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Lactobacillus Strain Diversity Based on Partial *hsp60* Gene Sequences and Design of PCR-Restriction Fragment Length Polymorphism Assays for Species Identification and Differentiation▽†

Giuseppe Blaiotta,[1]* Vincenzina Fusco,[1] Danilo Ercolini,[2] Maria Aponte,[1] Olimpia Pepe,[1] and Francesco Villani[1]

Department of Food Science, School of Agriculture,[1] and School of Biotechnological Sciences,[2] The University of Naples Federico II, via Università 100, 80055 Portici, Italy

Received 25 July 2007/Accepted 30 October 2007

A phylogenetic tree showing diversities among 116 partial (499-bp) *Lactobacillus hsp60* (*groEL*, encoding a 60-kDa heat shock protein) nucleotide sequences was obtained and compared to those previously described for 16S rRNA and *tuf* gene sequences. The topology of the tree produced in this study showed a *Lactobacillus* species distribution similar, but not identical, to those previously reported. However, according to the most recent systematic studies, a clear differentiation of 43 single-species clusters was detected/identified among the sequences analyzed. The slightly higher variability of the *hsp60* nucleotide sequences than of the 16S rRNA sequences offers better opportunities to design or develop molecular assays allowing identification and differentiation of either distant or very closely related *Lactobacillus* species. Therefore, our results suggest that *hsp60* can be considered an excellent molecular marker for inferring the taxonomy and phylogeny of members of the genus *Lactobacillus* and that the chosen primers can be used in a simple PCR procedure allowing the direct sequencing of the *hsp60* fragments. Moreover, in this study we performed a computer-aided restriction endonuclease analysis of all 499-bp *hsp60* partial sequences and we showed that the PCR-restriction fragment length polymorphism (RFLP) patterns obtainable by using both endonucleases AluI and TacI (in separate reactions) can allow identification and differentiation of all 43 *Lactobacillus* species considered, with the exception of the pair *L. plantarum*/*L. pentosus*. However, the latter species can be differentiated by further analysis with Sau3AI or MseI. The *hsp60* PCR-RFLP approach was efficiently applied to identify and to differentiate a total of 110 wild *Lactobacillus* strains (including closely related species, such as *L. casei* and *L. rhamnosus* or *L. plantarum* and *L. pentosus*) isolated from cheese and dry-fermented sausages.

Lactobacilli are some of the most important taxa involved in food microbiology and human nutrition owing to their role in food and feed production and preservation, including the probiotic properties exhibited by some strains. These traits are of increasing importance and have received attention in the food and feed industry (2).

At the beginning of 2005, the genus *Lactobacillus* was reported to include about 100 validly described species (10). However, the number of species is continually changing due to the description of new species and/or reclassification of others: indeed, at the beginning of 2007 this genus had reached about 120 species (http://www.bacterio.cict.fr/).

Their classification into three groups based on metabolism and physiological characteristics (30) is not in agreement with the results of phylogenetic studies by sequencing of 16S rRNA (7). Schleifer and Ludwig (49) reviewed the phylogeny of the genus *Lactobacillus* based on the 16S rRNA sequences but did not clarify the taxonomy of either the *L. casei* group or the *L. delbrueckii* subspecies. Recently, several systematic studies of lactobacilli have been carried out (10, 25, 40, 46, 60). With 16S rRNA-based phylogenetic analysis, at least eight phylogenetic groups have been recognized (10). Investigations by many authors using several molecular approaches focused on closely related species, such as the *L. casei*-related taxa (6, 9, 12, 38, 44, 47, 57, 61), the *L. acidophilus* group (19, 32, 41, 45, 47), the *L. delbrueckii* subspecies (20, 35, 36, 56), and the *L. plantarum*-related species (54). Nevertheless, the discrimination of very closely related species, especially of subspecies, is often critical (10). Thus, the sequencing of several other genes, such as the *tuf* gene (encoding elongation factor Tu, involved in protein biosynthesis), *mal* (encoding malolactic enzyme), the S-layer gene (encoding surface layer proteins), *pepC* (encoding aminopeptidase C), *pepN* (encoding aminopeptidase N), *htrA* (encoding stress-inducible trypsin-like serine protease), *recA* (encoding recombinase A), and *rpoB* (encoding RNA polymerase beta subunit), has been used for the discrimination of lactobacilli (4, 16, 22, 24, 42, 55, 58, 59). However, in these last studies, a small number of different *Lactobacillus* species were analyzed.

In this study, we evaluated the polymorphism within the *hsp60* (*groEL*, encoding a 60-kDa heat shock protein) genes of different *Lactobacillus* species, with a view to designing molecular systems allowing identification and differentiation of a broad number of species.

MATERIALS AND METHODS

Bacterial strains and culture media. Fifty reference (type and previously identified) strains, representing 30 different *Lactobacillus* species, were used in this study

* Corresponding author. Mailing address: Dipartimento di Scienza degli Alimenti, Università degli Studi di Napoli Federico II, Via Università 100, 80055 Portici (Naples), Italy. Phone: 39-081-2539451. Fax: 39-081-2539407. E-mail: blaiotta@unina.it.

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TABLE 1. Identification of the wild *Lactobacillus* strains isolated during manufacturing of Provolone del Monaco cheese by application of the PCR-RFLP assay designed during this study

Sample no.[a]	Isolation medium[b]	Isolation dilution	Strain(s)[c]	PCR-RFLP profile[d]		Species
				AluI	TacI	
1	Isolini	10^{-4}	137, 138^{S}, 147	A	a	*L. casei*
2	Isolini	10^{-5}	154^{P}	B	c	*L. plantarum*
	Isolini	10^{-5}	151	A	b	*L. rhamnosus*
4	Isolini	10^{-7}	221^{P,S}	B	c	*L. plantarum*
6	Isolini	10^{-7}	357, 358, 359^{S}	A	a	*L. casei*
	Isolini	10^{-6}	354^{P}	C	a	*L. helveticus*
	Isolini	10^{-7}	356	A	b	*L. rhamnosus*
7	Isolini	10^{-6}	426	A	a	*L. casei*
	Isolini	10^{-5}	428, 429, 430, 431, 432	A	a	*L. casei*
	Isolini	10^{-6}	423, 425	A	b	*L. rhamnosus*
	Isolini	10^{-5}	433	A	b	*L. rhamnosus*
8	Isolini	10^{-5}	488, 489^{S}, 490, 491, 492, 493, 494, 495, 496	A	a	*L. casei*
	Isolini	10^{-6}	497, 498, 499	A	a	*L. casei*
1	Rogosa	10^{-2}	283^{S}	A	b	*L. rhamnosus*
	Rogosa	10^{-2}	291^{P,S}	C	a	*L. helveticus*
2	Rogosa	10^{-6}	296, 297, 299, 300, 302	A	b	*L. rhamnosus*
3	Rogosa	10^{-6}	178,181	A	b	*L. rhamnosus*
4	Rogosa	10^{-7}	159, 164	A	b	*L. rhamnosus*
	Rogosa	10^{-6}	167, 168, 169, 170	A	b	*L. rhamnosus*
5	Rogosa	10^{-6}	260, 261, 262, 264	A	b	*L. rhamnosus*
	Rogosa	10^{-7}	265, 268, 269	A	b	*L. rhamnosus*
6	Rogosa	10^{-6}	338, 340, 342, 343, 344	A	b	*L. rhamnosus*
	Rogosa	10^{-7}	345, 346, 347	A	b	*L. rhamnosus*
7	Rogosa	10^{-5}	401, 402, 404, 406, 407, 408, 410	A	b	*L. rhamnosus*
	Rogosa	10^{-6}	411	A	b	*L. rhamnosus*
8	Rogosa	10^{-3}	478, 479, 480, 481	A	b	*L. rhamnosus*
	Rogosa	10^{-4}	482, 483, 484	A	b	*L. rhamnosus*
	Rogosa	10^{-5}	485^{S}, 486, 487	A	b	*L. rhamnosus*

[a] 1, milk; 2, curd at the beginning of ripening; 3, curd at the end of ripening; 4, cheese after stretching; 5, cheese in brine; 6, cheese at 1 month of ripening; 7, cheese at 5 months of ripening; 8, cheese at 9 months of ripening.

[b] Rogosa agar (Oxoid) or Isolini agar (29) was used.

[c] A superior P indicates that identification was confirmed by species-specific PCR assays (18, 55); a superior S indicates that the strain was confirmed by *hsp60* gene sequencing.

[d] Patterns: A, fragments of 330 and 169 bp (*L. salivarius*/*L. casei*/*L. rhamnosus*/*L. zeae*); B, fragments of 34, 63, 187, and 215 bp (*L. plantarum*/*L. pentosus*); C, fragments of 34, 138, and 327 bp (*L. acidophilus*/*L. helveticus*); a, undigested PCR product (499 bp); b, fragments of 4, 56, and 439 bp (*L. rhamnosus*); c, fragments of 221 and 278 bp.

(see Table S1 in the supplemental material). Moreover, to validate the implemented *hsp60* PCR-restriction fragment length polymorphism (RFLP) approach, 110 wild lactobacilli isolated during previous research in our department were included in the analysis. Of these strains, 80 were isolated during manufacturing of Provolone del Monaco cheese (Table 1), while the remaining 30 were isolated from different samples of dry-fermented sausages produced in Vallo di Diano (southern Italy) (Table 2). Both fermented products were traditionally manufactured without starter addition. All isolates were stored in MRS broth (Oxoid) cultures with 20% glycerol at −25°C. Working cultures were prepared in 10 ml of MRS broth (Oxoid) incubated anaerobically for 24 h at either 30 or 37°C.

DNA isolation. DNA extraction was carried out from a single colony grown on MRS agar (Oxoid) plates by using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA), following the conditions described by the supplier.

Amplification and sequencing of the *hsp60* gene. Universal *hsp60* oligonucleotide primers H279 and H280, previously described by Goh et al. (21), were used to amplify a 650-bp fragment internal to the *hsp60* gene.

PCR amplifications were performed with a 50-μl total volume including 5 μl of the target DNA, 5.0 μl of *Taq* DNA polymerase 10× buffer (Invitrogen, SG Milanese, Italy), 2.5 μl of 50 mM $MgCl_2$, 0.5 μl of a deoxynucleoside triphosphate mix (25 mM each), 0.125 μl of each primer (0.1 mM), and 0.5 μl of *Taq* DNA polymerase (5 U/μl) (Invitrogen). The PCR consisted of 40 cycles (30 s at 94°C, 30 s at 37°C, and 1 min at 72°C) and one additional, final cycle at 72°C for 5 min. The PCR amplification fragments were resolved by agarose (2%, wt/vol) gel electrophoresis at 100 V for 2 h. The gel was stained with ethidium bromide, and the bands were visualized under UV illumination at 254 nm.

After gel electrophoresis, the 650-bp PCR fragment was purified by using a QIAquick gel extraction kit (Qiagen S.p.A., Milan, Italy) and sequenced. In order to design a primer set for direct sequencing of the H279-H280 PCR products, the *Lactobacillus hsp60* gene sequences found in GenBank (http://www.ncbi.nlm.nih.gov/) or in the cpnDB database (http://cpndb.cbr.nrc.ca/) were aligned. Two primers, LB308F (TGAAGAAYGTNRYNGCYGG) and LB806RM (AANGTNCCVCGVATCTTGTT), were chosen from highly similar regions.

TABLE 2. Identification of the wild *Lactobacillus* strains isolated from different samples of dry-fermented sausages produced in Vallo di Diano (southern Italy) by application of the PCR-RFLP assay designed during this study

Sample	Strain(s)	PCR-RFLP profile[a]		Species[b]
		AluI	TacI	
A	ALAC1, ALAC2, ALAC3 ALAC4, ALAC5	D	D	*L. curvatus*
B	BLAC7, BLAC8, BLAC9, BLAC10	D	d	*L. curvatus*
	BLAC6	D	a	*L. sakei*
C	CLAC12, CLAC15	D	d	*L. curvatus*
	CLAC14	D	a	*L. sakei*
D	DLAC16, DLAC17, DLAC18, DLAC19, DLAC20	D	a	*L. sakei*
E	ELAC21, ELAC22, ELAC23, ELAC24	D	d	*L. curvatus*
	ELAC25	D	a	*L. sakei*
F	FLAC26, FLAC27, FLAC28, FLAC29, FLAC30	D	a	*L. sakei*
G	GLAC31	D	d	*L. curvatus*
	GLAC32, GLAC33, GLAC34, GLAC35	D	a	*L. sakei*
H	HLAC36, HLAC37, HLAC39	D	a	*L. sakei*
I	ILAC41, ILAC43, ILAC44, ILAC45	D	d	*L. curvatus*
	ILAC42	D	a	*L. sakei*
L	LLAC46, LLAC47, LLAC48, LLAC50	D	d	*L. curvatus*
	LLAC49	D	a	*L. sakei*

[a] Patterns: D, fragments of 75, 78, 170, and 177 bp (*L. curvatus*/*L. sakei*); a, undigested PCR product (499 bp); d, fragments of 110, 168, and 221 bp (*L. curvatus*).
[b] Identifications confirmed by species-specific PCR assays (3).

These primers target positions 308 to 328 and 806 to 787 of the *hsp60* gene, respectively (the nucleotide numbering is based on the *hsp60* gene of *L. acidophilus* CRL639, GenBank accession number AF300645).

The DNA sequences were determined by the dideoxy chain termination method by using a DNA sequencing kit (Perkin-Elmer Cetus, Emeryville, CA), according to the manufacturer's instructions. The sequences were analyzed by MacDNasis Pro v3.0.7 (Hitachi Software Engineering Europe S. A., Olivet Cedex, France), and research for DNA similarity was performed with GenBank and the EMBL database (1).

Phylogenetic analysis was performed using MEGA version 4.0 (50) after multiple alignment of data by ClustalW 1.8 (53). Distance matrix and neighbor-joining methods (48) were applied for tree construction.

PCR-RFLP of the 499-bp internal *hsp60* fragments. PCR-RFLP of 499-bp internal fragments of the *hsp60* genes was performed by digesting the PCR product obtained using primers LB308F and LB806RM. For this last PCR amplification, we used the reaction conditions described above, while the thermal condition consisted of 40 amplification cycles (30 s at 95°C, 40 s at 52°C, and 40 s at 72°C) and one additional, final cycle at 72°C for 5 min. Restrictions were performed by digesting 35 μl of a PCR product with 20 U of a restriction enzyme (Promega) in a total volume of 50 μl at 37°C for 5 h. Digested fragments were separated by agarose (2%, wt/vol) gel electrophoresis at 100 V for 3 h.

Nucleotide sequence accession numbers. All sequences determined in this study were deposited in GenBank under the following accession numbers: AY424311 to AY424357, AY571673 to AY571677, and AY700220.

RESULTS

Sequence analysis of *hsp60* gene sequences. Partial sequences (499 bp) of the *hsp60* genes of 52 *Lactobacillus* strains were determined during this study (see Table S1 in the supplemental material). Both strands of an *hsp60* PCR fragment of about 650 bp, obtained by using primers H279 and H280, which were previously described (21), were sequenced by primers LB308F and LB806RM, designed during this study. A total of 116 (including 64 sequences found in the chaperonin database [http://cpndb.cbr.nrc.ca]) *Lactobacillus hsp60* partial sequences, representing 50 different species, were compared. The results of neighbor-joining analysis of *hsp60* sequences of the 116 strains are shown in the dendrogram depicted in Fig. 1.

A total of 43 single species clusters were identified/detected. Due to their high sequence similarity (Fig. 1) and according to some recent systematic studies (11, 17, 31, 39), the pairs *L. cypricasei* and *L. acidipiscis* (cluster 27), *L. ingluviei* and *L. thermotolerans* (cluster 42), *L. vaccinostercus* and *L. durianis* (cluster 39), and *L. plantarum* and *L. arizonensis* (cluster 31) were considered synonyms. All strains of *L. casei* and *L. paracasei* showed very closely matched *hsp60* nucleotide sequences, with the exception of strain *L. casei* subsp. *casei* ATCC 4913, which displayed about 4% nucleotide divergence from members of the same cluster (cluster 19). These results are in agreement with those obtained by other studies (4, 9, 12, 13) that considered *L. casei* and *L. paracasei* members of the same species.

The topology of the diversity tree from *hsp60* nucleotide sequences showed a *Lactobacillus* species distribution similar, but not identical, to that based on the 16S rRNA gene sequence analysis reported by Dellaglio and Felis (10). In Fig. 1, the respective 16S rRNA affiliation group according to Dellaglio and Felis (10) is reported for each species. Our results slightly change the picture presented by Dellaglio and Felis (10) regarding phylogenetic groups. In particular, *L. homohiochii* (16S rRNA *L. buchneri* group) showed high levels of similarity to strains of *L. zeae* (16S rRNA *L. casei* group), *L. kefiranofaciens* (16S rRNA *L. delbrueckii* group) showed high levels of similarity to strains of *L. mali* (16S rRNA *L. salivarius*

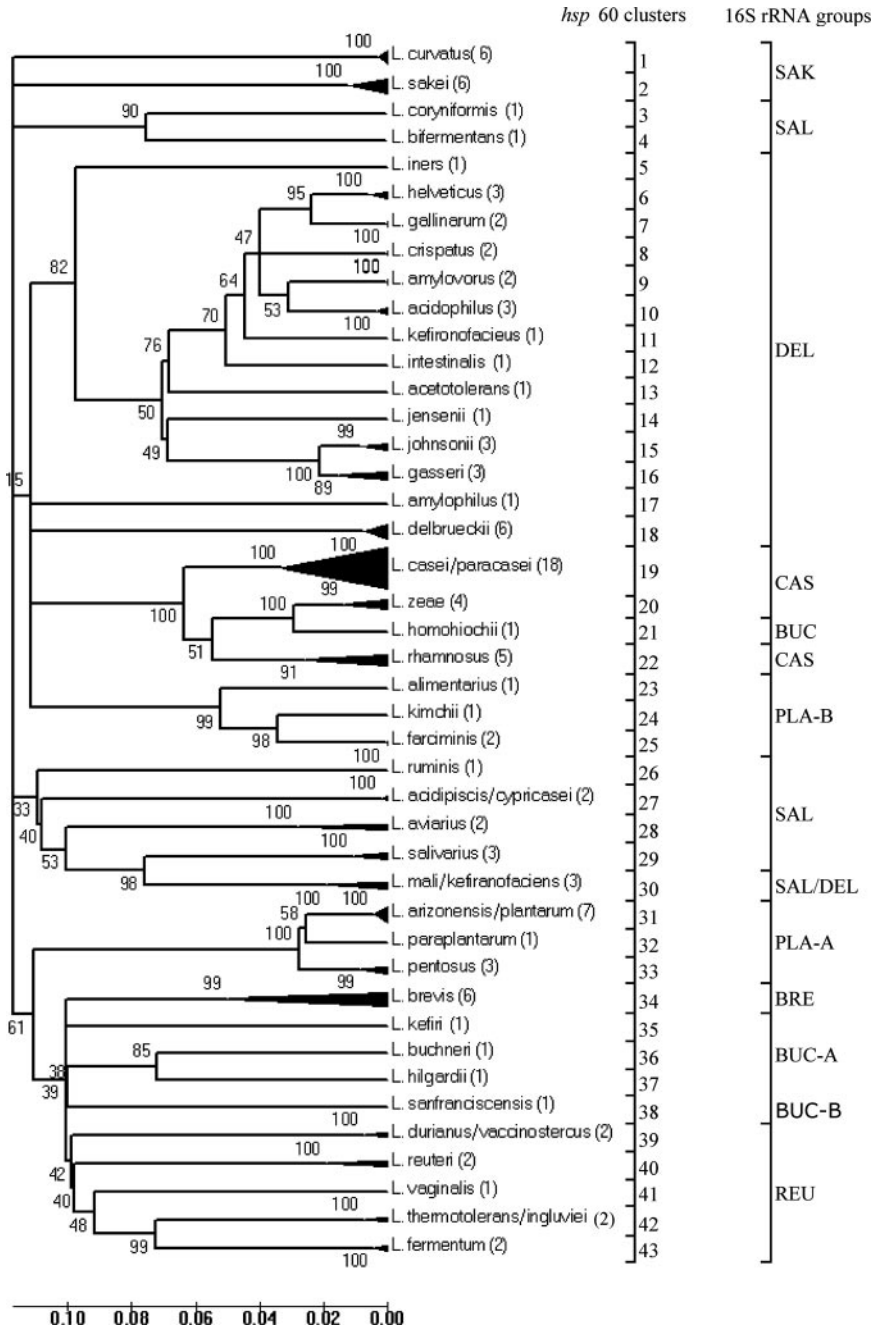

FIG. 1. Neighbor-joining tree based on comparison of 499-bp *hsp60* gene sequences showing the phylogenetic relationships between *Lactobacillus* species. Bootstrap values (expressed as percentages of 1,000 replications) greater than 50% are given at the nodes. The scale bar estimates the number of substitutions per site. The number of *hsp60* genes analyzed is reported in parentheses. Strains of each *hsp60* cluster are listed in Table S1 in the supplemental material. 16S rRNA groups are given according to Felis and Dellaglio (15): BRE, *L. brevis* group; BUC, *L. buchneri* group; CAS, *L. casei* group; DEL, *L. delbrueckii* group; PLA, *L. plantarum* group; REU, *L. reuteri* group; SAL, *L. salivarius* group; SAK, *L. sakei* group.

group), and strains of species of the 16S rRNA *L. salivarius* group were divided into two groups according to *hsp60* analysis (clusters 3 and 4 and clusters 26 to 30). Moreover, according to our results strains of the 16S rRNA *L. plantarum* group were divided into two groups (clusters 23 to 25 and clusters 31 to 33). These two groups correspond to 16S rRNA *L. plantarum* groups A (including *L. plantarum*, *L. pentosus*, and *L. paraplantarum*) and B (comprising *L. alimentarius*, *L. farciminis*, and *L. kimchii*) designed by Dellaglio and Felis (10). However, our results suggested that these two subgroups are very distant.

Computer-aided (in silico) restriction endonuclease analysis of all *Lactobacillus hsp60* partial sequences was also performed by MacDNasis Pro (v3.0.7.) software so as to evaluate their restriction polymorphisms. The endonucleases AluI, BamHI,

TABLE 3. Positions of AluI and TacI restriction sites in the 499-bp internal *hsp60* fragments of the different *Lactobacillus* species

Species	No. of sequences analyzed	Position(s) of indicated restriction site	
		AluI	TacI
L. mali	1	72	100
L. ruminis	1	72	
L. sanfranciscensis	1	72	56
L. jensenii	1	138	
L. kefiri	1	153	224, 376
L. fermentum	2	274	
L. salivarius	3	330	242
L. casei/*L. paracasei*	18	330	
L. rhamnosus	5	330	56, 495
L. zeae	4	330	56, 224, 242
L. kefiranofaciens	1	465	278
L. farciminis	1	138, 153, 330	56, 242, 338
L. kimchii	1	138, 153, 330, 465	56, 338
L. intestinalis	1	138, 153, 465	
L. acidophilus	3	138, 465	278
L. helveticus	3	138, 465	
L. vaccinostercus/*L. durianus*	2	150, 330	56, 124, 224, 338
L. coryniformis	1	153, 330	56, 242, 278
L. paraplantarum	1	173, 330	278, 338, 376
L. hilgardii	1	182, 279	85, 253
L. aviarius	1	21, 150	56, 142
L. acidipiscis/*L. cypricasei*	2	21, 63, 84, 186, 330	56, 242, 455
L. alimentarius	1	21, 72, 138, 330	80, 278
L. buchneri	1	250, 465	
L. brevis	5	250, 465	278
L. aviarius subsp. *aviarius*	1	27, 150, 330	56, 278
L. iners	1	27, 63, 120, 138, 465	242
L. ingluviei/*L. thermotolerans*	2	274, 330	56, 142
L. delbrueckii	6	330, 465	
L. acetotolerans	1	63, 138, 330, 465	
L. gallinarum	2	63, 153, 465	56
L. pentosus	3	63, 250, 465	278
L. plantarum/*L. arizonensis*	5	63, 250, 465	278
L. plantarum (type strain)	1	63, 465	278
L. crispatus	2	63, 72, 138, 465	
L. amylovorus	2	72, 138, 465	278
L. mali (*yamanashiensis*)	1	72, 153, 330	
L. bifermentans	1	72, 330	56, 242, 278
L. kefiranofaciens subsp. *kef.*	1	72, 330	
L. curvatus	6	75, 153, 330	110, 278
L. sakei	6	75, 153, 330	
L. johnsonii	3	75, 153, 430, 465	224, 278
L. gasseri	3	75, 153, 430, 465	278
L. homohiochii	1	75, 330	224, 242
L. reuteri	2	84, 274	
L. vaginalis	1	84, 274, 330, 465	278
L. amylophilus	1		224, 242, 278

CfoI, HaeIII, HincII, HindIII, MseI, Sau3AI, and TacI were considered. Since in 115 of the 116 *Lactobacillus hsp60* partial sequences analyzed, at least one AluI restriction site exists, this enzyme was initially selected (Table 3). In theory, on analysis of the AluI PCR-RFLP in silico patterns of the 499-bp *hsp60* LB308F-LB806RM PCR fragment, fewer than half of the species considered were differentiated. However, on coupling of the TacI PCR-RFLP in silico patterns with the AluI RFLP in silico pattern, all species were differentiated, with the exception of the *L. plantarum*/*L. pentosus* pair (Table 3). Finally, the latter species were differentiated by digesting the 499-bp *hsp60* LB308F-LB806RM PCR fragment with MseI or Sau3AI restriction enzymes (Table 4).

Identification of wild isolates by *hsp60* PCR-RFLP analysis. Two groups of wild isolates, from Provolone del Monaco cheese manufacturing and from dry-fermented sausages, were identified by analyzing their AluI and TacI *hsp60* PCR-RFLPs (Tables 1 and 2).

Among strains isolated during manufacturing of Provolone del Monaco cheese, three different AluI *hsp60* PCR-RFLPs were obtained: those showing fragments of 330 and 169 bp (pattern A); those displaying fragments of 215, 187, 63, and 34 bp (pattern B); and those showing fragments of 327, 138, and 34 bp (pattern C). Seventy-six strains showed pattern A, 2 strains pattern B, and 2 pattern C (Table 1). By analysis of the TacI *hsp60* PCR-RFLPs of the same strains, three different patterns were also obtained (Table 1): those showing only one fragment, of 499 bp (pattern a; undigested PCR product); those displaying fragments of 439, 56, and 4 bp (pattern b); and those showing fragments of 278 and 221 bp (pattern c). Considering the molecular weights of the fragments of both RFLPs and considering the results reported in Table 3, strains should be identified as follows (Table 1): *L. casei*, combined pattern A-a; *L. rhamnosus*, A-b; *L. plantarum*/*L. pentosus*, B-c; and *L. helveticus*, C-a. The two strains showing combined pattern B-c and displaying a Sau3AI *hsp60* PCR-RFLP containing fragments of 334, 104, and 61 bp were identified as *L. plantarum*. Further confirmation of *hsp60* PCR-RFLP-based identification was obtained, for some strains (Table 1), by performing *hsp60* partial sequence analysis and species-specific PCR assays (18, 55) (data not shown). Finally, from the identification reported in Table 1, it is evident that the *Lactobacillus* population occurring during the fermentation and ripening processes of the dairy product analyzed is mainly represented by strains of *L. casei* and *L. rhamnosus*. Moreover, the isolation medium also has an evident influence on the detection and enumeration of different species: all but one strain isolated on Rogosa agar were referable to *L. rhamnosus*, while strains isolated on Isolini agar medium were identified as *L. casei*, *L. rhamnosus*, *L. plantarum*, and *L. helveticus* species.

TABLE 4. Positions of the MseI and Sau3AI restriction sites in the 499-bp internal *hsp60* fragment of the *L. pentosus* and *L. plantarum*/*arizonensis* species

Species	No. of sequences analyzed	Position(s) of indicated restriction site	
		MseI	Sau3AI
L. pentosus	3	110, 175, 355	438
L. plantarum/*L. arizonensis*	6	110, 355	334, 438

A single AluI *hsp60* PCR-RFLP was obtained from 30 strains isolated from ready-to-eat samples of dry-fermented sausages produced in Vallo di Diano. On the basis of data reported in Table 3, this pattern, showing fragments of 75, 78, 170, and 177 bp, was related to *L. curvatus*/*L. sakei* strains. Moreover, these strains, after TacI *hsp60* PCR-RFLP analysis, were differentiated as follows: pattern a, *L. sakei*; pattern d, *L. curvatus*. Furthermore, also in this case, identification was confirmed by applying species-specific molecular assays (3) (data not shown).

DISCUSSION

Unambiguous identification of lactobacilli is a prerequisite for risk assessment (2). Identification of microbial species by use of phenotypic methods may sometimes be uncertain, complicated, and time-consuming. The use of molecular methods has revolutionized identification, improving its quality and ef-

ficacy (9). The techniques used for polyphasic analysis of lactobacilli have been reviewed elsewhere (5, 8). New, simple, and rapid procedures using genus- and species-specific primers for accurate identification of lactobacilli have been proposed. These molecular methods target mainly the rRNA genes or their spacer regions (5, 8). These approaches are particularly useful for rapid and reliable detection, identification, and/or monitoring of one or few *Lactobacillus* species in a particular environment. However, in the case of environments potentially hosting large numbers of *Lactobacillus* species, in order to choose the number of species-specific primers/probes to use, the preventive knowledge of the *Lactobacillus* species potentially occurring is needed. In these cases, more-general molecular approaches should be applied. In fact, many researchers compared partial or full 16S rRNA sequences, 16S rRNA gene PCR-RFLP patterns (43, 51, 59), or intergenic 16S-23S rRNA spacer region PCR-RFLP patterns (37) for the identification of *Lactobacillus* strains isolated from different environments. However, the 16S rRNA gene shows discrimination pitfalls in the identification of closely related species (10). Other target genes, present in only a single copy, such as the elongation factor Tu gene (*tuf*) (4, 58), the DNA repair recombinase gene (*recA*) (10), the RNA polymerase B subunit gene (*rpoB*) (42), and the chaperonin HSP60 gene (*cpn60*) (13), have been exploited for the differentiation of *Lactobacillus* species.

Recently, sequence analysis of *hsp60* (encoding a 60-kDa heat shock protein) genes allowed differentiation of species and/or subspecies in different taxa, such as *Staphylococcus*, *Macrococcus*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Vagococcus*, *Bifidobacterium*, *Campylobacter*, *Yersinia*, *Vibrio*, *Salmonella*, and *Shigella* species and *Escherichia coli* (14, 22, 23, 33, 34, 52, 62, 63). These studies highlight the significant advantage of this gene over 16S rRNA due to its higher level of species discrimination. In 2004, a new database based on chaperonin sequences from bacterial and eukaryotic species was established (http://cpndb.cbr.nrc.ca/) (26).

Hill et al. (27) compared ileum microbiota of pigs fed corn-, wheat-, or barley-based diets by chaperonin 60 sequencing and quantitative PCR. More recently, Dumonceaux et al. (15) described PCR primers specifically amplifying the *cpn60* genes of six individual species: *Clostridium perfringens*, *Enterococcus faecalis*, *Enterococcus cecorum*, *L. amylovorus*, *L. johnsonii*, and *Escherichia coli*. These data confirm the usefulness of conserved genes for comparing and differentiating related species. *hsp60* (*groEL*) sequence databases cover a considerable number of sequences from *Lactobacillus* species, and for this reason, these genes can be exploited as targets for detection and identification of organisms, providing a valuable resource in microbial ecology studies as well as in phylogenetic analyses. However, the real intra- and interspecific polymorphism of the *hsp60* gene in the *Lactobacillus* genus has not yet been evaluated. In this study, we designed a set of PCR primers for direct sequencing of the amplified *hsp60* gene sequences of *Lactobacillus* spp. The method described overcomes limitations of the existing PCR-based method applied by Hill et al. (27), which requires time-consuming and complex postamplification steps, such as the cloning of amplification products. A similar strategy was recently applied by Hill and coworkers (28) for direct sequencing of *Campylobacter* species. The results obtained by analyzing the *Lactobacillus hsp60* gene sequences demonstrate the potential utility of this tool for identifying *Lactobacillus* isolates. Moreover, a robust and rapid approach was applied for the identification and differentiation of *Lactobacillus* species. The protocol is based on restriction endonuclease analysis of the PCR-amplified *hsp60* gene sequences (*hsp60* PCR-RFLP). A sequential use of three endonucleases is proposed to simplify *Lactobacillus* strain identification: first, AluI; then, TaqI; and finally, if necessary, Sau3AI (or MseI). Our strategy is similar to the 16S rRNA PCR-RFLP and 16S-23S rRNA ITS PCR-RFLP methods. However, the latter methods require the use of more than three endonucleases (59) and are often unable to differentiate among closely related species, such as *L. casei* and *L. rhamnosus*; *L. acidophilus* and *L. crispatus* (51); *L. acidophilus*, *L. helveticus*, and *L. amylovorus* (37); and *L. plantarum* and *L. pentosus* (43). Moreover, it must be remembered that identification based on 16S rRNA genes may be misleading if closely related species are analyzed (10). 16S rRNA gene sequence analysis is not able to reveal significant differences between recently diverged species, such as *L. plantarum*, *L. paraplantarum*, and *L. pentosus* or *L. casei*, *L. rhamnosus*, and *L. zeae* (10). The *hsp60* PCR-RFLP technique is able to differentiate 45 *Lactobacillus* species. The amenability of this approach has been validated by the unambiguous identification of 110 wild *Lactobacillus* strains belonging to *L. casei*, *L. rhamnosus*, *L. plantarum*, *L. pentosus*, *L. helveticus*, *L. curvatus*, and *L. sakei*.

In conclusion, the high variability of the *hsp60* nucleotide sequences allows the discrimination of very closely related species, opens new possibilities for a rapid and reliable identification of lactobacilli, and offers good opportunities for development of assays based on hybridization (probe design), PCR (primer design), or DNA chip technologies (microarray design). The results herein obtained clearly highlight the higher usefulness of *hsp60* gene analysis than 16S rRNA or 16S-23S rRNA ITS analysis. In fact, *hsp60* sequence analysis has confirmed most of the recent systematic studies concerning the taxonomy of *Lactobacillus* species (10, 11, 17, 31, 39). Therefore, it can be utilized as a reliable marker for identification of either distant or very closely related taxa of the *Lactobacillus* genus. Moreover, in several species of bacteria, nucleotide sequence analysis of multiple protein-encoding loci has led to reliable phylogenies that have improved our understanding of population structure. A multigenic approach fulfills the recent recommendations of the ad hoc committee for the reevaluation of the definition of the bacterial species. The *hsp60* gene can be considered an excellent molecular marker for inferring the taxonomy and phylogeny of members of the genus *Lactobacillus* and may represent a good candidate in multilocus schemes designed for the identification and characterization of lactobacilli. Owing to its specificity, manageability, and rapidity, the *hsp60*-based PCR-RFLP approach proposed in this study can be considered a valid strategy for typing at the species level *Lactobacillus* strains isolated from food samples.

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