tested).

The fact that KCl reverses the inhibition by monensin of gram-negative bacteria injured by any of several methods is striking. Many resuscitation techniques are effective when cultures are subjected to one type of injury but are not effective when cells are injured in other ways (35).

It is not surprising that a variety of compounds that reportedly reverse inhibition of injured organisms were not effective in reversing the inhibition by monensin of freeze-injured *E. coli*. Some of these compounds are undoubtedly effective only with the particular combinations of culture media, organisms, and types of injury employed in the original study (35).

The results of pure-culture studies indicated that PMK agar was less inhibitory than were PBAC or CV-TTC agars for gram-negative bacteria, and experiments with food and environmental samples demonstrated that PMK agar was more selective than was PBAC agar against gram-positive bacteria (CV-TTC agar was not tested). However, not all gram-negative bacteria from naturally contaminated samples that grew on nonselective PCA also grew on PMK agar (Table 6). Both PMK and PBAC agars shared this shortcoming, but PMK agar recovered as many or more gram-negative bacteria than did PBAC agar in three of four instances. With a soil sample, however, although the percentages of gram-negative bacteria recovered from PBAC and PMK agars were about the same (98 and 100%, respectively), more colonies grew on PBAC agar. Soil bacteria may prefer the nutritionally poor base of PBAC agar to the richer base of PMK agar. Larkin (24) reported that 0.1% peptonized milk (Difco) agar (a nutritionally poor medium) recovered more organisms from soil than did Trypticase soy agar (a richer medium).

Particularly striking were the effects of including KCl in PCA and PM agar. Counts of raw milk and frozen vegetables were increased slightly to moderately on media supplemented with KCl, and counts of raw hamburger were increased substantially (Table 7). If these observations are confirmed, the routine addition of KCl to plating media would be warranted. Elucidation of the mechanism(s) behind these increased recoveries would be helpful in indicating other commodities and media with which KCl supplementation should be used. With water samples, however, inclusion of KCl in PCA or PM agar resulted in lower counts (Table 7). In retrospect, it is advisable that KCl should not be added when examining water samples with monensin-containing medium. Also, it may be advisable to include cycloheximide and amphotericin B in PMK agar when examining sample types that may contain relatively high numbers of yeasts or molds (such as raw milk and soil). When soil was examined, these two antibiotics were incorporated in PMK

TABLE 7. Effects of inclusion of 0.1 M KCl in PCA and monensin-containing medium

Sample type	No. of trials	Recovery of bacteria on the following medium:		% Increase or decrease	Recovery of bacteria on the following medium:		% Increase or decrease
		PCA	PCA plus 0.1 M KCl		PM	PMK	
Raw hamburger	4	5.4×10^{6a}	8.0×10^6	+48	3.9×10^6	5.3×10^6	+36
Frozen vegetables	2	2.4×10^{4b}	2.5×10^{4b}	+4	1.5×10^3	1.6×10^3	+7
Raw milk	4	3.2×10^3	3.5×10^3	+9	1.0×10^3	1.2×10^3	+20
Creekwater	2	1.2×10^5	5.2×10^4	−57	3.2×10^4	2.3×10^4	−28

[a] Geometric mean.
[b] Data for only one trial reported.

agar at the same levels as employed in PBAC agar; no fungi were recovered from either medium.

Identification of 393 of the strains isolated from four sample types by using PMK and PBAC agars was complicated and not entirely successful. Major problems stemmed from the fact that most commercial identification systems are based on data collected mainly from clinical isolates. Identification of environmental and food isolates by using such data bases is therefore hindered (3). However, determination of the flora recovered with a newly developed medium or technique is crucial and too often ignored (9). Many media actually select for different genera or species among the group of organisms for which the media are designed to enumerate; identification of the isolates would detect this.

Table 8 shows the flora recovered from raw hamburger. Many of the unidentified strains were classified by Minitek as *Moraxella* spp., *Alcaligenes* spp., or *Pseudomonas* spp. These nonsaccharolytic strains were similar to the *Alcaligenes* spp. and *Moraxella* spp. and *Moraxella*-like bacteria reported by several authors to occur in ground meats (7, 19). Examination of the floras recovered from hamburger with PMK and PBAC agars showed that more isolates of *P. fragi* were recovered on PMK agar, whereas more isolates of *P. fluorescens* were recovered on PBAC agar. Because freeze- or heat-injured *P. fragi* was partially inhibited by PBAC agar, and because PMK agar did not inhibit injured *P. fluorescens* (Tables 2 and 3), it seems that inhibition of some *P. fragi* by PBAC agar was responsible for the differences in percentage recoveries of these species on the two media.

Aeromonas hydrophila, a common water bacterium, was recovered from creekwater with PMK agar but not with PBAC agar (Table 10). The data in Tables 2 and 3 indicate that freeze- or heat-injured *Aeromonas hydrophila* is inhibited by PBAC agar. Likewise, more isolates of *Flavobacterium indologenes* (formerly CDC group IIb) were recovered from raw milk on PMK agar than on PBAC agar (Table 11). Freeze- or heat-injured *F. indologenes* was inhibited by PBAC agar (Tables 2 and 3). Injured *F. indologenes* was also inhibited by PMK agar (as compared with PCA), but PMK agar still recovered significantly more injured cells than did PBAC agar. More isolates of several biovars of *Acinetobacter calcoaceticus* were recovered from frozen vegetables and raw milk with PBAC agar than with PMK agar (Tables 9 and 11). However, when a strain of *Acinetobacter calcoaceticus* biovar *haemolyticus* isolated from frozen vegetables was injured with heat, it was not inhibited by PMK agar nor by PBAC agar (Table 3). When the same strain was injured by freezing, it was inhibited by PBAC agar but not by PMK agar (Table 2). It therefore seems that the greater frequency of acinetobacters on PBAC agar was caused by inhibition of nonacinetobacters by that medium.

Although Kilian and Bülow (21) reported that the 10 strains of *Y. enterocolitica* that they examined were GUD negative, the isolation of MUG-positive *Y. enterocolitica* in the present study agrees with the results of Massenti et al. (29), who reported that a minority of the 23 strains that they examined produced GUD. In the present study, control strains of yersiniae, obtained from three laboratories, were tested for GUD; all seven strains of *Y. enterocolitica* (with and without the 42-megadalton virulence-associated plasmid) were negative, as were three strains of *Yersinia frederiksenii*, two of *Yersinia intermedia*, and one of *Yersinia kristensenii*. Therefore, the infrequent occurrence of GUD-positive yersiniae should not be a problem when using PMK agar, especially in light of the fact that all of the GUD-pos-

TABLE 8. Gram-negative bacteria isolated from raw hamburger by using two media

Bacterium	No. (%) of isolates recovered on the following medium:	
	PMKT	PBAC
Acinetobacter calcoaceticus		
Biovar *alcaligenes*	1 (2)	1 (2)
Biovar *lwoffii*	1 (2)	0 (0)
Pseudomonas fluorescens		
Biovar I	8 (15)	12 (24)
Biovar III	3 (6)	7 (14)
Pseudomonas fragi	30 (57)	20 (40)
Unidentified oxidase-positive strains	10 (19)	10 (20)

TABLE 9. Gram-negative bacteria isolated from frozen mixed vegetables by using two media

Bacterium	No. (%) of isolates recovered on the following medium:	
	PMK	PBAC
Acinetobacter calcoaceticus		
Biovars *anitratus* and *haemolyticus*	29 (55)	32 (64)
Biovar *lwoffii*	2 (4)	0 (0)
Enterobacter agglomerans	1 (2)	0 (0)
Enterobacter sakazakii	2 (4)	2 (4)
Flavobacterium indologenes	2 (4)	3 (6)
Flavobacterium meningosepticum	3 (6)	0 (0)
Pseudomonas paucimobilis	2 (4)	3 (6)
Unidentified oxidase-positive strains	12 (23)	10 (20)

TABLE 10. Gram-negative bacteria isolated from surface water by using two media

Bacterium	No. (%) of isolates recovered on the following medium:	
	PMK	PBAC
Acinetobacter calcoaceticus biovar *lwoffii*	3 (6)	5 (11)
Aeromonas hydrophila	4 (7)	0 (0)
CDC group EF-4	0 (0)	2 (4)
Enterobacter agglomerans	4 (7)	4 (9)
Flavobacterium indologenes	1 (2)	0 (0)
Flavobacterium multivorum	0 (0)	3 (6)
Flavobacterium thalpophilum	0 (0)	2 (4)
Pseudomonas alcaligenes	8 (15)	11 (23)
Pseudomonas paucimobilis	0 (0)	3 (6)
Pseudomonas pseudoalcaligenes	3 (6)	6 (13)
Pseudomonas putrefaciens	2 (4)	0 (0)
Nonsaccharolytic oxidase-positive strains	9 (16)	0 (0)
Unidentified oxidase-positive strains	18 (33)	10 (21)
Unidentified oxidase-negative strains	2 (4)	1 (2)

TABLE 11. Gram-negative bacteria isolated from raw milk by using two media

Bacterium	No. (%) of isolates recovered on the following medium:	
	PMK	PBAC
Achromobacter xylosoxidans	0 (0)	1 (2)
Acinetobacter calcoaceticus biovar *alcaligenes*	0 (0)	3 (7)
Citrobacter diversus	0 (0)	2 (5)
Enterobacter agglomerans[a]	6 (14)	2 (5)
Enterobacter cloacae	0 (0)	1 (2)
Enterobacter gergoviae[b]	17 (41)	17 (39)
Flavobacterium indologenes	10 (24)	7 (16)
Pseudomonas maltophilia	0 (0)	1 (2)
Serratia marcescens	3 (7)	2 (5)
Unidentified oxidase-positive strains	6 (14)	8 (18)

[a] Several biogroups.
[b] Nitrate- and malonate-negative strain.

itive *Y. enterocolitica* isolated in the present study were recovered from a single sample.

Several points must be emphasized about the use of MUG in PMK agar to estimate *E. coli*. Results of the present study demonstrate that this technique can be used with raw hamburger, water, and raw milk but not with frozen vegetables. Obviously, if this medium is employed with other types of samples, the specificity of MUG for indicating *E. coli* must be tested and confirmed. It may be advantageous to determine the oxidase reaction of MUG-positive colonies because almost all of the false-positive reactions observed were caused by oxidase-positive strains. This is especially important if a large number of colonies become MUG positive between 24 and 48 h of incubation; indeed, counts of MUG-positive colonies from water should be determined after no longer than 24 h of incubation. If the oxidase test is not performed, any pigmented, MUG-positive colony should be disregarded. In addition, it is important to distinguish between the dull greenish fluorescence of some pseudomonad colonies and the brilliant blue fluorescence around GUD-positive colonies. Because *E. coli* usually constitutes only a small percentage of total gram-negative bacteria in most samples, MUG-positive colonies of *E. coli* appear on plates of relatively low dilutions. Thus, it is important to examine plates for fluorescence even if there are too many total colonies to count on those plates. Finally, PMK agar cannot be used for precise numerical determinations of the numbers of *E. coli* in most samples because of the great number of other gram-negative bacteria that grow on this medium. Rather, this technique will be useful in determining the approximate level or order of magnitude of contamination by *E. coli* (i.e., low, moderate, or heavy).

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