Strain typing with IS*Lpl1* in lactobacilli

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Abstract

Twenty-seven *Lactobacillus plantarum* ssp. *plantarum*, 11 *Lactobacillus paraplantarum* and five *Lactobacillus casei*-related strains, isolated from various autochthonous Serbian and Montenegro-fermented foods, were identified using phenotypical characterization and current PCR methods based on PCR of the *recA* gene or the 23S–5S rRNA gene intragenic spacer (IS) region. The strains were genotypically characterized by a new method based on the insertion sequence element IS*Lpl11* that grouped these lactobacilli into 10 IS-fingerprinting groups. Between six and 23 copies of the IS*Lpl1* were found in each strain and the IS*Lpl1*-fingerprint groups correlated well with the origin of the strains. The method proved suitable for strain typing of lactic acid bacteria at the infraspecies level.

Introduction

Lactic acid bacteria (LAB) are of great commercial value because they are widely used in fermented foods as probiotics and starter cultures. LAB strains isolated from traditional-fermented foods constitute a reservoir of unexplored potential in biotechnology despite the occurrence of genomic mining for biotechnological features in sequenced LAB (Nes *et al.*, 2004). For instance, among 1962 bacterial isolates from a smear-surface soft cheese (Munster cheese) screened for activity against *Listeria monocytogenes*, *Lactobacillus plantarum* WHE 92 was selected to naturally produce pediocin AcH (Ennahar *et al.*, 1996). Besides their functional characteristics that confer unique organoleptic and probiotic properties, reliable identification and high-resolution typing of LAB is essential for nutritional sciences, biotechnology and fundamental research. Strain typing implies the postulate that daughter cells of a unique ancestor share physiological and genetic features that differ from unrelated strains. These features will be pertinent in genotyping if they are diverse within a species and yet stable in clonal strains that have similar habitat constraints.

The use of molecular biology methods, in particular comparison of the molecular chronometer 16S rRNA gene-encoding sequences (Stiles & Holzapfel, 1997), has contributed to a major revision in taxonomy and a tremendous increase in our knowledge of lactobacilli diversity (Dykes & Holy, 1994; Klein *et al.*, 1998; Giraffa & Neviani, 2000). Phenotypic characters have been traditionally used as taxonomic tools for LAB. They include the investigation of cell morphology, physiological and biochemical properties such as growth at different temperatures, pH values, salt concentrations, antibiotic susceptibility, arginine degradation and carbohydrate catabolism (Kandler & Weiss, 1986). An important tool for identification of lactobacilli is the presence of fructose-1,6-biphosphate aldolase in fermentation reactions as a method to distinguish between obligate homofermenters, facultative heterofermenters and obligate heterofermenters (Kandler & Weiss, 1986). Current methods of microbial DNA-based typing have different discrimination power, ease of use and applications (reviewed by Olive & Bean, 1999). This prompted the emergence of polyphasic approaches that incorporate several methods for the determination of isolate relatedness in taxonomy, typing and evolutionary studies (Vandamme *et al.*, 1996; Bringel *et al.*, 2001; Gürtler & Mayall, 2001; Owen, 2004; Rodas *et al.*, 2005). Molecular typing techniques based on highly standardized fingerprints have been applied to *Lactobacillus*,

including sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell protein pattern analysis (Pot *et al.*, 1994), DNA pulse-field gel electrophoresis (PFGE) of whole chromosomal DNA (Chevallier *et al.*, 1994; Tynkkynen *et al.*, 1999; Roy *et al.*, 2000), Southern blotting and restriction fragment length polymorphism on genomic DNA or PCR-amplified fragments (Chevallier *et al.*, 1994; Bringel *et al.*, 2001), randomly amplified polymorphic DNA (RAPD) assays (Tynkkynen *et al.*, 1999; Cusick & O'Sullivan, 2000; Roy *et al.*, 2000; Bringel *et al.*, 2001), PCR amplification of repetitive DNA elements present within bacterial genomes (Rep-PCR) (Gevers *et al.*, 2001), and amplified fragment length polymorphism (AFLP) (Torriani *et al.*, 2001b), which is a genome fingerprinting technique based on the selective amplification subset of DNA fragments generated by restriction enzyme digestion and ligated. Recently, the diversity and phylogenetic relatedness has been explored in 20 strains of *L. plantarum* using DNA microarray (Molenaar *et al.*, 2005). This method has the advantage of being able to highlight genome segments of conserved or lost DNA in different isolates, but is not currently applicable to a large number of strains.

In this work, we propose a new strain-typing tool for lactobacilli based on fingerprinting of repeated elements like insertion sequences (IS). IS strain typing has been successfully used for *Salmonella abortusovis* (Schiaffino *et al.*, 1996) and *Mycobacterium avium* (Pestel-Caron & Arbeit, 1998) and has been described for strain differentiation of *Lactobacillus sanfranciscensis* (Ehrmann & Vogel, 2001). The mobile element IS*Lpl1* is an IS*30*-related element isolated from *L. plantarum* CCM 1904 (Nicoloff & Bringel, 2003). IS*Lpl1*-like elements were found on the chromosome, but also on episomes in different genera of LAB (*Lactobacillus*, *Pediococcus* and *Oenococcus*) with highly variable copy number from none to at least 18 copies (Nicoloff & Bringel, 2003). Among six sequenced IS*Lpl1*-like elements, three IS harbored truncated transposase (Nicoloff & Bringel, 2003) suggesting that at least half of the elements were cryptic. Therefore, IS*Lpl1* is a good candidate for IS fingerprinting in LAB. In this article, IS*Lpl1*-fingerprinting was successfully used to discriminate natural isolates at the infraspecies level within the *Lactobacillus* species.

Materials and methods

Reference strains and food isolates

The reference strains used in genotyping included type strains of *Lactobacillus pentosus* NCFB 363^T, *L. paraplantarum* CNRZ 1885^T, *L. plantarum* ssp. *plantarum* ATCC 14917^T and *L. plantarum* ssp. *argentoratensis* DSM 16365^T (Curk *et al.*, 1996; Bringel *et al.*, 2005). Other strains with clear taxonomical status included *L. casei*-related control strains such as *L. casei* NCIMB 3254, 5A and CIP 53.166 (Bringel & Hubert, 2003), *L. plantarum* ssp. *plantarum* NCIMB 8826, and *L. plantarum* WHE 92 (Ennahar *et al.*, 1996), and *L. plantarum* ssp. *plantarum* NCIMB 1401 and *Pediococcus acidilactici* H have been described as harboring 18 and three copies of IS*Lpl1*-like elements, respectively (Nicoloff & Bringel, 2003). Food isolates used in this work were recovered from native Serbian and Montenegro-fermented products. The sources of the food isolates are listed in Table 1.

Isolation of mesophilic lactobacilli

Food isolates were isolated from (i) the brine solutions from the last phase fermentation of traditionally prepared sauerkraut, (ii) homemade cheeses, and (iii) spontaneously fermented vegetables prepared from raw vegetables purchased from different areas and producers in Serbia and Montenegro. Vegetables such as paprika (yellow, green, red), beetroots, carrots, cucumbers, mushrooms (*Chantarellus cibarius*) and cabbage were washed in tap water, cut into slices, and mixed (weight in weight) with brine solution (sucrose 2%; NaCl 2.5%) in a 0.75 L glass jar. After 2 weeks of fermentation at room temperature (20 °C), LAB were isolated. For each food product, triplicate samples of 20 g (cheese and kajmak) or 20 mL of brine from fermented vegetables were mixed into 180 mL of saline solution (NaCl, 8.5 g L^{-1}) and subsequently used to inoculate de Man, Rogossa and Sharp (MRS) agar plates (Difco Laboratories, Richmond, CA). The MRS medium is a selective medium that supports growth of lactobacilli (Man *et al.*, 1960). After 5 days incubation at 30 °C in the presence of 0.05% cycloheximide (Sigma, France) to prevent yeast growth, five to 10 colonies from each sample were inoculated in MRS broth and then stored at −80 °C in MRS broth containing 60% glycerol. Strains were routinely propagated in MRS broth at 30 °C in a 4% CO_2-enriched atmosphere using a water-jacketed CH/P incubator (Forma Scientific, Marietta, OH).

Phenotypical characterization of isolates

The first tentative identification as LAB was performed using Gram staining and microscopic morphological investigation as well as catalase production (Harrigan & McCance, 1966). Catalase-negative Gram-positive rods were further identified as lactobacilli using standard physiological and biochemical tests (Kandler & Weiss, 1986). The ability of isolated lactobacilli to grow at different temperatures (15, 30, 37 and 45 °C) was determined in MRS broth incubated in a 4% CO_2-enriched atmosphere without agitation. To investigate the effect of NaCl on bacterial growth, cells were inoculated at 30 °C in MRS broth supplemented with 4%, 6%, 8% and 10% (weight in volume) NaCl in 4% CO_2-enriched atmosphere. The influence of pH on growth was assessed in filter-sterilized MRS broth adjusted to pH 5.0,

Table 1. Characteristics of the isolated homofermentative lactobacilli

Strain name and source		IS pattern relatedness*	PCR target (product size; bp)		Gas from†	
			RecA	5S–23S rRNA	Glucose	Gluconate
Lactobacillus casei-related						
5A	Lab reference strain	NT	None	250	–	+
CIP 53.166	Lab reference strain	NT	None	170; 250	–	+
NCIMB 3254	Lab reference strain	NT	None	250	–	+
SDB1.10	Sjenica cheese	S	None	170	–	+
SDB2.1	Sjenica cheese	NT	None	170	–	+
SDB2.6	Sjenica cheese	I	None	170; 250	–	+
SDB57	Sjenica cheese	I	None	170; 250	–	+
TKS9	Cheese	S	None	250	–	+
Lactobacillus paraplantarum						
CNRZ 1885^T	Type strain	NT	107	NT	–	+
SDB1.2	Sjenica cheese	II	107	NT	–	+
SDB1.5	Sjenica cheese	II	107	400; 125	–	+
SDB2.3	Sjenica cheese	II	107	NT	–	u
SDB2.4	Sjenica cheese	II	107	NT	–	+
SDB2.7	Sjenica cheese	NT	107	NT	–	+
TKR17A	Sauerkraut	S	107	NT	–	+
TKS13B	Acid-coagulated cheese	S	107	400; 125	–	+
TKS16A	Zlatar-ripened cheese	III	107	NT	–	+
TKS16B-1	Zlatar-ripened cheese	IV	107	400; 125	–	+
TKS16B-2	Zlatar-ripened cheese	IV	107	NT	–	+
TKS16C	Zlatar-ripened cheese	III	107	NT	–	u
Lactobacillus pentosus						
NCFB 363^T	Type strain	NT	218	125	–	+
Lactobacillus plantarum ssp. *argentoratensis*						
DSM 16365^T	Type strain	NT	120	400; 125	–	+
Lactobacillus plantarum ssp. *plantarum*						
ATCC 14917^T	Type strain	Nildoff & Bringel (2003)	318	400; 125	–	+
SDB1.1	Sjenica cheese	VII	318	NT	–	u
SDB1.4	Sjenica cheese	S	318	NT	–	+
SDB1.7-1	Sjenica cheese	VII	318	125	–	u
SDB1.7-2	Sjenica cheese	VII	318	NT	–	u
SDB1.9	Sjenica cheese	VIII	318	NT	–	u
SDB18	Sjenica cheese	VIII	318	NT	–	u
SDB2.2	Sjenica cheese	VIII	318	NT	–	u
TKK1	Fermented cabbage	X	318	NT	–	+
TKK2	Fermented cabbage	X	318	NT	–	+
TKKR	Fermented cucumber	S	318	NT	–	+
TKL1	Fermented mushroom	IX	318	NT	–	+
TKL2	Fermented mushroom	IX	318	NT	–	u
TKL3	Fermented mushroom	IX	318	NT	–	u
TKM1	Fermented carrots	VI	318	125	–	+
TKM2	Fermented carrots	X	318	NT	–	+
TKM4	Fermented carrots	S	318	NT	–	+
TKP1	Fermented paprika	VI	318	NT	–	+
TKP2	Fermented paprika	X	318	NT	–	+
TKR5A	Sauerkraut	V	318	NT	–	+
TKR6	Sauerkraut	V	318	NT	–	+
TKR7A	Sauerkraut	S	318	NT	–	+
TKR7B	Sauerkraut	NT	318	NT	–	+
TKR9B	Sauerkraut	V	318	NT	–	+
TKR12	Sauerkraut	S	318	NT	–	+
TKR13	Sauerkraut	V	318	NT	–	+
TKR16	Sauerkraut	S	318	NT	–	+
TKS11	White cheese in brine	S	318	400; 125	–	u

Strains were Gram-positive rods.

**HindIII*-digested DNA bands that hybridized to an IS*Lpl1* probe: pattern were specific to a given strain (S) or shared between strains (Roman numbers refer to similarity groups as defined in Table 3).

†Carbon dioxide production was reported as –, absent; +, present; u, uncharacterized for strains with no or weak growth in presence of gluconate.

7.0 or 9.6 using HCl or NaOH. Gas production from glucose in MRS broth lacking beef extract was used to discriminate homofermentators from heterofermentators. Gas production from pentose catabolism was tested in MRS broth lacking beef extract and glucose, and containing gluconate ($10\,g\,L^{-1}$). The carbon dioxide that was produced was trapped in Durham tubes. Arginine degradation by the arginine deiminase pathway was tested with two glucose concentrations (0.05% and 2%) as previously described (Curk *et al.*, 1996). Carbohydrate fermentation was determined using API 50 CHL strips with API 50 CHL medium (Bio-Merieux). The presence of exopolysaccharides (EPS) was evaluated by the ability of the strains to form viscous colonies on MRS agar plates.

PCR-based species identification

Lactobacillus plantarum, L. paraplantarum and *L. pentosus* were discriminated using *recA*-nested multiplex PCR assays (Torriani *et al.*, 2001a; Bringel *et al.*, 2005). *Lactobacillus casei*-related species were identified using PCR amplification of the 23S–5S rRNA intragenic spacer region (Chen *et al.*, 2000). To separate the low size PCR products, electrophoresis in a 1% agarose-2% Nusieve gel was performed.

16rRNA gene sequencing

The partial 16S rRNA gene sequence was amplified using primers fd1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rd1 (5′-AAGCTTAAGGAGGTGATCCAG-3′). The reaction mixture (25 μL) contained 30 ng of template DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM each primer and 1 U Taq DNA polymerase (Biolabs, Beverley, CA) in a standard reaction buffer. After an initial denaturation of 3 min at 95 °C, 35 cycles of 30 s at 94 °C, 45 s at 55 °C, 1.5 min at 72 °C and a final extension at 72 °C for 20 min were performed. The 1.5 kb amplification product (5 μL) was treated (37 °C for 15 min followed by 80 °C for 15 min) with 2 μL of the ExoSAP-IT (USB) nuclease and sequenced. The obtained sequence was aligned with the 16S rRNA gene sequence of the Ribosomal Database Project-II (http://rdp.cme.msu.edu/seqmatch).

Genotyping of isolates

DNA extracted from lysozyme-treated lactobacilli was separated from sodium perchlorate-precipitated cell debris and proteins by phenol-chloroform treatment, as previously described (Bringel *et al.*, 2001). Genomic DNA was *Hin*dIII restricted, separated by 16 h electrophoresis in a 0.8% agarose gel and transferred onto Hybond positively charged nylon membranes (Amersham Bioscience, France). Hybridization was conducted at 42 °C with the digoxigenin-dUTP-specific IS*Lpl1* probe prepared as previously described (Nicoloff & Bringel, 2003). Hybrids were detected using the alkaline phosphatase chemiluminescent substrate CDP-start (Roche Applied Sciences, Germany). The previously characterized IS*Lp1l* bands obtained with *L. plantarum* NCIMB 1406 (Nicoloff & Bringel, 2003) were used as reference.

Nucleotide sequence accession number

The nucleotide sequence of the 16S rRNA gene in *L. paraplantarum* TKR17B has been submitted to the EMBL database Library and assigned accession no AJ879739.

Results and discussion

Phenotypic characterization

The screening of the different fermented foods allowed us to isolate 64 clones able to grow on MRS agar plates at 30 °C. All clones tested were catalase-negative and Gram-positive rods with various shapes ranging from long rods to coccoid rods. The absence of gas production from glucose was used to select 43 homofermentative isolates that were further identified at the species level. Of them, 32 were facultative homofermentative as they grew without gas production when gluconate replaced glucose (Table 1). All homofermentative lactobacilli grew well at 15, 30 and at 37 °C. Only strain SDB2.1 grew well at 45 °C, which is a characteristic of some *L. casei*-related strains (Kandler & Weiss, 1986). All strains were tolerant to NaCl at concentrations of 4% and 6%, but not at 10%. In the presence of 8% NaCl, strains SDB1.2, SDB2.1, SDB57, TKKR, TKL2, TKL3, TKR5A, TKR6, TKS5, TKS6A, TKS7A and TKS9 were clearly unable to grow. Growth at different pH was tested. All strains grew at pH 5 and pH 7. No strain grew at pH 9.6, unlike a control *Enterococcus faecalis* lab strain. No tested homofermentative lactobacilli-degraded arginine, unlike the positive controls *L. sakei* 207 and *L. brevis* TKS8. The ability to produce exopolysaccharides was found in one-third of the strains (SB1.1, SB1.2, SB1.4, SB1.5, SB1.7-1, SB1.9, SB2.2, SB2.3, TKKR, TKR5A, TKR6, TKR13). A subset of 32 lactobacilli were further tested for their ability to ferment 49 carbohydrates using API CHL 50 strips. Their carbohydrate utilization profiles were compared with those of type strains of *L. pentosus*, *L. paraplantarum* and *L. plantarum* (Table 2), and analyzed by API CHL 50 tests with Lab plus software (version 5.0). Most strains had profiles similar to *L. plantarum* and *L. paraplantarum* strains. Strains SDB2.1 and SDB57 had carbohydrate abilities related to *L. rhamnosus* and *L. casei*, respectively.

Identification of the isolates at the species level

The phenotypes of most homofermentative lactobacilli were similar to those of reference-type strains of *L. plantarum*

Table 2. Carbohydrate utilization profiles of isolated homofermentative lactobacilli and type strains of *Lactobacillus plantarum*, *Lactobacillus paraplantarum* and *Lactobacillus pentosus*

	Utilization of*																								
Strain	Glycerol	D-arabinose	L-arabinose	D-xylose	L-xylose	L-sorbose	Rhamnose	Dulcitol	Sorbitol	α-methyl-D-mannoside	α-methyl-D-glucoside	Amygdalin	Lactose	Melibiose	Sucrose	Trehalose	Melezitose	D-raffinose	Starch	β-gentiobiose	D-turanose	D-tagatose	D-arabitol	Gluconate
Lactobacillus casei-related																								
SDB2.1	−	−	−	−	−	++	++	++	++	−	++	++	++	−	+	++	++	−	−	++	−	+	−	++
SDB57	−	−	−	−	−	−	−	−	−	−	−	++	++	−	−	++	++	−	−	+	++	++	−	+
TKS9	−	−	−	−	−	−	−	−	++	−	+	−	++	++	+	++	++	++	−	−	+	++	−	+
Lactobacillus paraplantarum																								
CNRZ 1885^T	−	−	−	−	−	−	−	−	−	−	−	++	++	++	++	++	++	++	−	++	−	−	−	++
SDB1.2	+	−	−	−	−	−	−	−	−	−	−	++	++	+	++	++	++	++	−	+	++	−	+	+
SDB1.5	−	−	++	−	−	−	+	−	++	−	++	++	++	−	++	++	+	−	−	++	−	−	+	+
SDB2.4	−	−	++	−	−	−	+	−	++	−	++	++	++	++	+	++	++	−	−	++	−	−	−	++
SDB2.7	−	−	−	−	−	−	+	−	−	−	++	++	++	++	++	++	++	++	−	++	−	−	+	−
TKS13B	−	−	++	−	−	−	−	−	−	−	−	++	++	++	−	++	+	+	−	++	++	−	+	+
TKS16A	−	−	++	−	−	−	+	−	++	−	++	++	++	−	++	++	−	−	−	++	−	−	+	+
TKS16C	−	−	++	−	−	−	++	−	++	−	++	++	++	++	++	++	++	+	−	++	−	−	+	+
TKS16B-2	+	−	−	−	−	−	−	−	++	−	−	++	++	++	+	++	++	++	−	++	++	−	+	+
TKR17B	+	−	++	−	−	−	−	−	−	−	−	++	++	++	++	++	++	++	−	++	−	−	+	+
Lactobacillus pentosus																								
NCFB 363^T	++	−	++	++	−	−	−	−	++	−	−	++	++	++	++	++	−	++	−	++	++	−	−	++
Lactobacillus plantarum ssp. *plantarum*																								
ATCC 14917^T	−	−	++	−	−	−	−	−	++	+	−	++	+	++	++	++	++	+	−	++	++	−	+	++
SDB1.1	−	−	−	−	−	−	−	−	−	−	++	++	++	++	++	++	++	++	−	++	+	−	+	+
SDB1.4	+	+	−	−	+	−	−	−	++	−	++	++	++	++	++	++	++	−	−	+	−	−	+	+
SDB1.9	−	−	−	−	−	−	−	−	−	−	−	++	++	++	++	++	++	+	−	++	−	−	+	+
SDB2.2	−	−	−	−	−	−	−	−	−	−	−	++	++	++	+	++	++	++	−	+	+	−	+	+
TKK1	+	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	++	+	−	+	+
TKK2	−	−	−	−	−	++	−	−	++	++	−	++	++	++	++	++	++	++	+	++	−	−	++	++
TKL1	−	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	++	−	++	+	+
TKL2	−	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	++	−	−	−	−
TKM1	+	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	+	++	−	−	+	++
TKM2	−	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	+	++	−	−	+	++
TKM4	+	−	−	−	−	−	−	−	++	++	++	++	++	++	++	++	++	++	+	++	++	−	+	++
TKP1	−	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	+	−	++	+	++
TKP2	−	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	+	++	−	−	++	++
TKR5A	−	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	−	−	++	−	−	−	++
TKR7A	+	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	++	−	−	−	++
TKR9B	−	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	++	−	−	+	+
TKR12	−	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	++	−	−	−	++
TKR13	+	−	−	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	++	−	−	+	++
TKR16	+	−	++	−	−	−	−	−	++	++	−	++	++	++	++	++	++	++	−	+	++	−	+	+
TKS11	−	−	+	−	−	−	−	−	−	−	−	++	++	++	−	−	−	−	−	++	−	−	−	−

*++, positive reaction; +, weak positive reaction; −, negative reaction. None of the strains utilized the following compounds: erythritol, β-methyl-xyloside, adonitol, inositol, inulin, glycogen, xylitol, D-fucose, L-fucose, D-lyxose, 2 keto-gluconate, 5 keto-gluconate, L-arabitol. All the strains utilized the following compounds: ribose, galactose, D-glucose, D-fructose, D-mannose, mannitol, *N*-acetylglucosamine, arbutin, esculin, salicin, cellobiose, maltose, lactose.

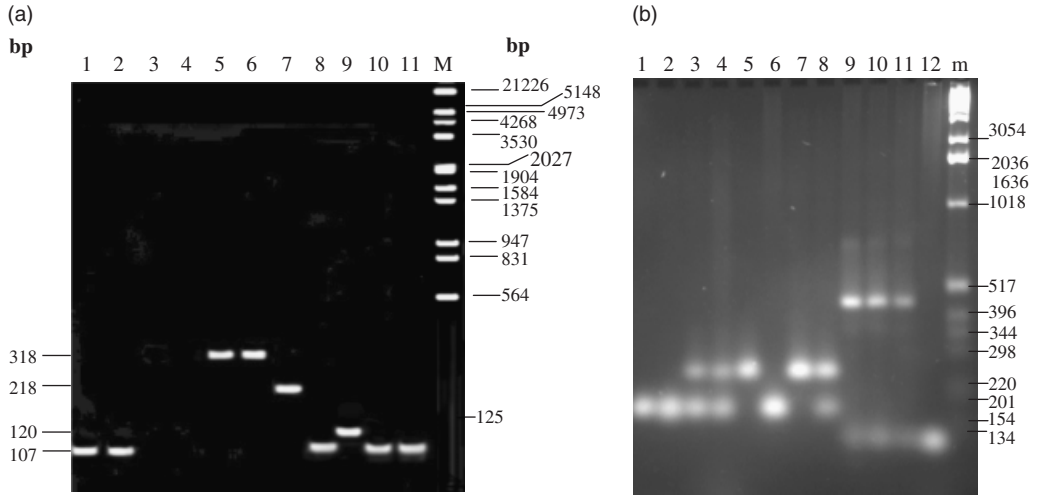

Fig. 1. PCR-based lactobacilli identification. (a) *rec*A nested multiplex PCR assays. PCR products obtained with different strains are shown in lane 1, SDB16B-2; 2, TKS16C; 3, SDB 1.10; 4, TKS9; 5, TKP2; 6, *Lactobacillus plantarum* ssp. *plantarum* ATCC 14917^{T}; 7, *Lactobacillus pentosus* NCFB 363^{T}; 8, *Lactobacillus paraplantarum* CNRZ 1885^{T}; 9, *L. plantarum* ssp. *argentoratensis* DSM 16365^{T}; 10, TKS16B-1; 11, SDB2.4. (b) PCR amplification targeting 23S–5S rRNA gene spacer region. The different lanes correspond to PCR products with different strains. In lanes 1–5, *Lactobacillus casei* related isolates are shown: SDB1.10; SDB2.1; SDB2.6; SDB57 and TKS9; in lanes 6–12, reference strains, *L. casei* NCIMB 3254, 5A and CIP 53.166, *L. plantarum* ssp. *plantarum* ATCC 14917^{T}, *L. plantarum* ssp. *argentoratensis* DSM 16365^{T}, *L. paraplantarum* CNRZ 1885^{T}, and *L. pentosus* NCFB 363^{T} are shown; lane m, DNA molecular weight marker X (Roche Diagnostics) with band size in base pairs.

ATCC 14917^{T}, *L. pentosus* NCFB 363^{T} and *L. paraplantarum* CNRZ 1885^{T}. *L. plantarum*, *L. pentosus* and *L. paraplantarum* display quasi-identical 16S rRNA encoding genes (Curk *et al*., 1996; Bringel *et al*., 2001). Thus, nested multiplex PCR assays based on *recA* were used to differentiate these species (Torriani *et al*., 2001a; Bringel *et al*., 2005). Neither a band of 120 bp specific to *L. plantarum* ssp. *argentoratensis* (Fig. 1a, lane 9 and Table 1), nor a band of 218 bp specific for *L. pentosus* NCFB 363^{T} (Fig. 1a, lane 7) was detected in the tested homofermentative lactobacilli. A band of 318 bp specific to *L. plantarum* ssp. *plantarum* was found in 27 strains as well as in the reference strain ATCC 14917^{T} (Fig. 1a, lane 6 and Table 1). A band of 107 bp specific to *L. paraplantarum* was obtained in the reference strain CNRZ 1885^{T} (Fig. 1a, lane 8) and in 11 other isolates (Table 1 and Fig. 1a, lanes 1, 2, 10 and 11). To test our identification scheme, the 16S rRNA gene of a representative strain (TKR17B) was sequenced and found to show 99% nucleic acid identity with the corresponding gene in *L. paraplantarum*-type strain.

No *recA* amplification was detected (Fig. 1a, lanes 3 and 4) in the five remaining isolates (SDB1.10, SDB2.1, SDB2.6, SDB57 and TKR9). The corresponding DNA samples were successfully used in PCR amplifications of the 16S rRNA gene (data not shown), demonstrating that the DNA matrices were suitable for PCR amplifications. We concluded that they did not belong to *L. paraplantarum*, *L. pentosus* or *L. plantarum* species. Of them, strains SDB2.1 and SDB57 had carbohydrate profiles related to *L. casei* (Table 2). To identify *L. casei*-related species, a 23S–5S rRNA intragenic spacer region PCR assay was performed (Chen *et al*., 2000). In strains SDB1.10 and SDB2.1 (Fig. 1b, lanes 1–2), a band of 170 bp was obtained as with the control, the *L. casei* NCIMB 3254 reference strain (Fig. 1b, lane 6). In strain TKS9 (Fig. 1b, lane 5), a band of 250 bp was obtained, like the control strain *L. casei* 5A (Fig. 1b, lane 7). In strains SDB2.6 and SDB57, two bands of 170 and 250 bp were found (Fig. 1b, lanes 3–4), like *L. casei* CIP 53.166 reference strain (Fig. 1b, lane 8). Controls were performed using *L. paraplantarum*, *L. plantarum* and *L. pentosus* type strains (bands of 400 or 125 bp; Fig. 1b, lanes 9–12). Thus, as deduced from the 170/250 bp bands obtained, strains SDB1.10, SDB2.1, SDB2.6, SDB57 and TKR9 were related to *L. casei*.

IS*Lpl1* fingerprints and origin of isolation

Insertion sequence fingerprinting based on Southern hybridization with the insertion element IS*Lpl1* probe (Nicoloff & Bringel, 2003) was tested for its use in strain genotyping on most of the homofermentative lactobacilli isolated from Serbian and Montenegro-fermented foods. Their IS fingerprints were compared with reference strains with known IS*Lpl1*-like copy number such as the *L. plantarum* sequenced strain NCIMB 8826 (none), *L. plantarum*-type strain ATCC 14917 (one copy), *L. plantarum* ATCC 8014 (one copy), *L. plantarum* WHE 92 (four copies), *L. plantarum* NCIMB

Table 3. IS*Lpl1*-fingerprints, characterization of the *Hind*III bands detected with the *ISLpl1* probe

Strain	IS pattern group	Band number	Size in kb
Lactobacillus casei			
SDB2.6; SDB57	I	10	≥20; 8.4; 6.8; 6.4; 4.6; 4.0; 3.5; 2.9; 1.2; 1.0
SDB1.10		10	12; 7.0; 6.2; 4.7; 4.4; 3.2; 2.7; 2.4; 1.9; 1.5
TKS9		16	11; 7.8; 7.4; 6.8; 6.2; 6.0; 5.6; 5.3; 5.0; 4.0; 3.4; 3.0; 2.7; 2.4; 1.7; 1.4
Lactobacillus paraplantarum			
TKR17A		7	8.1; 7.1; 5.4; 4.3; 3.4; 3.0; 2.6
SDB2.4	II	14	9.3; 8.6; 6.8; 6.2; 5.6; 5.1; 4.8; 4.3; 4.1; 3.9; 2.6; 1.9; 1.7; 1.3
SDB1.2	II	16	12; 10; 9.8; 7.5; 6.8; 6.2; 5.5; 5.1; 4.5; 4.1; 3.8; 3.6; 3.0; 2.4; 1.9; 0.7
SDB1.5	II	16	≥20; 14; 12; 8.6; 7.5; 6.8; 5.7; 5.3; 4.9; 4.4; 3.7; 3.0; 2.7; 2.5; 1.9; 1.7
SDB2.3	II	21	≥20; 16; 14; 12; 10; 9.3; 8.8; 8.4; 7.5; 6.2; 5.8; 5.4; 4.8; 4.4; 3.6; 3.0; 2.6; 2.4; 2.2; 1.9; 1.3
TKS16A	III	17	13; 9; 7.4; 6.1; 5.5; 4.9; 4.5; 4.1; 3.7; 3.3; 2.9; 2.6; 2.1; 1.8; 1.5; 1.3; 1.0
TKS16C	III	18	13; 10; 9; 7.6; 7.2; 6.6; 6.2; 5.5; 4.9; 4.5; 4.1; 3.9; 3.3; 3.0; 2.1; 1.7; 1.3; 1.0
TKS16B-2	IV	20	16.5; 15; 13; 11; 9.5; 9.0; 8.1; 6.8; 5.9; 5.5; 5.1; 4.7; 4.4; 3.7; 3.5; 3.2; 2.9; 2.6; 2.0; 1.8
TKS16B-1	IV	21	16.5; 15; 13; 12; 9.5; 9.0; 8.1; 6.8; 6.3; 5.9; 5.5; 5.1; 4.7; 4.4; 3.7; 3.5; 3.2; 2.9; 2.6; 2.0; 1.8
TKS13B		21	≥25; 20; 16.5; 15.3; 14; 12.7; 11.5; 10.5; 9.8; 8.2; 7.1; 6.2; 5.8; 5.2; 4.9; 4.7; 3.5; 3.2; 3.0; 2.8; 2.5
Lactobacillus plantarum			
NCIMB 8826		0	
ATCC 8014		1	3.4
ATCC 14917^T		1	5.0
WHE92		7	11; 10; 7.5; 6.5; 4.7; 2.9; 1.8
TKKR		6	6.1; 5.3; 4.8; 4.3; 2.7; 0.8
SDB1.4		7	7.2; 6.7; 5.1; 4.3; 2.7; 1.7; 0.8
TKM4		7	10; 4.0; 3.6; 2.9; 2.5; 2.3; 1.3
TKR13	V	8	9.4; 6.4; 5.4; 5.1; 4.3; 3.6; 2.9; 2.7
TKR5A	V	8	7.0; 6.5; 6.2; 4.9; 4.2; 3.5; 2.1; 1.6
TKR6	V	8	13; 11; 6.3; 5.6; 4.8; 4.3; 2.8; 0.8
TKR9B	V	9	11; 7.5; 6.2; 5.5; 4.4; 3.6; 2.9; 2.8; 2.7
TKP1	VI	10	≥20; 11.5; 8.2; 6.5; 5.5; 4.3; 3.8; 3.1; 2.8; 2.5
TKM1	VI	12	≥20; 11.5; 10; 8.2; 5.5; 4.3; 4.1; 3.8; 3.1; 2.8; 2.5; 1.4
SDB1.1	VII	11	≥20; 14; 11; 7.4; 6.5; 5.0; 4.2; 3.5; 3.0; 2.9; 1.4
SDB1.7-1	VII	13	≥20; 14; 11; 9.0; 8.4; 7.4; 6.5; 5.0; 4.2; 3.5; 3.0; 2.9; 1.4
SDB1.7-2	VII	18	≥20; 14; 13; 11; 7.4; 7.1; 6.5; 5.9; 5.5; 5.0; 4.6; 4.2; 3.9; 3.5; 3.2; 2.9; 2.7; 1.4
TKR7A		11	14; 12; 9.3; 7.9; 7.2; 6.5; 5.5; 4.4; 3.8; 3.0; 2.8
TKR12		12	7.7; 7.0; 5.3; 4.7; 4.2; 3.8; 3.6; 3.2; 2.9; 2.6; 2.3; 2.0
TKR16		14	11; 8.9; 7.0; 6.2; 5.9; 5.2; 4.7; 4.3; 4.1; 2.4; 1.4; 1.3; 0.9; 0.8
SDB1.9	VIII	14	16; 14; 13; 11; 9.4; 6.2; 5.3; 4.9; 4.8; 3.9; 3.0; 2.8; 2.6; 1.2
SDB2.2	VIII	15	14; 11; 8.9; 7.6; 6.6; 6.2; 5.9; 5.3; 4.9; 4.7; 3.0; 2.8; 2.5; 1.9; 1.2
SDB18	VIII	16	16; 14; 11; 9.9; 8.7; 7.5; 6.6; 6.2; 5.3; 4.9; 4.7; 4.5; 3.8; 3.0; 2.8; 1.3
TKS11		16	≥20; 11; 8.2; 6.9; 5.7; 5.2; 4.7; 4.2; 3.8; 3.2; 2.7; 2.4; 2.3; 2.0; 1.7; 1.6
TKL2; TKL3	IX	19	≥20; 16; 14.5; 13; 10.5; 9.4; 8.1; 7.6; 6.2; 5.2; 4.7; 4.1; 3.3; 2.9; 2.7; 2.4; 2.1; 1.8; 1.6
TKL1	IX	23	≥20; 16; 14.5; 10.5; 8.0; 7.6; 7.1; 6.6; 6.2; 5.6; 5.4; 4.7; 4.5; 4.1; 3.3; 3.1; 2.9; 2.6; 2.4; 2.3; 2.1; 1.8; 1.6
NCIMB 1406		18	18.5; 12.5; 11; 9.0; 8.4; 7.7; 6.9; 5.9; 5.5; 5.3; 4.9; 4.1; 3.4; 3.0; 2.8; 2.1; 1.7; 1.2
TKM2	X	14	17.5; 17; 15; 14; 13; 9.8; 9.2; 8.0; 7.4; 6.9; 6.4; 6.0; 5.6; 4.8
TKP2	X	21	17.5; 16; 15; 14; 13; 12; 9.7; 9.2; 8.0; 7.4; 6.9; 6.4; 6.0; 5.6; 4.7; 4.4; 3.7; 3.4; 2.8; 2.5; 2.4
TKK1	X	22	17.5; 16; 15; 14; 13; 12; 9.7; 8.9; 8.0; 7.6; 7.0; 6.4; 6.0; 5.6; 4.6; 4.4; 4.2; 3.7; 3.5; 2.8; 2.5; 2.3
TKK2	X	22	17.5; 16; 15; 14; 13; 12; 9.7; 8.0; 7.4; 6.8; 6.5; 6.0; 5.6; 4.9; 4.6; 4.4; 3.8; 3.5; 2.8; 2.5; 2.3; 1.3
Pediococcus acidilactici			
H		3	15; 6.2; 4.8

IS, insertion sequences.

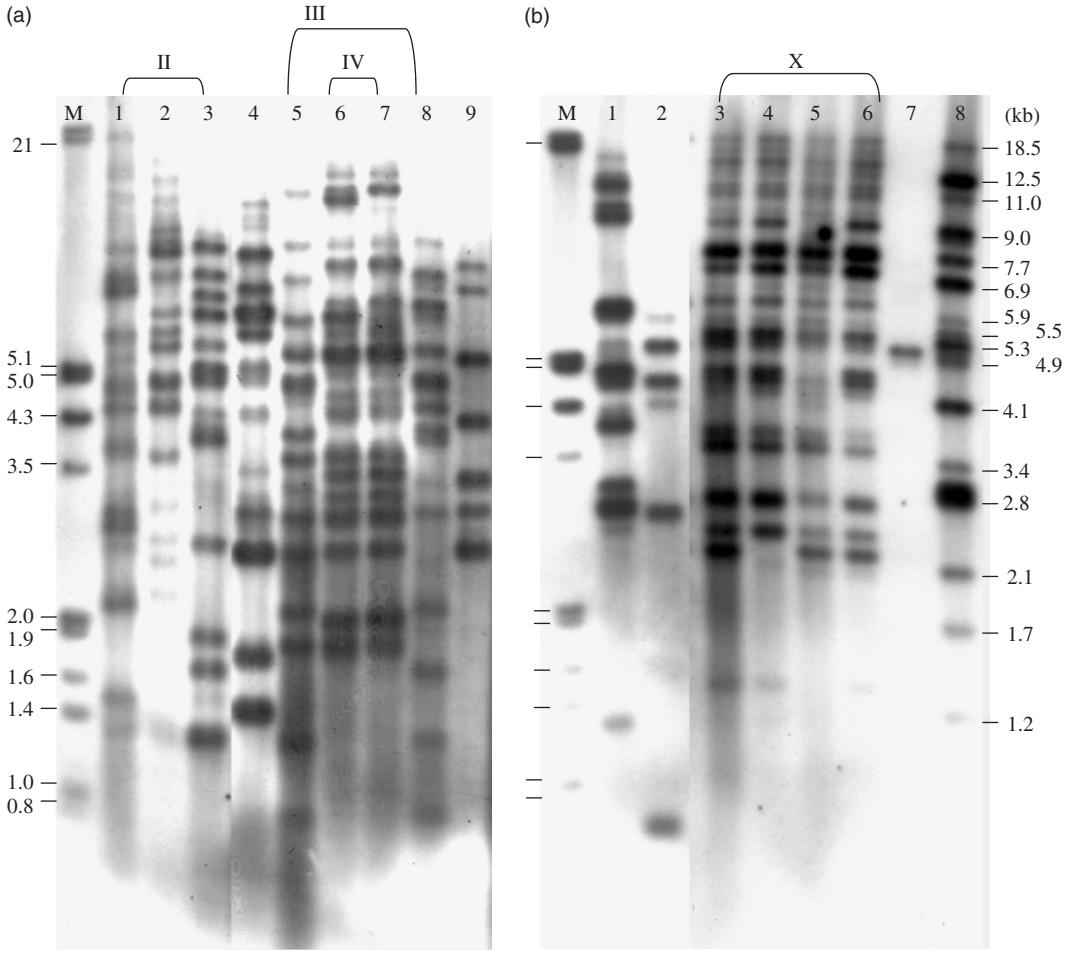

Fig. 2. Differentiation of *Lactobacillus* strains by Southern hybridization with an *ISLpl1* probe. For each strain, the hybridization pattern obtained with the IS*Lpl1*-*Hin*dIII detected bands were compared and grouped according to relatedness [roman numbers on top of the figure indicate names of the insertion sequence (IS) fingerprint groups as defined in Table 3]. The bands of the molecular size marker (M) are indicated in kb. (a) IS fingerprints of *Lactobacillus paraplantarum* isolates are shown, respectively, in lanes 1–9: SDB1.5, SDB2.3, SDB2.4, TKS13B, TKS16A, TKS16B-1, TKS16B-2, TKS16C and TKR17B. (b) IS fingerprints of *L. plantarum* isolates are shown, respectively, in lanes 1–6: SDB1.9, TKKR, TKM2, TKP2, TKK1 and TKK2. In lane 7, the heterofermentative lactic acid bacteria TKS12B is shown. In lane 8, *L. plantarum* NCIMB 1408 IS*Lpl1* fingerprint, is shown as previously described (Nicoloff & Bringel, 2003).

1406 (18 copies) and *P. acidilactici* H (three copies) (Nicoloff & Bringel, 2003). IS*Lpl1*-related sequences were detected in all the tested isolates from Serbian and Montenegro-fermented products (Table 3 and Fig. 2). Thus, IS*Lpl1*-like elements are widespread among these mesophilic lactobacilli. As the restriction enzyme *Hin*dIII does not cut within IS*Lpl1*, the number of detected bands should correspond to the number of IS copies present. Between six and 23 different bands were found. The size and the number of bands were compared between strains. Where two strains harbored at least two thirds of the bands in common, they were assigned to the same IS group. The relative band intensity was not used as a discriminative criterion. Ten different IS-related groups were found and numbered from I to X, and did not include the reference strains. Similar IS fingerprints may be found in strains that derive from a common parental strain. A correlation between the IS fingerprint and the origin of isolation was established in several instances. Strains isolated from Sjenica cheese fell into three groups: *L. casei* group I (strains SDB2.6 and SDB57), *L. paraplantarum* group II (SDB1.2, SDB1.5, SDB2.3; SDB2.4) and *L. plantarum* group VII (SDB1.1, SDB1.7-1 and SDB1.7-2). Similarly isolates from different producers of ripened Zlatar cheese belonged to *L. paraplantarum* group III (TKS16A and TKS16C, Fig. 2a, compare lanes 5 and 8) and *L. paraplantarum* group IV (TKS16B-1 and TKS16B-2, Fig. 2a, compare lanes 6 and 7). Furthermore, some strains isolated from sauerkraut clustered in *L. plantarum* group V, and most strains isolated from fermented mushrooms belonged to *L. plantarum* group IX (Table

1). The strains from the group X were isolated from various fermented vegetable matrices: cabbage, carrots and paprika (Table 1). Strain TKK1 was unable to degrade starch unlike strains TKK2, TKM2 and TKP2 (Table 2). Only strain TKK2 catabolized L-sorbose. In group VI, *L. plantarum* TKM1 isolated from spontaneously fermented carrots and *L. plantarum* TKP1 isolated from spontaneously fermented paprika differed in their ability to degrade starch, glycerol and D-tagatose (Table 2); this may correspond to strain adaptation to different fermentation conditions.

IS*Lpl1* fingerprints as a method for infraspecies lactobacilli typing

IS*Lpl1*-typing requires standard molecular biology equipment and 1-week labor from culture to the final IS fingerprint (2 days for culture and DNA extraction; 1 day for genomic DNA *Hin*dIII-restriction and electrophoresis; 2 days for hybridization and hybrid detection). Compared with other genotyping fingerprinting procedures such rep-PCR, AFLP or RAPD (Cusick & O'Sullivan, 2000), no fastidious prerequisite standardization is required. Like RAPD analysis, ribotyping and PFGE (Tynkkynen *et al*., 1999), IS fingerprinting is primarily a method that discriminates at the infraspecies level, but also has the potential to give species-specific information. Ribotyping targets a molecule found in all microorganisms, whereas IS typing can only be applied to LAB that harbor IS*Lpl1*-like elements. Such IS elements were not found in sequenced genomes of *L. acidophilus* NCFM, *L. johnsonii* NCC 533, *E. faecalis* V583 and *Lactococcus lactis* ssp. *lactis* IL1403. On the other hand, inactive IS highly similar to IS*Lpl1* have been found in the sequenced genomes of *Oenococcus oeni* PSU-1 (98% nucleic acid identity; accession no NZ_AABJ03000002) and *Pediococcus pentosaceus* ATCC 25745 (two copies with respectively 86% and 98% nucleic acid identity; accession no ZP_00322972 and ZP_00322988). We found that IS*Lpl1*-like elements were commonly encountered in the three *Lactobacillus* species as 44 out of 45 tested lactobacilli harbored IS*Lpl1*-like elements [*L. paraplantarum* ($n = 10$); *L. casei*-related strains ($n = 4$); *L. plantarum* ($n = 31$); Table 3]. Recently, IS*Lpl1*-like elements were also found in *L. paracasei* ATCC 25302, in *L. rhamnosus* strains (Suokko *et al*., 2005) and in the genome of *L. casei* ATCC 334 (accession no ZP_00386524). Thus, IS typing would be suitable for strain typing in these *Lactobacillus* species. IS*Lpl1* is a mobile element often present in several copies (Table 3) raising the question of its stability in the host. IS fingerprint stability has previously been investigated after 12 rounds of serial growth in liquid-rich media (Nicoloff & Bringel, 2003). Discrete IS pattern changes with an average of four band shifts for the 18 IS copies present in the parental strain *L. plantarum* strain NCIMB 1406 were observed after 110 generations in liquid media MRS in clones that had acquired the ability to grow faster than the parental strain (Nicoloff & Bringel, 2003). In the same conditions, stable IS fingerprints (seven bands) were observed in *L. plantarum* WHE 92 (F. Bringel, unpublished data). Thus, IS fingerprint stability depends on the host genetic context (IS copy number; functionality of the IS-encoded transposase, etc.). IS elements are efficient tools for monitoring changes in evolving populations including both divergence from an ancestor and diversity within a population (Papadopoulos *et al*., 1999). As suggested by this work, strain typing using IS*Lpl1* fingerprint offers a new tool to evaluate strain stability and the ecological distribution of related lactobacilli.

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