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Genotypic Analyses of Lactobacilli with a Range of Tannase Activities Isolated from Human Feces and Fermented Foods

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Running title: **GENOTYPIC ANALYSES OF TANNNASE PRODUCING LACTOBACILLI**

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Summary

A total of 77 tannase producing lactobacilli strains isolated from human feces or fermented foods were examined for their genotypic profiles and intensities of tannase production. With a PCR-based assay targeting *recA* gene, all strains

except one isolate were assigned to either *Lactobacillus plantarum*, *L. paraplantarum*, or *L. pentosus* whereas a 16/23S rDNA targeted PCR-based assay identified all except 6 isolates (inclusive of the above one isolate) as one of the closely related species. Subsequent DNA/DNA hybridization assays revealed that these 6 exceptional isolates showed low homology (between 1.2% and 55.8% relative DNA binding) against type strains of the three species. Supplemental carbohydrate fermentation profiles on the 6 isolates indicated that two of them were identified as *L. acidophilus*, one as *Pediococcus acidilactici*, one as *P. pentosaceus*, and two remained unidentifiable. The evidence suggests that the 16/23S rDNA targeted PCR assay can be used as a reliable identification tool for the closely related lactobacilli, and that the tannase gene is widely distributed within members of the Lactobacillaceae family. Meanwhile, a randomly amplified polymorphism DNA (RAPD) analysis revealed that all except 8 isolates were well allocated in 4 major RAPD clusters, though not species specific, consisting of two *L. plantarum* predominant clusters, one *L. paraplantarum* predominant, and one *L. pentosus* predominant. The RAPD patterns of the 8 non-clustered isolates, which consisted of the 6 unidentifiable isolates and 2 isolates identified as *L. pentosus*, were < 40% similarity to those belonging to the 4 clusters. A quantitative assay of the tannase activities showed that there was a marked variation in the activities among the strains, which did not correlate with either species identification or clustering by RAPD.

Key words: Tannase producing – *Lactobacillus plantarum* – *Lactobacillus paraplantarum* - *Lactobacillus pentosus* – Genotypic analyses

Introduction

Hydrolyzable tannins are polyphenolic substances produced by many plants as their defense mechanism against pathogens and herbivores [24] since they bind readily with proteins or starch to form indigestible complexes [30]. Tannase (tannin acylhydrolase, EC 3.1.1.20.) that specifically cleaves galloyl ester bonds of the tannins has been first reported for fungi [1, 20]. Over the past decade,

several studies reported that bacterial species such as *Streptococcus gallolyticus* and *Lonepinealla koalarum* gen. nov. sp. nov. that were isolated from feces of koalas, goats or sheep [13,16,19, 26] showed tannase activity. More recently, this enzymatic activity was found in lactobacilli strains isolated from human feces and fermented foods, and in *Lactobacillus plantarum*, *L. paraplantarum*, or *L. pentosus*, which were identified by PCR-based analysis [17].

Lactobacillus plantarum, *L. paraplantarum*, and *L. pentosus* have been reported to play an important role in the production of many fermented foods including dairy products, silage, pickled vegetables, meat and fish products [2, 7, 32], thus being proposed as a potential probiotic [25]. Furthermore, it has been speculated that their tannase activities in the human alimentary tract have significant effects on pharmacological aspects of dietary tannins that are prevalent in beverage and tea [17]. However, identification of these three species is problematic since they have very few distinguishing phenotypic characteristics [4]. This difficulty extends to molecular methods, in which they share almost an identical 16 S rRNA sequence [6, 21]. In this context, Berthier *et al.* [3] developed a PCR based identification method, which employs a set of primers complementary to species-specific partial sequences in the 16S/23S rDNA spacer regions, claiming that the method can distinguish these closely related species. Likewise, Torriani *et al.* [28] developed another PCR-based method, which differentiated the three species on the basis of differences in gene sequence encoding the RecA protein implicated in homologous DNA recombination [9]. Meanwhile, Bringel *et al.* [5] reported that randomly amplified polymorphic DNA fingerprinting (RAPD) successfully identified strains at the species level.

Over the past two years, we have isolated a number of tannase producing lactobacilli from fermented foods and human feces, and observed that clear zone formation around colonies growing on tannin-treated agar plates varied in their intensities among the isolates, suggesting an appreciable variation in their abilities to produce tannase. In the present study, we have evaluated use of the above PCR-based or RAPD assays for species identification. Subsequently we quantified tannase enzymatic activities of the isolates in order to determine whether differences in activity correlate with the respective species.

Materials and Methods

Isolates and strains used. Forty-two tannase producing lactobacilli strains were newly isolated from human feces (11 strains) and fermented foods (31strains), following the isolation technique described elsewhere [17]. Briefly, Swab samples (ca. 0.1g wet weight each) of fresh human feces and food samples (ca. 1g wet weight each) were each transferred to a tube containing 30 ml of MRS broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) and thoroughly mixed aseptically with a homogenizer and a vortex test-tube mixer. The broth was then incubated anaerobically in Anaero-Pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37・C for 48h. After incubation, one loopfull (ca. 10μl) of each culture was streaked onto tannin-treated brain heart infusion agar (T-TBHIA) [15]. The inoculated plates were incubated anaerobically in Anaero-Pack (Mitsubishi) at 37・C for 5 days. After incubation, colonies with a clear zone extending beyond their edges with various intensities on the T-TBHIA (Fig. 1) were subcultured onto MRS agar (Oxoid) plates, and their tannase activities were further confirmed by a qualitative assay described elsewhere [19] for subsequent use in this study.

In addition to the above isolates, we used 3 human fecal isolates (KLH 1, 2, and 3[17]) 23 food isolates (KOG 1 to KOG 25 [17]) that had been tested qualitatively for their tannase activities [17], and 9 lactobacilli strains obtained from established culture collections including type strains of *L. plantarum* (ATCC 14917^T), *L. paraplantarum* (ATCC 700211^T), and *L. pentosus* (ATCC 8041^T) (Table 1) as reference strains. The isolates and reference strains used in the present investigation are listed in Table 1 together with information of their sources of isolation.

DNA preparation. For subsequent genotypic analyses, whole genomic DNA from each isolate was prepared essentially following the method of Marmur [12]. The purity and the amount of DNA in each preparation was estimated colorimetrically and stored at 4・C until use.

16S/23S rDNA targeted PCR assay. A PCR assay reported by Berthier *et al.* [3] that was designed to amplify species-specific sequences in the 16S/23S rDNA spacer regions of the three *Lactobacillus* species was performed on the prepared DNAs. Briefly, three separate sets of primers were used: 1st set, 16 (16S rRNA gene, 3' end, forward; 5'-GCTGGATCACCTCCTTTC-3') and Lpl (16S/23S rDNA spacer region, *L. plantarum* specific; 5'-ATGAGGTATTCAACTTATG-3'), specific to *L. plantarum*; 2nd set, 16 and Lpapl (16S/23S rDNA spacer region, *L. paraplantarum* specific; 5'-ATGAGGTATTCAACTTATT-3'), specific to both *L. plantarum* and *L. paraplantarum*; 3rd set, 16 and Lpe (16S/23S rDNA spacer region, *L. pentosus* specific; 5'-GTATTCAACTTATTACAACG-3'), specific to *L. pentosus.* PCR was performed on a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems, Foster City, Ca., USA) with a cycle consisting of denaturation at 94・C for 1 min, primer annealing 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 in a 1% agarose gel and subsequently visualized by UV illumination for specifically amplified fragments (approx. 200 bp in size for all primer sets) after ethidium bromide staining.

recA gene targeted multiplex PCR assay. Another PCR assay was performed as described by Torriani *et al.* [28]. The assay was designed to amplify partial *recA* sequences each specific to *L. plantarum*, *L. paraplantarum*, or *L. pentosus*. Briefly, the assay employed three primers, paraF (5'-GTC ACA GGC ATT ACG AAA AC-3'), pentF (5'-CAG TGG CGC GGT TGA TAT C-3'), planF (5'-CCG TTT ATG CGG AAC ACC TA-3'), and pREV (5'-TCG GGA TTA CCA AAC ATC AC-3'). PCRs were performed on a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems) with initial denaturation at 94・C for 3 min, followed by a cycle consisting of denaturation at 94・C for 30 s, annealing at 56・C for 10 s, and elongation at 72・C for 30 s; the cycle was repeated 30 times, and finally extension at 72・C for 5 min. The PCR products were electrophoresed in a 2% agarose gel and subsequently visualized by UV illumination for detecting specifically amplified fragments (318 bp for *L. plantarum*, 218 bp for *L. pentosus*, and 107 bp for *L. paraplantarum*) after ethidium bromide staining.

Whole genomic DNA/DNA hybridization assay. As will be shown later, the results obtained from the above two PCR-based assays were not consistent for some of the isolates. A non-radioisotope DNA/DNA hybridization assay as described by Yaeshima *et al.* [30] was thus performed on these isolates in order to determine their taxonomic identities. Briefly, heat-denatured DNAs of type strains of *L. plantarum* (ATCC 14917^T), *L. paraplantarum* (ATCC 700211^T), *L. pentosus* (ATCC 8041^T) were fixed each onto the bottom of a well of 96-well microplate (Immuno Plate F96 Maxisorp; Nunc, Roskilde, Denmark). DNAs of the isolates in question were photobiotinylated, denatured, and subsequently hybridized to the 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, Ca.., USA) to obtain DNA similarity values of the isolates against the type strains.

Carbohydrate fermentation profiling. For the isolates not unequivocally identified by PCR, we used a commercially available identification kit, API 50 CHL kit (bioM**é**rieux, Lyon, France) based on carbohydrate fermentation profile in order to determine their tentative taxonomic identities.

RAPD assay. RAPD assay for genome fingerprinting of the isolates and the reference strains was performed following essentially the method described by Bringel *et al.* [5] with some modifications in PCR conditions. Briefly, three different oligonucleotide primers, primer P1 (5・-TGCTCTGCCC-3・), P2 (5・-GGTGACGCAG-3・) and P3 (5・-CTGCTGGGAC-3・) were used. The reaction mixture was heated at 94・C for 5 min prior to 30 cycles of PCR amplification in a DNA thermal cycler (GeneAmp PCR System 2700; Applied Biosystems): one cycle PCR with P1 consisted of denaturation at 94・C for 1 min, primer annealing at 36・C for 2 min, and extension at 72・C for 2 min; with P2, denaturation at 94・

C for 1 min, annealing at 40・C for 2 min, and extension at 72・C for 1 min; with P3, denaturation at 94・C for 1 min, annealing at 45・C for 2 min, and extension at 72・C for 1 min. The PCR products were electrophoresed in a 2 % agarose gel and were visualized by UV illumination for specifically amplified fragments after ethidium bromide staining. The three RAPD patterns thus obtained were tandemly merged and subsequently analyzed by the GelComparII program (Applied Maths, Kortrijk, Belgium) to generate a dendrogram based on the Dice coefficient [8], using the unweighted pair group method with 1% position tolerance.

Quantitative assay for tannase activity. Tannase activities of the isolates and reference strains was quantified by a colorimetric method as described elsewhere [14]. Briefly, the isolates and the reference strains grown on MRS agar (Oxoid) were each harvested with sterile cotton swabs and suspended in 1ml of substrate medium (pH 5.0) containing 33 mM NaH_2PO_4 and 5 mM methyl gallate at final concentration to prepare a dense suspension whose absorbance at 660 nm was adjusted to 0.4 (= ca. 1.4 to 1.6 x 10^8 CFU/ml). The bacterial suspension was incubated aerobically at 37・C for 24 h. After incubation, 1 ml of each suspension was dispensed into a microtube and centrifuged (8,000 x g, 5 min). Then 100 μl of supernatant was mixed with an equal volume of the saturated $NaHCO_3$ solution (pH 8.6) and incubated at 37 ^oC for 2 h. The mixture was vortexed and centrifuged (8,000 x g, 20 s). Next, 100 □l of each supernatant was dispensed into a well of a 96-well microplate (ELISA plate/ flat bottom; Iwaki Co. Ltd., Tokyo, Japan) and its absorbance at 450 nm was read on a microplate reader (Model 550; Bio-Rad). The test was performed in triplicate for each strain.

Results and Discussion

Taxonomic identities as determined by the PCR based assays.

It has been reported for several bacterial genera such as *Bifidobacterium* [11] and *Burkholderia* [29] that polygenetic analysis of partial *recA* gene sequences provided useful information for identifying closely related species remaining unidentified by a conventional 16S rDNA analysis. Consequently, Torriani *et al.*

[28] have developed a *recA*-based PCR test that can differentiate *L. plantarum*, *L. paraplantarum*, and *L. pentosus*, claiming that *recA* gene is an excellent phylogenetic marker for closely related species. Identification of reference strains and the isolates as determined by 16/23S rDNA spacer region targeted PCR assay [3] and by *recA* gene targeted PCR [28] assay are listed in Table 1. Both assays gave consistent results for identities of the references strains and most of the isolates (Figures 2A and 2B). There were, however, 5 isolates (KB 96, KB 249, NOS 122, NOS 151, and NOS 152) yielding conflicting results, in which the former PCR did not yield any amplicons although the latter PCR did produce 218 bp sized amplicon specific to *L. pentosus*. An isolate (NOS 141) did not produce any detectable amplicon by either assay.

DNA/DNA homology values of the above 6 isolates in question were well below 70% against any of the type strains tested (Table 2). The carbohydrate fermentation profiles of the isolates and the type strains are shown in Table 3. Based on the profiles, KB 96 and KB 249 were identified as *L. acidophilus* (98.3 and 95.2 % confidence by the API system [bioMérieux], respectively), NOS 122 as *Pediococcus acidilactici* (99.8 %), and NOS 141 as *P. pentosaceus* (99.9 %) but the remaining two isolates, NOS 151 and NOS 152, could not be identified. The evidence suggests that the 16/23S rDNA spacer region targeted PCR assay rather than the *recA*-targeted PCR is a reliable method for identification of the three lactobacilli species; not giving them false taxonomic designations. This in turn suggests that any PCR-based assay targeting a single phylogenetic marker has limited use for bacterial identification unless results of the assay are consistent with those of DNA/DNA homology using a large numbers of strains.

Genotypes as determined by RAPD analysis

RAPD has been reported to be a useful species identification tool for several genera including *Brucella* [27], *Legionella* [23], and *Lactococcus* [22]. Bringel *et al.* [5] performed RAPD assays on strains of *L. plantarum* and closely related species, demonstrating that this approach can classify strains into four species-specific groups, consisting of one group corresponding to *L. pentosus*, one to *L. paraplantarum*, and two to *L. plantarum*. Our RAPD results were essentially consistent with their findings if we accepted the taxonomic identities of strains or isolates as determined by the 16/23S rDNA targeted PCR. The

combined RAPD profiles of the reference strains and the isolates are shown in Fig. 3. Four RAPD clusters, A, B, C, and D were identified at the 40 % similarity level according to a dendrogram generated by the GelComparII program (Applied Maths)(Fig. 2; see also Table 1 for each strain's or isolate's cluster designation).

RAPD cluster A was composed of 14 strains. These consisted of all reference strains of *L. paraplantarum* (ATCC 700211T and 61D) and 10 isolates that were identified as *L. paraplantarum* by both PCR-based identification assays, together with two isolates (KOG 7 and KOG 8) which were identified as *L. plantarum* by the same assays. RAPD cluster B was composed of 18 strains. These included not only all reference strains of *L. paraplantarum,* 13 isolates that were identified as *L. plantarum* by both PCR-based assays, and one isolate, KOG 17 identified as *L. pentosus* by the same assays. RAPD cluster C consisted of all reference strains of *L. pentosus* and one isolate (KOG 9) that was identified as *L. pentosus* by the same assays. RAPD cluster D was composed of 33 isolates that were all identified as *L. plantarum* by both PCR-based assays. It should be noted that 10 of the 14 human fecal isolates were included in this cluster. The remaining 8 strains whose RAPD profiles showed low similarity to those of any of the clusters consisted of the 5 isolates (KB 96, KB 249, NOS 122, NOS 151, and NOS 152) giving conflicting results by the PCR-based assays, one isolate (NOS 141) that could not be identified by both assays, and two human fecal isolates (KB 206 and KB 232) that were identified as *L. pentosus* by both assays.

Based on the above results, the RAPD analysis may be a useful but not absolutely reliable tool for distinguishing closely related lactobacilli species. Nevertheless, our approach can be used as an important tool for ecological investigations on these species. For example, human fecal isolates of *L. plantarum* were found invariably in cluster D but not cluster B, comprising a distinct sub-cluster within the former. The view should be confirmed with more lactobacilli strains of established taxonomic identities.

Variations in tannase activity.

The tannase activities measured for the reference strains and the isolates varied markedly between 5.99 mU/ml to less than 0.1 mU/ml. The measured

activities of the strains are presented from the perspective of their taxonomic identities*, L. plantarum, L. paraplantarum*, or *L. pentosus*, as agreed by the two PCR-based identification methods [3, 28] and summarized in Table 3. The activity varied markedly even within each species. For example, *L.paraplantarum* ATCC 700211T expressed an enzymatic activity at least 50 times higher *than L. paraplantarum* strain 61D. Similar observations were made for *L. plantarum* and *L. pentosus*. Although *L. plantarum* showed a relatively low tannase activity on average as compared to *L. paraplantarum* and *L. pentosus*, the difference could not be statistically verified (Student *t*-test, $p > 0.05$). Likewise, the variation was not related to the RAPD cluster difference (Student *t*-test, $p > 0.05$, data not shown). Although highly speculative, there may be a gene regulatory system (e.g. activator, repressor) that directs expression of the gene encoding tannase in these lactobacilli. Such a system has been described for varied extracellular protease production among *Staphylococcal aureus* strains [10]. Alternatively, the variation may simply reflect differences in copy number of the gene. More research is also needed to investigate other strain characteristics that may be linked to this marked difference in tannase production.

The present results indicate that tannase production was a phenotypic trait present not only in *L. plantarum*, *L. paraplantarum*, and *L. pentosus* but also in all species and genera of the family Lactobacillaceae. In the light of our results and those of previous studies [13, 16, 17, 18], the tannase gene may be widely distributed in members of the mammalian intestinal microflora, thereby interacting with dietary tannins. Further analysis on 16S rDNA sequences of those unidentifiable isolates is currently in progress in order to determine their taxonomic identities.

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Legends of Figures

FIG. 1. Growth of tannase-producing lactobacilli strains with various intensities of clear zone formation around their colonies on tannin-treated BHIA plate after 5 days of anaerobic incubation at 37·C. (a) *L. plantarum* ATCC 14917 ^{T} with marked clear zone formation, (b) *L. plantarum* CNRZ 184, with vaguely formed clear zones, and (c) *L. paraplantarum* 61D with less obvious formation of clear zones.

FIG. 2. Representative PCR amplifications by two different species-specific PCR assays on tannase producing lactobacilli. (A) PCR assay developed by Berthier *et al.* [3] using 16/Lpl (lanes 1 4, 7, and 10), 16/Lpapl (lanes 2, 5, 8, and 11), and 16/Lpe (lanes 3, 6, 9, and 12) on DNAs of *L. plantarum* ATCC 14917 ^{T} (lanes 1, 2, and 3), *L. paraplantarum* ATCC 700211^{T} (lanes 4,5, and 6), *L. pentosus* ATCC 8041^{T} (lane 7, 8, and 9) and a human fecal isolate KB 96 (lanes 10, 11, and 12). (B) multiplex PCR assay developed by Torriani *et al.* [28] on *L. plantarum* ATCC 14917 ^{T} (lane 1), *L. paraplantarum* ATCC 700211^{T} (lane 2), *L. pentosus* ATCC 8041^{T} (lane 3) , NOS 122 (lane 4), NOS 141 (lane 5), NOS 151 (lane 6), NOS 152 (lane 7), KB 96 (lane 8) and KB 249 (lane 9).

FIG. 3. RAPD profiles of 77 strains tannase producing lactobacilli, and deduced dendrogramme generated by the GelComparII program (Applied Maths) of tandemly aligned RAPD patterns obtained with three primer sets, P1, P2, and P3. The RAPD patterns shown are negative digitized images of RAPD products separated on ethidium bromide stained agarose gels. RAPD patterns are clustered in 4 major groups (A, B, C, and D) at the 40% similarity

level.

Table 1a. The lactobacilli isolates and the reference strains with various tannase activities, their taxonomic identities as determined by two different PCR based assays and the RAPD classification.

Strain[a]	Source	Taxon determined by PCR assay developed by		RAPD Cluster[b]	Tannase activity (mU/ml)[c]
		Berthier *et al.* [3]	Torriani *et al.* [27]		
The reference strains from established culture collections					
L. plantarum ATCC 14917^T	Pickled cabbage	*L. plantarum*	*L. plantarum*	**B**	5.73
L. plantarum CNRZ 184	Unknown	*L. plantarum*	*L. plantarum*	**B**	0.77
L. plantarum CNRZ 1228	Cheese	*L. plantarum*	*L. plantarum*	**B**	4.35
L. plantarum ATCC 8014	Unknown	*L. plantarum*	*L. plantarum*	**B**	2.88
L. paraplantarum ATCC 700211^T	Beer contaminant	*L. paraplantarum*	*L. paraplantarum*	**A**	5.99
L. paraplantarum 61D	Human feces	*L. paraplantarum*	*L. paraplantarum*	**A**	<0.1
L. pentosus ATCC 8041^T	Silage	*L. pentosus*	*L. pentosus*	**C**	4.40
L. pentosus CNRZ 1544	Fermented olives	*L. pentosus*	*L. pentosus*	**C**	3.58
L. pentosus CNRZ 1561	Fermented olives	*L. pentosus*	*L. pentosus*	**C**	<0.1
The isolates reported previously (18)					
KLH 1	Human feces	*L. plantarum*	*L. plantarum*	**D**	0.49
KLH 2	Human feces	*L. plantarum*	*L. plantarum*	**D**	5.10
KLH 3	Human feces	*L. plantarum*	*L. plantarum*	**D**	1.32
KB 90	Human feces	*L. plantarum*	*L. plantarum*	**D**	1.17
KB 96	Human feces	**Unidentifiable**	*L. pentosus*	**Unclassified**	1.41
KB 175	Human feces	*L. plantarum*	*L. plantarum*	**D**	1.47
KB 179	Human feces	*L. plantarum*	*L. plantarum*	**D**	1.46
KB 183	Human feces	*L. plantarum*	*L. plantarum*	**D**	1.15
KB 201	Human feces	*L. plantarum*	*L. plantarum*	**D**	0.58
KB 205	Human feces	*L. plantarum*	*L. plantarum*	**D**	0.77
KB 206	Human feces	*L. pentosus*	*L. pentosus*	**Unclassified**	3.96
KB 232	Human feces	*L. pentosus*	*L. pentosus*	**Unclassified**	0.83
KB 247	Human feces	*L. plantarum*	*L. plantarum*	**D**	1.35
KB 249	Human feces	**Unidentifiable**	*L. pentosus*	**Unclassified**	5.11
KOG 1	Pickled rice bran	*L. paraplantarum*	*L. paraplantarum*	**A**	4.16
KOG 2	Turnip pickled with rice bran	*L. plantarum*	*L. plantarum*	**B**	2.13
KOG 3	Korean cabbage kimchi	*L. paraplantarum*	*L. paraplantarum*	**A**	2.88
KOG 4	Cucumber pickled with rice bran	*L. plantarum*	*L. plantarum*	**B**	0.62
KOG 5	Chinese cabbage pickled with rice bran	*L. plantarum*	*L. plantarum*	**B**	0.69
KOG 7	Pickled eggplant and cucumber	*L. plantarum*	*L. plantarum*	**A**	1.92
KOG 8	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**A**	2.01
KOG 9	Chinese cabbage pickled with rice bran	*L. pentosus*	*L. pentosus*	**C**	3.21
KOG 11	Chinese cabbage pickled with rice bran	*L. plantarum*	*L. plantarum*	**B**	1.52
KOG 12	Pickled radish and tan	*L. plantarum*	*L. plantarum*	**B**	1.45
KOG 13	Korean radish kimchi	*L. plantarum*	*L. plantarum*	**B**	4.01
KOG 14	Pickled eggplant	*L. plantarum*	*L. plantarum*	**B**	1.42
KOG 15	Korean cabbage kimchi	*L. paraplantarum*	*L. paraplantarum*	**A**	4.07
KOG 16	Korean cabbage kimchi	*L. paraplantarum*	*L. paraplantarum*	**A**	2.73
KOG 17	Pickled vegetables assorted	*L. pentosus*	*L. pentosus*	**B**	3.03
KOG 18	Turnip pickled with rice bran	*L. plantarum*	*L. plantarum*	**B**	0.92

a ATCC, American Type Culture Collection, Manassas, Va.; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France.

b see Fig. 3 for the cluster allocation

c Average tannase activity per ca. 1.5 x 10^8 CFU/ml measured in triplicate tests

Table 1 continued

Strain[a]	Source	Taxon determined by PCR assay developed by		RAPD Cluster[b]	Tannase activity (mU/ml)[c]
		Berthier *et al.* [3]	Torriani *et al.* [27]		
The isolates reported previously (18)					
KOG 19	Mixed vegetables pickled with rice bran	*L. plantarum*	*L. plantarum*	**B**	<0.1
KOG 20	Pickled cucumber and radish	*L. paraplantarum*	*L. paraplantarum*	**A**	1.24
KOG 21	Pickled vegetables assorted	*L. plantarum*	*L. plantarum*	**B**	1.27
KOG 22	Chinese cabbage pickled with rice bran	*L. plantarum*	*L. plantarum*	**B**	0.32
KOG 23	Korean radish kimchi	*L. plantarum*	*L. plantarum*	**B**	0.34
KOG 24	Cheese	*L. plantarum*	*L. plantarum*	**B**	<0.1
KOG 25	Korean cabbage kimchi	*L. paraplantarum*	*L. paraplantarum*	**A**	1.54
The isolates collected in this study					
NOS 118	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	2.69
NOS 119	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 120	Korean cabbage kimchi	*L. paraplantarum*	*L. paraplantarum*	**A**	1.56
NOS 121	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	1.21
NOS 122	Korean cabbage kimchi	**Unidentifiable**	*L. pentosus*	**Unclassified**	<0.1
NOS 123	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	1.01
NOS 124	Korean cabbage kimchi	*L. paraplantarum*	*L. paraplantarum*	**A**	3.16
NOS 125	Korean radish kimchi	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 126	Korean radish kimchi	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 128	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 129	Korean cucumber kimchi	*L. plantarum*	*L. plantarum*	**D**	1.25
NOS 131	Korean radish kimchi	*L. plantarum*	*L. plantarum*	**D**	1.23
NOS 132	Korean leek kimchi	*L. plantarum*	*L. plantarum*	**D**	1.46
NOS 133	Korean leek kimchi	*L. plantarum*	*L. plantarum*	**D**	1.49
NOS 134	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	1.26
NOS 135	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	1.41
NOS 138	Korean octopus kimchi	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 139	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	1.88
NOS 140	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	1.41
NOS 141	Korean cabbage kimchi	**Unidentifiable**	**Unidentifiable**	**Unclassified**	<0.1
NOS 142	Korean radish kimchi	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 143	Korean cabbage kimchi	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 144	Cucumber pickled with rice bran	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 145	Cucumber pickled with rice bran	*L. plantarum*	*L. plantarum*	**D**	0.13
NOS 146	Cucumber pickled with rice bran	*L. paraplantarum*	*L. paraplantarum*	**A**	0.29
NOS 147	Pickled Chinese cabbage	*L. paraplantarum*	*L. paraplantarum*	**A**	<0.1
NOS 148	Pickled Chinese cabbage	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 149	Pickled Chinese cabbage	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 150	Pickled Chinese cabbage	*L. plantarum*	*L. plantarum*	**D**	<0.1
NOS 151	Pickled vegetables assorted	**Unidentifiable**	*L. pentosus*	**Unclassified**	<0.1
NOS 152	Pickled vegetables assorted	**Unidentifiable**	*L. pentosus*	**Unclassified**	<0.1

a ATCC, American Type Culture Collection, Manassas, Va.; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France.

b see Fig. 3 for the cluster allocation

c Average tannase activity per ca. 1.5 x 10^8 CFU/ml measured in triplicate tests

Table 2. DNA relatedness between the three type strains of *L. plantarum* , *L. paraplantarum* and *L. pentosus* and the lactobacilli isolates with inconsistent results by two diferent PCR-based identification assays and their taxon as detemined by API 50CHL.

Strain	Taxon determined by PCR assay developed by:		% Relative binding with DNA from:			Taxon as determied by API 50 CHL[a]
	Berthier *et al.* (3)	Torriani *et al.* (28)	*L. plantarum* ATCC 14917^T	*L. paraplantarum* ATCC 700211^T	*L. pentosus* ATCC 8041^T	
L. plantarum **ATCC 14917^T**	***L. plantarum***	***L. plantarum***	**100**	**69.5**	**53.3**	***L. plantarum***
L. paraplantarum **ATCC 700211^T**	***L. paraplantarum***	***L. paraplantarum***	**69.5**	**100**	**52.2**	**Not determined[b]**
L. pentosus **ATCC 8041^T**	***L. pentosus***	***L. pentosus***	**53.3**	**52.2**	**100**	***L. pentosus***
KB 96	**Unidentifiable**	***L. pentosus***	**1.2**	**3.7**	**3.4**	***L. acidophilus***
KB 249	**Unidentifiable**	***L. pentosus***	**9.7**	**20.8**	**11.7**	***L. acidophilus***
NOS 122	**Unidentifiable**	***L. pentosus***	**31.8**	**55.8**	**31.1**	***Pediococcus acidilactici***
NOS 141	**Unidentifiable**	**Unidentifiable**	**5.6**	**14.5**	**4.4**	***P. pentosaceus***
NOS 151	**Unidentifiable**	***L. pentosus***	**2.1**	**4.4**	**2**	**Unidentifiable**
NOS 152	**Unidentifiable**	***L. pentosus***	**2.1**	**5.3**	**2.4**	**Unidentifiable**

[a] **see Table 3 for detailed Api profiles**

[b] **undetermined because the Api identification system did not include the taxon *L. paraplantarum***

Table 3. Carbohydrate fermentation profile of the lactobacilli isolates with inconsistent results by two diferent PCR-based identification assays.

Carbohydrate fermentation profile as determined with the API 50 CHL system

Strain	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 Acetyl glucosamine	Amygdaline	Arbutine
L. plantarum ATCC 14917[T]	–	–	–	+	+	–	–	–	–	+	+	+	+	–	–	–	–	+	+	+	–	+	+	+
L. paraplantarum ATCC 700211[T]	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	+	–	–	–	+	+	+
L. pentosus ATCC 8041[T]	+	–	–	+	+	+	–	–	–	+	+	+	+	–	–	–	–	+	+	–	–	+	+	+
KB 96	–	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
KB 249	–	–	–	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	+	+
NOS 122	–	–	–	+	+	+	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	+	–	–
NOS 141	–	–	–	+	+	+	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+
NOS 151	–	–	–	+	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	+	–	+	+	+
NOS 152	–	–	–	+	+	–	–	–	–	+	+	+	+	–	–	–	–	–	–	+	–	+	+	+

Strain	Esculine	Salicine	Cellobiose	Maltose	Lactose	Melibiose	Saccharose	Trehalose	Inuline	Melezitose	D-Raffinose	Amidon	Glycogene	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 ATCC 14917[T]	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–
L. paraplantarum ATCC 700211[T]	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–
L. pentosus ATCC 8041[T]	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
KB 96	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
KB 249	+	+	+	+	+	–	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
NOS 122	+	+	+	–	–	–	–	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
NOS 141	+	+	+	+	–	–	–	+	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–
NOS 151	+	+	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–
NOS 152	+	+	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–

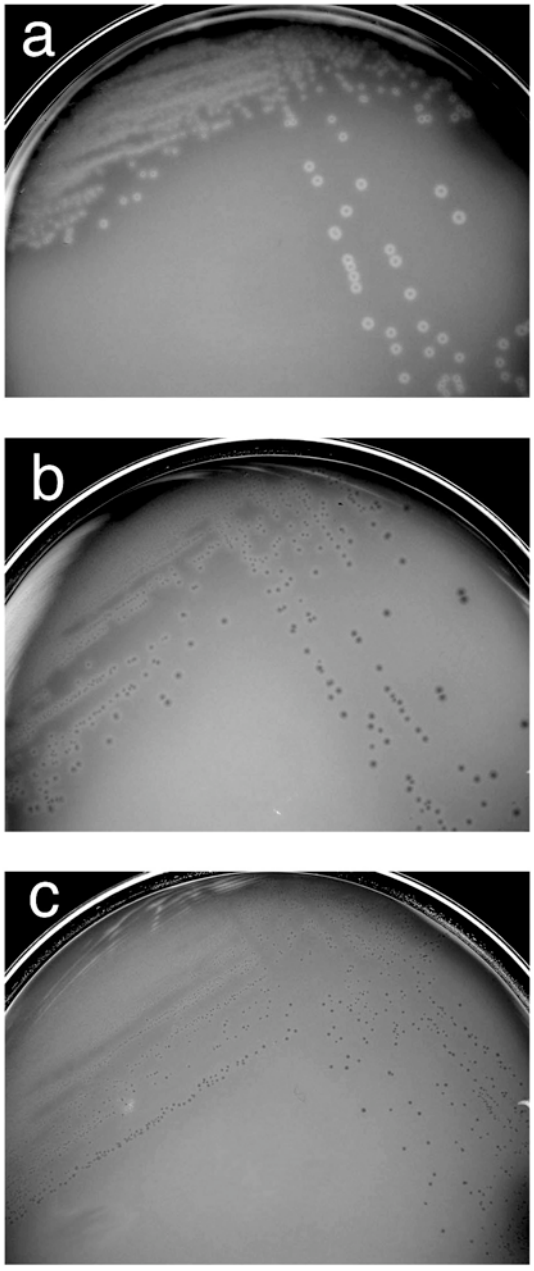

FIG. 1

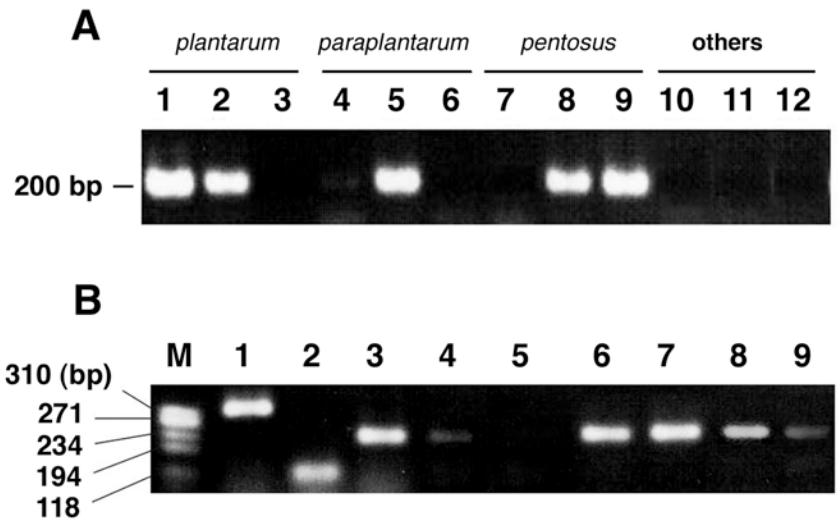
A
plantarum
paraplantarum
pentosus
others
1 2 3 4 5 6 7 8 9 10 11 12
200 bp
B
M 1 2 3 4 5 6 7 8 9
310 (bp)
271
234
194
118

FIG. 2

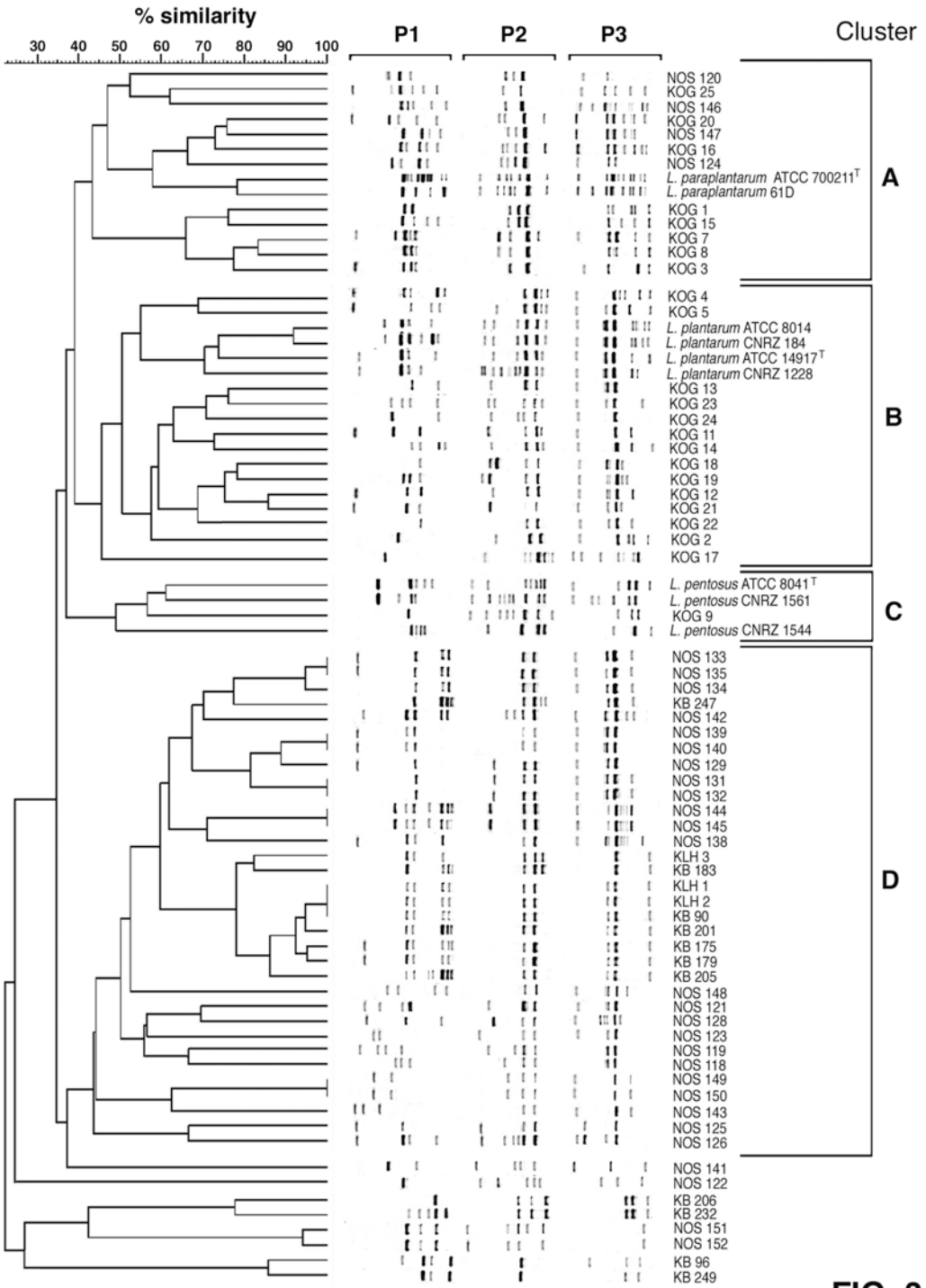
% similarity
30 40 50 60 70 80 90 100
P1
P2
P3
Cluster
NOS 120
KOG 25
NOS 146
KOG 20
NOS 147
KOG 16
NOS 124
L. paraplantarum ATCC 700211T
L. paraplantarum 61D
KOG 1
KOG 15
KOG 7
KOG 8
KOG 3
KOG 4
KOG 5
L. plantarum ATCC 8014
L. plantarum CNRZ 184
L. plantarum ATCC 14917T
L. plantarum CNRZ 1228
KOG 13
KOG 23
KOG 24
KOG 11
KOG 14
KOG 18
KOG 19
KOG 12
KOG 21
KOG 22
KOG 2
KOG 17
L. pentosus ATCC 8041T
L. pentosus CNRZ 1561
KOG 9
L. pentosus CNRZ 1544
NOS 133
NOS 135
NOS 134
KB 247
NOS 142
NOS 139
NOS 140
NOS 129
NOS 131
NOS 132
NOS 144
NOS 145
NOS 138
KLH 3
KB 183
KLH 1
KLH 2
KB 90
KB 201
KB 175
KB 179
KB 205
NOS 148
NOS 121
NOS 128
NOS 123
NOS 119
NOS 118
NOS 149
NOS 150
NOS 143
NOS 125
NOS 126
NOS 141
NOS 122
KB 206
KB 232
NOS 151
NOS 152
KB 96
KB 249
A
B
C
D

FIG. 3