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Improved Medium for Selective Isolation and Enumeration of *Bifidobacterium*

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Petuely's selective medium for *Bifidobacterium* was improved by addition of riboflavin, nucleic acid bases, pyruvic acid, and nalidixic acid. The modified medium, when examined under strictly anaerobic conditions for efficient isolation of *Bifidobacterium* from human fecal samples, exhibited selective and high viable counts that were close to those found on the usual nonselective medium.

In 1956, Petuely (12) proposed a simple synthetic medium consisting of lactose, ammonium acetate, cystine, biotin, and pantothenic acid and indicated that the medium enabled the selective isolation of *Bifidobacterium* from human fecal samples. Two years later, Gyllenberg and Carlberg (4) demonstrated that riboflavin and nucleic acid bases were essential for growth of some strains of bifidobacteria after the isolation of three different nutritional types and examined the effects of these supplements. In the present study, we attempted to improve Petuely's synthetic medium (PSM) in order to obtain more quantitative recovery and higher selectivity for *Bifidobacterium*. We developed modified PSM supplemented with nalidixic acid in addition to essential components such as riboflavin, nucleic acid bases, and pyruvic acid. The new selective medium (MPN medium) for *Bifidobacterium* was evaluated in experiments carried out with human fecal samples under strictly anaerobic conditions.

MATERIALS AND METHODS

Strains. One hundred and fifty-four strains and eight reference strains of *Bifidobacterium* were used; the eight reference strains, i.e., *B. bifidum* E319, *B. infantis* S12, *B. infantis* subsp. *lactentis* 659, *B. infantis* subsp. *liberorum* S76e, *B. breve* S1, *B. breve* subsp. *parvulorum* aS 50, *B. adolescentis* aE 194a, and *B. longum* E194b, were kindly supplied by T. Mitsuoka, Institute of Physical and Chemical Research, Wako, Saitama, Japan. The strains were divided as follows: *B. bifidum*, 33; *B. infantis*, 4; *B. infantis* subsp. *lactentis*, 1; *B. infantis* subsp. *liberorum*, 15; *B. breve*, 19; *B. breve* subsp. *parvulorum*, 55; *B. adolescentis*, 12; and *B. longum*, 17. All of these strains were isolated from infant feces in our institute. In addition, the following strains of other genera were also used: *Eubacterium aerofaciens* VPI 1003, *E. contortum* VPI 8700, *E. cylinderoides* VPI 8072, *E. eligens* c15-48, *E. limosum* VPI 6684, *Bacteroides fragilis* ATCC 23745 and 25285, *B. ovatus* VPI 10649, *B. distasonis* VPI 4243, *B. vulgatus* VPI 8482, *B. hypermegas* VPI 2366-1, *Fusobacterium necrophorum* ATCC 25286, *F. russi* VPI 0307, *F. varium* ATCC 8501, *Clostridium bifermentans* NCTC 504, *C. butyricum* IFO 3315, *C. innocuum* ATCC 14501, *C. perfringens* ATCC 10543, *C. sorderii* ATCC 9714, *C. sporogenes* ATCC 3584, *Lactobacillus catenaforme* VPI 1553-1, *L. crispatus* VPI 3199, *Streptococcus durans* IFO 13131, *S. equinus* IFO 12553, *S. faecalis* IFO 3826, 3971, 12964, and *Escherichia coli* IFO 12734.

Anaerobic techniques. Medium preparation, dilution, and inoculation were anaerobically carried out according to the modified Hungate method (1, 6).

Media. Modified VL-G medium (MVL-G) (1) was used as a nonselective medium which could support growth of all strains used. The ingredients of this medium were as follows: 7.5 ml of 0.1% K_2HPO_4 (solution I); 7.5 ml of salt solution II, consisting of 0.6% KH_2PO_4, 1.2% $(NH_4)_2SO_4$, 1.2% NaCl, 0.12% $MgSO_4 \cdot 7H_2O$, and 0.12% $CaCl_2 \cdot 2H_2O$; 0.1% resazurin, 0.1 ml; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 1 g; yeast extract (Difco Laboratories, Detroit, Mich.), 0.5 g; meat extract (BBL), 0.2 g; glucose, 0.5 g; 8% Na_2CO_3, 5 ml; 3% cysteine-HCl, 1 ml; and distilled water, 79 ml. Agar (2 g per 100 ml of medium) was added for the solid medium. Diluent for cultures and fecal specimens contained the following in 100 ml: 7.5 ml each of salt solution I and salt solution II; 0.1% resazurin, 0.1 ml; 10% Na_2CO_3, 3.0 ml; 5% cysteine-HCl, 1.0 ml; and distilled water, 81 ml. The medium and the diluent were adjusted to pH 6.8 and sterilized at 121°C for 15 min.

The ingredients in 100 ml of MPN were as follows: lactose, 2 g; $(NH_4)_2SO_4$, 0.5 g; K_2HPO_4, 0.1 g; 0.5 ml of a salt solution consisting of 10 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $FeSO_4 \cdot 7H_2O$, 0.4 g of $MnSO_4 \cdot 2H_2O$, and 0.5 g of NaCl in 250 ml of distilled water; 0.1% resazurin, 0.1 ml; biotin, 0.01 mg; pantothenic acid, 0.2 mg; riboflavin, 0.1 mg; adenine, guanine, xanthine, and uracil, 0.1 mg each; Tween 80, 0.1 g; 10% pyruvic acid, 0.1 ml; 8% Na_2CO_3, 5.0 ml; 3% cysteine-HCl, 1 ml; nalidixic acid, 10 mg; 1.6% bromocresol purple, 0.1 ml; and agar, 2 g. The pH of PSM and MPN medium was adjusted to 6.8. Portions (5 ml) of the media in roll tubes were sterilized at 100°C for 30 min and stored at 5°C until used. Autoclaving was unnecessary, probably because the media were relatively simple synthetic, prereduced preparations.

Evaluation of growth. Test strains were grown in MVL-G broth for 24 to 48 h at 37°C. Cultures were

washed twice and diluted with anaerobic diluents. Volumes (0.5 ml) of the diluted suspension were inoculated in duplicate into roll tubes containing the MVL-G and test media. After incubation at 37°C for 3 to 5 days, the colonies in the tube were counted. Usually a culture showing a colony count ranging between 30 and 300 was used for enumeration. Growth in the test medium was read as positive when the ratio of colony count in the test medium against that in nonselective MVL-G medium was more than 50%.

Test for fecal sample. A 1-g (wet weight) portion of fecal sample was added to the test. A 0.5-ml amount of a 10^{-8} or 10^{-9} dilution was transferred into MPN medium and subjected to roll tube culture. After incubation at 37°C for 5 days, 70 to 80% of the developed colonies were transferred into MVL-G medium. Isolates were analyzed biochemically and for identification of the genus *Bifidobacterium*. The pure cultures were first tested for oxygen sensitivity. Biochemical identification of *Bifidobacterium* was performed according to the *Anaerobe Laboratory Manual* (5). Routine identification was based on Gram stain, morphological characteristics and analysis of fermentation end products by gas chromatography (5). The ability of isolates to ferment various carbohydrates, except for glucose, was tested as described by Mitsuoka (9). Determination of species was performed according to the method of Mitsuoka (9, 10).

The number of *Bifidobacterium* in fecal samples was calculated from the colony counts identified as *Bifidobacterium* in MVL-G and MPN media.

RESULTS

Effects of nutritional requirements. PSM was supplemented with riboflavin, nucleic acid bases, or pyruvic acid to obtain enhanced growth of bifidobacteria. Of 162 strains of 8 bifidobacterial species cultured on PSM, only 114 (70.4%) were growth positive. When riboflavin was added to PSM, the growth of 142 strains (87.7%) was positive. Additional supplementation with nucleic acid bases had little effect, but 154 strains (95%) showed positive growth when pyruvic acid was added in the presence of riboflavin and the nucleic acid bases (Table 1).

Effects of antibiotics. Forty strains of bifidobacteria arbitrarily selected were tested first in the presence of 200 μg of nalidixic acid, polymixin B, or neomycin per ml for growth in nonselective MVL-G medium. We found that nalidixic acid, polymyxin B, and neomycin allowed positive growth of 31 (77.5%), 23 (57.5%), and 6 (15.0%) strains, respectively. Since the results showed that nalidixic acid was the least inhibitory, we chose this antibiotic for our selective media in the following experiments. In addition, six arbitarily selected strains of *Bifidobacterium* and nine strains of other genera were examined for growth responses to MPN medium with or without nalidixic acid after 5 days of incubation. The maximal optical density (OD) at 660 nm obtained with bifidobacterial strains was 1.07 ± 0.78 (mean ± standard deviation) and 0.87 ± 0.84 in the presence of 100 and 200 μg of nalidixic acid per ml, respectively. In contrast, the OD obtained in the control media, MPN and MVL-G, was 1.25 ± 0.92 and 1.84 ± 0.31, respectively. No species other than those of *Bifidobacterium* grew in MPN medium without nalidixic acid except one strain of *Bacteroides fragilis* ATCC 23745, although the maximal OD of the strain was reduced significantly from 2.44 to 0.08 in the presence of 100 μg of nalidixic acid per ml. Based on these findings, we propose a new modified Petuely's medium (MPN) which includes riboflavin, nucleic acid bases, pyruvic acid, and nalidixic acid. The selection efficiency of this

TABLE 1. *Effects of riboflavin, nucleic acids, and pyruvic acid on the growth of Bifidobacterium*[a]

Species	No. of strains tested	No. of strains showing positive growth in PSM supplemented with:			
		None	Riboflavin	Riboflavin + nucleic acid	Riboflavin + nucleic acid + pyruvate
B. bifidum	33	28	32	32	32
B. infantis	5	4	4	4	5
subsp. *liberorum*	16	16	16	16	16
subsp. *lactentis*	1	1	1	1	1
B. breve	20	13	15	15	18
subsp. *parvulorum*	56	37	49	49	53
B. adolescentis	18	5	14	14	16
B. longum	13	10	11	11	13
Total	162	114 (70.4)[b]	42 (87.7)	142 (87.7)	154 (95.1)

[a] For evaluation for positive growth, refer to the text. Riboflavin, 100 μg, nucleic acids (adenine, guanine, uracil, and xanthine), 100 μg of each, and pyruvic acid, 10 mg, were added to 100 ml of medium. In preliminary experiments, each of the concentrations as indicated above was determined on the basis of growth responses (maximal OD) and compared with the recommended concentrations of Gyllenberg and Carlberg (4) except for pyruvic acid.

[b] Numbers in parentheses are percentages.

medium for bifidobacteria was examined by using 28 strains of *Bifidobacterium* and 30 strains of other strict and facultative anaerobes (Table 2). All of the *Bifidobacterium* strains were growth positive and formed colonies greater than 1 mm in diameter on the 5th day of incubation, whereas none of 30 cultures of other species formed colonies, except one strain each of *E. contortum* VPI 8700 and *C. innocuum* ATCC 14501; these colonies were less than 0.1 mm in diameter.

Further study revealed that nalidixic acid at a concentration of 100 μg/ml in MPN medium was also effective for suppressing all of the following strains: *E. aerofaciens* (VPI 1003), *E. limosum* (VPI 6684), *B. fragilis* (ATCC 23745), *B. ovatus* (VPI 10649), and *C. perfringens* (ATCC 10543).

Enumeration of bifidobacteria in feces. A qualitative and quantitative analysis of bifidobacteria in human fecal samples was performed with MPN medium. Fifteen fecal samples of five healthy adults were anaerobically cultured on MPN medium, and 175 strains were established from 175 colonies on the medium and then submitted to the test for identification. Of 175 strains, 164 (94%) were identified as *Bifidobacterium*. Of these 89 strains (51%) were identified as *B. adolescentis*, 40 (23%) as *B. longum*, and 31 (18%) as *B. bifidum*; 4 strains (2%) were unidentified *Bifidobacterium* (Table 3). The remaining isolates of the other genus consisted of strains of *Eubacterium* and *Peptostreptococcus*.

TABLE 2. *Growth on MPN medium of different species of Bifido bacterium and other strict or facultative anaerobes*

Species	No. of strains tested	Growth on MPN[a]
Bifidobacterium bifidum	3	3
B. infantis	4	4
subsp. *liberorum*	4	4
subsp. *lactentis*	1	1
B. breve	3	3
subsp. *parvulorum*	5	5
B. adolescentis	4	4
B. longum	4	4
Total	28	28
Eubacterium sp.	7	1[b]
Bacteroides sp.	6	0
Fusobacterium sp.	3	0
Clostridium sp.	6	1
Lactobacillus sp.	2	0
Streptococcus sp.	6	0
Escherichia coli	3	0
Total	30	2

[a] Incubation period, 5 days.

[b] Colonies formed were less than 0.1 mm in diameter.

TABLE 3. *High recovery ratio on MPN medium of Bifidobacterium from 15 human fecal samples*

Genus	No. of isolates	Species
Bifidobacterium and non-*Bifidobacterium*	175	
Bifidobacterium	164 (94)[a]	*B. adolescentis*, 89 (51) *B. longum*, 40 (23) *B. bifidum*, 31 (18) Unidentified, 4 (2)
Non-*Bifidobacterium*	11 (6)	*Eubacterium* spp., 5 (3) *Peptostreptococcus* spp., 6 (3)

[a] Numbers in parentheses are percentages.

Another quantitative analysis for bifidobacteria in human fecal samples of 19 adults was performed, using MPN and the nonselective MVL-G media. A total of 678 colonies, that is, over 70% of the total colonies developed on MVL-G medium at a 10^{-9} dilution of fecal suspension, were identified to the genus level. Of these, 206 (30.4%) were identified as strains of *Bifidobacterium*. The total number of *Bifidobacterium* on MVL-G medium was calculated from the recovery ratio of the colonies identified as *Bifidobacterium*. The total number of *Bifidobacterium* per gram (wet weight) of human fecal samples was 10.39 ± 0.19 ($\log_{10}$ colony-forming units; 95% confidence limit) for cultures on MVL-G medium and 10.30 ± 0.16 for cultures on MPN medium (Fig. 1). The finding that no significant difference in the recovery between the two media was found by the paired *t*-test suggests the high efficiency of selection for *Bifidobacterium* by MPN medium.

DISCUSSION

Several media are currently available for selective isolation of bifidobacteria (3, 11). In most of these media, an antibiotic such as neomycin, kanamycin, or nalidixic acid was used as the primary selective agent. Finegold et al. (3) showed that any antibiotic system in conventional nonselective media was not fully selective and in some cases failed to promote growth of *Bifidobacterium*. In view of these results, we attempted to improve Petuely's synthetic medium by supplementing to meet previously reported nutritional requirements (4, 5). We preferred nalidixic acid to other antibiotics because it inhibited growth of *Bifidobacterium* least and because it effectively inhibited the growth of strains of the other genera (8, 13). Our later experiments disclosed that the inclusion of nalidixic acid at a concentration of 100 μg/ml in our MPN medium sufficiently suppressed almost all

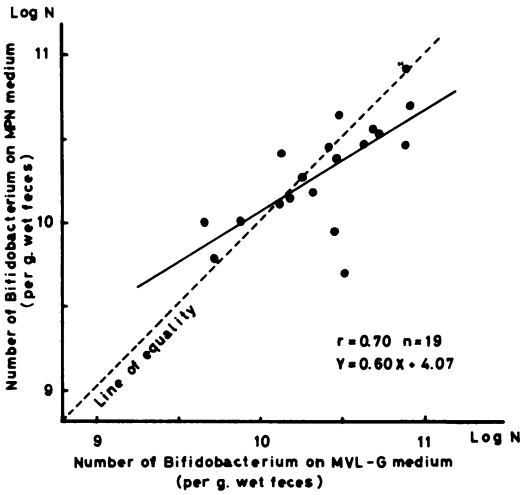

FIG. 1. *Comparative recovery of Bifidobacterium by MPN and nonselective MVL-G media from fecal specimens of 19 healthy adults.*

non-*Bifidobacterium* strains. Furthermore, as for *Bifidobacterium* flora of human adults, the total number and the species pattern of bifidobacteria obtained from MPN medium were in general agreement with the recent report of Mitsuoka and Kaneuchi (10). Fecal culture samples from the new medium gave rise to *Peptostreptococcus* and *Eubacterium*, but in very low numbers.

Evidence is accumulating which suggests that the predominant bacteria of human feces have relatively simple nutritional requirements (2, 14). The present study shows that bifidobacteria can grow abundantly with ammonia as the sole nitrogen source if strictly anaerobic conditions are provided. In addition, Matteuzzi et al. (7) indicated that a large number of bifidobacteria preferred ammonia to organic nitrogen compounds such as amino acids as the nitrogen source. This information would be of considerable interest in assessing the role of intestinal bifidobacteria in relation to ammonia metabolism in the human ecosystem. The relatively simple synthetic medium described here shows promise for meeting the nutritional requirements of *Bifidobacterium*. Further work is now in progress with the strains that did not grow in PSM to explore other nutritional requirements such as various amino acids, peptides, B vitamins, and certain *N*-substituted glucosamine derivatives such as *N*-acetyl-D-glucosamine. Our experiments with fecal samples under strictly anaerobic conditions confirmed the efficacy of the new medium for selective isolation and enumeration of bifidobacteria.

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