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Isolation of Tannin-Degrading Bacteria Isolated from Feces of the Japanese Large Wood Mouse, *Apodemus speciosus*, Feeding on Tannin Rich Acorns

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Running title: TANNIN DEGRADING FECAL BACTERIA FROM WOOD MICE

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Summary

Bacteria with tannase activity were isolated from the feces of the Japanese large wood mouse, *Apodemus speciosus.* They were largely classified into two groups: Gram positive cocci and Gram positive bacilli. Genotypic analysis using a species-specific PCR assay as well as biochemical tests identified all cocci as *Streptococcus gallolyticus*. A PCR assay targeting a genus -specific sequence in the 16S/23S rDNA spacer region and additional 16S rDNA sequencing indicated that the bacilli belong to the genus *Lactobacillus*, with *L. animalis* and *L. murinus* being closely related taxa. Subsequent estimation of guanine-plus-cytosine content, amplified ribosomal DNA restriction analysis, and DNA/DNA hybridization assay confirmed that the bacilli are homologous to each other but different from *L. animalis* or *L. murinus*. Consequently, a novel species of the genus *Lactobacillus* may be proposed. To date, this study is the first to report on the isolation of tannase-positive bacteria from the feces of a rodent species. These bacteria may play an essential role for the host organism in digesting tannin-rich acorns available in their natural habitats, thereby endowing them with a greater ecological advantage.

Key words: Novel tannase producing lactobacilli – *Lactobacillus animalis* – *Lactobacillus murinus*- *Streptococcus gallolyticus*

Introduction

Tannins comprise a large group of polyphenolic compounds widely distributed in the plant kingdom [26]. They are largely divided into two basic groups: the hydrolyzable tannins (HT) and the condensed tannins: HT are polymers of gallic or ellagic acid esterified with a core molecule, commonly glucose or alcohol and the condensed tannins are flavonoid polymers [26]. Both group of tannins have the ability to bind proteins and form insoluble tannin-protein complexes, which are not readily degraded by mammalian digestive enzymes, thus adversely affecting the digestibility of plant materials fed by the animals [7, 14].

Tannase (tannin acylhydrolase) that is commonly found in some fungal species hydrolyzes the galloyl esters of HTs and is thus capable of degrading the tannin-protein complexes [1]. Over the past decade, however, several studies [15, 22] have reported the presence of bacterial species with tannase activity in the guts or feces of many animals. These have included *Streptococcus gallolyticus* [20] and *Lonepinella koalarum* [10] that are frequently isolated from the gut-microflora of the koala, an Australian marsupial feeding almost exclusively on tannin-rich eucalyptus leaves. These bacteria are also capable of decarboxylating gallic acid that is released after tannin hydrolysis to pyrogaroll [20, 21]. Evidence suggests that there is a symbiotic relationship between the host animal and the bacteria to counter the anti-nutritional effect of dietary tannins.

The Japanese large wood mouse (*Apdemous specious* [referred to as wood mouse hereafter]) inhabits various types of forests in Japan [16]. Although the animal has been reported to be omnivorous [31], acorns (seeds of oak trees, the genus *Quercus*) are its predominant food item in autumn and winter [30, 31]. However, the acorns eaten by the mice contain considerable amounts (11.7%) of tannins [28]. A major part of the tannins in acorns is thought to be HT [2]. Recently Shimada and Saitoh [29] have demonstrated that the higher tannin content in acorns had a more negative influence on the animal's apparent digestive capability, resulting in marked body weight loss. Nevertheless, wood mice in wild are believed to rely greatly on acorns as a staple diet for "wintering" [9, 32]. In this context we postulated that the wood mice overcome the negative effects of dietary tannins by harboring some tannase-producing bacteria in their guts. We report here the isolation of bacteria with tannase activity from the feces

of wild wood mice, and describe their phenotypic and genotypic characteristics, which indicate that these represent a novel species belonging to the genus *Lactobacillus*.

Materials and Methods

Isolates and reference strains used. Fresh fecal pellets were collected from a total of 8 wild wood mice from the forests of Kyotanabe in the central part of Japan (34^{o} 46' N, 135^{o} 46' E) during autumn 2003. The fecal sample (ca. 0.2g - 0.5g wet weight) of each mouse was transferred to a tube containing sterile physiological saline and thoroughly emulsified by a homogenizer and a vortex test-tube mixer. A series of 10-fold dilutions (10^{-1} to 10^{-5}) of the emulsified sample was made in saline and, from each dilution, 0.1 ml samples were spread onto tannic acid treated-brain heart infusion agar (T-TBHIA) plates, which had opaque surface due to formation of tannin-protein complex, as described elsewhere [18]. The inoculated plates were incubated anaerobically in Anaero-Pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37・C for 5 days. After incubation, colonies with a clear zone extending beyond their edges with various intensities on the T-TBHIA, indicating apparent bacterial degradation of tannin-protein complex, were subcultured onto brain heart infusion agar (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) or MRS agar (Oxoid) plates, and their tannase and gallate-decarboxylating activities were further confirmed by qualitative assays described elsewhere [23, 24]. These assays are colorimetric methods, which are designed to detect gallic acid released from methyl gallate due to bacterial tannase activity or pyrogallol converted from gallic acid due to bacterial gallate-decarboxlylase. We used type strains of *S. gallolyticus* ACM 3611^{T}, *Lactobacillus animalis* JCM 5670^{T} and *L. murinus* JCM 1717^{T} as reference strains for subsequent phenotypic and genotypic comparisons with the isolates.

In addition, we collected several samples (ca. 10g each) of surface soil from the floor where the wood mice lived. The soil samples were treated in the same manner as the above fecal samples for possible isolation of tannin-degrading bacteria.

Phenotypic characterization of the isolates. For characterization of phenotypic properties of the isolates, we used commercially available identification kits, API20 STREP (bioM**é**rieux, Lyon, France) for the Gram-positive cocci and API 50 CHL kit (bioM**é**rieux) for the Gram-positive bacilli. Growth under aerobic and anaerobic conditions was also tested for the isolates.

DNA preparation. For subsequent genotypic characterization, whole genomic DNAs from the isolates and reference strains were prepared essentially following the method of Marmur [13]. The purity and the amount of DNA in each preparation was estimated colorimetrically and stored at 4·C until use.

Genotypic identification of the isolates. A PCR-based assay described by Sasaki et al. [27] that was designed to amplify species-specific sequences in the manganese-dependent superoxide dismutase gene (*sodA*) of *S. gallolyticus* was performed on the prepared DNAs of the Gram-positive cocci. Briefly, a set of primers, SgsodA-F(forward; 5'-CAATGACAATTCACCATGA-3') and SgsodA-R (reverse; 5'-TTGGTGCTTTTCCTTGTG-3')were used. PCR was performed on a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems, Foster City, Calif.) with a cycle consisting of an initial denaturation at 94·C for 5 min; 30 cycles of denaturation at 94·C for 60 s, annealing at 54·C for 30 s, and extension at 72·C for 30 s; and a final extension at 72·C for 7 min. The PCR products were electrophoresed in a 1% agarose gel and subsequently visualized by UV illumination for specifically amplified fragments (approx. 408 bp in size for all primer sets) after ethidium bromide staining.

Another PCR-based described by Dubernet et al. [4] that was designed to amplify a genus-specific sequence in the 16S/23S rDNA spacer region of the genus *Lactobacillus* was performed on the prepared DNAs of the Gram-positive bacilli, essentially following their methodology.

Sequencing of 16S rRNA of the Gram-positive bacilli. Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, purification of PCR products and electrophoresis of sequence reactions as described previously [25] were performed on one of the Gram-positive bacilli, ASR 1. The phylogenetic

analyses were done applying the ARB software package including a current release of the small subunit rRNA database [12].

Amplified ribosomal DNA restriction analysis (rrn-ARDRA) on the Gram-positive bacilli. The rrn-ARDRA analysis was performed on the genomic DNAs of the Gram-postive bacilli as well as *L. animalis* JCM 5670^T and *L. murinus* JCM 1717^T, essentially following the method described by Ibrahim *et al.* [8]. Briefly, PCR amplification targeting complemented rRNA gene cluster including the 16S-23S intergenic spacer region was performed by a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems) using primers, univ f (5'-GAGTTTGATCCTGGCTCA-3') and univ r (5'-CCGGTCCTCTCGTACT-3'). The PCR products were then digested with either of the restriction endonucleases, *Hha*I, *Mbo*II, or *Sau*3A. The restricted fragments were analyzed electrophoretically using 1.5 % agarose gel and the fragment pattern was visualized by UV illumination.

The rrn-ARDRA patterns were converted to PICT files and entered into the GelCompar II program (Applied Maths, Kortrijk, Belgium) to generate a dendrogram based on the Dice coefficient [3], using the unweighted pair group method with 1% position tolerance.

Estimation of guanine-plus-cytosine (G+C) content of the DNA of the Gram-positive bacilli. The G+C content of DNA extracted from one of the Gram-positive bacilli, ASB 1, was determined by high-performance liquid chromatography described by Katayama-Fujimura et al. [11].

Whole genomic DNA/DNA hybridization assay on the Gram-positive bacilli.
A non-radioisotope DNA/DNA hybridization assay as described by Yaeshima *et al.* [33] was performed on the Gram-positive bacilli in order to determine their taxonomic identities. Briefly, heat-denatured DNAs of one of the bacilli, ASB 1, and type strains of *L. animalis* JCM 5670^T and *L. murinus* JCM 1717^T were fixed each onto the bottom of a well of 96-well microplate (Immuno Plate F96 Maxisorp; Nunc, Roskilde, Denmark). DNAs of all gram-positive bacilli as well as the type strains were photobiotinylated, denatured, and subsequently hybridized to ASB 1 or type strains' DNAs in the wells. The hybridizations were performed

at 33.2 ・C for 16h. After hybridization, the wells were washed and incubated for 10 min with an aliquot of streptavidin-alkaline phosphatatse. After the enzymatic treatment, the wells were washed and then incubated for 30 min with a *p*-nitrophenylphosphate solution. After incubation, 5M NaOH solution was added to the wells and the absorbance at 405 nm was measured with a microplate reader (Model 550; Bio-Rad Laboratories, Richmond, Calif.) to obtain DNA similarity values of the isolates against ASB 1.

Results and Discussion

Colony and cellular morphology of HT degrading isolates. After the five days anaerobic incubation, smooth-surfaced white colonies, 3 to 5 mm in diameter, with distinct, clearing zones around clolonies grew on the T-TBHIA plates for four out of the eight mice subjected. There were also colonies 2 to 3 mm in diameter with less distinct clear zones for all eight mice (Fig. 1a). The former colonies were gram-positive cocci (ca. 1.0 μm in diameter [Fig. 1b]) and the latter Gram-positive bacilli (ca. 1.0 μm x 5~7 μm [Fig. 1c]). A single representative colony of the cocci and the bacilli was randomly selected from the plate for each mouse giving 4 gram-positive cocci (ASC 1 to 4) and 8 gram-positive bacilli (ASB 1 to 8) which were characterized phenotypically and genotypically. Average numbers of colony forming units of ASC and ASB (in logarithmic form) per gram of fresh feces of the mice were 6.5 and 8.4, respectively. Clear zone forming bacterial colonies were seldom isolated from any of the soil samples.

Phenotypic and genotypic identity of the HT degrading Gram-positive cocci. Phenotypic and genotypic characteristics of the HT degrading cocci are summarized in Table 1. All of them were positive for tannase and gallate-decarboxylating activities. Biochemical tests by the API 20 STREP (API System) revealed that they showed an almost identical profile to *S. gallolyticus* ACM 3611^T. The PCR-based assay specific to *S. gallolyticus* also identified all isolates as *S. gallolyticus*.

Phenotypic characteristics of the HT degrading Gram-positive bacilli. Phenotypic characteristics of the HT degrading bacilli are summarized in Table 2. They are

all positive for tannase activity but only ASB 6 was positive for gallate-decarboxylation. They grew aerobically but more vigorously under anaerobic conditions. The carbohydrate fermentation patterns as determined in API 50 CHL (API System) kits revealed that the isolates had profiles comparable to those shown by *L. animalis* JCM 5670^T and *L. murinus* JCM 1717^T. It should be noted, however, that the reference strains were negative for both tannase and gallate-decarboxylating activities.

Genotypic characteristics of the HT degrading gram-positive bacilli. The PCR-based assay specific to the genus *Lactobacillus* confirmed that the HT degrading bacilli belong to this genus. The similarities of the 16S rRNA gene sequence of ASB 1 to *L. animalis* JCM 5670^T and *L. murinus* JCM 1717^T were 96.8 % and 99.0 %, respectively, and phylogenetic position of ASB 1 is shown in Fig. 2. The rrn-ARDRA profiles of the bacilli showed that they form a cluster at the 90 % similarity level that is markedly distinct from *L. animalis* JCM 5670^T and *L. murinus* JCM 1717^T (Fig. 3). The G+C content of ASB 1 was 38.5 %. The DNA/DNA homology values of all 8 HT degrading bacilli against ASB 1 was above 70% whereas those of *L. animalis* JCM 5670^T and *L. murinus* JCM 1717^T were less than 20% (Table 3). Likewise, the values of any of the HT degrading bacilli against *L. animalis* JCM 5670^T and *L. murinus* JCM 1717^T were under 30% (Table 3).

Taxonomic consideration of the HT degrading gram-positive bacilli. Our phenotypic and genotypic analyses on the gram-positive bacilli with tannase activity have indicated that they belong to the genus *Lactobacillus*. Recently, Nishitani et al. [17] have described the classification of the lactobacilli with tannase activity isolated from fermented foods and human feces, which are identified as *L. plantarum*, *L. paraplantarum*, and *L. pentosus*. In the present study, the 16S rDNA sequence of the bacilli were different from the above three species, with *L. animalis* and *L. murinus*, being the closest. However, the type strains of the latter two species did not show tannase activity. Furthermore, the rrnARDRA profiles of the new isolates were quite different from these type strains. The G+C content (38.5 %) of one of the bacilli was also lower than that of *L. animalis* (41-44% [10]) and *L. murinus* (43-44% [9]). Subsequent DNA/DNA

hybridization testing revealed that they are quite homologous to each other but markedly different from the type strains of *L. animalis* and *L. murinus*. From the evidence provided, the HT degrading *Lactobacillus* strains are considered to represent a new species of the genus *Lactobacillus*, in which tannase activity may be a valid biochemical property to differentiate it from closely related species. Further phylogenetic analyses and phenotypic characterization of the bacilli in comparison with more strains of *L. animalis* and *L. murinus* strains and those of other established *Lactobacillus* species would be necessary to confirm its novel taxonomic status.

Ecological consideration. The wood mice are considered one of the most important animals in the temperate forests, both as seed dispersers and as seed consumers [31, 32]. Conversely, the acorns are an important nutrient resource for the wood mice although they contain considerable amounts of tannins [28]. Through their feeding experiments, however, Shimada and Saitoh [29] have demonstrated that the acorn tannins and tannic acid cause marked body weight loss and negative effect on digestibility of nitrogen in the wood mice tested. To minimize such adverse effects of tannins, the wood mice in the wild can select for less taniferous forage available in their habitats. But if such forage is limited, which is most likely to be the case during winter, having tannin-degrading bacteria as normal members of their gut-microflora may endow the wood mice with some ecological advantage over other forest-dwelling wildlife. This is indeed the case with the koala, an Australian herbivorous marsupial, which feeds almost exclusively on tannin-rich eucalyptus leaves [5]. The animal harbors a large numbers of at least two different taxa of tannin-degrading bacteria in their alimentary tracts; *S. gallolyticus* [20] and *Lonepinella koalarum* [21]. Although *S. gallolyticus* was reported to occur occasionally in the feces of other herbivores (deer, cattle, horse, and sheep) and omnivores (guinea pig and pig) [22], this is the first report of its frequent occurrence in a rodent species. In addition, we have isolated yet another group of tannin degrading Gram-positive bacilli from the wood mice feces, which seem to be more prevalent than *S. gallolyticus*.

The evidence obtained from the present study suggests that, like koalas, the wood mice have a symbiotic relationship with tannin-degrading bacteria, thereby enabling them to overcome the anti-nutritional effects of acorn tannins.

Interestingly, such bacteria were never isolated from the floor soil samples of forests, where the wood mice were captured. This in turn suggests that the wood mice do not acquire the bacteria by accidental ingestion of the soils. A possible way of the acquisition may be vertical transfer of the bacteria from the maternal feces as observed in koalas [19]. Further studies are in progress in order to evaluate the above possibilities.

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Legends of Figures

FIG. 1. Growth of hydrolyzable tannin degrading bacteria forming clear zones around colonies on T-TBHIA and cellular morphology.

An appropriate dilution of wild wood mouse feces was spread on the plate and incubated anaerobically for 5 days at 37 ^{o}C. (a) Black and white arrowheads indicate several example colonies of HT-degrading cocci and bacilli, respectively. Gram-stained cells of the cocci (b) and the bacilli (c). Bar, 10 μm.

Fig. 2. Phylogenetic dendrogram illustrating the position of strain ASB 1 amongsome closely related Lactobacillus species.

The tree is based on a collection of almost complete 16S rRNA gene sequences and was reconstructed using the neighbour-joining method as implemented in

the ARB package. Phylogenetic distances were calculated as described by Felsenstein [6]. Only bootstrap values above 50% (1000 bootstrap re-samplings for each node) are shown. The sequence of Lactobacillus delbrueckii was used as an outgroup (not shown). Bar, 5% estimated sequence divergence.

FIG. 3. rrn-ARDRA profiles of the gram-positive bacilli with tannase activity from wood mice feces and *L. animalis* JCM 5670^T and *L. murinus* JCM 1717^T.

The rrn-ARDRA patterns were obtained after digestion with *Hha*I, *Mbo*II, and *Sau*3A, and ethidium bromide staining. The patterns shown are negative digitized images of the gels. Dendrogram generated by the GelCompar II program (Applied Maths) from the tandemly aligned patterns.

Table 1. Phenotypic and genotypic characteristics of the gram positive cocci isolated from wild wood mice with the type strain of *S. gallolyticus* as a reference.

Strain	Tannase	Gallate decarboxylase	Catalase	VP reaction	Hippurate hydrolysis	Esculin hydrolysis	Pyrrolidonyl arylamidase	α-galactosidase	β-gluccuronidase	β-galactosidase	Alkaline phosphatase	Leucine arylamidase	Arginine dihydrolase	Acid produced from: Ribose	L-arabinose	Mannitol	Sorbitol	Lactose	Treharose	Inulin	Raffinose	Starch	Glycogen	*S. gallolyticus* species-specific *sodA* gene [a]
ASC 1	+	+	-	+	-	+	-	-	-	-	-	+	-	-	-	+	-	+	+	-	+	+	+	+
ASC 2	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	+	+	+
ASC 3	+	+	-	+	-	+	-	-	-	-	-	+	-	-	-	+	-	+	+	-	+	+	+	+
ASC 4	+	+	-	+	-	+	-	-	-	-	-	+	-	-	-	+	-	+	+	-	+	+	+	+
S. gallolyticus ACM[b] 3611[T]	+	+	-	+	-	+	-	+	-	-	-	+	-	-	-	+	-	+	+	-	+	+	+	+

[a] determiend by the PCR-based assay described by Sasaki et al. (26).

[b] ACM, Australian Collection of Microorganisms, University of Queensland, Australia

Table 2. Phenotypic characteristics of the gram positive bacilli isolated from wild wood mice with the type strains of *L. animalis* and *L. murinus* as references.

Strain	Tannase	Gallic acid decarboxylase	Anerobic growth	Aerobic growth	Carbohydrate fermentation profile[a]																
					Galactose	D-Glucose	D-Fructose	D-Mannose	Mannitol	N Acetyl glucosamine	Arbutine	Esculine	Salicine	Cellobiose	Maltose	Lactose	Melibiose	Saccharose	Trehalose	D-Raffinose	ß Gentiobiose
ASB 1	+	–	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+	–
ASB 2	+	–	+	+	+	+	+	+	–	+	+	+	+	–	+	+	+	+	+	+	–
ASB 3	+	–	+	+	+	+	+	+	–	+	+	+	+	–	+	+	+	+	+	+	–
ASB 4	+	–	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	+	–
ASB 5	+	–	+	+	+	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	–
ASB 6	+	+	+	+	+	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	–
ASB 7	+	–	+	+	–	+	+	+	–	+	+	+	–	–	+	+	+	+	+	+	–
ASB 8	+	–	+	+	+	+	+	+	–	+	+	+	–	+	+	+	+	+	+	+	–
***L. animalis* JCM[b] 5670[T]**	–	–	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	+	–	+	–
***L. murinus* JCM 1717[T]**	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+

[a] Showing only carbohydrates with at least one strain giving positive fermentation result as determined by Api 50CHL

[b] JCM, Japan Collection of Microorganisms, RIKEN, Japan

Table 3. Levels of total DNA hybridization of the gram-positive bacilli with tannase activity isolated from feces of the wood mice and type strains of *L. animalis* and *L. murinus* .

Strain	% Relative binding with DNA from:		
	ASB 1	*L. animalis* JCM 5670^T	*L. murinus* JCM 1717^T
ASB 1	100.0	7.4	5.4
ASB 2	82.3	8.7	3.8
ASB 3	100.0	12.9	12.6
ASB 4	85.1	5.7	1.2
ASB 5	74.8	12.3	10.5
ASB 6	73.8	10.2	8.3
ASB 7	95.1	13.1	8.7
ASB 8	97.3	13.9	10.2
L. animalis JCM 5670^T	10.1	100.0	48.0
L. murinus JCM 1717^T	9.7	48.0	100.0

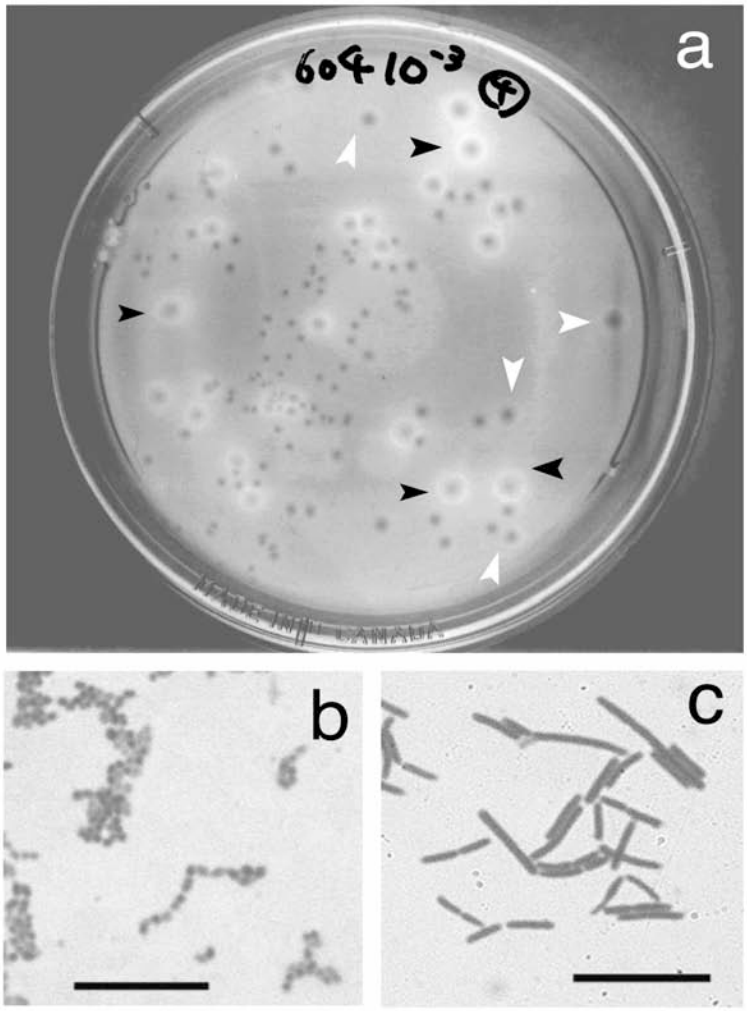

FIG. 1

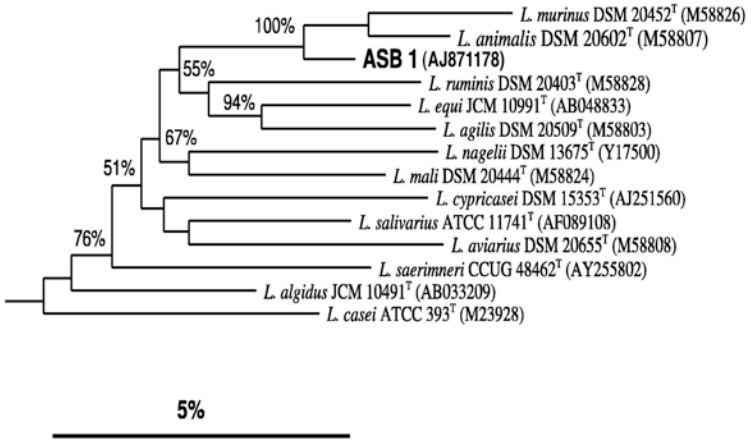
100%
55%
94%
67%
51%
76%
L. murinus DSM 20452T (M58826)
L. animalis DSM 20602T (M58807)
ASB 1 (AJ871178)
L. ruminis DSM 20403T (M58828)
L. equi JCM 10991T (AB048833)
L. agilis DSM 20509T (M58803)
L. nagelii DSM 13675T (Y17500)
L. mali DSM 20444T (M58824)
L. cypricasei DSM 15353T (AJ251560)
L. salivarius ATCC 11741T (AF089108)
L. aviarius DSM 20655T (M58808)
L. saerimneri CCUG 48462T (AY255802)
L. algidus JCM 10491T (AB033209)
L. casei ATCC 393T (M23928)
5%

Fig. 2

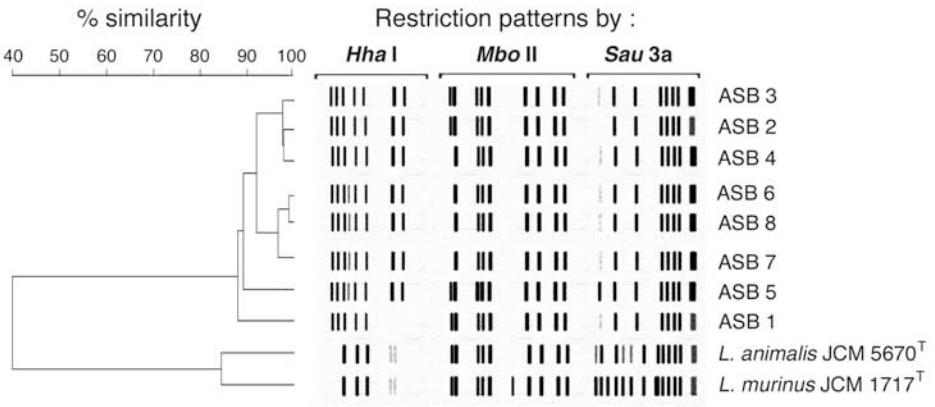
% similarity
40 50 60 70 80 90 100
Restriction patterns by :
Hha I
Mbo II
Sau 3a
ASB 3
ASB 2
ASB 4
ASB 6
ASB 8
ASB 7
ASB 5
ASB 1
L. animalis JCM 5670T
L. murinus JCM 1717T

Fig. 3