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Isolation of Tannin-Degrading Lactobacilli from Humans and Fermented Foods

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Lactobacilli with tannase activity were isolated from human feces and fermented foods. A PCR-based taxonomic assay revealed that the isolates belong to *Lactobacillus plantarum*, *L. paraplantarum*, and *L. pentosus*. Additional studies on a range of *Lactobacillus* species from established culture collections confirmed that this enzymatic activity is a phenotypic property common to these three species.

Hydrolyzable tannins, such as gallotannin and ellagitannin, are widely distributed in the plant kingdom (17). These tannins bind readily with proteins to form indigestible complexes, and they are thus considered effective antinutritional compounds for herbivorous animals (19). Tannase (tannin acylhydrolase) specifically breaks the galloyl ester bonds of tannins, thereby inhibiting their protein-binding properties (3). The enzyme is common not only in fungal strains (1, 16) but also in several taxonomically novel bacterial species which are frequently found in alimentary tracts of koalas (11, 12) and of goats and sheep fed tannin-rich forage (8, 18). These findings suggest that the bacteria help the animals digest the tanniferous leaves.

During quantitative and qualitative studies of the tannase-producing bacteria in the intestinal microflora of various mammalian species (9, 13), another novel type of tannin-degrading bacteria from human fecal samples and fermented foods was isolated. We present here a brief report on the ecological prevalence, phenotypic characteristics, and identities of these tannin-degrading bacteria.

A swab sample (ca. 0.1 g [wet weight]) of fresh human feces was taken from a total of 35 healthy Japanese individuals. Food samples (ca. 1 g [wet weight]) were taken from 61 samples of fermented foods commercially available in Japan. These foods included 46 pickled vegetables from different producers and 15 different commercial brands of cheeses. Samples were transferred to tubes containing 30 ml of MRS broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and thoroughly mixed aseptically using a homogenizer and a vortex mixer. The mixture was then incubated anaerobically in an Anaero-Pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C for 48 h. After incubation, one loopful (ca. 10 μl) of each culture was streaked onto tannin-treated brain heart infusion agar (10). The inoculated plates were incubated anaerobically in an Anaero-Pack (Mitsubishi) at 37°C for 72 h. After incubation, colonies with a distinct clear zone extending just beyond their edges were subcultured onto MRS agar plates. Cultures of the isolates were considered to be pure after three successive subcultures on MRS agar plates. As a result, we obtained 3 tannin-protein complex-degrading isolates from the feces of 3 individuals and 25 isolates from 25 food samples (24 fermented vegetables and one brand of cheese) (Table 1).

Tannase activity of the isolates was confirmed by a visual reading method described elsewhere (14). Briefly, fresh cultures on MRS agar plates were harvested with sterile cotton swabs and suspended in 1 ml of substrate medium (pH 5.0) containing NaH_2PO_4 (33 mmol/liter) and methylgallate (20 mmol/liter) (Wako Pure Chemical Industries Ltd., Osaka, Japan) to prepare a dense suspension (at least equivalent to a no. 3 McFarland turbidity standard). The substrate medium was then incubated aerobically at 37°C for 24 h. After incubation, the sample was alkalinized with an equal amount of saturated $NaHCO_3$ solution (pH 8.6) and exposed to the atmosphere at room temperature (23°C) for 1 h. Green to brown coloration of the medium was judged as a positive indicator of tannase activity. All 28 isolates showed positive results for tannase activity (Table 1).

Several tannase-positive bacteria, such as *Streptococcus gallolyticus* sp. nov. (11) and *Lonepinella koalarum* (12), have distinct tannase activity and also show gallate decarboxylation of gallic acid to pyrogallol. We determined the gallate decarboxylase activity in the isolates using a simple colorimetric test described elsewhere (15). Briefly, 50 μl of an overnight culture of the isolate in MRS broth (Oxoid) was inoculated into 10 ml of MRS broth containing 10 mmol of gallic acid (Wako) per liter (final concentration) and incubated anaerobically in an Anaero-Pack (Mitsubishi) at 37°C for 3 days. After incubation, the culture was alkalinized with equal amounts of saturated $NaHCO_3$ solution (pH 8.6) and incubated aerobically at 37°C for 1 h. Light yellow to brown coloration of the medium was judged as a positive result for gallate decarboxylase activity, and all but two isolates, KOG 4 and KOG 11, were positive (Table 1).

Gram stains of the isolates showed gram-positive rods. Subsequent biochemical tests with a commercially available identification kit, API 50 CHL (API System, Montalieu, Vercieu, France), revealed that all three human fecal isolates (KHL 1, 2, and 3) belonged to *Lactobacillus plantarum*. Of the food isolates, 20 belonged to *L. plantarum*, 2 belonged to *L. pentosus*, and 3 remained unidentified (Table 1). However, a recent taxonomic study (4) claimed that the phenotypic differentiation of *L. plantarum* and *L. pentosus* is difficult. Furthermore, *L. paraplantarum*, a species phenotypically indistinguishable but taxonomically distinct from the above two species has been proposed by other investigators (6).

A reliable PCR-based method for distinguishing among the lactobacilli has been developed (2). The method is designed to amplify species-specific sequences in the 16S-23S ribosomal DNA (rDNA) spacer regions of these three *Lactobacillus* species. We performed this PCR assay on total DNAs extracted

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TABLE 1. Tannase and gallate decarboxylase activities, carbohydrate utilization profiles, and identities of *Lactobacillus* isolates

Strain	Source	Tannase	Gallate decarboxylase	Carbohydrate utilization profile as determined with the API 50 CHL system																						
				Glycerol	Erythritol	D-Arabinose	L-Arabinose	Ribose	D-Xylose	L-Xylose	Adonitol	β-Methyl-xyloside	Galactose	D-Glucose	D-Fructose	D-Mannose	L-Sorbose	Rhamnose	Dulcitol	Inositol	Mannitol	Sorbitol	α-Methyl-D-mannoside	α-Methyl-D-glucoside	*N*-Acetylglucosamine
KLH 1	Human feces	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	+
KLH 2	Human feces	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	−	+	−	−	+	+	+	−	+
KLH 3	Human feces	+	+	−	−	−	+	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	+
KOG 1	Pickled rice bran	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 2	Turnip pickled with rice bran	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	+
KOG 3	Korean cabbage kimchi	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	+
KOG 4	Cucumber pickled with rice bran	+	−	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	+	−	+	+	−	−	+
KOG 5	Chinese pickled with rice bran	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+
KOG 6	Radish pickled with rice bran	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 7	Pickled eggplant and cucumber	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 8	Korean cabbage kimchi	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	+
KOG 9	Chinese cabbage pickled with rice bran	+	+	+	−	−	+	+	+	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 10	Eggplant pickled with rice bran	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	+
KOG 11	Chinese cabbage pickled with rice bran	+	−	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 12	Pickled radish and tang	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	+
KOG 13	Korean radish kimchi	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+	+
KOG 14	Pickled eggplant	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 15	Korean cabbage kimchi	+	+	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 16	Korean cabbage kimchi	+	+	−	−	−	−	+	−	−	−	−	−	+	+	+	−	−	−	−	+	+	−	−	+
KOG 17	Pickled vegetables	+	+	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 18	Turnip pickled with rice bran	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	+
KOG 19	Mixed vegetables pickled with rice bran	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	+
KOG 20	Pickled cucumber and radish	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	+	+
KOG 21	Pickled vegetables	+	+	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	+
KOG 22	Chinese cabbage pickled with rice bran	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	+
KOG 23	Korean radish kimchi	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	−	+
KOG 24	Cheese	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	+	−	−	+
KOG 25	Korean cabbage kimchi	+	+	−	−	−	−	+	−	−	−	−	+	+	+	+	−	−	−	−	+	−	−	−	+

[a] With the API analytical profile indices (>99.0% identity) expressed as "good identification" or "excellent identification."
[b] Described by Berthier and Ehrlich (2).

(7) from the isolates. The PCR used three separate sets of primers: the primer set 16 (16S rRNA gene, 3′ end, forward; 5′-GCTGGATCACCTCCTTTC-3′) and Lpl (16S-23S rDNA spacer region, *L. plantarum* specific; 5′-ATGAGGTATTCAA CTTATG-3′), specific to *L. plantarum*; the primer set 16 and Lpapl (16S-23S rDNA spacer region, *L. paraplantarum* specific; 5′-ATGAGGTATTCAACTTATT-3′), specific to both *L. plantarum* and *L. paraplantarum*; and the primer set 16 and Lpe (16S-23S rDNA spacer region, *L. pentosus* specific; 5′-GTATTCAACTTATTACAACG-3′), specific to *L. pentosus*. The PCR consisted of denaturation at 94°C for 1 min, hybridization at 53°C for 1 min, and elongation at 72°C for 1 min; this cycle was repeated 30 times. The PCR products were electrophoresed on an agarose gel and were visualized by UV illumination for specifically amplified fragments (approximately 200 bp in size for all specific primer sets) after ethidium bromide staining. The results of the PCR assay correlated with those obtained using the API 50 CHL system for 20 isolates identified as *L. plantarum* and 2 isolates identified as *L. pentosus*. However, four isolates tentatively identified as *L. plantarum* with the API system were found to be *L. paraplantarum* by PCR (Table 1). In addition, two (KOG 15 and KOG 25) of the three isolates whose identities could not be determined by the API system due to their irregular carbohydrate utilization patterns were assigned to *L. paraplantarum*, and a remaining isolate (KOG 24) was assigned to *L. plantarum* (Table 2).

Subsequently, we examined a range of *Lactobacillus* species obtained from established culture collections for tannase and gallate decarboxylase activities. These included the type strains of *L. plantarum* (ATCC 14917), *L. paraplantarum* (ATCC 700211), and *L. pentosus* (ATCC 8041) and reference strains belonging to these species (Table 2). The strains were also assayed by the method described above to confirm their taxonomic identities. The results of the assays are summarized in Table 2. All strains received as *L. plantarum*, *L. paraplantarum*, and *L. pentosus* were positive for tannase activity and their identities were reconfirmed by the PCR assay (2). All of them except for *L. plantarum* CNRZ 184 were positive for gallate decarboxylase activity. Gallate decarboxylase activity was also observed in two strains of *L. gasseri*, although these strains were negative for tannase activity. The rest of the strains, belonging to 14 different *Lactobacillus* species, were negative for both tannase and gallate decarboxylase activities. The present study indicated that tannase activity is common in *L. plantarum*, *L. paraplantarum*, and *L. pentosus*. This enzymatic property may have an ecological advantage for these *Lactobacillus* species, as they are often associated with fermentation of plant materials (4). For example, the observed occurrence

TABLE 1—*Continued*

Carbohydrate utilization profile as determined with the API 50 CHL system																									Putative taxon by the API 50 CHL system[a]	Taxon as determined by PCR assay[b]
Amygdalin	Arbutin	Esculin	Salicin	Cellobiose	Maltose	Lactose	Melibiose	Sucrose	Trehalose	Inulin	Melezitose	D-Raffinose	Starch	Glycogen	Xylitol	β-Gentiobiose	D-Turanose	D-Lyxose	D-Tagatose	D-Fucose	L-Fucose	D-Arabitol	L-Arabitol	Gluconate	2-Keto-gluconate	5-Keto-gluconate		
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	−	+	+	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	−	+	+	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. paraplantarum*
+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. paraplantarum*
+	+	+	+	+	+	+	+	+	+	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−	+	−	−	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−	+	−	−	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−	*L. pentosus*	*L. pentosus*
+	+	+	+	+	+	−	−	+	+	−	+	−	−	−	−	+	−	−	−	−	−	+	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	−	−	−	−	+	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. paraplantarum*
+	+	+	+	+	+	−	−	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	Undetermined	*L. paraplantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	*L. pentosus*	*L. pentosus*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	+	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	+	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. paraplantarum*
+	+	+	+	+	+	−	+	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−	*L. plantarum*	*L. plantarum*
+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	Undetermined	*L. plantarum*
+	+	+	+	+	+	−	+	+	+	−	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	Undetermined	*L. paraplantarum*

rates of these species in the present study were indeed higher in fermented vegetables (52.2%) than in fermented milk products (6.3%).

This is the first study reporting the occurrence of lactobacilli capable of degrading hydrolyzable tannin in human gut microflora and foodstuffs. Since humans do not rely entirely on tannin-rich diets, the role played by these lactobacilli in human nutrition is probably marginal. Nevertheless, many beverages and teas that are routinely consumed in our society have been reported to contain various hydrolyzable tannins with marked pharmacological activities (5). The presence of lactobacilli with distinct tannase activity in the human alimentary tract may thus have a significant effect on the medicinal properties of tannins. Further study is necessary to evaluate this speculation.

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TABLE 2. Tannase and gallate decarboxylase activities of *Lactobacillus* strains of established culture collections

Name as received	Strain	Source	Tannase	Gallate decarboxylase	Taxon confirmed by PCR[a] assay
L. plantarum	ATCC[b] 14917[T]	Pickled cabbage	+	+	*L. plantarum*
	ATCC 8014	Unknown	+	+	*L. plantarum*
	CNRZ[c] 184	Unknown	+	−	*L. plantarum*
	CNRZ 1228	Cheese	+	+	*L. plantarum*
L. paraplantarum	ATCC 700211[T]	Beer contaminant	+	+	*L. paraplantarum*
	61D[d]	Human feces	+	+	*L. paraplantarum*
L. pentosus	ATCC 8041[T]	Silage	+	+	*L. pentosus*
	CNRZ 1544	Fermented olives	+	+	*L. pentosus*
	CNRZ 1561	Fermented olives	+	+	*L. pentosus*
L. gasseri	F191[e]	Human feces	−	+	ND[f]
	JCM[g] 5343	Unknown	−	+	ND
	JCM 5344	Vaginal tract	−	−	ND
	JCM 1131[T]	Human intestine	−	−	ND
	ATCC 332	Human feces	−	−	ND
L. reuteri	DSM[h] 20016	Human intestine	−	−	ND
	DSM 20015	Manure	−	−	ND
	DSM 20053	Human feces	−	−	ND
L. helveticus	ATCC 15009	Cheese	−	−	ND
	ATCC 10797	Cheese starter	−	−	ND
L. crispatus	JCM 1185	Unknown	−	−	ND
	F199[e]	Human feces	−	−	ND
L. salivarius	JCM 1231	Human saliva	−	−	ND
	JCM 1150	Saliva	−	−	ND
L. murinus	ATCC 1717	Rat intestine	−	−	ND
L. animalis	D-170[e]	Dog feces	−	−	ND
	JCM 5670	Dental plaque of baboon	−	−	ND
L. johnsonii	F133[e]	Calf feces	−	−	ND
	5F49[e]	Mouse feces	−	−	ND
L. acidophilus	JCM 1023	Rat feces	−	−	ND
	JCM 2123	Turkey feces	−	−	ND
	JCM 2010	Hog small intestine	−	−	ND
	ATCC 314	Unknown	−	−	ND
L. amylovorus	JCM 1126	Cattle waste-corn fermentation	−	−	ND
L. gallinarum	F41[e]	Chicken feces	−	−	ND
L. casei	JCM 1133	Saliva of child	−	−	ND
	ATCC 393	Cheese	−	−	ND
L. brevis	ATCC 8287	Fermented olives	−	−	ND
L. ruminis	JCM 1152	Bovine rumen	−	−	ND
L. aviarius	JCM 5666	Chicken feces	−	−	ND

[a] PCR assay as described by Berthier and Ehrlich (2).
[b] ATCC, American Type Culture Collection, Manassas, Va.
[c] CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France.
[d] Obtained from F. Bringel, Institut de Botanique, Centre National de la Recherche Scientifique, Strasbourg, France.
[e] Obtained from T. Fujisawa, Kanagawa Prefectural Health Laboratory, Yokohama, Japan.
[f] ND, not determined.
[g] JCM, Japan Collection of Microorganisms, Saitama, Japan.
[h] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

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