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Development of Genus/Species-Specific PCR Analysis for Identification of *Carnobacterium* Strains

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Abstract. Heterofermentative lactic acid bacteria belonging to the genus *Carnobacterium* are currently divided into seven different species, *C. piscicola*, *C. mobile*, *C. gallinarum*, *C. inhibens*, *C. divergens*, *C. funditum*, and *C. alterfunditum*. 16S rDNA-targeted PCR assay was carried out for the identification of the genus *Carnobacterium*. In addition, type strains of all *Carnobacterium* species were analyzed by 16S–23S rDNA intergenic spacer analysis in comparison with type strains of phylogenetically related lactic acid bacteria. These methods enabled the identification and the discrimination among *Carnobacterium* species and the other phylogenetically related lactic acid bacteria. Likewise, analogous results were obtained by restriction analysis of amplified 16S rDNA performed with *Hae*III and *Hin*fI as restriction enzymes.

The genus *Carnobacterium* proposed by Collins et al. in 1987 [2] includes heterofermentative lactic acid rods, isolated from a wide variety of foods, such as poultry, meat, cheese, and fish [10, 11, 16, 21], and from anoxic waters [8]. On the basis of their phenotypic traits, *Carnobacterium* species can be differentiated from other lactic acid bacteria by their ability to produce only the L (+) lactic acid enanthiomeric form, by the presence of meso-diaminopimelic acid in cell wall, and by their ability to growth in alkaline, but not in acid, environment [9]. However, phenotypical characteristics do not allow an easy identification at species level within the genus *Carnobacterium*. Specifically, the two species most frequently isolated in food products, *Carnobacterium divergens* and *Carnobacterium piscicola* [7], can hardly be differentiated on the basis of their phenotypic traits. In this context, Montel et al. [13] proposed an identification key based on physiological and biochemical tests to differentiate *Carnobacterium divergens* from *Carnobacterium piscicola*, but without the comparative inclusion of the other *Carnobacterium* species in the study. Because of the difficulties encountered with methods based on phenotypical characters, many authors proposed the use of molecular methods [1, 12, 16, 22]. Despite the importance of *Carnobacterium* strains as food-related lactic acid bacteria, the seven species constituting this genus could be differentiated by time-consuming or by poorly reproducible techniques such as DNA/DNA relatedness and RAPD analysis, respectively [7, 12]. Moreover, up to now, the seven currently described *Carnobacterium* species have never been involved in a comparative genetic characterization. The aim of this study was to develop rapid and reliable tools for the discrimination and identification of all *Carnobacterium* species. For this reason, we have focused our attention on the ribosomal operon, whose importance as a target genomic locus in the development of genus/species or strain-specific PCR-based techniques has been well documented in several studies [4, 5, 14, 15, 20]. Within the ribosomal operon, the internal transcribed spacer region (ITS) located between the 16S and the 23S genes also provides an excellent tool for fine identification at species/strain level [6, 17], while ribosomal genes (16S rDNA, 23S rDNA) may lose resolution at the species or lower taxa level. In this context, the important group of food-related lactic acid bacteria represented by *Carnobacterium* species was studied by analyzing both the

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ITS region and the 16S rDNA, with the aim to develop PCR-based genus- and species-specific protocols.

Materials and Methods

Bacterial strains and media. Cultures of *Carnobacterium piscicola* DSM 20722^T, *C. divergens* DSM 20623^T, *C. gallinarum* DSM 4847^T, *C. mobile* DSM 4848^T, *C. funditum* DSM 5971^T, *C. alterfunditum* DSM 5973^T, *C. inhibens* CCUG 31728^T, *Vagococcus salmonarium* DSM 6633^T, *V. fluvialis* DSM 5731^T, *Lactobacillus maltaromicus* DSM 20342^T, *Enterococcus hirae* DSM 20160^T, *E. faecium* NCDO 1229^T, *E. faecalis* NCDO 588^T, *S. thermophilus* DSM 20617^T, and *Desemzia incerta* DSM 20581^T were routinely maintained at 4°C after growth at 25–37°C for 12 or 24 h in the appropriate medium [M17 (Difco), Trypticase soy yeast extract medium: Tripticase soy broth 30 g L^{-1}, yeast extract 3 g L^{-1}, pH 7.0±0.2]. For longer-term maintenance, stock cultures were stored in 20% (vol/vol) glycerol, 80% (vol/vol) of appropriate liquid medium at −20°C and −80°C.

DNA extraction and PCR protocols. Total DNA extraction was performed on 100 μl of overnight broth culture as previously described [15]. DNA solutions were stored at −20°C. All PCR reactions were performed in a volume of 50 μl containing: 2 μl of bacterial genomic DNA solution, 5 μl of 10 × PCR reaction buffer, 200 μM of each dNTP, 2 mM of $MgCl_2$, 0.5 μM of each primer, and 0.5 U of Taq polymerase (Amersham-Pharmacia). Amplification of the 16S rDNA was carried out with the primer set 16SF–16SR (16SF, 5′-AGAGTTTGATCCTGGCTCAG-3′; 16SR, 5′-CTACGGCTACCTTGTTACGA-3′) and the following temperature profile: primary DNA denaturation step at 94°C for 2 min, followed by 5 cycles of 45 s at 94°C, 45 s at 50°C, and 1 min at 72°C; 30 additional cycles were carried out increasing the annealing temperature to 55°C. A DNA 16S–23S intergenic spacer (ITS) region amplification was performed with the primer set ITSF-ITSR (ITSF 5′-GTCGTAACAAGGTAGCCGTA-3′; ITSR 5′-CAAGGCATCCACCGT-3′) and the following temperature profile: 5 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and 30 cycles consisting of 92°C for 45 s, 60°C for 45 s, 72°C for 2 min. Primer set Cb1-Cb2 was used for the genus-specific amplification of a 340-bp 16S rDNA fragment as described by Yost and Nattress [22]. A selection of five primers—Carno1R (5′-AGCCACCTTTCCTTCAAG-3′), Carno2R (5′-AGCCACCTTTCATCCGTTC-3′), Carno3R (5′-AGCGGTAGCCGAAGCCAC-3′), Carno4R (5′-AACCGTCTTTTATCCATCC-3′), and DesemR (5′-AACCGTCTTTCTTCTTTCTG-3′)— was used with the forward primer 16SF for the selective amplification of a 16S rDNA fragment of about 230 bp from species belonging to the genus *Carnobacterium* and *Desemzia* with the following temperature profile: primary DNA denaturation step at 94°C for 2 min followed by 35 cycles of 45 s at 94°C, 45 at 55°C, and 40 at 72°C. For all amplification cycles the final extension was continued for 7 min at 72°C. All amplification reactions were performed in a Gene Amp PCR System 2400 (Perkin-Elmer). Following amplification, 5 μl of product was electrophoresed at 5 V cm^{-1} (1.5% agarose gel, 0.2 μg of ethidium bromide ml^{-1}) in TAE buffer.

Dot blot hybridization. Approximately 200 ng of amplified 16S rDNA of each lactic acid bacteria strains used in this work was spotted to a nylon membrane (Boehringer) according to the manufacturer's instructions. The DIG DNA Labeling and Dectection Kit (Boehringer) was used for digoxigenin labeling of 16S rDNA amplified from *Carnobacterium mobile*. Hybridization, with the same kit, was also performed according to the manufacturer's recommendations with prehybridization and hybridization steps in 50% (wt/vol) formamide at 37–42°C and stringency washes in 0.1 × SSC, at 40–50°C.

16S rDNA sequence analysis. After amplification of the 16S rDNA from DNA of the strains *C. mobile* DSM 4848^T, PCR product was purified (NucleoSpin Extract, Machery-Nagel), and the first 500 bp were sequenced with the 16SF as primer in a model 310 automatic DNA sequencer (Applied Biosystems, Foster City, CA) with fluorescent dideoxy chain terminators. The 16S rDNA sequences were manually aligned with the published sequence of the 16S rDNA of *Carnobacterium* and other phylogenetically related lactic acid bacteria. The 16S rDNA sequences accession numbers are the following: *Carnobacterium piscicola* (AF184247), *C. gallinarum* (X54269), *C. divergens* (X54270), *C. alterfunditum* (L08623), *C. funditum* (S86170), *C. inhibens* (Z73313), *C. mobile* (AJ317963), *Desemzia incerta* (Y17300), *Enterococcus faecium* (AJ3011830), *E. faecalis* (AJ271855), *E. hirae* (Y17302), *Vagococcus salmonarium* (X54272), *V. fluvialis* (X54258).

Restriction analysis. Restriction digestion of the amplified fragments was carried out for 5 h at 37°C in 20 μl reaction mixture containing 300–400 ng of the PCR product solution, an appropriate dilution of incubation buffer, and 10 U of one of the following restriction enzymes: *Hae*III, *Hin*fI (Amersham Pharmacia Biotech). Restriction digestions were subsequently analyzed by agarose electrophoresis (3% wt/vol agarose gel, containing 0.2 μg of ethidium bromide ml^{-1}, TAE buffer). The gels were run at 5 V cm^{-1} in an appropriate buffer, stained in a solution containing 0.5 μg of ethidium bromide ml^{-1}, and photographed in UV light.

Computation of strain similarities. A computer similarity analysis, carried out on 16S rDNA restriction patterns, was estimated by means of the Jaccard coefficient [19], and clustering of strains was based on unweighted pair group method with arithmetic average (UPGMA). The NTSYS–PC computer program (version 1.30) [18] was used in the data analysis.

Results and Discussion

Molecular identification of the genus *Carnobacterium*. The 16S rDNA amplified fragments from all *Carnobacterium* and related lactic acid bacteria species were used as target DNA in dot blot hybridization experiments with the genus-specific probe CB1 (5′ digoxigenin labeled) described by Nissen et al. [16]. Hybridization assays were performed with the aim to verify the identification at a genus level of the seven *Carnobacterium* species. The results obtained (Table 1) showed positive hybridization signals in six of the seven *Carnobacterium* species analyzed. Specifically, the probe CB1 failed to hybridize with the amplified 16S rDNA of *Carnobacterium mobile* DSM 4848^T also when low-stringency conditions were applied. No hybridization signals were detected for *Enterococcus faecalis*, *E. faecium*, *E. hirae*, *Desemzia incerta*, *Vagococcus fluvialis*, *V. salmoniarum*, and *Streptococcus thermophilus*. In addition, positive hybridization signal was detected for *Lactobacillus maltaromicus*, confirming the high phylogenetic relationship between this species and *Carnobacterium piscicola* as reported by Collins and co-workers [3]. A hybridization experiment performed on the same membrane but with the 16S rDNA of *Carnobacterium mobile* DSM 4848^T as probe was also carried out as a positive hybridization

Table 1. Dot blot hybridization and PCR results obtained for *Carnobacterium* and related lactic acid bacteria

Strain		Probes		PCR primer set		
		16S rDNA	CB1	Cb1-Cb2r	16SF-Carn1-4R[a]	16S-DeseR
Carnobacterium piscicola	DSM[b]20722^T	+	+	+	+	−
Carnobacterium divergens	DSM20623^T	+	+	+	+	−
Carnobacterium gallinarum	DSM 4847^T	+	+	+	+	−
Carnobacterium mobile	DSM 4848^T	+	−	+	+	−
Carnobacterium funditum	DSM 5971^T	+	+	+	+	−
Carnobacterium alterfunditum	DSM 5973^T	+	+	+	+	−
Carnobacterium inhibens	CCUG[c]31728^T	+	+	+	+	−
Vagococcus salmonarium	DSM 6633^T	+	−	−	−	−
Vagococcus fluvialis	DSM 5731^T	+	−	−	−	−
Lactobacillus maltaromicus	DSM20342^T	+	+	+	+	−
Enterococcus hirae	DSM20160^T	+	−	W+[e]	−	−
Enterococcus faecium	NCDO[d] 1229^T	+	−	−	−	−
Enterococcus faecalis	NCDO 588^T	+	−	W+	−	−
Streptococcus thermophilus	DSM20617^T	+	−	−	−	−
Desemzia incerta	DSM20581^T	+	−	+	−	+

[a] The PCR was carried out with the 16SF as forward primer and Carn1R, Carn2R, Carn3R, and Carn4R as reverse primers.
[b] Deutsche Sammlung Von Mikroorganismen und Zellkulturen, Germany.
[c] National Collection of Dairy Organisms, UK.
[d] Culture Collection of Göteborg, Sweden.
[e] W: weakly positive amplification signal.

control. The results showed that all spotted 16S rDNA gave positive signals (Table 1).

The strain *C. mobile* DSM 4848^T, which failed to hybridize with the genus-specific probe CB1, was analyzed by partial sequencing of 16S rDNA. Sequence analysis confirmed that the strain belongs to the species *C. mobile*, but revealed a single mismatch in the target region of the probe CB1 that could justify the absence of a hybridization signal in *C. mobile* when low-stringency conditions were used. In this context, it should be noted that two mismatches did not allow the hybridization between the probe CB1 and the target region in the 16S rDNA of *Enterococcus* and *Desemzia* species when the same stringency conditions were applied. The discordance between these results and those obtained by Nissen and co-workers [16] regarding the species *C. mobile* could be justified by the presence of intra-species ribosomal gene sequence diversity, which has been detected often in other lactic acid bacteria [15]. In conclusion, our data suggest that the probe CB1 was not suitable for the identification of the genus *Carnobacterium*. Likewise, the use of the primer set Cb1-Cb2r in a genus-specific PCR assay, performed as described by Yost and Nattress [22], did not allow the identification of the genus *Carnobacterium*, because amplification products of the expected dimension were also obtained for *Enterococcus* species and *Desemzia incerta* (Table 1, and Fig. 1A). This result was expected because *Carnobacterium* species, *Lactobacillus maltaromicus*, and *Desemzia incerta* showed the same sequence in the target region of primers Cb1, Cb2r, and a high level of homology was also present between the primers' sequences and the target region of *Enterococcus* species. New sets of primers targeted to the variable region II of the 16S rDNA were designed to obtain the specific identification of the genus *Carnobacterium*. The sequences of the new sets of primers were preliminarily checked against other sequences deposited in the EMBL database by using the Wu-blastn program with the aim to verify whether significant matches were present with 16S rDNA sequences of other lactic acid bacteria. Four reverse primers designated Carn1r, Carn2r, Carn3r, and Carn4r were used together with the forward universal primer 16SF to obtain amplification fragments of about 230 bp in the seven *Carnobacterium* species. As expected, experimental data revealed that the primer Carn1r was also suitable for the amplification of the species *C. divergens* despite the fact that one mismatch and one deletion were present between primer and target sequence. The results obtained (Fig. 1B) showed the expected amplification fragments only in *Carnobacterium* strains and in *Lactobacillus maltaromicus*. In this context the high 16S rDNA sequence similarity between *Lactobacillus maltaromicus* and *Carnobacterium piscicola* reported by Collins et al. [3] suggested that these taxa should constitute the same species and that they certainly should belong to the same genus. In addition, the use of the primer set 16SF-Deser allowed the amplification of about 230-bp product only

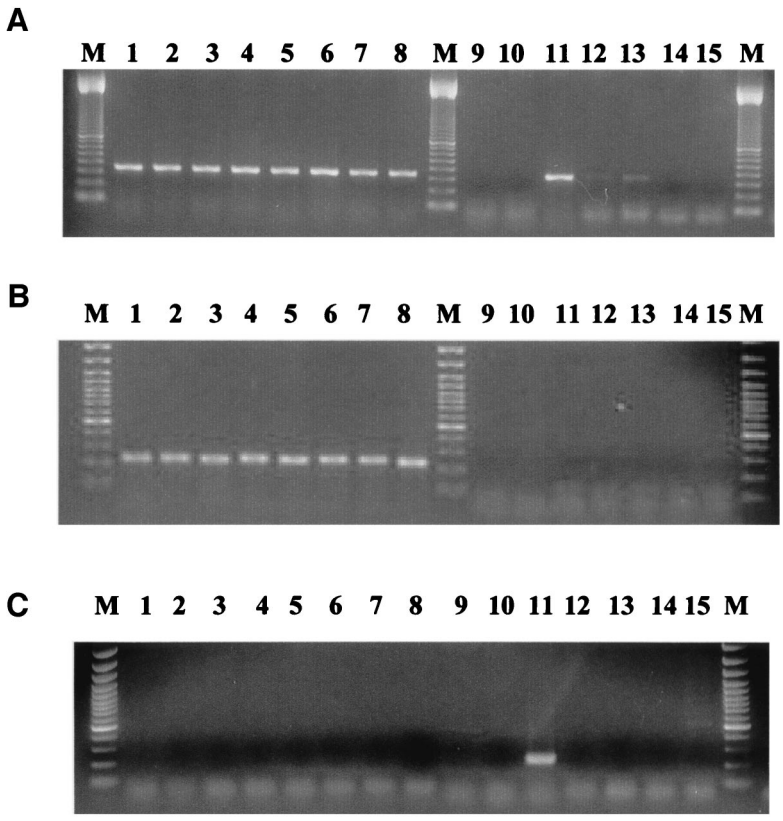

Fig. 1. (**A**) Agarose gel electrophoresis showing the genus-specific amplification of the 340-bp fragment for *Carnobacterium* and related species obtained with the primer set Cb1-Cb2r [22]. (**B**) Agarose gel electrophoresis showing the genus-specific amplification of the 230-bp fragment for *Carnobacterium* and related species obtained with the primer set 16SF (Carn1R, Carn2R, Carn3r, Carn4r). (**C**) Agarose gel electrophoresis showing the species-specific amplification of the 280-bp fragment for *Desemzia incerta* and related lactic acid bacteria. Lane 1, *Lactobacillus maltaromicus* DSM 20342^T; lane 2, *Carnobacterium piscicola* DSM 20722^T; lane 3, *Carnobacterium mobile* DSM 4848^T; lane 4, *Carnobacterium funditum* DSM 5971^T; lane 5, *Carnobacterium divergens* DSM 20623^T; lane 6, *Carnobacterium alterfunditum* DSM 5973^T; lane 7, *Carnobacterium inhibens* CCUG 31728^T; lane 8, *Carnobacterium gallinarum* DSM 4847^T; lane 9, *Vagococcus fluvialis* DSM 5731^T; lane 10, *Vagococcus salmoniarum* DSM 6633^T; lane 11, *Desemzia incerta* DSM 20581^T; lane 12, *Enterococcus hirae* DSM 20160^T; lane 13, *Enterococcus faecalis* NCDO 588^T; lane 14, *Enterococcus faecium* NCDO 1229^T; lane 15, *Streptococcus thermophilus* DSM 20617^T. M-molecular weight marker 1-kb ladder (MBI).

when DNA from *Desemzia incerta* was used (Fig. 1C, Table 1).

Restriction analysis of amplified 16S rDNA and computation of strain similarities. The amplified 16S rDNA analysis of *Carnobacterium* species and related lactic acid bacteria was subjected to restriction analysis with *Hae*III and *Hin*fI as restriction enzymes. The enzymes were chosen on the basis of theoretical restriction analysis of the deposited 16S rDNA sequences of *Carnobacterium* species. The dimensions of the restriction fragments obtain with both enzymes were in accordance with the theoretical restriction of the published 16S rDNA sequences. *Hae*III restriction analysis (data not shown) generated four different restriction profiles among the seven *Carnobacterium* species and allowed their discrimination from all the other related lactic acid bacteria. A species-specific restriction profile was obtained only for *C. alterfunditum*, while it was not possible to discriminate among *C. divergens*, *C. inhibens*, and *C. gallinarum*, between *C. mobile* and *C. funditum* and between *C. piscicola* and *L. maltaromicus*. However, *Hinf*I restriction analysis allowed the discrimination between *C. inhibens* and *C. gallinarum*, between *L. maltaromicus* and *C. piscicola*, and between *C. mobile* and *C. funditum*. As a consequence, the double restriction *Hae*III/*Hin*fI (Fig. 2A) generated species-specific restriction profiles that allowed the identifcation of each *Carnobacterium* species. Furthermore, few differences were also detected between the restriction profiles of *Lactobacillus maltaromicus* and *Carnobacterium piscicola*.

The graphic representation, derived from the computation of strain similarity of *Hae*III/*Hin*fI 16S rDNA restriction analysis (Fig. 2B), shows a clear clustering of all *Carnobacterium* species in a main branch separated at a similarity level of 0.23% from the other phylogenetically closely related lactic acid bacteria. In addition, *Carnobacterium* species are separated from each other at a similarity level ranging from 0.33% to 0.75%, underlining the species-specific differentiation power of *Hae*III/*Hinf*I 16S rDNA restriction analysis.

ITS analysis. Amplification of the internal transcribed spacer (ITS) between the 16S and the 23S rDNA was carried out for all *Carnobacterium* species and related lactic acid bacteria. The results obtain are shown in Fig 3. On the basis of the amplification fragments obtained, *Carnobacterium* strains were clustered in seven different ITS profiles, and they were clearly differentiated from

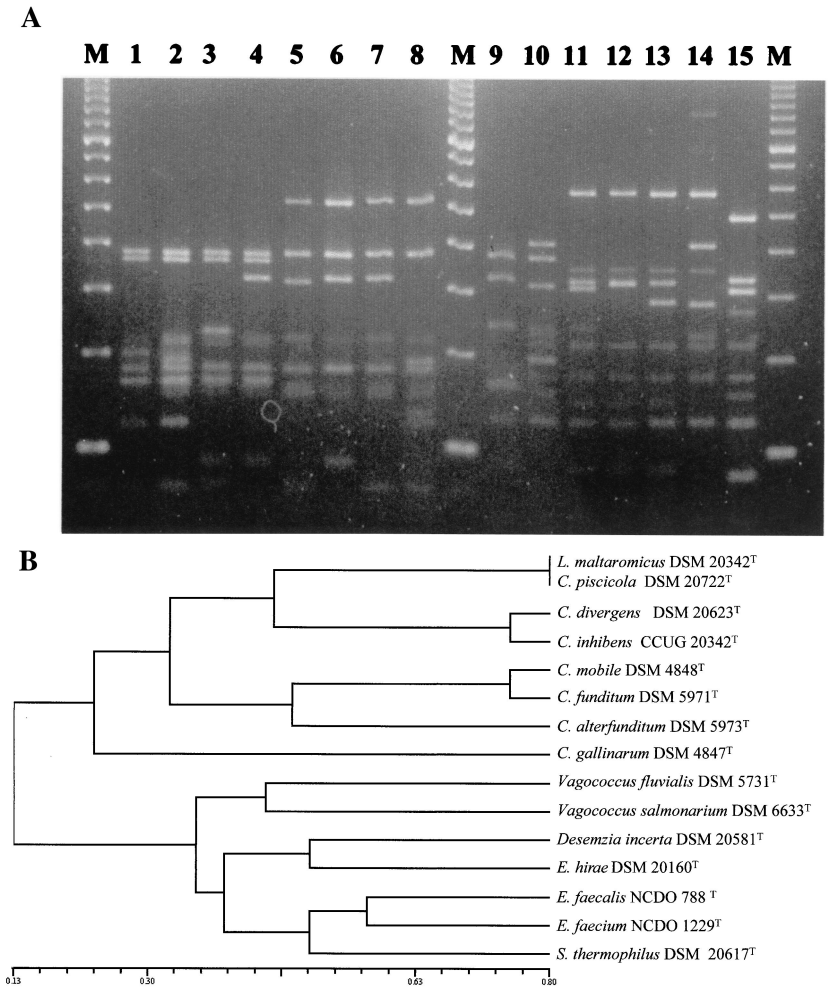

Fig. 2. (**A**) Agarose gel electrophoresis showing the *Hae*III/*Hin*fI 16S rDNA restriction analysis of *Carnobacterium* and related lactic acid bacteria. Lane 1, *Lactobacillus maltaromicus* DSM 20342^T; lane 2, *Carnobacterium piscicola* DSM 20722^T; lane 3, *Carnobacterium mobile* DSM 4848^T; lane 4, *Carnobacterium funditum* DSM 5971^T; lane 5, *Carnobacterium divergens* DSM 20623^T; lane 6, *Carnobacterium alterfunditum* DSM 5973^T; lane 7, *Carnobacterium inhibens* CCUG 31728^T; lane 8, *Carnobacterium gallinarum* DSM 4847^T; lane 9, *Vagococcus fluvialis* DSM 5731^T; lane 10, *Vagococcus salmoniarum* DSM 6633^T; lane 11, *Desemzia incerta* DSM 20581^T; lane 12, *Enterococcus hirae* DSM 20160^T; lane 13, *Enterococcus faecalis* NCDO 588^T; lane 14, *Enterococcus faecium* NCDO 1229^T; lane 15, *Streptococcus thermophilus* DSM 20617^T. M-molecular weight marker 100 bp (Pharmacia). (**B**) UPGMA dendrogram derived from similarity coefficients calculated by the Jaccard method, showing the relationship among *Carnobacterium* and related lactic acid bacteria analyzed by amplified 16S rDNA restriction analysis.

the other lactic acid bacteria analyzed. *C. mobile* was characterized by a main amplification fragment of 290 bp, *C. divergens* showed a main fragment at 280 bp, and a fragment of 300 bp was peculiar to *C. divergens*; *C. alterfunditum* was characterized by a 310-bp fragment, and *C. inhibens* showed a main amplified fragment at

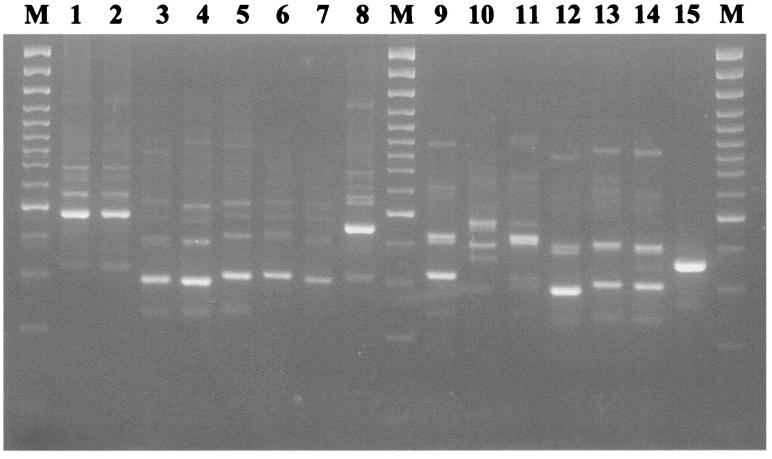

Fig. 3. Agarose gel electrophoresis showing the amplified 16S–23S rDNA spacers of *Carnobacterium* and related lactic acid bacteria. Lane 1, *Lactobacillus maltaromicus* DSM 20342^T; lane 2, *Carnobacterium piscicola* DSM 20722^T; lane 3, *Carnobacterium mobile* DSM 4848^T; lane 4, *Carnobacterium funditum* DSM 5971^T; lane 5, *Carnobacterium divergens* DSM 20623^T; lane 6, *Carnobacterium alterfunditum* DSM 5973^T; lane 7, *Carnobacterium inhibens* CCUG 31728^T; lane 8, *Carnobacterium gallinarum* DSM 4847^T; lane 9, *Vagococcus fluvialis* DSM 5731^T; lane 10, *Vagococcus salmoniarum* DSM 6633^T; lane 11, *Desemzia incerta* DSM 20581^T; lane 12, *Enterococcus hirae* DSM 20160^T; lane 13, *Enterococcus faecalis* NCDO 588^T; lane 14, *Enterococcus faecium* NCDO 1229^T; lane 15, *Streptococcus thermophilus* DSM 20617^T. M-molecular weight marker 1kb ladder (MBI).

about 295 bp; *C. gallinarum* was characterized by a 420-bp fragment, and *C. piscicola* and *L. maltaromicus* showed a common main fragment at 480 bp. Secondary amplified fragments ranging from 300 bp to 700 bp were also useful to highlight the differences among the ITS profiles of each *Carnobacterium* species. The high similarity observed between the ITS profiles of *C. piscicola* and *L. maltaromicus* was completely in accordance with the results previously obtained analyzing the 16S rDNA and strongly confirms that a revision of the taxonomic position of *L. maltaromicus* should be considered. Our data also confirm the ability of ITS analysis in generating species-specific profiles.

Despite the importance of *Carnobacterium* species as food-related lactic acid bacteria [10, 11, 16, 21], few attempts have been made to develop rapid and reliable tools for their accurate identification at a genus/species level [1, 7, 12, 16, 22]. In this study, representative strains of *Carnobacterium* species and closely related lactic acid bacteria were studied by analyzing both the ITS region and the 16S rDNA. A specific PCR assay 16S rDNA targeted was developed for the identification of strains belonging to the genus *Carnobacterium*. In addition, species-specific PCR-based protocols were carried out for the identification of each *Carnobacterium* species. Specifically, the identification of *Carnobacterium* species was obtained both by *Hae*III/*Hinf*I restriction analysis of the 16S rDNA and by the amplification of the internal transcribed spacer between the 16S and the 23S rDNA. We suggest this kind of approach for the identification of *Carnobacterium* species as an alternative solution to the use of more time-consuming techniques such as the determination of the DNA-DNA homology or the 16S rDNA sequencing, and as a complementation of the described species-specific PCR assays.

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