Development of a Selective Enterococcus Medium Based on Manganese Ion Deficiency, Sodium Azide, and Alkaline pH

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Rogosa broth, without its salt supplement and dissolved in deionized water, was adapted for the selective isolation and enumeration of enterococci. This medium supported good growth of enterococci, but it suppressed growth of other lactic acid bacteria. The sensitivity and specificity of the medium were tested after addition of various increasing concentrations of NaN_3 against known strains of enterococci and other bacteria. Many strains of *Streptococcus faecium* showed low azide tolerance; optimal growth was obtained at a concentration of 0.01% NaN_3, which totally, or partially inhibited unrelated species of lactic acid bacteria. The selectivity of the medium was further increased by pH adjustment to 9.6. Carbonate and Tween 80 were added to overcome partial inhibition of enterococcal growth by the new combination of selective conditions. The final medium was evaluated in agar form in isolations from human and animal feces, polluted water, meat, and dairy products. Counts were obtained after 16 to 17 h of incubation at 37 C. The isolates satisfactorily conformed to the group characteristics of enterococci.

Depletion of available Mn^{2+} levels in nutrient media results in a significant reduction of growth by lactobacilli (7) and pediococci (6), but has no effect on the growth of enterococci (6). Since the former groups of bacteria frequently occur in nature together with enterococci, the utilization of Mn^{2+} deficiency might facilitate the isolation of enterococci from mixed microfloras. A selective medium utilizing this premise was developed in the present study. The sensitivity and specificity of the medium was tested against known strains of bacteria and in isolations from various natural sources.

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MATERIALS AND METHODS

Cultures. Authenticated strains of enterococci were used for the initial evaluation of the medium. They comprised *Streptococcus faecalis*, American Type Culture Collection (ATCC) 11700, 14428, and strain 19-1, obtained from W. H. Seeley, Cornell University; *S. faecium* typical strains, ATCC 6057, and 13 other strains from the culture collection maintained in this laboratory. The latter strains had been previously isolated from dairy products and identified according to Deibel (3, 4); *S. faecium* atypical strains, FCMA-2 (var. *casseliflavus*) and FCMA-11, were obtained from J. O. Mundt, Univer-

sity of Tennessee, and T-15 (a Langston strain isolated from grass silage) was received from H. W. Seeley. In addition, we included three strains of *S. bovis*, SS-752, 963, and 964, and three strains of *S. equinus*, SS-945, 946, and 947, received from R. R. Facklam, Center for Disease Control, Atlanta, Ga.

Other lactic acid bacteria, not related to fecal streptococci, included: *S. lactis* ATCC 7962, *S. cremoris* ATCC 9625, *S. diacetilactis* DRC-1, and two group N strains received from Rebecca Lancefield, Rockefeller University, New York; *Leuconostoc mesenteroides*, two strains obtained from J. O. Mundt, *L. dextranicum* Ld688 and *L. citrovorum* 91404, obtained from W. E. Sandine, Oregon State University, and *L. citrovorum* A1, obtained from T. W. Keenan, Purdue University; *Pediococcus cerevisiae* ATCC 8081, 8042, 10981 and strain no. 25, obtained from H. W. Seeley; *Lactobacillus bulgaricus* G5 and *L. jugurti* G2, isolated from Greek yogurt by C. J. Efthymiou; and one strain each of *L. casei* and *L. brevis*, obtained from the Midwestern Culture Service, Terre Haute, Ind. Five strains of different gram-negative and gram-positive organisms with cytochrome- and catalase-dependent metabolism (see Table 3) were also included to test the selective capacity of the NaN_3-containing medium.

Culture media. As basal medium we used a modified Rogosa broth (6); the salt supplement of the complete medium was deleted; all ingredients were dissolved in deionized, all-glass-distilled water. NaN_3 was added at different, increasing amounts: 0.01 0.015, 0.02, 0.025, 0.03, and 0.05%. The resultant media were evaluated against our test cultures. The concentration adopted for the final medium was 0.01% NaN_3. Various initial pH values were also

examined, and we finally selected pH 9.6 as the most suitable; in conjunction with this modification, 5.3 g of Na_2CO_3 per liter was added.

The final selective medium of this study (Table 1) was prepared in the following manner: Trypticase, yeast extract, tryptose, ammonium citrate, Tween 80, sodium acetate, and glucose were dissolved in 150 ml of water; the K_2HPO_4 salt was dissolved in 50 ml of water; the carbonate was dissolved in 50 ml of water and was autoclaved in a screw-capped bottle with its cap tightened. All three solutions, autoclaved for 15 min at 15 lb/in^2 (121 C), were mixed aseptically at room temperature, and the pH was adjusted to 9.6 with sterile 2 N NaOH. Five milliliters of 2% Seitz-filtered NaN_3 was added. The temperature of the medium was then raised to 70 C in a water bath, and a sterile solution of agar (15 g dissolved in 740 ml of water) was added and mixed well. After mixing, the pH was rechecked and, if necessary, readjusted. Avoiding direct exposure to sunlight, the medium was poured into plates, allowed to solidify, and stored in a refrigerator. The medium was also tested as broth (agar omitted). All dissolved ingredients making up the broth were mixed aseptically at room temperature. For application, a sterile membrane filter (pore size 0.22 μm) was aseptically placed in a sterile filter holder. Ten milliliters of each cheese solution tested was introduced into the funnel and vacuum was applied. The funnel wall was rinsed with 50 ml of sterile phosphate buffer (pH 7.2). After filtration, the filter was aseptically transferred to a plastic petri dish containing an absorbent pad already saturated with about 2 ml of broth, and incubated. Enterococcus counts of various natural samples were determined on this medium. Total viable counts of the same samples were obtained on standard methods agar (BBL).

Culture methods. The selective medium was tested in both liquid and agar forms. Inocula of the test organisms were prepared by culturing each strain in basal broth. One drop of a 24-h culture was added from a sterile Pasteur pipette to each tube containing 8 ml of medium. In those cases where gram-negative organisms were tested (Table 3), the inocula were alternatively prepared by diluting the 24-h cultures 1:100 in phosphate-buffered saline (pH 7.0). For the

inoculation of agar plates, one drop of culture was spread in a standard three-way streak on the surface of the medium; the plates were incubated aerobically. All test media were incubated at the optimal temperature for each species tested. All enterococcus cultures and the isolation plates inoculated with natural specimens were incubated at 37 C, except for some tests, which, as indicated, were incubated at 45 C.

Isolation of enterococci from natural sources. Fecal samples from cattle, sheep, swine, chickens, and ducks were obtained at the farm of the State University of New York, Farmingdale, N.Y. Human and dog fecal specimens were also used. Freshly voided samples were taken in sterile bottles and refrigerated until analysis. Five-gram samples were weighed, transferred aseptically into 100 ml of sterile phosphate-buffered saline (pH 7.0), dispersed for 45 s in a Waring blender, and then serially diluted. Portions of 0.1 ml were spread on the surface of agar plates with a bent glass rod. Water samples were taken from rivers, creeks, and nearshore points in the environs of New York City and Long Island. Volumes of 50 ml were centrifuged at high speed, the supernatants were siphoned off, and the sediments were taken up in 2 ml of sterile distilled water. Portions were deposited on agar plates and spread as described. Food samples included meat sausages and three cheese varieties purchased at a local New York market. The preparation of these samples for analysis followed established procedures (9, 13). After enumeration at 24 and 48 h, colonies from all specimens cultured on the selective agar were picked and seeded in basal broth. The isolates were examined for conformity to the characteristics of enterococci (Sherman's criteria). They were also checked for catalase production and for group D antigen. The latter test was carried out microscopically by using a fluorescein-conjugated, group D-specific antiserum (Sylvana).

RESULTS

The addition of increasing concentrations of NaN_3 to the nutrient base afforded an initial evaluation of sensitivity. Table 2 presents the effect of such additions of NaN_3 on the growth of 20 enterococci and 18 lactic acid bacteria. At a NaN_3 level of 0.02%, S. faecium grew weakly or not at all; when the azide level was decreased to 0.015%, most typical S. faecium strains grew well, and at 0.01% azide, even atypical variants isolated from plants grew well. S. faecalis, as expected, exhibited greater tolerance toward NaN_3, as all strains grew at concentrations 0.02 to 0.05%. Certain of the tested lactobacilli, pediococci, and lactic streptococci showed equal or greater resistance than S. faecium to NaN_3. The basal medium, supplemented with 0.01% NaN_3, provided a maximum limit for satisfactory growth of the typical and atypical strains of S. faecium (Fig. 1 and 2), but 0.01% NaN_3 was insufficient to completely inhibit other lactic

TABLE 1. *Composition of the enterococcus selective agar*

Component	Amount (g)
Trypticase	10
Yeast extract, dehydrated	5
Tryptose	3
$(NH_4)_2$ citrate	2
Tween 80	1
Sodium acetate	1
Glucose	20
K_2HPO_4	3
Na_2CO_3	5.3
NaN_3	0.1
Agar (Oxoid Ionagar no. 2)	15
Water, all-glass-distilled and deionized, to 1,000 ml (pH adjusted to 9.6)	

cocci and bacilli. Incubation at 45 C, tried as a supplementary selective factor, proved unsatisfactory, since some of our enterococci failed to grow within 24 h and, in some cases, even 48 h.

All enterococci grew well in the basal medium adjusted to pH 9.6, whether in the presence or absence of 0.01% NaN₃ (Table 3). The high alkaline pH inhibited growth in all six strains of non-enterococcal group D streptococci. To ascertain the alkaline growth limits of these strains, we varied the pH of the medium and adjusted it to lower values. We found that they

TABLE 2. *Tolerance to NaN₃ shown by enterococci and other lactic acid bacteria*[a]

Species	No. of strains tested	NaN₃ concn in broth (%)			
		0	0.01	0.015	0.02
Streptococcus faecalis	3	+	+	+	+
S. faecium (typical)	14	+	+	13 (+)	W
S. faecium (plant isolates)	3	+	+	–	–
Streptococcus sp. (group N)	5	+	1 (+)	–	–
Leuconostoc sp.	5	+	–	–	–
Pediococcus sp.	4	+	+	1 (+)	–
Lactobacillus sp.	4	+	2 (+)	1 (+)	1 (W)

[a] Determined in the basal broth medium supplemented with the indicated concentrations of NaN₃; +, good growth; W, weak growth; –, no growth; ( ), good growth limited to some strains only. Observations: after 24 h of incubation at optimal temperature for each species. *S. faecalis* strains tolerated NaN₃ concentrations higher than 0.02% well.
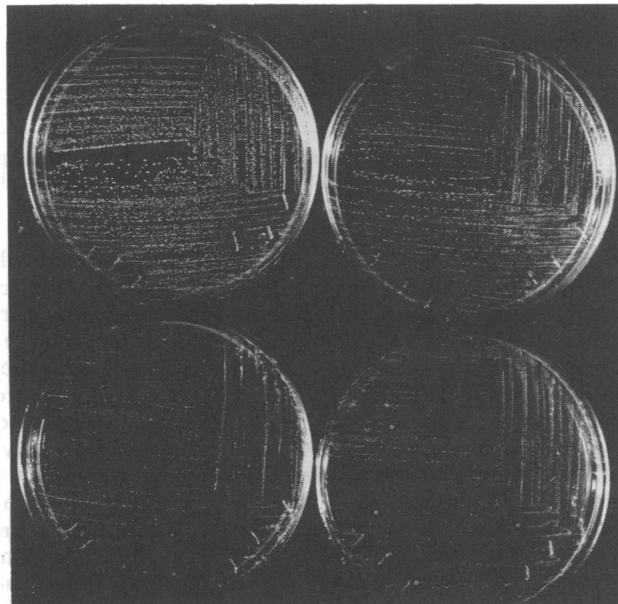
could grow well at pH 8.9. The 10 strains of lactic cocci grew slowly in the alkaline, NaN₃-free medium within a 72-h period; only half of these strains could show some growth in the alkaline medium supplemented with 0.01% NaN₃. The pediococci and lactobacilli were unable to produce visible growth even after 72 h, either in the alkaline, NaN₃-free medium, or in the alkaline, 0.01% NaN₃-containing medium. The five cytochrome- and catalase-containing, gram-positive or gram-negative strains demonstrated a slight retardation of growth in the alkaline, NaN₃-free medium, but they showed a marked retardation of growth and no visible growth before 24 h of incubation in the alkaline medium containing 0.01% NaN₃. This partial inhibition became more distinct when we used diluted (1:100) inocula.

On the alkaline agar medium (pH 9.6 plus 0.01% NaN₃), the enterococcus counts from cured meat, cheese, water, and feces (Table 4) varied between less than one enterococcus per ml of water to 20×10^6 per gram of sheep feces. The ratios of enterococci to the total count also varied; in the chicken feces, the enterococci were nearly as numerous as the total count, whereas in the dog feces the ratio was 1:60,000. Colonies of gram-negative bacteria that predominated in most of the fecal specimens, when grown on standard methods agar, were effectively suppressed by the selective medium (Fig.

FIG. 1. *Restrictive effect of increasing concentrations of NaN₃ on colony formation by S. faecium SJU-28. Upper left, right; lower left, right: 0; 0.01; 0.015; 0.02% NaN₃, respectively. All additions were made into basal agar. Note poor readability of colonies at the 0.015 and 0.02% NaN₃ level.*
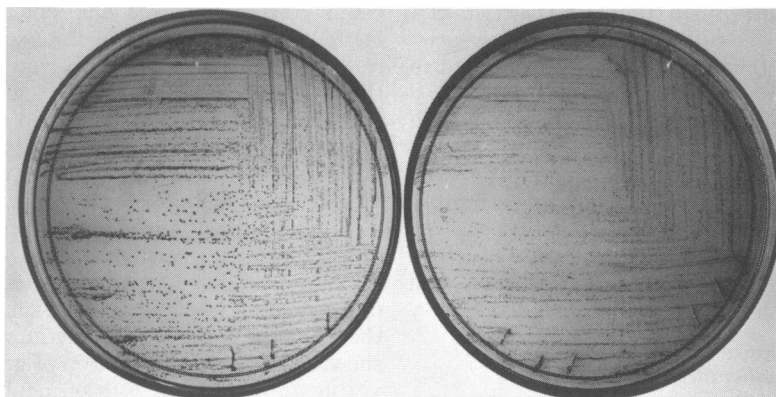
FIG. 2. *Restrictive effect of 0.01% NaN₃ (right) on colony formation by S. faecium var. casseliflavus FMA-2. Medium on the left contains 0% NaN₃ (control).*

TABLE 3. *Synergistic inhibition of various bacteria by NaN₃ and alkalinity[a]*

Species tested	No. of strains	Growth in basal broth plus								
		pH 6.8 (control)			pH 9.6			pH 9.6 plus 0.01% NaN₃		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Streptococcus sp. (enterococci)	20	+	+	+	+	+	+	+	+	+
Streptococcus sp. (group D, non-enterococci)	6	+	+	+	−	−	−	−	−	−
Streptococcus sp. (group N) and Leuconostoc sp.	10	+	+	+	−	−	+	−	−	5 (+)
Pediococcus sp.	4	+	+	+	−	−	−	−	−	−
Lactobacillus sp.	4	+	+	+	−	−	−	−	−	−
Bacillus sp.	1	+	+	+	±	W	+	−	W	+
Serratia marcescens	1	+	+	+	±	W	+	−	W	+
Escherichia coli	1	+	+	+	±	W	+	−	W	+
Proteus mirabilis	1	+	+	+	±	W	+	−	W	+
P. vulgaris	1	+	+	+	±	W	+	−	W	+

[a] +, Good growth; W, weak growth; ±, very slight growth; −, no growth; ( ), good growth limited to some strains only. Observations were made after 24, 48, and 72 h of incubation at optimal temperature for each species.

3). On the standard methods agar, the food samples yielded mostly small or pinpoint-size colonies, typical of gram-positive bacteria. On the selective medium, the colonies exhibited uniform morphology, whereas microscopically, they yielded gram-positive cocci. Upon prolonged incubation, i.e., 48 to 72 h, on some of the selective agar plates that were seeded with cheese samples, some hazy growth appeared around the well-developed enterococcus colonies (Fig. 4). Microscopic examinations revealed pleomorphic gram-positive bacilli. The medium used in these experiments was prepared with common-grade bacteriological agar. When purified, demineralized agar (Oxoid, Ion Agar no. 2) was substituted, the secondary growth was not observed. The observation indicates that inorganic ion depletion enhances the selectivity of the enterococcus medium. The round surface colonies obtained on the selective medium reached an average diameter of 1 to 2 mm after 16 to 17 h of incubation at 37 C.

One hundred ninety-four colonies isolated at random from the 14 analyzed specimens (Table 4) conformed substantially to the Sherman criteria for enterococci; few possessed catalase, and they reacted well with group D, fluorescein-labeled antiserum. Among 151 isolates from the fecal and water samples, there were six catalase-positive strains. Such strains were not encountered at all among the isolates from the fermented meat and dairy products. Thirty-eight out of 42 strains, tested with the group D antiserum, produced reactions of a 2+ or higher

intensity, whereas four showed a weaker reaction or no reaction at all. A few isolates showed nonconformity with one or another of the Sherman characteristics. Lack of agreement in more than one of the Sherman characteristics was observed in few of these variants.

DISCUSSION

About 20 years ago, Reinhold et al. (8) developed a selective medium containing 20 g of sodium citrate per liter and 0.01% NaN_3. At this concentration, the NaN_3 had generally no adverse effect upon the numbers of enterococcus colonies that developed on the agar plates, although a slight inhibition of some unidentified enterococci was noticed. In higher concentrations (0.015 to 0.025%) a decrease in colony size was noted. At 0.03% NaN_3, both the size and number of colonies were markedly inhibited. According to Reinhold et al. the 0.01% level of NaN_3 showed highest sensitivity, not only in terms of recovery rates, but also in readability of colonies on the agar plates. We adopted the 0.01% concentration of NaN_3 as the desired level of chemical inhibitor for our basal medium because of its least inhibitory effect on the growth and colony development of diverse forms of enterococci (Table 2, Fig. 1 and 2). We included a moderate concentration (2 g/liter) of citrate; as an energy source, we primarily used glucose. For a suppression of lactobacilli and physiologically related bacteria, we depended in part on Mn^{2+} depletion (5, 7).

Adjustment of pH to 9.5 to 9.6 was utilized originally by Sherman and Stark (10) as a tolerance test for the identification of *S. faecalis*, and later by Smith and Sherman (11)and others as a general differential characteristic of the entire group of enterococci. Certain forms of enterococci, however, grew poorly or not at all at this pH, especially after short incubation periods. Chesbro and Evans (2) studied the factors affecting the growth of enterococci in highly alkaline media. They found that the addition of carbonate and oleate (Tween 80) markedly stimulated the growth of enterococci in media with a pH higher than 9. When our basal medium was supplemented with carbonate, the high alkalinity of the modified medium (pH 9.6) did not interfere with the growth of any of the 20 strains tested (Table 3). It is noted that Burkwall and Hartman (1) found that the addition of
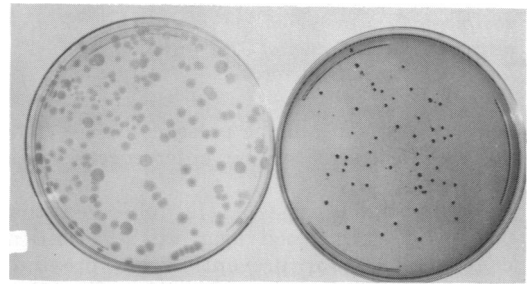

FIG. 3. *Growth from a human fecal specimen. Left, Standard methods agar, dilution 1:10⁶; right, the selective agar medium, dilution 1:10⁴.*

TABLE 4. *Isolation of enterococci from natural sources on the developed selective medium*

Description of sample analyzed	Counts/g or ml		No. of strains studied	Conformity to enterococcus characteristics				
	Total viable	Entero-cocci		10 C	45 C	60 C, 30 min	6.5% NaCl	Catalase act
Sausage	19×10^9	12×10^6	7	+	+	+	+	−
Cheese								
Cheddar	72×10^6	50×10^3	6	+	+	+	+	−
Feta	64×10^9	44×10^5	10	+	+ (9)[b]	+	+ (9)	−
Kefalotyri	15×10^8	19×10^7	20	+	+	+	+	−
Water								
East River	NT[a]	3	25	+ (23)	+ (22)	+ (24)	+ (23)	− (24)
Little Neck Bay	NT	1	8	+ (7)	+	+	+ (7)	− (7)
Brookville	NT	1	2	+	+	+	+	−
Feces								
Dog	88×10^7	15×10^3	6	+	+	+	+	−
Chicken	10×10^9	40×10^8	10	+	+	+	+ (9)	− (9)
Pig	15×10^7	40×10^6	10	+ (9)	+ (9)	+ (9)	+ (9)	− (9)
Duck	78×10^7	50×10^6	30	+	+ (25)	+ (29)	+ (29)	− (29)
Human 1	44×10^7	11×10^6	20	+ (18)	+	+ (19)	+ (18)	−
Human 2	18×10^7	70×10^4	20	+	+	+	+	−
Sheep	14×10^9	20×10^6	20	+ (14)	+	+	+	− (18)

[a] NT, Not tested.

[b] Numbers in parenthesis signify number of strains agreeing with the indicated characteristic.
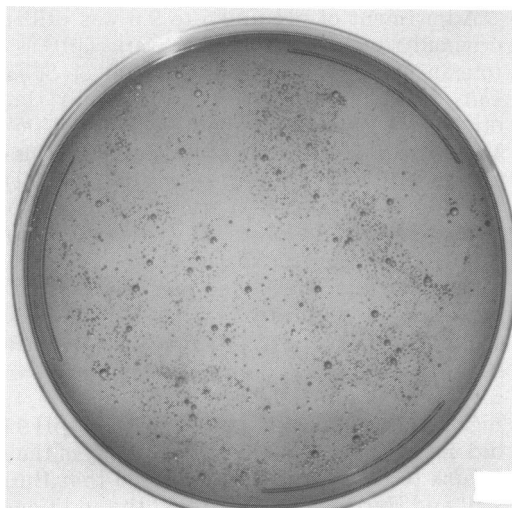
FIG. 4. *Hazy growth of lactobacilli that grew from a sample of Feta cheese on the selective agar medium after a 72-h incubation at 37 C (dilution 1:10⁶). The larger colonies are enterococci.*

sodium carbonate in the presence of Tween 80 enhanced the sensitivity of enterococcus media containing NaN_3.

Snyder and Lichstein (12) observed that 0.01% NaN_3 in blood agar was the critical concentration for prevention of spreading growth of *Proteus* sp. and coliform bacteria, since a 0.008% level of NaN_3 allowed such growth to occur. The delayed growth of the cytochrome-possessing strains in our alkaline plus 0.01% NaN_3-containing medium suggests that the nutrients of this medium may favor a certain degree of bacterial resistance. Raising the level of NaN_3 to higher than 0.01% levels would probably eliminate the growth of these strains. Nevertheless, we did not attempt such an increase since we intended to maintain the established sensitivity for enterococci at the 0.01% level, and also because isolations from fecal samples (Fig. 3, Table 4) indicated an efficient, for all practical purposes, suppression of gram-negative bacilli.

In the isolations of enterococci from various natural habitats (Table 4), the enterococcus counts were determined within 16 to 17 h of incubation. This early enumeration proved useful from the selection point of view. Bacteria unrelated to enterococci that were able to grow on the experimental medium slowly or marginally (Table 3, Fig. 4) did not interfere with the presumptive count and isolation of the

enterococci. Partial identification of the isolated strains disclosed an overwhelming proportion of confirmed enterococci (Table 4).

The environmental stress, to which enterococci are subjected in nature and particularly in foods, requires optimal growth conditions during isolation. Our results indicate that such optimal conditions needed for cell resuscitation may be very important for biotypes of enterococci with low tolerance toward the selective agents used in the isolations. The initial evaluation of the medium indicates a favorable performance on this essential point. A comparison of the developed medium with several standard enterococcus selective media is the subject of another report (5).

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