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Bile Salt Hydrolase Activity and Resistance to Toxicity of Conjugated Bile Salts Are Unrelated Properties in Lactobacilli

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Bacteria of numerous species isolated from the human gastrointestinal tract express bile salt hydrolase (BSH) activity. How this activity contributes to functions of the microorganisms in the gastrointestinal tract is not known. We tested the hypothesis that a BSH protects the cells that produce it from the toxicity of conjugated bile salts. Forty-nine strains of numerous *Lactobacillus* spp. were assayed to determine their capacities to express BSH activities (taurodeoxycholic acid [TDCA] hydrolase and taurocholic acid [TCA] hydrolase activities) and their capacities to resist the toxicity of a conjugated bile acid (TDCA). Thirty of these strains had been isolated from the human intestine, 15 had been recovered from dairy products, and 4 had originated from other sources. Twenty-six of the strains expressed both TDCA hydrolase and TCA hydrolase activities. One strain that expressed TDCA hydrolase activity did not express TCA hydrolase activity. Conversely, in one strain for which the assay for TDCA hydrolase activity gave a negative result there was evidence of TCA hydrolase activity. Twenty-five of the strains were found to resist the toxicity of TDCA. Fourteen of these strains were of human origin, nine were from dairy products, and two were from other sources. Of the 26 strains expressing both TDCA hydrolase and TCA hydrolase activities, 15 were resistant to TDCA toxicity, 6 were susceptible, and 5 gave inconclusive results. Of the 17 strains that gave negative results for either of the enzymes, 7 were resistant to the toxicity, 9 were susceptible, and 1 gave inconclusive results. These findings do not support the hypothesis tested. They suggest, however, that BSH activity is important at some level for lactobacillus colonization of the human intestine.

Bile acids are synthesized from cholesterol and conjugated to either glycine or taurine in the liver (20). They then pass into the intestine, where the amino acid may be hydrolyzed from the conjugated bile acid by bacterial enzymes. These enzymes constitute a class collectively known as conjugated bile salt hydrolases (BSHs). They are expressed by gastrointestinal bacteria of several genera, including *Bacteroides*, *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus* (15).

How the capacity to express BSHs contributes to the functions of bacteria in the human gastrointestinal tract has been speculated about for several years (6, 12, 19, 23); two major hypotheses have been advanced. One hypothesis states that bacteria of some species that are able to deconjugate bile salts may be able to use the amino acid taurine as an electron acceptor. Evidence which supports this hypothesis has been obtained for certain *Clostridium* species (12, 24). The second hypothesis states that BSHs decrease the toxicity of conjugated bile acids for bacteria (19). Compared with their conjugated counterparts, deconjugated bile acids have decreased solubility and diminished detergent activity and may, therefore, be less toxic to bacteria in the intestine (6). It has also been suggested that BSHs are detergent shock proteins that protect the bacteria that produce them from the toxicity of bile acids in the gastrointestinal tract (1, 8). We used strains of several species of the genus *Lactobacillus* to test the hypothesis that BSHs protect bacteria against the toxicity of conjugated bile acids. In this study we compared strains isolated from the human intestine and from dairy products.

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MATERIALS AND METHODS

Media and chemicals. MRS (Becton Dickinson) broth and agar were used in all experiments. Sodium salts of taurodeoxycholic acid (TDCA) and taurocholic acid (TCA) were obtained from Sigma. [24-^{14}C]TCA was obtained from New England Nuclear.

Bacterial strains. Forty-nine *Lactobacillus* strains isolated from the human intestine, dairy products, or other sources were obtained from culture collections in Belgium, Germany, Japan, and the United States (Table 1). They were grown anaerobically in MRS broth at 37°C. Stock cultures stored at −80°C were prepared from overnight cultures grown in MRS to which 15% glycerol was added just prior to freezing.

BSH assays. The strains were tested for TDCA hydrolase activity with an assay involving MRS agar plates supplemented with 0.5% TDCA (MRS-TDCA) (5, 18). Overnight MRS broth cultures were inoculated onto the agar medium, which was then incubated anaerobically for 5 days. BSH activity was present when deoxycholic acid precipitated in the agar medium below and around a colony. When necessary, a stereoscope was used to detect the precipitate. The strains were tested for TCA hydrolase activity with a radiochemical assay (7, 18). This assay was positive when [^{14}C]cholic acid was detected by liquid scintillation counting in supernatant solutions from cultures to which [^{14}C]TCA had been added.

Assay for toxicity of conjugated bile salt. The strains were tested for the capacity to resist the bactericidal activity of a conjugated bile salt (TDCA) with an assay modified from the assay described by De Smet et al. (6). A stationary-phase culture inoculum (1%) was added to MRS broth supplemented with TDCA at a concentration of 0, 1, 3, or 5 mM. At zero time and after 1, 5, and 10 h of anaerobic incubation at 37°C, dilutions of the bacterial suspensions were prepared. Aliquots of the dilutions were smeared onto MRS agar plates, which were then incubated anaerobically at 37°C for 48 h. Population estimates were made from viable counts.

Statistical analysis. Population estimates made in assays to determine the capacities of the strains to resist the toxicity of TDCA were analyzed by analysis of variance and linear regression analysis with programs posted at http://faculty.vassar.edu/~lowry/VassarStats.html.

TABLE 1. *Lactobacillus* strains tested, their origins, their capacities to express TDCA hydrolase and TCA hydrolase activities, and their capacities to resist toxicity of TDCA

Group	*Lactobacillus* species or subspecies[a]	Strain[b]	Origin	TDCA hydrolase activity[c]	TCA Hydrolase activity[d]	Resistance to TDCA toxicity[e]
Human isolates	*L. acidophilus*	ATCC 4356	Human	P	P	R
		ATCC 4357	Human	P	P	R
		ATCC 53544	Infant	P	P	R
		JCM 1028	Intestine	P	P	A
		JCM 1034	Intestine	P	P	R
		JCM 1229	Intestine	P	P	S
	L. amylovorus	JCM 2125	Intestine	P	P	S
		JCM 5811	Feces	P	P	A
	L. brevis	ATCC 14869	Feces	N	N	R
		BCCM 7944	Feces	P	P	R
	L. buchneri	JCM 1068	Intestine	N	N	R
		JCM 1069	Intestine	P	N	R
	L. crispatus	JCM 8778	Feces	P	P	S
		JCM 8779	Feces	P	P	S
		BCCM 12003	Infant feces	P	P	R
	L. fermentum	ATCC 11976	Infant	P	P	R
		ATCC 23271	Intestine	N	N	R
	L. gasseri	ATCC 19992	Feces	N	N	S
		JCM 1025	Intestine	P	P	A
		BCCM 9203	Human	P	P	A
	L. johnsonii	JCM 1022	Intestine	P	P	S
		JCM 8791	Feces	P	P	A
	L. paracasei subsp. *paracasei*	ATCC 27092	Feces	N	N	S
		JCM 1109	Intestine	N	N	S
	L. reuteri	ATCC 23272	Feces	P	P	R
		BCCM 13046	Feces	P	P	R
	L. rhamnosus	BCCM 10770	Bowel drain	N	N	S
	L. ruminis	ATCC 25644	Intestine	P	P	R
	ND[f]	ATCC 11146	Infant feces	N	N	S
		ATCC 53103	Feces	N	N	S
Dairy isolates	*L. acidophilus*	BCCM 8151	Acidophilus milk	0	N	R
	L. brevis	BCCM 11998	Dairy starter	P	P	R
		BCCM 18022	Zabady (yogurt)	P	P	R
	L. curvatus subsp. *curvatus*	BCCM 9198	Milk	N	N	S
	L. delbrueckii subsp. *bulgaricus*	ATCC 11842	Bulgarian yogurt	0	N	S
		BCCM 12168	Homemade yogurt	0	N	R
		DSM 20080	Yogurt	0	N	S
	L. kefir	BCCM 9480	Kefir grains	N	P	A
	L. maltaromicus	BCCM 6903	Raw milk	N	N	R
	L. paracasei subsp. *paracasei*	BCCM 11407	Milk	N	N	S
	L. plantarum	BCCM 18021	Milk	P	P	R
		BCCM 18027	Laban Rayeb	P	P	R
		BCCM 18035	Milk	N	N	R
	L. rhamnosus	BCCM 18028	Laban Rayeb	N	N	A
		BCCM 18030	Zabady (yogurt)	N	N	R
Other isolates	*L. acidophilus*	ATCC 700396 (= NCK 1070)	Unknown	P	P	R
	L. fermentum	BCCM 6902	Fermented beets	N	N	S
		DSM 20050	Unknown	N	N	R
	L. gasseri	NCK 99	Unknown	P	P	S

[a] The species or subspecies as determined by the culture collection was assumed to be correct.

[b] ATCC, American Type Culture Collection; JCM, Japanese Collection of Microorganisms; BCCM, Belgian Coordinated Collection of Microorganisms; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; NCK, Todd Klaenhammer, North Carolina State University.

[c] Capacity to express TDCA hydrolase activity as detected when precipitates of deoxycholic acid appeared below bacterial colonies growing on MRS-TDCA. P, positive; N, negative; 0, no growth.

[d] Capacity to express TCA hydrolase activity as detected when [^{14}C]cholic acid could be extracted from supernatant solutions of *Lactobacillus* cultures in MRS medium containing [^{14}C]TCA. P, positive; N, negative.

[e] Estimated by examining bacterial populations growing for 0, 1, 5, and 10 h in MRS containing TDCA at concentrations of at 0, 1, 3 and 5 mM (see Materials and Methods). R, resistant; S, sensitive; A, ambiguous (statistical analysis was not able to resolve resistance or sensitivity, the result was ambiguous [see Table 2]).

[f] ND, not determined.

TABLE 2. Representative data from assays of 49 *Lactobacillus* strains to determine their capacities to resist the toxicity of TDCA at different concentrations and statistical methods used to analyze the data for each strain[a]

Strain	Resistance[b]	TDCA concn (mM)	Population size (10^7 cells ml^{-1}) after incubation for:				ANOVA *P* value[c]	Linear regression analysis[d]	
			0 h	1 h	5 h	10 h		*r* value	*P* value
JCM 1068	R	0	11.8[e]	12.8	23.1	31.9	0.99	0.98	0.01
		1	13.5	13.0	24.8	35.4			
		3	10.7	12.2	22.2	35.4			
		5	14.7	10.9	24.3	36.6			
ATCC 25644	R	0	13.9	14.5	31.7	33.1	0.14	0.91	0.05
		1	12.6	12.2	26.7	28.6			
		3	16.9	12.6	23.9	25.6			
		5	4.9	4.1	11.0	18.3			
JCM 1229	S	0	9.9	10.5	24.4	31.3	0.14	−0.95	0.03
		1	10.4	12.1	13.6	12.1			
		3	11.2	12.7	12.2	7.7			
		5	11.8	11.6	10.2	5.7			
JCM 1025	S	0	3.3	3.5	7.4	9.3	0.17	−0.86	0.20
		1	3.5	5.0	7.8	17.3			
		3	4.7	5.0	3.6	2.6			
		5	4.9	3.2	2.1	1.1			
BCCM 9203	A	0	5.5	5.2	13.3	20.8	0.60	0.18	0.49
		1	8.0	7.6	13.3	18.1			
		3	6.9	6.6	12.3	15.8			
		5	6.3	6.5	10.4	5.9			

[a] Assayed by examining the populations in MRS broth medium containing TDCA at the concentrations indicated. The population sizes were estimated from the viable counts on MRS agar at the times indicated.

[b] R, Resistant; S, susceptible; A, ambiguous.

[c] One-way analysis of variance (ANOVA) for four independent samples. The *P* value indicates the probability that the means and variance around the means for populations grown in MRS lacking TDCA differ from the means and variance around the means for populations grown in MRS containing TDCA at concentrations of 1, 3, and 5 mM (identity, $r = 1.0$).

[d] *r* is the Pearson correlation coefficient, the coefficient of correlation for data that generated slopes of lines from estimates of population-size after 0, 1, 5, and 10 h of incubation in MRS lacking and containing 5 mM TDCA (identity, $r = 1.0$). *P* is the probability (one tailed) that the correlation coefficient indicates nonidentity of the slopes of the lines generated (identity, $P = 0.00$).

[e] Estimated population size.

RESULTS

BSH activities. Four of the 49 *Lactobacillus* strains tested failed to produce colonies on MRS-TDCA. Of the 45 strains that grew on this medium, 27 expressed TDCA hydrolase activity (Table 1); 21 of these 27 strains had been isolated from the human intestine, 4 were from dairy products, and 2 were from other sources. All 49 strains were tested for TCA hydrolase activity with the radiochemical assay (Table 1). Twenty-seven of the strains also exhibited this type of activity; 20 of these 27 strains had been isolated from human sources, 5 were from dairy products, and 2 were from other sources. One isolate from a human intestine, *Lactobacillus buchneri* JCM 1069, was able to express TDCA hydrolase activity but did not express TCA hydrolase activity. Conversely, a strain from the dairy product Kefir grains, *Lactobacillus kefir* BCCM 9480, was positive in the assay for TCA hydrolase activity but negative in the assay for TDCA hydrolase activity (Table 1).

Toxicity of conjugated bile salt. The 49 strains were also assayed to determine their capacities to resist the toxicity of the conjugated bile salt TDCA (Table 1). Sample data from the assays and the methods used for statistical analysis are shown in Table 2. Of the 49 isolates, 25 were resistant, 17 were susceptible, and 7 gave inconclusive (statistically ambiguous) results (Table 1). Fourteen of the 30 human isolates were resistant, compared to nine of the dairy strains. Two of the strains from sources other than human specimens and dairy products also resisted the toxicity of TDCA. Of the 26 strains that were able to express both TDCA hydrolase and TCA hydrolase activities, 15 resisted TDCA toxicity, 6 were sensitive to it, and 5 gave inconclusive results. Of the 17 strains that gave negative results in both enzymatic assays, 7 were resistant to the bactericidal activity, 9 were sensitive to it, and 1 gave a statistically ambiguous result (Table 1).

DISCUSSION

Strains of certain species of the genus *Lactobacillus* are well known to be members of the indigenous gastrointestinal microbiota of humans and other animals (21). Numerous factors are thought to be involved in the capacities of these bacteria to colonize the intestinal tract. Some of these factors are the capacity to adhere to intestinal epithelium or to colonize the mucous gel overlaying this epithelium (2, 4, 11, 14, 16), the capacity to withstand low pH (6, 9), and the capacity to express certain proteins (16, 17). Some of these proteins may have BSH activities. Conjugated bile salts are periodically released into the intestinal environment (22). These salts are known to be toxic to bacteria (6, 10, 23). Therefore, bacteria in the intestine may express BSHs to protect themselves from this

toxicity (1, 8). As noted above, we examined this hypothesis in this study. Our findings do not support the hypothesis. The capacities of lactobacilli to resist the toxicity of TDCA and the capacities of lactobacilli to express TDCA hydrolase and TCA hydrolase activities appear to be independent properties. Several years ago, using technologies different from our technology, Gilliland and Speck (10) and Tannock et al. (23) reached a similar conclusion.

The conclusion described above has been disputed, however, by findings obtained in experiments conducted by De Smet et al. (6). In their experiments, these investigators used some genetically engineered, isogenic strains derived from a wild-type isolate of *Lactobacillus plantarum*. Using assays similar to some of the assays employed in our study, they obtained data which indicated that the capacity of *L. plantarum* to resist the toxicity of TDCA is related to its ability to express TDCA hydrolase activity (6). These data are consistent with our findings obtained with two isolates of *L. plantarum* (BCCM 18201 and BCCM 18207) but are inconsistent with our data obtained with another strain of this species (BCCM 18035) (Table 1). Moreover, they are inconsistent with our overall conclusion in this study and the conclusions of Gilliland and Speck (10) and Tannock et al. (23). Only further research may resolve the discrepancies.

A side benefit of our study was an observation made with two strains concerning specificity in the catalytic activities of BSHs (6, 13). One of the strains, *L. buchneri* JCM 1069, expressed TDCA hydrolase activity but not TCA hydrolase activity. Conversely, the other strain, *L. kefir* BCCM 9480, expressed TCA hydrolase activity but not TDCA hydrolase activity. The specificity of BSHs may be influenced either by the amino acid in the conjugate or by other side chains on the steroid moiety (10, 13). The TDCA and TCA used in our assays for BSH activities both have taurine as their amino acid moiety. They differ, however, at the 7α position of their steroid moieties. TDCA lacks a hydroxyl group at the 7α position that is present in TCA. Presumably, therefore, the hydrolase expressed by BCCM 9480 recognizes this 7α hydroxyl group, while the hydrolase expressed by JCM 1069 recognizes the 7α position lacking the group. Whatever the underlying mechanism, our observations confirm the findings of other investigators that BSHs can exhibit substrate specificity dictated by the structure of the steroid moiety of the bile salt conjugate (6).

Lactobacilli have long been used as probiotics in humans (17). A problem facing probiotic use is survival and colonization of the intestine by the bacteria after oral consumption. Probiotics must survive the low pH of the stomach and the conjugated bile acids in the duodenum (17). The introduced bacteria need to overcome major obstacles, therefore, in order to travel through the stomach and small intestine to the large bowel. In the latter region, moreover, they must compete for the resources available with the intestinal microbiota. Such obstacles may explain the finding that introduced lactobacilli disappear from human feces after oral administration has ended (16).

Producers of probiotic bacteria may be able to improve the survivability of probiotic lactobacilli by manipulating genes that encode properties which enable the bacteria to survive the obstacles in the gastrointestinal tract (3). One such property may be the capacity to express a BSH. Our findings do not support the hypothesis that the capacity to express such an enzyme is related to the capacity of lactobacilli to resist the toxicity of conjugated bile salts. They do support a hypothesis, however, that the capacity to express a BSH is on some level important for the bacteria living in the human intestine (17). A high proportion of strains isolated from the human intestine express both TDCA hydrolase and TCA hydrolase activities. Therefore, BSH activity may be important in some way for the bacteria to survive in and colonize the intestine. At this time, however, the function remains obscure. Given the potential importance of the enzymes, however, genes encoding them may be important targets for genetic manipulation (17).

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REFERENCES

1. **Adamowicz, M., P. M. Kelley, and K. W. Nickerson.** 1991. Detergent (sodium dodecyl sulfate) shock proteins in *Escherichia coli*. J. Bacteriol. **173:**229–233.
2. **Alander, M., R. Satokari, R. Korpela, M. Saxelin, T. Vilpponen-Salmela, T. Mattila-Sandholm, and A. von Wright.** 1999. Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG, after oral consumption. Appl. Environ. Microbiol. **65:**351–354.
3. **Chassy, B. M.** 1985. Prospects for improving economically significant *Lactobacillus* strains by 'genetic technology.' Trends Biotechnol. **3:**273–275.
4. **Coconnier, M. H., T. R. Klaenhammer, S. Kerneis, M. F. Bernet, and A. L. Servin.** 1992. Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. Appl. Environ. Microbiol. **58:**2034–2039.
5. **Dashkevicz, M. D., and S. D. Feighner.** 1989. Development of a differential medium for bile salt hydrolase-active *Lactobacillus* spp. Appl. Environ. Microbiol. **55:**11–16.
6. **De Smet, I., L. Van Hoorde, M. Vande Woestyne, H. Christiaens, and W. Verstraete.** 1995. Significance of bile salt hydrolytic activities of lactobacilli. J. Appl. Bacteriol. **79:**292–301.
7. **Elkins, C. A., and D. C. Savage.** 1998. Identification of genes encoding conjugated bile salt hydrolase and transport in *Lactobacillus johnsonii* 100-100. J. Bacteriol. **180:**4344–4349.
8. **Flahaut, S., J., Frere, P. Boutibonnes, and Y. Auffray.** 1996. Comparison of the bile salts and sodium dodecyl stress responses in *Enterococcus faecalis*. Appl. Environ. Microbiol. **62:**2416–2420.
9. **Gardiner, G., R. P. Ross, J. K. Collins, G. Fitzgerald, and C. Stanton.** 1998. Development of a probiotic cheddar cheese containing human-derived *Lactobacillus paracasei* strains. Appl. Environ. Microbiol. **64:**2192–2199.
10. **Gilliland, S. E., and M. L. Speck.** 1977. Deconjugation of bile acids by intestinal lactobacilli. Appl. Environ. Microbiol. **33:**15–18.
11. **Greene, J. D., and T. R. Klaenhammer.** 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. Appl. Environ. Microbiol. **60:**4487–4494.
12. **Huijghebaert, S. M., J. A. Mertens, and H. J. Eyssen.** 1982. Isolation of a bile salt sulfatase-producing *Clostridium* strain from rat intestinal microflora. Appl. Environ. Microbiol. **43:**185–192.
13. **Huijghebaert, S. M., and A. F. Hoffman.** 1986. Influence of the amino acid moiety on deconjugation of bile acid amidates by cholyglycine hydrolase of human fecal cultures. J. Lipid Res. **27:**742–752.
14. **Huis In't Veld, J. H. J., and C. Shortt.** 1996. Selection criteria for probiotic microorganisms. R. Soc. Med. Int. Congr. Symp. Ser. **219:**27–36.
15. **Hylemon, P. B.** 1985. Metabolism of bile acids in intestinal microflora, p. 331–334. *In* H. Danielson and J. Svövall (ed.), Steroids and bile acids: new comprehensive biochemistry, vol. 12. Elsevier Publishing Inc., Amsterdam, The Netherlands.
16. **Jacobsen, C. N., V. Rosenfeldt Neilsen, A. E. Hayford, P. L. Møller, K. F. Michaelsen, A. Pærregaard, B. Sandström, M. Tvede, and M. Jakobsen.** 1999. Screening of probiotic activities of forty-seven strains of *Lactobacillus* spp. by in vitro techniques and evaluation of the colonization ability of five selected strains in humans. Appl. Environ. Microbiol. **65:**4949–4956.
17. **Lee, Y.-K., and S. Salminen.** 1995. The coming of age of probiotics. Trends Food Sci. Technol. **6:**241–245.
18. **Lundeen, S. G., and D. C. Savage.** 1990. Characterization and purification of bile salt hydrolase from *Lactobacillus* sp. strain 100-100. J. Bacteriol. **172:** 4171–4177.
19. **Savage, D. C.** 1992. Gastrointestinal microbial ecology: possible modes of action of direct-fed microbials in animal production—a review of the literature, p. 11–81. *In* Direct-fed microbials in animal production. National Feed Ingredients Association, West Des Moines, Iowa.

20. **Savage, D. C., S. G. Lundeen, and L. T. O'Connor.** 1995. Mechanisms by which indigenous microorganisms colonise epithelial surfaces as a reservoir of the lumenal microflora in the gastrointestinal tract. Microecol. Ther. **21:**27–36.
21. **Savage, D. C.** 1999. Mucosal microbiota, p. 19–30. *In* P. L. Ogra, J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, and J. R., McGhee (ed.), Mucosal immunology, 2nd ed. Academic Press, San Diego, Calif.
22. **Stevens, C. E., and I. D. Hume.** 1995. Comparative physiology of the vertebrate digestive system, p. 245–246. Cambridge University Press, Cambridge, United Kingdom.
23. **Tannock, G. W., M. P. Dashkevicz, and S. D. Feighner.** 1989. Lactobacilli and bile salt hydrolase in the murine intestinal tract. Appl. Environ. Microbiol. **55:**1848–1851.
24. **Van Eldere, J., J. Robben, G. De Pauw, R. Merckx, and H. Eyssen.** 1988. Isolation and identification of intestinal steroid-desulfating bacteria from rats and humans. Appl. Environ. Microbiol. **54:**2112–2117.