APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1989, p. 11–16
0099-2240/89/010011-06$02.00/0

Vol. 55, No. 1

Development of a Differential Medium for Bile Salt Hydrolase-Active *Lactobacillus* spp.

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Received 22 August 1988/Accepted 30 September 1988

An agar plate assay was developed to detect bile salt hydrolase activity in lactobacilli. On *Lactobacillus*-selective MRS or Rogosa SL medium supplemented with taurodeoxycholic, taurocholic, or taurochenodeoxycholic acids, bile salt hydrolysis was manifested at two intensities: (i) the formation of precipitate halos around colonies or (ii) the formation of opaque granular white colonies. Sixty-six lactobacilli were tested for bile salt hydrolase activity by both the plate assay and a sensitive radiochemical assay. No false-positive or false-negative results were detected by the plate assay. Based on results of experiments with *Eubacterium lentum* and *Bacteroides* species, the plate assay was dependent on two factors: (i) the presence of bile salt hydrolytic activity and (ii) the ability of the organism to sufficiently acidify the medium to protonate free bile acids. The availability of a differential medium for determination of bile salt hydrolase activity will provide a rapid method for determining shifts in a specific functional activity of intestinal *Lactobacillus* species and provide a rapid screening capability for identifying bile salt hydrolase-deficient mutants. The latter application should allow bile salt hydrolase activity to be used as a marker enzyme in genetic experiments.

We have been investigating the hypothesis that the improvement in growth characteristics of monogastric animals fed subtherapeutic levels of feed additive antibiotics is caused by a reduction in the level of toxic metabolites generated from bile acids by bacteria in the intestine. This possibility was initially suggested by Eyssen and colleagues (3, 5, 6) following their investigations on the effects of virginiamycin on lipid metabolism in poultry. Recently, Fuller and colleagues (2, 10, 11) have suggested that growth depression in chickens is due to deconjugation of bile salts by selected *Enterococcus* (*Streptococcus*) *faecium* strains adhering to duodenal epithelial cells. We have demonstrated that subtherapeutic levels of feed additive antibiotics decrease bile salt hydrolase activity, the first step in bile acid transformations, in homogenates of chicken small intestines (7). Conversely, we have shown that the growth depression in poultry elicited by dietary carbohydrates (i.e., rye or sucrose) is associated with elevated levels of bile salt hydrolase activity in intestinal homogenates (8). These homogenates reflect the composite bile salt hydrolase activities of the attached and nonattached intestinal microflora.

Salanitro et al. (24) showed that the small intestine of chickens was colonized with a significant anaerobic flora which included members of the genera *Lactobacillus*, *Enterococcus* (*Streptococcus*), *Eubacterium*, *Propionibacterium*, *Clostridium*, *Gemminger*, and *Fusobacterium*. Bacteria capable of catalyzing the hydrolysis of bile acid conjugates include members of the following genera: *Lactobacillus*, *Enterococcus* (*Streptococcus*), *Clostridium*, *Peptostreptococcus*, *Bifidobacterium*, *Fusobacterium*, *Eubacterium*, and *Bacteroides* (14, 17, 18). *Lactobacillus* species predominate in the chicken small intestine from a few days posthatching and throughout the life of the chicken (21, 22, 26, 27). More specifically, Morishita et al. (23) have shown that *Lactobacillus acidophilus*, *Lactobacillus salivarius*, and *Lactobacillus fermentum* are the most common species in the chicken small intestine. In addition, Sarra et al. (25) have demonstrated that *L. acidophilus* is the predominant homofermentative lactobacillus and that *Lactobacillus reuteri* (a new species derived from *L. fermentum* [19]) is the predominant heterofermentative lactobacillus in chickens.

The predominance of lactobacilli in the small intestine of poultry, the capability of some of these species or strains to catalyze the hydrolysis of conjugated bile acids (12), and our previous work showing a correlation between growth promotion and bile salt hydrolase activity (7, 8) led us to attempt the development of a medium selective for lactobacilli which would also permit rapid differentiation between hydrolase-positive and -negative strains. A differential and selective medium of this type would allow us to monitor the bile salt hydrolase activity of a specific gastrointestinal microbial population in chickens fed feed additive antibiotics. In this report we describe a modification of MRS and Rogosa SL selective media which imparted a differential capability for the detection of bile salt hydrolase-elaborating strains.

(Portions of this work were presented at the Annual Meeting of the American Society for Microbiology, March 1987, Atlanta, Ga. [M. P. Dashkevicz and S. D. Feighner, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, Q72, p. 294].)

MATERIALS AND METHODS

Culture techniques. *Lactobacillus* strains were obtained from the Merck culture collection (MB numbers) or from the collection of lactobacilli of L. DeVries, which were isolated from the intestines of farm animals (AP numbers [4]). The cultures, which were received as lyophiles, were revived and subcultured at least 3 times in anaerobic MRS broth (Difco Laboratories, Detroit, Mich.) before further use. The MRS broth was rehydrated according to the directions of the manufacturer, dispensed into screw-cap tubes, autoclaved, and placed, while still hot, into an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) containing an atmosphere of 10% H_2 and 5% CO_2 in nitrogen. All transfers and manipulations were performed anaerobically either in the anaerobic chamber or by use of the modification of Bryant and Burkey (1) of the Hungate technique. Agar plates

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were prepared by adding 1.7% (wt/vol) granulated agar (Difco) to broth medium and poured into sterile plastic petri dishes (60 by 15 mm). Once solidified, the plates were inverted and placed in the anaerobic chamber for at least 48 h before use. All plates were inoculated from an overnight culture by using a 10-μl loop. Plates were incubated in glass screw-cap jars at 37°C in the anaerobic chamber for 72 h. Precipitated bile acid could be seen around colonies representing highly active strains within 48 h. All other organisms were obtained from the Merck culture collection and were maintained on supplemented brain heart infusion broth or agar (1.7% [wt/vol]) (16). All other procedures and manipulations were done as described above.

Bile salt plates. Test plates were prepared with 0.5% (wt/vol) of the sodium salt of taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), or taurodeoxycholic acid (TDCA). After boiling and steam sterilization, the plates were handled as described above.

Thin-layer chromatography. Agar plugs were cut from the appropriate plates after 72 h of incubation by using the blunt end of a Pasteur pipette and were dissolved in 1.0 ml of 0.01 N HCl with gentle heating. The resulting solution was extracted with 1.0 ml of ethyl acetate to partition the free bile acids into the organic phase, while the taurine conjugates remained in the aqueous phase (7). The ethyl acetate extract was dried under a stream of nitrogen in a 55°C water bath, suspended in a small amount of ethyl acetate, and spotted onto silica gel IB2 thin-layer chromatography plates (J. T. Baker Chemical Co., Phillipsburg, N.J.). The plates were developed with isooctane-isopropyl ether-isopropanol-acetic acid (2:1:1:1) (13). Once they were developed, the plates were dried and sprayed with sulfuric acid-dichromate (1:1 [vol/vol]; Sigma Chemical Co., St. Louis, Mo.) and charred in an oven at 90°C for 3 to 5 min.

Radiochemical assay. To assess taurine-conjugated bile salt hydrolase activity by pure cultures, 0.5 ml of fresh MRS broth and 0.5 ml of an overnight *Lactobacillus* culture were added to a 1-dram vial (1 fluidram = 3.696 ml) which contained 1.0×10^5 cpm [*carboxy*-^{14}C]TCA and 4.0 μmol of TCA as substrate (final specific activity, 28.0 dpm/nmol of TDCA), which was prepared as described previously (7). The resulting mixture was incubated under anaerobic conditions for 24 h at 37°C. The reaction was terminated by the addition of 6.0 N HCl (final pH, 2.0). Ethyl acetate (1 ml) was added and the vials were shaken vigorously. Samples of the organic phase (0.5 ml) containing cholic acid, the product of bile salt hydrolysis, were removed and added to 10 ml of Aquasol-2 (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) in glass scintillation vials. Radioactivity was measured in a liquid scintillation counter (1215 Rack-beta II; LKB Instruments, Inc., Rockville, Md.). Counts per minute were corrected with an external standard channel ratio and a ^{14}C quench curve.

Recovery experiments. Recovery experiments were conducted to gauge the effects of TDCA on growth and to determine plating efficiencies. Cocultures were composed of bile salt hydrolase-positive and -negative species which exhibited different colony morphologies. The cultures were spread onto MRS–0.5% TDCA plates, which were scored after 72 h of incubation at 37°C under anaerobic conditions. The results are expressed as the log (cell number/milliliter of original suspension); thus, percent efficiency was defined as 10^x, where $x = \{[\log(\text{cell number/milliliter})_{trt} - \log(\text{cell number/milliliter})_{ctrl}] + 2\}$, where trt and ctrl are treated and control, respectively.

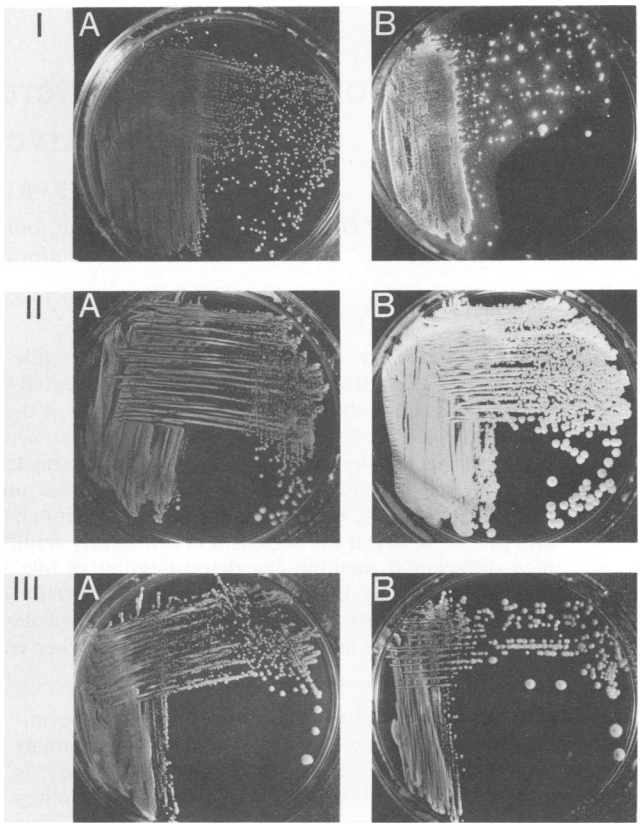

FIG. 1. Manifestation of bile salt hydrolase activity by lactobacilli on solid MRS medium. Plates were incubated anaerobically for 72 h at 37°C. Panels labeled A are control plates; those labeled B contain 0.5% TDCA. Series I, *L. brevis* AP905aC; series II, *L. plantarum* AP13b; series III, *L. plantarum* ATCC 10241.

RESULTS

When bile salt hydrolase-producing lactobacilli were streaked out on MRS or Rogosa SL plates containing 0.5% TDCA, the taurine-conjugated bile acid was deconjugated, producing deoxycholic acid. The deconjugation activity of *Lactobacillus* colonies was manifested in two forms. In the first case (depicted in Fig. 1, panel IB), copious amounts of deoxycholic acid precipitated around active colonies and diffused into the surrounding medium. In the second case, bile salt hydrolase-active strains produced opaque white colonies without precipitate halos, when grown in the presence of TDCA (Fig. 1, panel IIB). Bile salt hydrolase-inactive strains produced similar colony types on plates with or without TDCA (Fig. 1, panel IIIA and IIIB). In strains which produced precipitate halos (e.g., *L. brevis* AP905aC; Fig. 1, panel IB), TDCA seemed to produce the most sharply defined halos, while TCA and TCDCA were slightly less effective (data not shown). When an equivalent amount of taurolithocholic acid was added to the agar medium, it precipitated after sterilization as the medium cooled.

The bile salt hydrolase activity observed on plates was verified by thin-layer chromatography of agar plugs taken from various plates (Fig. 2). Deoxycholic acid was detected in agar plugs from plates containing TDCA which were inoculated with strains producing either precipitate halos or white opaque colonies. No deoxycholic acid was detected in agar plugs from either uninoculated bile salt plates or the corresponding control plates.

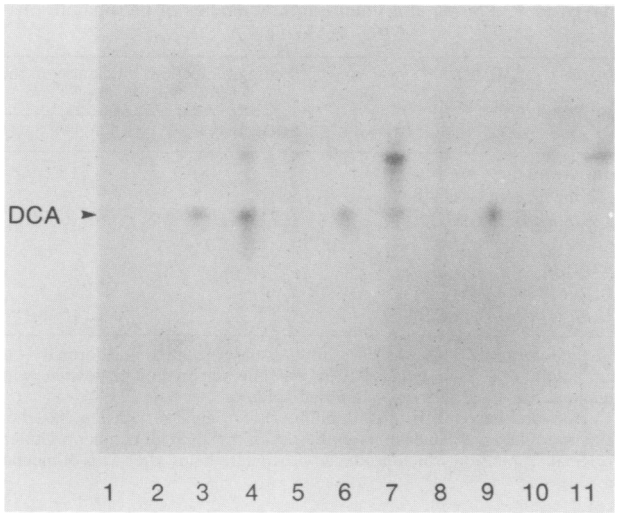

FIG. 2. Thin-layer chromatogram of ethyl acetate-extracted agar plugs. When grown on MRS plates containing 0.5% TDCA, *L. acidophilus* AP943bP produced white colonies with precipitate halos similar to those shown in Fig. 1, panel IB. *L. plantarum* AP8a produced white colonies without a surrounding precipitate similar to those shown in Fig. 1, panel IIB, but *L. fermentum* AP13a did not produce either white colonies or precipitate halos similar to those shown in Fig. 1, panel IIIB. Lane 1, AP13a colonies from a bile salt plate; lane 2, AP13a colonies from a control plate; lanes 3, 6, and 9, deoxycholic acid (DCA) standard (25 μg); lane 4, AP943bP colonies from a bile salt plate; lane 5, AP943bP colonies from a control plate; lane 7, AP8a colonies from a bile salt plate; lane 8, AP8a colonies from a control plate; lane 10, uninoculated bile salt plate; lane 11, uninoculated control plate.

In order to gauge the potential utility of the differential media, 66 *Lactobacillus* strains were tested for bile salt hydrolase activity on MRS agar plates supplemented with 0.5% TDCA. Each strain was also tested for bile salt hydrolase activity by using a sensitive radiochemical bile salt hydrolase assay (Tables 1 and 2). Based on our survey of *Lactobacillus* strains, the ability to hydrolyze taurine-conjugated bile acids was associated with several species representing both homolactic acid and heterolactic acid fermenters (Table 3).

The applicability of the modified MRS medium for differentiating bile salt hydrolase-positive and -negative strains was tested by preparing a mixed culture of two *Lactobacillus* species differing in bile salt hydrolase activity and colony morphology. The total number of bacteria plated onto control plates, the number of colonies appearing on 0.5% TDCA-containing plates, and the number expressing bile salt hydrolase activity were determined (Table 4).

An additional survey of various intestinal organisms was conducted to extend our observations on the lactobacilli. *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Enterococcus*, and *Fusobacterium* strains were streaked onto brain heart infusion agar–0.5% TDCA plates (Table 5). The manifestation of bile salt hydrolase activity on this medium was not as dramatic as that seen with the lactobacilli. The clostridia and fusobacteria tested did not possess bile salt hydrolase activity. Interestingly, *Eubacterium lentum* CDC 15681 and all of the *Bacteroides* species tested were bile salt hydrolase negative in the plate assay, while they were positive radiochemically.

TABLE 1. Bile salt hydrolase activity in homofermentative *Lactobacillus* species

Strain	Isolation source	Plate assay[a]	Radiochemical assay[b]
L. acidophilus			
ATCC 832, MB311	Rat	+	+
ATCC 4355, MB312	Rat	+	+
ATCC 4356, MB313	Human	+	+
ATCC 4357, MB314	?	+	+
ATCC 4796, MB316	?	+	+
ATCC 314, MB319	?	+	+
MB323	?	+	+
MB391	?	−	−
MB1316	Poultry	+	+
MB1933	?	+	+
AP20b	Cattle	+	+
AP18b	Cattle	+	+
AP14a	Cattle	+	+
AP25b	Cattle	+	+
AP596b	Swine	+	+
AP866b	Swine	+	+
AP943bP	Swine	+	+
AP975b	Swine	+	+
L. casei			
ATCC 4646, MB315	Caries	−	−
ATCC 4961, MB318	?	−	−
ATCC 393, MB320	Cheese	−	−
ATCC 4224, MB324	?	−	−
ATCC 4007, MB326	?	−	−
ATCC 4940, MB347	?	0	−
L. casei subsp. *rhamnosus* ATCC 7469, MB340	?	−	−
L. lactis			
ATCC 10697, MB367	?	0	−
ATCC 8000, MB368	?	−	−
L. leichmannii ATCC 7830, MB538	?	−	−
L. plantarum			
ATCC 4008, MB325	?	+	+
ATCC 8041, MB346	?	−	−
ATCC 10241, MB348	Cabbage	−	−
ATCC 10012, MB355	?	+	+
AP8a	Cattle	+	+
AP13b	Cattle	+	+
MB68	?	−	−
MB70	?	−	−
L. salivarius			
AP25a	Swine	+	+
AP18a	Swine	+	+
AP9b	Swine	+	+
AP6a	Swine	+	+
AP8b	Swine	+	+
AP12b	Swine	+	+
AP9a	Swine	+	+
AP1a	Poultry	+	+
AP3a	Poultry	+	+
AP17b	Poultry	+	+

[a] A plus sign indicates either precipitate halos or white granular opaque colonies. A zero indicates that the strain did not grow in the presence of 0.5% TDCA.

[b] Reactions were positive if >1.0% transformation was detected after correction for extraction efficiency (7).

TABLE 2. Bile salt hydrolase activity of heterofermentative *Lactobacillus* species

Strain	Isolation source	Plate assay[a]	Radiochemical assay[b]
L. brevis			
ATCC 367, MB329	?	+	+
AP888bP	Poultry	+	+
AP942c	Poultry	+	+
AP17a	Cattle	–	–
AP905aC	Swine	+	+
L. fermentum			
ATCC 9338, MB342	?	0	–
AP701b	Swine	+	+
AP701aP	Swine	+	+
AP615a	Swine	+	+
AP1143c	Swine	+	+
AP980bB	Swine	+	+
AP1138a	Swine	+	+
AP19a	Poultry	–	–
AP22a	Poultry	+	+
AP15c	Poultry	+	+
AP13a	Poultry	–	–
AP886b	Cattle	+	+
AP887b	Cattle	+	+
AP1137bC	Cattle	+	+
Lactobacillus sp. strain ATCC 9857, MB349	Vaginal tract	+	+

[a] A plus sign indicates either precipitate halos or white granular opaque colonies. A zero indicates that the strain did not grow in the presence of 0.5% TDCA.

[b] Reactions were positive if >1.0% transformation was detected after correction for extraction efficiency (7).

DISCUSSION

In the analysis of any complex ecosystem, the availability of selective media provides a necessary tool for the enumeration of various bacterial groups. In many situations the enumeration of populations at the genus level provides sufficient information to address a problem (e.g., the enumeration of fecal coliforms to determine water quality). In other cases, the enumeration of bacterial numbers at the genus level merely quantifies the magnitude of the problem, and subsequent tests are required to reach the final goal (e.g., the determination of antibiotic resistance patterns). In the latter case the availability of media which are both selective and differential is requisite to a successful answer. In our attempt to further elucidate the effects of feeding subtherapeutic levels of feed additive antibiotics on bile acid metabolism, media were developed which allowed the detection of bile salt hydrolase-elaborating *Lactobacillus* strains.

TABLE 3. Frequency of bile salt hydrolase activity exhibited by lactobacilli

Species	No. active	No. tested
L. acidophilus	17	18
L. casei	0	7
L. lactis	0	2
L. leichmannii	0	1
L. plantarum	4	8
L. salivarius	10	10
L. brevis	4	5
L. fermentum	11	14

TABLE 4. Plating and detection efficiencies of lactobacilli on MRS–0.5% TDCA

Culture and organism[a]	Plating (detection) efficiency on the following media:[b]	
	MRS	MRS–0.5% TDCA
Monoculture		
L. plantarum MB348	7.81	7.73
L. acidophilus AP20b	6.71	6.67 (6.64)
Coculture		
L. plantarum MB348	7.45	7.31
L. acidophilus AP20b	6.37	6.22 (6.20)

[a] *L. plantarum* MB348 was bile salt hydrolase negative and formed 1- to 2-mm colonies. *L. acidophilus* AP20b was bile salt hydrolase positive with precipitate halos and formed 5- to 6-mm colonies.

[b] Values are the log (cell number/milliliter) of original suspension, based on colony morphology. Values in parentheses are the log (cell number/milliliter) of original suspension which produced precipitate halos after 72 h of incubation.

By incorporating TDCA into commercially available *Lactobacillus*-selective media (MRS and Rogosa SL), bile salt-hydrolyzing colonies could be detected. TCA and TCDCA were slightly less effective for this purpose (data not shown). The usefulness of these di- and trihydroxy taurine-conjugated bile acids in these media is probably related to their pK_a values. The taurine conjugates exhibit an apparent pK_a of 1.9 in aqueous solution (28), whereas the pK_a of the unconjugated species is approximately 5.0 (9, 28). Thus, at pH values achievable by acidic fermentative metabolism, unconjugated bile acids would be protonated and precipitate, while taurine conjugates would remain completely ionized and remain in solution. This interpretation is consistent with the observation that *E. lentum* CDC 15681 exhibited bile salt hydrolase activity in the radiochemical assay but not in the plate assay (Table 5). *E. lentum* metabolism is notable in that growth occurs without lowering the pH (15). Glycine-conjugated bile acids exhibit pK_a values of approximately 3.9 (9) and could be partially precipitated without hydrolysis at fermentative pH values. Attempts to use taurolithocholic acid failed because of its extremely low solubility at room temperature.

The *Lactobacillus* differential plating medium (MRS–0.5% TDCA) permitted identification of all bile salt hydrolase-positive and -negative strains. On the basis of the sensitive radiochemical bile salt hydrolase assay, the plating medium did not yield false-positive or false-negative results (Tables 1 and 2). Three strains, *L. lactis* ATCC 10697, *L. casei* ATCC 4940, and *L. fermentum* ATCC 9338, did not grow on the plates in the presence of 0.5% TDCA. They were, however, negative in the radiochemical bile salt hydrolase assay. The recovery of *Lactobacillus* species in medium containing TDCA was approximately 80% (Table 4). However, differentiation between bile salt hydrolase-positive and -negative colonies which did appear approached 95%, thus making the medium useful for quantitating proportions of a population exhibiting a particular biochemical trait, although it would slightly underestimate the total *Lactobacillus* population (Table 4).

Bile salt hydrolase activity was measured in 71% of the *Lactobacillus* cultures tested, representing both homo- and heterofermentative species. Nothing is known of the genetics of bile salt hydrolysis in lactobacilli or any other bacterial species. The observation that some strains of a particular species do not possess the activity, although others do (e.g., *L. acidophilus*, *L. plantarum*, *L. brevis*, and *L. fermentum*),

TABLE 5. Survey of bile salt hydrolase activity of various intestinal bacteria on agar plates

Organism	Plate assay[a]	Radiochemical assay
Bacteroides fragilis		
MB3273	−[b]	+[c]
MB4087	−	+
MB4270	−	+
Bacteroides vulgatus ATCC 8482, MB3340	−	+
Clostridium pectiovorum MB1386	−	−
Enterococcus faecalis		
MB118	+	+
MB120	+	+
MB821	+	+
Enterococcus faecium		
ATCC 9790, MB1801	+	+
MB116	+	+
Eubacterium lentum		
CDC 15681,MB3279	−	+
ATCC 25559, MB3343	−	−
Eubacterium limosum ATCC 8486, MB3344	+	+
Fusobacterium mortferum ATCC 25557, MB3345	−	−
Fusobacterium necrophorum CDC 5164, MB3276	−	−
Fusobacterium nucleatum ATCC 25586, MB3277	−	−

[a] Supplemented with brain heart infusion agar–0.5% TDCA Positive strains produced precipitate halos.

[c] Reactions were positive if >1.0% transformation was detected after correction for extraction efficiency.

may suggest that the activity is plasmid mediated. Alternatively, bile salt hydrolase activity may represent a biochemical marker for species which can now only be separated on the basis of DNA homology (e.g., *L. gasseri* from *L. acidophilus* [20] and *L. reuteri* from *L. fermentum* [19]). The availability of this medium should make it possible to easily address the considerations given above. More importantly, however, the results in Table 5 with *Enterococcus* species and *Eubacterium limosum* demonstrate that bile salt hydrolase plate assay media could be devised for species other than *Lactobacillus*. Furthermore, by the appropriate choice of the bile salt concentration and fermentation substrate, it is conceivable that results similar to those observed with lactobacilli are obtainable with other species.

ACKNOWLEDGMENTS

We thank L. A. Devriese (Faculty of Veterinary Medicine, Casinoplein 24, B-9000 Gent, Belgium) and S. A. Currie (Merck & Co., Inc., Rahway, N.J.) for supplying the strains used in this study. We also thank Karen Rosa for typing the manuscript.

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