

Lactobacilli and Bile Salt Hydrolase in the Murine Intestinal Tract

GERALD W. TANNOCK,¹ MICHAEL P. DASHKEVICZ,² AND SCOTT D. FEIGNER^{2*}

Department of Microbiology, University of Otago, Dunedin, New Zealand,¹ and Department of Growth Biochemistry and Physiology, Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065-0900²

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Mice that have a complex intestinal microflora but that do not harbor lactobacilli were used to determine the contribution of lactobacilli to the total bile salt hydrolase activity in the murine intestinal tract. Bile salt hydrolase activity in the ileal contents of these mice was reduced 86% in the absence of lactobacilli and by greater than 98% in the absence of lactobacilli and enterococci compared with samples from conventional mice. Bile salt hydrolase activities were lower in ileal and cecal contents from lactobacillus-free mice colonized with enterococci than in samples from lactobacillus-free mice colonized with lactobacilli. Bile salt hydrolase activity in the duodena, jejunum, ilea, and ceca of reconstituted lactobacillus-free mice colonized by lactobacilli was similar to that in samples from the intestinal tracts of conventional mice. We conclude from these studies that lactobacilli are the main contributors to total bile salt hydrolase activity in the murine intestinal tract.

Lactobacilli are the numerically predominant bacteria detected in the proximal digestive tracts of mice. Many strains of lactobacillus of rodent origin can associate with epithelial surfaces lining the esophagus and forestomach of the mouse and form a relatively thick layer of bacterial cells on the tissue surface. Lactobacilli shed from these sites, as well as strains inhabiting the gastrointestinal lumen, permeate all regions of the digestive tract (12, 14, 23). Historically, lactobacilli have been considered beneficial residents of the intestinal ecosystem, although rigorous scientific investigation of the influence of lactobacilli on their animal hosts has been lacking (19). The derivation of colonies of mice, maintained by the gnotobiotic method, which have a complex intestinal microflora yet do not harbor lactobacilli permits the detection and investigation of lactobacillus-associated traits in animals under microbiologically constant conditions (20). Lactobacilli isolated from the proximal gastrointestinal tracts of mice have the ability to deconjugate taurocholic acid (G. W. Tannock, unpublished observation). The availability of lactobacillus-free (LF) mice has thus permitted us to evaluate the contribution that lactobacilli make to the total bile salt hydrolase activity of the microbial consortia in the intestinal tracts of mice. This evaluation is of particular significance in the light of recent evidence that bile salt hydrolase-producing bacteria contribute to "growth depression" in production animals, a condition which can be alleviated by the administration of subtherapeutic concentrations of feed additive antibiotics. We have previously demonstrated in poultry that the administration of subtherapeutic levels of growth-permitting antibiotics correlated with a decrease in bile salt hydrolase activity in intestinal homogenates (5) and that the growth depression induced by incorporation of "poor" carbohydrate sources into poultry diets was positively correlated with an elevation of bile salt hydrolase activity in intestinal homogenates (6). In addition, it has been shown that most lactobacilli from the intestinal tracts of livestock are capable of deconjugating taurocholic and taurodeoxycholic acids (3, 9). We have also investigated in this study the influence of enterococci on bile salt hydrolase activity in the murine intestinal tract because of the known ability of these bacteria to deconjugate taurocholate

(18) and because of their putative role in the growth depression of poultry (2, 4, 8).

MATERIALS AND METHODS

Mice. The derivation of the LF BALB/c mouse colony has been reported previously (20). These animals harbor a complex intestinal microflora, but lactobacilli, enterococci, filamentous ileal microbes, and some anaerobic components of the cecal microflora are absent (20). Reconstituted LF (RLF) mice have been derived from LF mice by inoculation with specific bacterial cultures, harvested noncultivable microbes, and cecal homogenates from chloramphenicol-treated conventional mice. RLF mice have an intestinal microflora functionally equivalent to that of conventional mice but do not harbor lactobacilli (21, 22). All mice, including conventional animals, were maintained in isolators under identical conditions using the standard gnotobiotic method described previously (13). LF, RLF, and conventional mice provided the initial basis for comparison of the effect of lactobacilli on bile salt hydrolase activity. In later experiments, LF or RLF mice were inoculated with cultures of lactobacilli or enterococci. The bacterial cultures were used to contaminate the food, fur, and bedding of animals in isolators. The mice were killed by carbon dioxide anesthesia and cervical dislocation 3 weeks after inoculation. Specimens of gastrointestinal organs were collected and used for culture and biochemical assays. Male and female mice about 7 weeks old were used in our investigations.

Bacteria. Enumeration of lactobacilli and enterococci was accomplished by homogenizing forestomach, duodenal, jejunal, ileal, or cecal samples in sterile distilled water to give a 10-fold dilution (wt/vol). The homogenates were further diluted and cultured on an appropriate selective agar medium. Lactobacilli in gastrointestinal samples were enumerated on medium 10 agar plates (16) incubated anaerobically in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.). Enterococci were enumerated on aerobically incubated methylene blue agar plates (15). Bacterial strains used to inoculate mice were isolated from rodent gastrointestinal samples during a previous study (*Lactobacillus reuteri* 100-23) (25) or during the present study (*Lactobacillus* strains 100-18, 100-20, and 100-21 and enterococci). *Lactobacillus* strains 100-18, 100-20, and 100-21 represent the three biotypes of lactobacilli that can be detected on medium 10 agar

* Corresponding author.

cultures of the stomachs of our conventional mice. The biotypes were recognized by determining the fermentation profiles of 45 isolates from medium 10 cultures by using API CH galleries (A.P.I. System S.A., La Balme les Grottes, France) and API CHL suspending medium according to the instructions of the manufacturer. The three biotypes of lactobacilli detected represent *Lactobacillus delbrueckii* (strains 100-18 and 100-21) and *Lactobacillus fermentum* (strain 100-20). Strain 100-21 ferments galactose, but strain 100-18 does not. Enterococci were cultured from RLF cecal samples on methylene blue agar, subcultured as described previously (22) on brain heart infusion agar (Difco Laboratories, Detroit, Mich.), and used to inoculate LF mice. *Enterococcus faecalis* and *Enterococcus faecium* form the enterococcal inhabitants of the mouse cecum (17).

Bile salt hydrolase activity of bacterial cultures. Bile acid-deconjugating ability of lactobacillus strains was detected by culturing the bacteria on Lactobacilli MRS agar (Difco) containing 0.5% (wt/vol) taurodeoxycholic acid (wt/vol; sodium salt) incubated under anaerobic conditions in GasPak jars for 48 to 72 h (3). Deconjugation of taurodeoxycholate results in a white precipitate of deoxycholate in the vicinity of bacterial colonies. In addition, the lactobacilli and 20 enterococcal isolates were screened for deconjugating ability by anaerobic culture for 24 h in Lactobacilli MRS broth containing 0.1% taurocholate (wt/vol; sodium salt). Bile acid was extracted from 3-ml volumes of culture by adding 0.6 ml of 4 N sodium hydroxide and 3.6 ml of ethyl acetate. After vigorous mixing, the emulsion was allowed to separate into phases, and 10 μ l of the ethyl acetate phase was applied to an ITLC-SG thin-layer chromatography sheet (Gelman Instrument Co., Ann Arbor, Mich.). A mobile phase of isooctane-isopropyl ether-glacial acetic acid (50:20:10) was used in a Gelman chromatography system. Cholic acid resulting from deconjugation of taurocholate was detected by heating the chromatogram after spraying it with 50% sulfuric acid. A cholic acid standard (1 mg/ml) was applied to each chromatographic sheet for comparative purposes.

Bile salt hydrolase activity in intestinal samples. Intestinal contents were homogenized in sterile Milli-Q water (Millipore S.A., Molsheim, France) to form a 10-fold dilution (wt/vol). The homogenates were lyophilized in glass ampoules for 24 h; the ampoules were sealed and then dispatched for analysis. The ampoules were opened and placed into an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) containing an atmosphere of 10% H₂-5% CO₂ in nitrogen. The lyophilized homogenates were suspended in anaerobic acetate buffer (5 mM, pH 5.6) containing 0.336% EDTA, 0.156% 2-mercaptoethanol, and 0.25% Triton X-100 to a concentration of 50 mg/ml. The whole cells in the intestinal homogenate were permeabilized with the Triton X-100 and a freeze-thaw cycle in a dry-ice-acetone bath (6). Bile salt hydrolase activity in luminal contents was measured radiochemically by quantitating the amount of [carboxy-¹⁴C]cholic acid hydrolyzed from tauro[carbonyl-¹⁴C]cholic acid (6). Reaction mixtures contained taurocholic acid and sufficient [¹⁴C]taurocholic acid to yield a final concentration of 2.0 mM, with a specific radioactivity of 0.05 μ Ci/ μ mol. Test extract (10 to 15 mg) was added, and the mixture was incubated at 37°C for 30 min.

All reactions were terminated by lowering the pH to approximately 2.0 with 6 N HCl. Ethyl acetate (2.0 ml) was added to partition the [¹⁴C]cholic acid into the organic phase, and 1.0-ml samples were removed and added to 10 ml of Aquasol-II (Du Pont Co., Wilmington, Del.) in glass scintil-

TABLE 1. *Lactobacillus* and enterococcal populations in the ilea and ceca of mice

| Mouse group | No. of ^a : | | | |
|--------------------------------------|-----------------------|-----------|-----------------|-----------|
| | Lactobacilli in: | | Enterococci in: | |
| | Ileum | Cecum | Ileum | Cecum |
| I (LF) | None | None | None | None |
| II (LF + 100-23 and 100-21) | 8.2 (0.1) | 8.3 (0.1) | None | None |
| III (LF + enterococci) | None | None | 4.5 (0.1) | 6.2 (0.2) |
| IV (RLF) | None | None | 4.3 (0.2) | 4.8 (0.2) |
| V (RLF + 100-18, 100-20, and 100-21) | 7.6 (0.2) | 7.7 (0.2) | 4.2 (0.4) | 4.2 (0.6) |
| VI (conventional) | 8.0 (0.2) | 8.4 (0.2) | 3.4 (0.3) | 4.0 (0.3) |

^a Mean log₁₀ count per gram of organ (standard error of the mean). Five mice per group.

lation vials. Counts per minute were corrected by using an external-standard-channels ratio and a ¹⁴C quench curve.

The bile salt hydrolase data were analyzed by using a robust analysis of variance on the natural logarithm-transformed (+1.0) data (1). The transformed means were compared by the Duncan multiple range test, since all pairwise comparisons within experiments were of interest.

RESULTS

In vitro bile salt hydrolase activity of cultures. All of the enterococcal isolates from the ceca of RLF mice deconjugated taurocholate, as determined by thin-layer chromatography. *Lactobacillus* strains 100-23 and 100-20 did not deconjugate taurodeoxycholate by the agar plate method, whereas strains 100-18 and 100-21 did. All of the *Lactobacillus* strains, however, deconjugated taurocholate when tested by thin-layer chromatography.

Lactobacillus and enterococcal populations. Population levels of lactobacilli and enterococci in the ilea and ceca of the various mouse groups are given in Table 1. Lactobacilli were detected only in cultures from conventional mice and from LF and RLF mice that had been inoculated with lactobacillus cultures. Population levels of lactobacilli were similar in all of these groups (the Student *t* test, *P* < 0.01). Enterococci were detected in cultures from conventional and RLF mice and from LF mice inoculated with enterococcal culture. Population levels of enterococci in group III mice (LF and inoculated with enterococci) were significantly higher than those detected in conventional and RLF animals (Table 1; the Student *t* test, *P* < 0.05). An additional experiment in which *Lactobacillus* populations present in the forestomachs, duodena, jejunum, ilea, and ceca of RLF mice colonized by lactobacilli and in those of conventional mice were determined was carried out. The number of lactobacilli present in each region of the gastrointestinal tract was similar in both groups of animals (Table 2).

Bile salt hydrolase activity in colonized mice. Bile salt hydrolase activity was measured in intestinal contents from the various mouse groups described in Table 1. The data collected from two independent experiments are shown in Table 3. Bile salt hydrolase activity in ileal contents was reduced 86% in the absence of lactobacilli (RLF) and >98% in the absence of lactobacilli and enterococci (LF) compared with that of conventional mice. Hydrolase activity in cecal contents was reduced 74 and 94%, respectively, compared

TABLE 2. *Lactobacillus* populations in the gastrointestinal tracts of conventional and RLF mice inoculated with *Lactobacillus* strains 100-18, 100-20, and 100-21

| Organ | <i>Lactobacillus</i> population in mice ^a | |
|-------------|--|--------------------------|
| | Conventional | RLF + lacto ^b |
| Forestomach | 8.7 (0.3) | 8.5 (0.3) |
| Duodenum | 6.6 (0.3) | 6.5 (0.5) |
| Jejunum | 7.5 (0.1) | 7.8 (0.2) |
| Ileum | 7.8 (0.3) | 8.2 (0.3) |
| Cecum | 8.3 (0.3) | 8.5 (0.1) |

^a Mean log₁₀ count per gram of organ (standard error of the mean). Five mice per group.

^b RLF mice inoculated with *Lactobacillus* strains 100-18, 100-20, and 100-21.

with that in conventional mice. Levels of bile salt hydrolase activity were significantly ($P \leq 0.05$) lower in ileal and cecal contents from LF mice colonized with enterococci (group III) than in LF mice colonized with lactobacilli (group II). The levels of bile salt hydrolase activities in ileal and cecal contents from LF and RLF mice colonized with lactobacilli (group II and group V, respectively) were not significantly different ($P \leq 0.05$) and approached the levels of activity found in conventional mice (group VI). Since the results in Table 3 were from two independent experiments which did not share a common group, the data across all treatments could not be grouped for statistical analyses. An additional experiment was carried out to compare the levels of bile salt hydrolase activity in contents of different intestinal segments from RLF mice colonized by lactobacilli and from conventional mice (Table 4). In each group of mice, the hydrolase activity increased distally through the intestinal tract, with the highest levels detected in the cecum. Moreover, within each intestinal segment, the levels of bile salt hydrolase activity in RLF mice harboring lactobacilli and in conventional mice were not different ($P \leq 0.05$).

DISCUSSION

The derivation of mouse colonies maintained by gnotobiotic methods in which the animals harbor a complex intestinal microflora from which lactobacilli are absent afforded us the opportunity to evaluate the influence of lactobacilli on bile salt hydrolase activity in the intestinal contents. Bile salt hydrolase activity in intestinal samples from LF mice colonized by lactobacilli and from RLF mice colonized by lactobacilli was numerically similar and statistically no different ($P \leq 0.05$) from that present in samples from conventional mice. Enterococci are not major contributors to the total bile salt hydrolase activity in the murine intestinal tract,

TABLE 4. Bile salt hydrolase activities in intestinal contents from RLF mice inoculated with *Lactobacillus* strains 100-18, 100-20, and 100-21 and from conventional mice

| Group | Sp act in the ^a : | | | |
|---------------------|------------------------------|-------------|-------------|-------------|
| | Duodenum | Jejunum | Ileum | Cecum |
| RLF + lacto-bacilli | 225 | 373 (52) | 732 (145) | 4,731 (443) |
| Conventional | 400 | 1,120 (137) | 1,533 (323) | 3,991 (476) |

^a Specific activity = nanomoles of cholic acid released per 30 min per g of contents (wet weight). Five animals per group. There were no significant differences ($P \leq 0.05$). Values within parentheses are standard errors of the mean. Contents were pooled from the five animals for the duodenum samples.

since LF mice inoculated with enterococci harbored a larger population of these bacteria than did their conventional counterparts (Table 1) yet exhibited only 5% of the bile salt hydrolase activity detected in ileal contents from LF mice colonized by lactobacilli (Table 3). We conclude, therefore, that lactobacilli are the major bile salt hydrolase-producing bacteria in the murine intestinal tract. Probably because lactobacilli inhabit the forestomach of mice, bile salt hydrolase activity can be detected in all regions of the small bowel, including the duodenum.

The significance of bile salt hydrolase to the lactobacilli is not known. It is probably not a detoxification mechanism, since free bile acids are more inhibitory than conjugated bile salts to anaerobes and gram-positive aerobes (7). It can be speculated that lactobacilli utilize the amino acid portion of the hydrolyzed bile salt. Van Eldere et al. (24) and Huijghebaert et al. (11), for example, have reported that certain strains of clostridia utilize the taurine portion of bile salts as electron acceptors and have demonstrated that growth rates are improved in the presence of taurine and taurine-conjugated bile salts. In the one *Lactobacillus* species that has been tested, however, taurine or taurine conjugates did not affect bacterial growth (24). The lactobacilli do not utilize the steroid moiety of the bile salt for cellular precursors, since incubation of lactobacilli with conjugated bile salts radiolabeled in the steroid nucleus yielded only two spots when thin-layer chromatography plates were autoradiographed. These spots corresponded to the free bile acid and the unhydrolyzed substrate, indicating that neither ring cleavage nor subsequent metabolism occurred (S. D. Feighner and M. P. Dashkevich, unpublished observations).

The significance to the murine host of bile salt hydrolase activity along the entire length of the small bowel is unknown. Colonization of the proximal small bowel of humans with bile salt-hydrolyzing bacteria, however, is a feature of pathophysiological conditions grouped together as the contaminated small bowel syndrome (10). Deconjugated bile

TABLE 3. Bile salt hydrolase activity in ileal and cecal contents from LF, RLF, and conventional mice

| Expt no. | Mouse group | Sp act in ^a : | |
|----------|--------------------------------------|--------------------------|----------------------------|
| | | Ileum | Cecum |
| 1 | I (LF) | 60 (10; $n = 7$) b | 760 (30; $n = 6$) b |
| 2 | II (LF + 100-23 and 100-21) | 3,490 (240; $n = 8$) B | 5,130 (310; $n = 8$) B |
| 2 | III (LF + enterococci) | 180 (0.4; $n = 8$) C | 980 (70; $n = 8$) C |
| 1 | IV (RLF) | 540 (40; $n = 10$) c | 3,120 (140; $n = 10$) c |
| 2 | V (RLF + 100-18, 100-20, and 100-21) | 1,990 (150; $n = 10$) B | 6,180 (260; $n = 10$) B |
| 1 | VI (conventional) | 3,970 (410; $n = 6$) d | 11,960 (1,000; $n = 6$) d |

^a Specific activity = nanomoles of cholic acid released per 30 min per g of contents (wet weight). Values in parentheses are standard errors of the means and numbers of animals per group. In each column, different letters within experiment 1 or 2 following entries indicate a significant difference ($P \leq 0.05$).

acids, present in the intestinal lumen as a result of bile salt hydrolase activity, are involved in these pathological conditions, since the free bile acids are toxic to enterocytes and since they impair carbohydrate and protein absorption results. Lipid absorption is impaired because, owing to bile salt hydrolase activity, concentrations of conjugated bile acid molecules suboptimal for adequate micelle formation are present in the intestinal lumen (10). It is possible, therefore, that intestinal bile salt hydrolase activity due to lactobacilli imposes a physiological burden on the animal host. We plan to utilize our unique LF mouse colonies to investigate this possibility.

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