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Differentiation of Closely Related *Carnobacterium* Food Isolates Based on 16S-23S Ribosomal DNA Intergenic Spacer Region Polymorphism

Petia Kabadjova, Xavier Dousset,* Virginie Le Cam, and Hervé Prevost

Laboratoire de Microbiologie Alimentaire et Industrielle, ENITIAA, F-44322 Nantes Cedex 3, France

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A novel strategy for identification of *Carnobacterium* food isolates based on restriction fragment length polymorphism (RFLP) of PCR-amplified 16S-23S ribosomal intergenic spacer regions (ISRs) was developed. PCR amplification from all *Carnobacterium* strains studied always yielded three ISR amplicons, which were designated the small ISR (S-ISR), the medium ISR (M-ISR), and the large ISR (L-ISR). The lengths of these ISRs varied from one species to another. *Carnobacterium divergens* NCDO 2763^T and *C. mobile* DSM 4849^T generated one major S-ISR band (ca. 400 bp) and minor M-ISR and L-ISR bands (ca. 500 and ca. 600 bp, respectively). The ISRs amplified from *C. gallinarum* NCFB 2766^T and *C. piscicola* NCDO 2762^T were larger (S-ISR, ca. 600 bp; M-ISR, ca. 700 bp; and L-ISR, ca. 800 bp). The L-ISR contained two tDNAs coding for tRNA^{Ile} and tRNA^{Ala} genes. The M-ISR included one tRNA^{Ala} gene, and the S-ISR did not contain a tDNA gene. The RFLP scheme devised involves estimation of variable PCR product sizes together with *Hin*fI, *Taq*I, and *Hin*dIII restriction analysis. Forty-two isolates yielded four unique band patterns that correctly resolved these isolates into four *Carnobacterium* species. This method is very suitable for rapid, low-cost identification of a wide variety of *Carnobacterium* species without sequencing.

Lactic acid bacteria (LAB) have been the focus of extensive research due to their value in the food-processing industry (14, 27). One of the most recent taxonomic additions to the LAB group is the genus *Carnobacterium*. Members of this genus are widespread in nature, and the habitats of these organisms range from foods, such as poultry, meat, cheese, and seafood, to fish intestines and anoxic Antarctic lake water (2, 10, 16, 26, 29). The four species of *Carnobacterium* isolated from food are *Carnobacterium divergens*, *C. piscicola*, *C. gallinarum*, and *C. mobile* (6). Most research on the genus *Carnobacterium* has been focused on the production of bacteriocins and the regulation of metabolic enzymes (9, 28). In the last few years, many ways to differentiate *Carnobacterium* species have been developed. The phylogenetic interrelationships of species in the genus *Carnobacterium* have been investigated by using numerical taxonomic matrices and sequencing of the 16S *rrn* segments encoding mature rRNAs (19, 41). The *Carnobacterium* species form a phylogenetically coherent group, which is quite distinct from all other LAB. However, the number of polymorphic sites in the 16S rRNA of *Carnobacterium* species is rather low, since some species have the same sequence (the sequences of *C. piscicola* and *C. gallinarum* are 98% identical) and other species exhibit very high degrees of sequence similarity (the sequences of *C. divergens* and *C. mobile* are 96% similar) (41). Thus, it is difficult to define specific 16S RNA *rrn* sequences that can be used for differentiation of these closely related species. Carnobacteria have been identified at the genus level by nucleic acid hybridization by using 16S rRNA-targeted genus-specific probes (32). Species-specific primers have been designed by using the domains that exhibit low homology in the 16S ribosomal DNA (rDNA) sequences of species of *Carnobacterium* (1, 4). However, the PCR primers used in these studies were not specific enough to differentiate *Carnobacterium* spp. from other genera of LAB. Data obtained with the randomly amplified polymorphic DNA PCR technique enable only *C. divergens* to be differentiated from other *Carnobacterium* species (20).

In view of this, a study of the more variable sequences in the rRNA operon of phylogenetically closely related *Carnobacterium* species could be useful. In most prokaryotes, the ribosomal genes form an operon with the order 5′-16S-23S-5S-3′, and the genes are separated by two intergenic spacer regions (ISRs). ISRs, especially those located between the 16S and 23S rDNAs, are thought to be under less evolutionary pressure and, therefore, to provide greater genetic variation than rRNA coding regions (13, 17). Thus, the 16S-23S ISR is an important tool for developing DNA-based typing methods because it varies in length and sequence from one species to another (11, 31, 37, 40). The heterogeneity of rRNA operons is mainly due to the presence of several functional units within the operons, such as tRNA genes (18, 43). Therefore, the genetic variations in ISRs are not only interstrain variations but also intercistronic variations (7). Analysis of the sequences of the ribosomal operons (*rrn*) is a method of choice for determining phylogenetic relationships among organisms. Even so, the high expense makes sequencing unacceptable for general use. This leads to problems in the development of simpler analysis methods, such as PCR-restriction fragment length polymorphism (RFLP) analysis of the 16S-23S ISR.

The aim of the present study was to investigate the suitability of the 16S-23S ISR in the four closely related *Carnobacterium* species that are of interest to the food industry, *C. divergens*, *C.*

* Corresponding author. Mailing address: Laboratoire de Microbiologie Alimentaire et Industrielle, Département Sciences des Aliments, ENITIAA, rue de la Géraudière, BP 82225, 44322 Nantes Cedex 3, France. Phone: 33-2-51785525. Fax: 33-2-51785455. E-mail: dousset@enitiaa-nantes.fr.

piscicola, *C. gallinarum*, and *C. mobile*, for establishing a rapid PCR-RFLP-based identification scheme. The first goal was to design universal primers for genus-specific amplification and evaluation of the ISR length polymorphism in these species. Restriction analysis of the ISRs was used to develop a reliable diagnostic algorithm for identification of a broad spectrum of *Carnobacterium* species with one to three endonucleases. The degree of interspecies divergence and the intraspecies discriminatory power of ISR sequences were investigated by using 42 strains belonging to four species. 16S-23S ISRs of the type strains of four *Carnobacterium* species were sequenced to examine the variation found in wider phylogenetic gaps.

MATERIALS AND METHODS

Bacterial strains. The strains of the genus *Carnobacterium* used in this study are listed in Table 1. *C. divergens* NCDO 2763, *C. piscicola* NCDO 2762, *C. gallinarum* NCFB 2766, and *C. mobile* DSM 4849 are type strains. Thirty-eight *Carnobacterium* strains isolated from different habitats were tested in order to evaluate the molecular differentiation and degree of intraspecific diversity in the genus. LAB other than *Carnobacterium* were chosen in order to improve our PCR strategy. The bacteria tested in this study represented 175 strains (32 reference strains and 143 isolates) belonging to 40 defined species in seven genera (Table 2). The strains were stored as 15% (wt/vol) glycerol stock cultures at −80°C in MRS medium. Cultures were grown at 30°C for 24 to 36 h in brain heart infusion medium, M17, or MRS medium (Biokar). *Carnobacterium* strains were identified by phenotypic tests as described by Montel et al. (30).

Escherichia coli INV αF′ (Invitrogen), which was used for cloning procedures, was grown in Luria-Bertani broth (0.1% tryptone, 0.1% sodium chloride, 0.05% yeast extract; pH 7.0) for 16 h at 37°C. Transformed *E. coli* was grown on Luria-Bertani agar plates containing 100 μg of ampicillin/ml, 0.5 mM isopropyl-β-D-thiogalactopyranoside, and 80 μg of 5-bromo-4-chloro-3-indolyl-β-galactopyranoside/ml.

PCR primers for ISR amplification. The ISRs were amplified with primers 16S/p2 (5′-CTTGTACACACCGCCCGTC-3′) and 23S/p10 (5′-CCTTTCCCTC ACGGTACTG-3′), which anneal to positions 1388 to 1406 of the 16S rRNA gene and to positions 456 to 474 of the 23S rRNA gene (*E. coli* numbering; GenBank accession number V00331), respectively. The resultant PCR products contained the complete 16S-23S ribosomal ISR and parts of the flanking rDNAs (ca. 150 bp of 16S rDNA and 474 bp of 23S rDNA).

In order to improve the specificity of 16S-23S ISR PCR amplification, the PCR products were used as templates in a second PCR (nested PCR) with primers 16S/p4 (5′-GCTGGATCACCTCCTTTCT-3′) and 23S/p7 (5′-GGTACTTAGA TGTTTCAGTTC-3), which anneal to positions 1526 to 1542 of the 16S rRNA gene and positions 207 to 189 of the 23S rRNA gene (*E. coli* numbering), respectively. Primer 23S/p7 was designed by using sequences of the 23S gene conserved in various bacteria (11). The DNA that was PCR amplified with primers 16S/p4 and 23S/p7 contained the complete 16S-23S ribosomal ISR and parts of the flanking rDNAs (ca. 17 bp of 16S rDNA and 207 bp of 23S rDNA). The $tRNA^{Ala}$ primer (5′-TAGCTCAGCTGGGAGAGC-3′) was designed by using a conserved sequence of the $tDNA^{Ala}$ gene located in 16S-23S ISR of *Oenococcus oeni* (22). The PCR products obtained after amplification with the $tRNA^{Ala}$ and 23S/p10 primers were used for ISR-RFLP analysis.

DNA isolation and PCR. Chromosomal DNA was isolated as previously described by Tudor et al. (39). The resultant DNA samples were used for PCR amplification (ca. 50 ng of DNA per PCR mixture) performed with a PTC-100 thermocycler (MJ Research). The PCR mixture (total volume, 50 μl) contained 5 μl of 10× PCR buffer, 2 μl of a mixture containing each deoxynucleoside triphosphate at a concentration of 0.25 mM, each primer at a concentration of 0.3 μM, 2.5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Appligene). The amplification reaction consisted of a 60-s denaturation step at 94°C, a 75-s annealing step at 60°C, and a 75-s extension step at 72°C. The first cycle was preceded by incubation for 5 min at 94°C. After 30 cycles, there was a final 7-min extension at 72°C. Negative controls containing no DNA template were included in parallel. PCR products were purified with a QIA-quick PCR purification kit (Qiagen) used according to the manufacturer's protocol.

Restriction enzyme analysis and computer-assisted analysis of rDNA restriction patterns. The following enzymes were used: *Alu*I, *Rsa*I, *Hha*I, *Hin*fI, *Hin*dIII, and *Taq*I. Digestion was performed in a 25-μl (final volume) mixture at the optimal temperature according to the manufacturer's protocols (BioLabs). The total digested products were separated by electrophoresis in a 2% (wt/vol) agarose gel. PCR-amplified products were separated by horizontal electrophoresis in 1.5% (wt/vol) agarose gels in Tris-borate-EDTA buffer. Gels were stained with 0.5 μg of ethidium bromide per ml and visualized with UV light. Gel images were digitized with a charged-coupled device video camera (Sony) and stored as TIFF files. These files were converted, normalized with the molecular size markers in a 100-bp DNA ladder (BioLabs), and analyzed with Bio Profile software (Vilbert Lourmat). For ISR-RFLP analysis, a band-matching algorithm was used to calculate pairwise similarity matrices with the Dice coefficient (15). A band-matching tolerance of 5% was chosen.

16S-23S ISR DNA cloning and sequencing. Clone libraries of the carnobacterial PCR-amplified 16S-23S rDNA ISRs obtained with primers 16S/p4 and

TABLE 1. *Carnobacterium* strains used in this study

Species	Strain[a]	Source (reference)
C. divergens	NCDO 2763^T	Meat (30)
C. divergens	INRA 541	Meat (30)
C. divergens	INRA 695	Meat (30)
C. divergens	INRA 507	Meat (30)
C. divergens	INRA 548	Meat (30)
C. divergens	INRA 733	Meat (30)
C. divergens	INRA 508	Meat (30)
Identified as *C. piscicola*		This study
C. divergens	INRA 524	Meat (30)
C. divergens	INRA 586	Meat (30)
Identified as *C. piscicola*		This study
C. divergens	INRA 687	Meat (30)
C. divergens	INRA 515	Meat (30)
Identified as *C. piscicola*		This study
C. divergens	INRA 337	Meat (30)
C. divergens	ENITIAA V41	Fish (34)
C. mobile	DSM 4849^T	Poultry
C. mobile	CIP 103159	Poultry
C. piscicola	NCDO 2762^T	
C. piscicola	IFREMER 644	Smoked salmon (23)
C. piscicola	IFREMER 665	Smoked salmon (23)
C. piscicola	IFREMER 666	Smoked salmon (23)
C. piscicola	IFREMER 692	Smoked salmon (23)
C. piscicola	IFREMER 694	Smoked salmon (23)
C. piscicola	IFREMER 668	Smoked salmon (23)
C. piscicola	INRA 545	Meat (30)
Identified as *C. divergens*		This study
C. piscicola	INRA 725	Meat (30)
C. piscicola	INRA 722	Meat (30)
Identified as *C. divergens*		This study
C. piscicola	INRA 501	Meat (30)
C. piscicola	INRA 527	Meat (30)
C. piscicola	INRA 572	Meat (30)
Identified as *C. divergens*		This study
C. piscicola	INRA 543	Meat (30)
C. piscicola	INRA 528	Meat (30)
C. piscicola	ENITIAA V1	Trout (34)
C. piscicola	INRA 336	Meat (30)
C. piscicola	INRA 338	Meat (30)
C. piscicola	INRA 526	Meat (30)
C. piscicola	ENSAIA 1	Cheese (29)
C. piscicola	ENSAIA 7	Cheese (29)
C. divergens	ENSAIA 13	Cheese (29)
Identified as *C. piscicola*		This study
C. piscicola	ENSAIA 16	Cheese (29)
C. piscicola	ENSAIA 29	Cheese (29)
C. piscicola	ENSAIA 32	Cheese (29)
C. gallinarum	NCDO 2766^T	Poultry (6)
C. gallinarum	INRA 680	Poultry (6)

[a] DSM, Deutsche Sammlung von Microorganismen and Zelkulturen GmbH, Braunschweig, Germany; NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; CIP, Collection de l'Institut Pasteur, Paris, France; INRA, Institut National de Recherche Agroalimentaire, Theix, France; ENSAIA, Ecole Nationale Superieure d'Agronomie et des Industries Alimentaires, Nancy, France; ENITIAA, Ecole Nationale des Ingénieurs des Techniques des Industries Agricoles et Alimentaires, Nantes, France; IFREMER, Institut Français de Recherche pour l'Exploitation de la Mer, Nantes, France.

TABLE 2. Numbers and lengths of PCR-amplified 16S-23S ISRs obtained with primers 16S/4 and 23S/7 from 175 strains of LAB isolated from food

Species or subspecies		Reference strain(s)	No. of isolates	No. of ISR PCR products	Length(s) of PCR product(s) containing ISR (bp)
Carnobacterium	*divergens*	NCDO 2763^T	14	3	400, 500, 600
Carnobacterium	*piscicola*	NCDO 2762^T	24	3	600, 700, 800
Carnobacterium	*gallinarum*	NCDO 2766^T	2	3	600, 700, 800
Carnobacterium	*mobile*	DSM 4849^T	2	3	400, 500, 600
Enterococcus	*faecium*	ATCC 19434	2	2	550, 650
Enterococcus	*faecalis*	CIP 103015, JH 22	2	2	450, 600
Lactobacillus	*sakei*	ATCC 15531, ATCC 15521, ATCC 3453	12	2	450, 650
Lactobacillus	*acidophilus*	ATCC 4356	5	2	450, 650
Lactobacillus	*alimentarius*	DSM 20181	8	2	450, 650
Lactobacillus	*brevis*	CIP 102806^T, ATCC 3548	17	2	450, 650
Lactobacillus	*buchneri*	ATCC 9460	2	2	450, 650
Lactobacillus	*curvatus*	DSM 20019, CIP 102986^T	7	2	450, 650
Lactobacillus	*delbrueckuii* subsp. *bulgaricus*	CIP 101027	2	2	450, 650
Lactobacillus	*delbrueckuii* subsp. *lactis*	CIP 101028	2	2	450, 650
Lactobacillus	*farciminis*	DSM 20180	3	2	450, 650
Lactobacillus	*kefir*	ATCC 35411	6	2	450, 650
Lactobacillus	*helveticus*	CIP 103146^T	4	2	450, 650
Lactobacillus	*kefiranofaciens*	ATCC 43761	2	2	450, 650
Lactobacillus	*parakefiri*	CIP 104242^T	2	2	450, 650
Lactobacillus	*kefirgranum*	CIP 104241^T	2	2	450, 650
Lactobacillus	*casei*	ATCC 11578^T	4	2	450, 650
Lactobacillus	*pentosus*	ATCC 8041	2	2	450, 650
Lactobacillus	*plantarum*	ATCC 14917, NCDO 343	19	2	450, 650
Lactobacillus	*graminis*	CIP 105164^T	1	2	450, 650
Lactococcus	*lactis* subsp. *lactis*	ATCC 11454	3	1	500
Lactococcus	*lactis* subsp. *cremoris*	CNRZ 117	3	1	500
Leuconostoc	*mesenteroides* subsp. *mesenteroides*	DSM 20200, DSM 20240, DSM 20241	8	1	600
Leuconostoc	*mesenteroides* subsp. *dextranicum*	DSM 20484	4	1	600
Leuconostoc	*pseudomesenteroides*	DSM 20193	2	1	600
Leuconostoc	*lactis*	DSM 20192	2	1	600
Weissella	*confusa*	DSM 20186	2	3	450, 550, 650
Streptococcus	*thermophilus*	CNRZ 216	5	1	550

[a] DSM, Deutsche Sammlung von Microorganismen and Zelkulturen GmbH, Braunschweig, Germany; NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; ATCC, American Type Culture Collection, Rockville, Md.; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France; CIP, Collection de l'Institut Pasteur, Paris, France.

23S/p7 were constructed by using a pCR^{R}2.1. TA cloning kit (Invitrogen). From each clone library, 30 white colonies were picked randomly. Clones were screened by PCR for the presence of rDNA ISR inserts and for the sizes of inserts by restriction mapping. For each strain, three independent clones of each 16S-23S ISR identified in *Carnobacterium* were selected and sequenced. Double-stranded DNAs from the recombinant plasmids of the positive clones obtained in the screening assays described above were purified by using a QIAprep Spin Miniprep kit (Qiagen). The nucleotide sequences of the cloned 16S-23S ISRs were determined by the dideoxynucleotide chain termination method (35) with an ABI 370 automated sequencer by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer). The M13 primers flanking the multiple cloning site of pCR^{R}2.1 DNA were used to sequence both DNA strands.

DNA analysis. Sequences were submitted to the National Center for Biotechnology Information (Bethesda, Md.) for similarity searches with the GenBank database. The computer program CLUSTALW (38) was used for sequence alignment, and the BLAST 2 program (36) was used to represent ISR similarities for sequences which did not include 16S or 23S rDNA.

Nucleotide sequence accession numbers. The 16S-23S ISR rDNA sequences determined in this study have been deposited in the EMBL, GenBank, and DDBJ nucleotide databases under the following accession numbers: AF374286, AF374287, AF374288, AF374289, AF 374290, AF374291, AF374292, AF374293, AF374294, AF374295, AF374296, and AF374297.

RESULTS

Length polymorphism of the carnobacterial 16S-23S ISR. PCR amplification with primers 16S/p2 and 23S/p10 designed by using the flanking terminal sequences of the 16S and 23S genes was performed with chromosomal DNA isolated from 42 *Carnobacterium* strains. Amplification yielded nearly identical band patterns containing three fragments. To verify that amplified products were specific to the 16S-23S ISR region, a second PCR was performed. Nested PCR amplification of these fragments with primers 16S/p4 and 23S/p7 resulted in similar profiles. PCR products from each *Carnobacterium* strain always contained three ISR amplicons, which were designated the small ISR (S-ISR), the medium ISR (M-ISR), and the large ISR (L-ISR) spacer region, and their lengths varied from one species to another (Fig. 1). *C. divergens* NCDO 2763^T and *C. mobile* DSM 4849^T generated a major S-ISR band (ca. 400 bp) and minor M-ISR and L-ISR bands (ca. 500 and ca. 600 bp, respectively). The ISRs amplified from *C. gallinarum* NCFB 2766^T and *C. piscicola* NCDO 2762^T were larger (S-ISR, ca. 600 bp; M-ISR, ca. 700; and L-ISR, ca. 800 bp). Extensive investigations of a large number of *Carnobacterium* strains gave similar results (data not shown). Polymorphism in the length of the 16S-23S amplicon for different *Carnobacterium* species was evident, and two groups were distinguished. The first group, designated group A, included *C. divergens* and *C. mobile*. The second group (group B) contained all strains of *C. piscicola* and *C. gallinarum*. The three different 16S-23S ISR PCR products obtained for each strain indicated that there are at least three types of *rrn* operons in *Carnobacterium* species. The variations in ISR length observed could be due in part to variations in the number and type of tRNA sequences found in

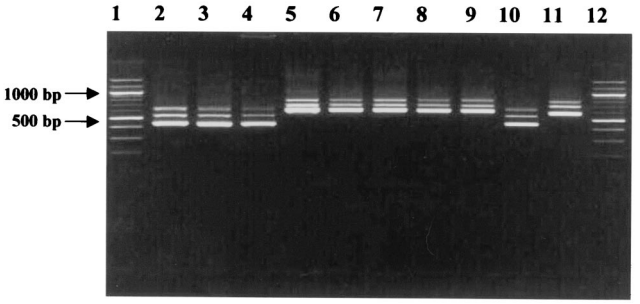


FIG. 1. Electrophoresis in a 1.5% agarose gel of PCR-amplified 16S-23S ISRs of *C. divergens*, *C. mobile*, *C. gallinarum*, and *C. piscicola.* Lanes 1 and 12, molecular weight marker (100-bp DNA ladder); lane 2, *C. divergens* NCDO 2763^T; lane 3, *C. divergens* INRA 541; lane 4, *C. divergens* V41; lane 5, *C. piscicola* NCDO 2762^T; lane 6, *C. piscicola* V1; lane 7, *C. piscicola* SF 644; lane 8, *C. gallinarum* NCDO 2766^T; lane 9, *C. gallinarum* INRA 680; lane 10, *C. mobile* DSM 4849^T; lane 11, *C. piscicola* INRA 501.

this region. The 16S-23S ISRs were PCR amplified from LAB related to *Carnobacterium*. The LAB strains used in this study included reference strains and independent isolates which are geographically and temporally distinct (Table 2). 16S-23S ISR PCR amplification with primers 16S/4 and 23S/7 of 175 LAB strains resulted in very different patterns (in terms of the size and number of bands). Two ISR fragments that were approximately 450 and 650 bp long were amplified for all *Lactobacillus* strains. A single amplified fragment was obtained for *Lactococcus* (ca. 500 bp), *Leuconostoc* (ca. 600 bp), and *Streptococcus thermophilus* (ca. 550 bp) strains. Polymorphism in the lengths of ISRs was found for strains of *Enterococcus*. Two bands at ca. 450 and 600 bp were observed for *Enterococcus faecalis*, and two fragments at ca. 550 and 650 bp were found for *Enterococcus faecium*. The variation in PCR amplicon number and length was useful for differentiation of the *Carnobacterium* strains at the genus level in the LAB group (Table 2).

Nucleotide sequence analysis of the 16S-23S rDNA ISR. The polymorphism of *Carnobacterium* 16S-23S ISRs was investigated by sequencing the corresponding three amplicons of type strains *C. divergens* NCDO 2763, *C. mobile* DSM 4849, *C. gallinarum* NCFB 2766, and *C. piscicola* NCDO 2762. For each strain, the three ISR amplicons cloned in recombinant plasmid pCR^{R}2.1 were screened by PCR amplification. Three independent clones containing the insert rDNA corresponding to each of three PCR ISR amplicons were identified and sequenced. Anticipated errors of PCR and sequencing reactions were avoided by sequencing both strands from each cloned fragment obtained from separate PCR experiments. Sequences at the 3′ end of the 16S coding region and at the 5′ end of the 23S coding region were recognized by comparison with previously published sequences in the GenBank database. The sequence alignments for S-ISR, M-ISR, and L-ISR are shown in Fig. 2.

Sequence analysis of the S-ISRs of the four *Carnobacterium* species revealed four different sequences. Two short sequences, 204 and 218 bp long, were found for *C. mobile* and *C. divergens*, respectively. Two longer S-ISR sequences (391 and 362 bp) were amplified from *C. piscicola* and *C. gallinarum*, respectively. These four S-ISRs showed 40.7% homology and displayed multiple microheterogeneities. The unexpected lengths of the *C. piscicola* and *C. gallinarum* sequences are explained by the presence of one short insertion in the *C. piscicola* S-ISR (ca. 25 bp long, located at positions 68 to 93) and one long insertion in both S-ISRs (located at positions 184 to 340 and 212 to 370, respectively). In the strains examined, none of the S-ISR fragments contained a tDNA gene. A 103-bp fragment located at the 3′ end of the four S-ISRs exhibited 83 to 88% identity with the S-ISR sequences of *Enterococcus muntidii, Enterococcus faecalis, Lactobacillus pentosus*, and *Lactobacillus plantarum* (GenBank accession numbers X87188, AJ301836, U97141, and U97139).

Sequence analysis of the M-ISRs amplified from the type strains also revealed four different sequences, with 50.8% similarity. The 16S-23S M-ISRs were found to contain 326, 312, 473, and 484 bp in *C. divergens*, *C. mobile*, *C. piscicola*, and *C. gallinarum*, respectively. In all strains, the M-ISR invariably contained an insertion with a gene encoding tRNA^{Ala} (anticodon, UGC) beginning at position 90 or 99 and ending at position 162 or 171. The central region was flanked by sequences that were identical or almost identical to those of the corresponding S-ISRs. The M-ISRs of the four *Carnobacterium* species exhibited 36% identity with the ISR sequences of *Enterococcus durans* and *Enterococcus faecium* (GenBank accession numbers X87177 and AF082294, respectively). The M-ISRs of *Carnobacterium* species are organized like the ISRs of *Enterococcus*, *Lactococcus*, *Leuconostoc*, and *Streptococcus*, which contain a unique tRNA^{Ala} gene (21, 22, 31, 33, 44).

Sequence analysis of the L-ISRs of the type strains revealed four different sequences. The L-ISR amplicons of *C. piscicola* and *C. gallinarum* consisted of 604 and 599 nucleotides, respectively. In contrast, *C. divergens* and *C. mobile* had shorter versions (440 and 441 bp). All L-ISRs contained two tDNA genes, coding for tRNA^{Ile} (located at position 104, 106, or 111; anticodon, GAU) and tRNA^{Ala} (located at position 204, 214, or 221), which were 73 to 74 bp long. The difference in length between groups A and B was attributed to an approximately 149-bp deletion in *C. mobile* and *C. divergens*, located between the end of the tDNA^{Ala} gene and the start of the 23S gene. The four carnobacterial L-ISRs showed 56.5% similarity and 36.7% identity with the L-ISR sequences of *Lactobacillus graminis*, *Lactobacillus curvatus*, and *Lactobacillus sakei* (GenBank accession numbers U97136, U97135, and U97137). The L-ISRs of *Carnobacterium* species are organized like those of the *Lactobacillus* species (3). They are composed of the corresponding S-ISRs and M-ISRs, interrupted by sequences (which are 213 to 237 bp long) containing two tDNA genes (Fig. 3).

The primary structures of the two tDNA genes found in M-ISR and L-ISR were similar in the four *Carnobacterium* species. We detected only two microheterogeneities in both tDNA genes. The tDNA^{Ile} of *C. divergens* NCDO 2763^T differed by a single T-C substitution at position 154. At the tDNA^{Ala} level, only one G-A heterogeneity was suspected in the L-ISR of *C. mobile* DSM 4849^T (at position 264). The results of a BLAST search of tRNAs are summarized in Table 3. The highest levels of similarity of carnobacterial tDNA^{Ala} and tDNA^{Ile} genes were found with *Bacillus*, *Lactobacillus*, and *Listeria* strains.

PCR-based restriction analysis. The variation in 16S-23S ISR length among the four *Carnobacterium* species was not substantial. The limitations of the ISR PCR method are evident, and this leads to problems in the development of simple

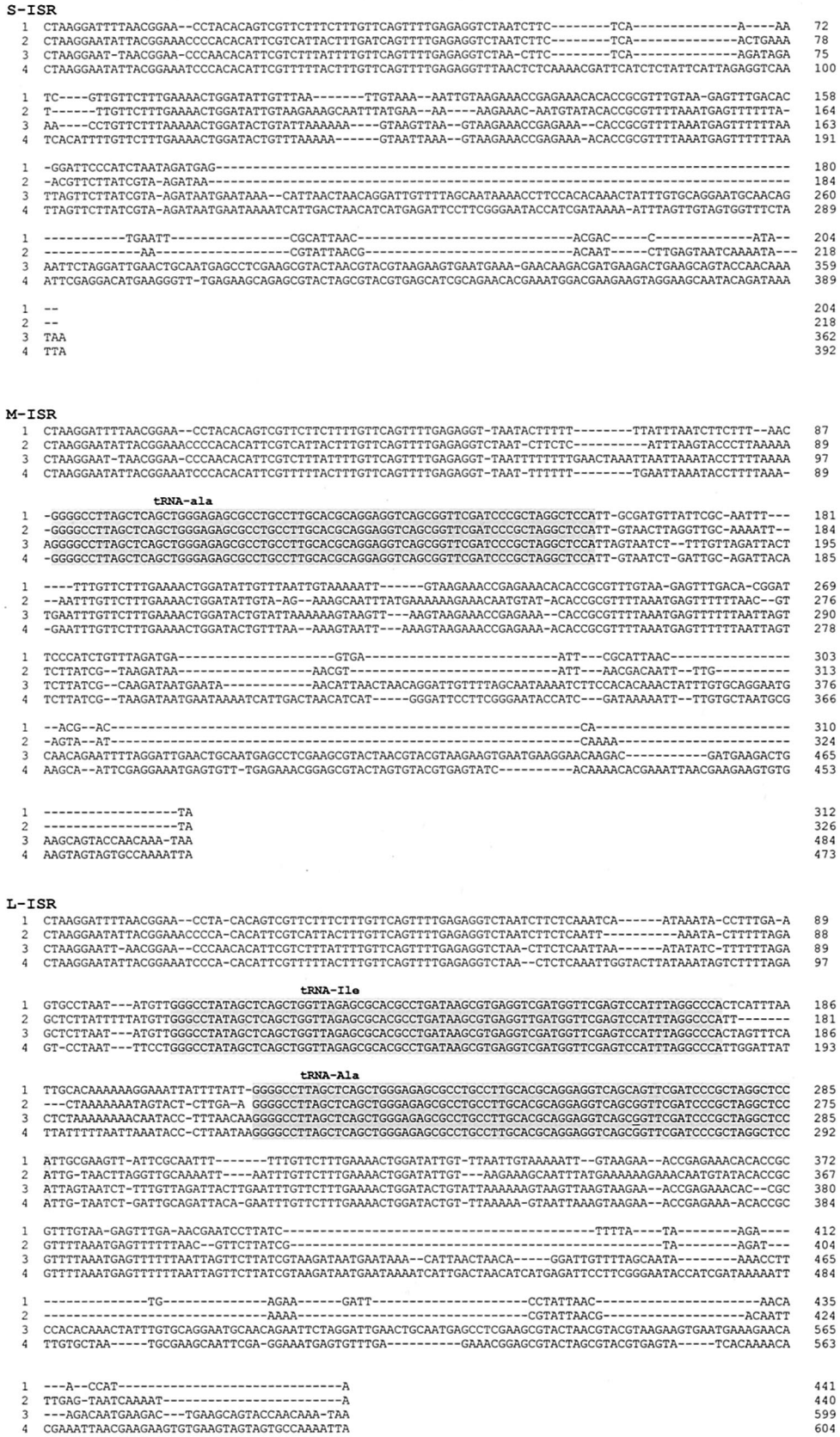


FIG. 2. Alignment of the nucleotide sequences of the *Carnobacterium* 16S-23S ISRs. Line 1, *C. mobile*; line 2, *C. divergens*; line 3, *C. gallinarum*; line 4, *C. piscicola*. GenBank accession numbers of the complete sequences are listed in Materials and Methods. The tRNAs are indicated by grey boxes.

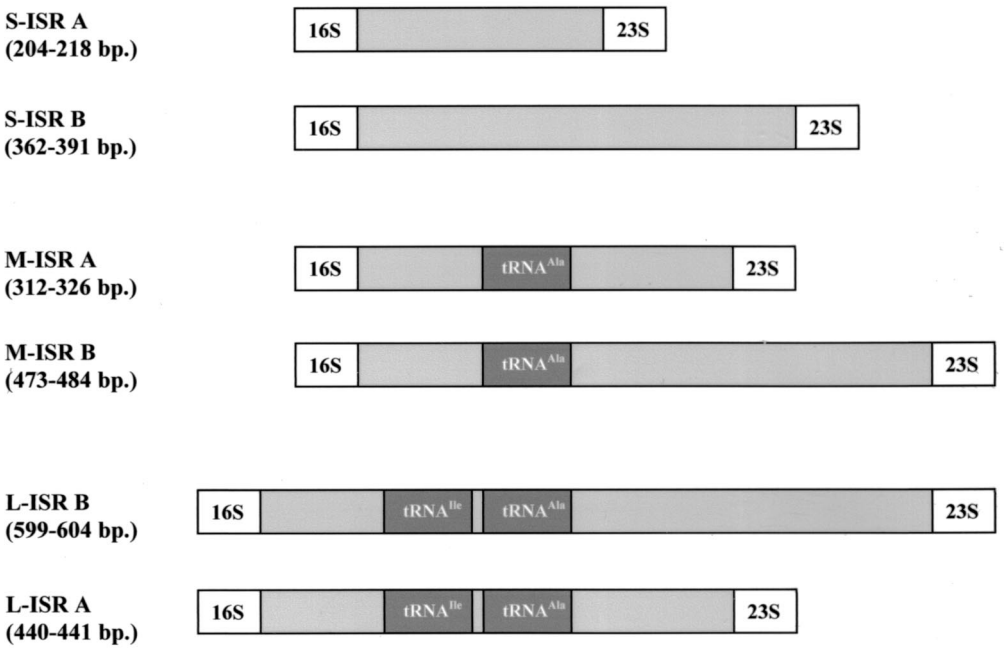


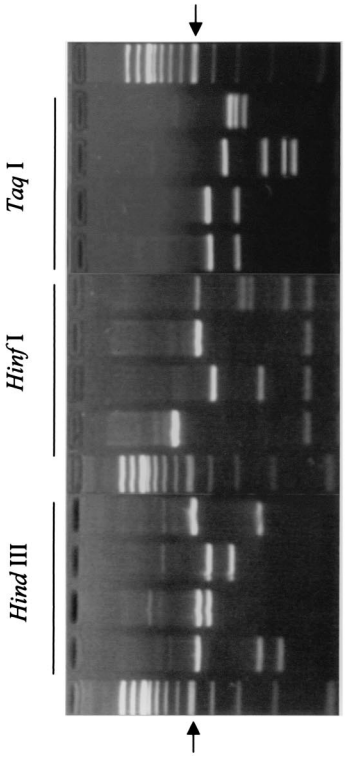
FIG. 3. Schematic representation of S-ISR, M-ISR, and L-ISR of 16S-23S rDNA in the genus *Carnobacterium*. Group A is the group containing *C. divergens* and *C. mobile*; group B is the group containing *C. gallinarum* and *C. piscicola*. The positions of both tRNAs ($tRNA^{Ile}$ and $tRNA^{Ala}$) in M-ISR and L-ISR are indicated.

sequence analysis methods, such as PCR-RFLP. Since PCR amplification with primers 16S/p4 and 23S/p7 yields three ISR amplicons of different lengths, it was difficult to obtain clear patterns in a PCR-RFLP analysis. The L-ISR and M-ISR sequence analysis showed that there is polymorphism in the region located between $tRNA^{Ala}$ and 23S DNA. The gene encoding $tRNA^{Ala}$ contains an 18-nucleotide sequence which is conserved in all the $tRNA^{Ala}$ sequences compared, making this region a suitable target for performing PCR inside the ISR. Primer 23S/p10 is therefore the recommended primer, as it is the primer most likely to detect all copies of the spacer region and hence to determine spacer and sequence variation

TABLE 3. Sequence similarities of tRNAs found in 16S-23S ISRs of *Carnobacterium* species

ISR	tRNA	Organism(s)	Matching organisms	% Similarity[a]
M-ISR	$tRNA^{Ala}$ (UGC)	*C. mobile, C. gallinarum, C. piscicola, C. divergens*	*Bacillus megaterium*	100 (0/73)
			Bacillus pseudomycoides	100 (0/73)
			Bacillus mycoides	100 (0/73)
			Bacillus thuringiensis	100 (0/73)
			Streptococcus difficile	98 (1/73)
			Lactococcus lactis subsp. *lactis*	98 (1/73)
L-ISR	$tRNA^{Ii}$ (GAu)	*C. mobile, C. gallinarum, C. piscicola*	*Lactobacillus sakei*	100 (0/74)
			Bacillus sporothermodurans	100 (0/74)
			Bacillus pseudomycoides	100 (0/74)
			Listeria innocua	100 (0/74)
			Listeria monocytogenes	100 (0/74)
			Lactobacillus graminis	100 (0/74)
			Lactobacillus curvatus	100 (0/74)
			Bacillus subtilis	97 (2/74)
		C. divergens	*Lactobacillus sakei*	98 (1/74)
			Bacillus sporothermodurans	98 (1/74)
			Bacillus megaterium	95 (3/74)
	$tRNA^{Ala}$ (UGC)	*C. mobile*	*Bacillus megaterium*	98 (1/73)
			Alcanivorax borkumensis	98 (1/73)
		C. divergens, C. gallinarum, C. piscicola	*Bacillus megaterium*	100 (0/73)
			Bacillus cereus	100 (0/73)
			Alcanivorax borkumensis	100 (0/73)
			Lactococcus lactis subsp. *lactis*	98 (1/73)

[a] The numbers in parentheses are the number of different nucleotides/total number of nucleotides. The Genbank accession numbers for the $tRNA^{Ala}$ and $tRNA^{Ile}$ genes are as follows: *Alcanivorax borkumensis*, AF197901; *Bacillus cereus*, AF416570; *Bacillus megaterium,* AF142677; *Bacillus mycoides,* AJ420067; *Bacillus pseudomycoides,* AJ420068; *Bacillus sporothermodurans,* AF071855; *Bacillus subtilis,* Z99119; *Bacillus thuringiensis,* AJ420060; *Lactobacillus curvatus,* U97135; *Lactobacillus graminis,* U97136; *Lactobacillus sakei,* U97137; *Lactococcus lactis,* U32972; *Listeria innocua,* AL596170; *Listeria monocytogenes,* AL591983; and *Streptococcus difficile,* AF064741.



Carnobacterium species	Fragment size (bp.)	Number of tested strains
Size marker	500	
C. gallinarum	322, 300, 266	2
C. piscicola	348, 209, 169, 150	22
C. divergens	419, 295	15
C. mobile	419, 295	2
C. mobile	469, 284, 262, 180, 134	2
C. divergens	469, 134	15
C. piscicola	389, 234, 134	22
C. gallinarum	626, 134	2
Size marker		
C. mobile	465, 235	2
C. divergens	422, 308	15
C. piscicola	460, 408	22
C. gallinarum	460, 228, 198	2
Size marker	500	

FIG. 4. Gel electrophoresis of PCR-amplified 16S-23S ISR fragments of the *Carnobacterium* species digested with *Taq*I, *Hin*fI, and *Hin*dIII. Algorithms of RFLP pattern species are presented by molecular size. The position of the 500-bp fragment of the 100-bp DNA ladder is indicated by arrows.

(11). The $tRNA^{Ala}$ gene is located the same distance from 23S rRNA in M-ISR and L-ISR (Fig. 3). For this reason, PCR performed with primers $tRNA^{Ala}$ and 23S/p10 allowed amplification of two amplicons of the same length, which appeared as a single DNA fragment at about 740 bp for group A organisms (*C. divergens* and *C. mobile*) and at about 900 bp for group B organisms (*C. gallinarum* and *C. piscicola*). These amplicons included polymorphism of the two targets (M-ISR and L-ISR). The PCR products derived from each strain were digested separately with six endonucleases, *Alu*I, *Rsa*I, *Hha*I, *Hin*fI, *Hin*dIII, and *Taq*I. These restriction enzymes were selected as first-line enzymes that, together with information about amplicon sizes and sequence polymorphism of the 16S-23S ISR, would produce the most discriminatory RFLP patterns. Digestion with *Alu*I, *Hha*I, and *Rsa*I generated two genotypes, corresponding to group A (*C. divergens* and *C. mobile*) and group B (*C. gallinarum* and *C. piscicola*) (data not shown). The two groups required further analysis with additional endonucleases for accurate identification.

The observed individual *Taq*I, *Hin*fI, and *Hin*dIII RFLP patterns of reference strains are shown in Fig. 4. *Taq*I digestion revealed three genotypes with well-resolved bands. In this case, polymorphism in the closely related organisms *C. piscicola* and *C. gallinarum* was found. The three genotypes produced the following patterns: four bands at 150, 169, 209, and 348 bp for C. *piscicola*, three bands at 266, 300, and 322 bp for *C. gallinarum* and two bands at 295 and 419 bp for *C. divergens* and *C. mobile*.

The four *Carnobacterium* species were finally distinguished by digestion of the ISR amplicon with *Hin*fI and *Hin*dIII. *Hin*fI digestion revealed four genotypes with two to five bands. Two bands at 134 and 469 bp were distinguished for *C. divergens*, two bands at 134 and 626 bp were distinguished for *C. gallinarum*, three bands at 134, 234, and 389 bp were distinguished for *C. piscicola*, and five bands at 134, 180, 262, 284, and 469 bp were distinguished for *C. mobile.*

The results showed that the total length of the *Hin*fI DNA fragments was much more than 740 bp only for *C. mobile* (1,329 bp). On the basis of the alignment of the M-ISR and L-ISR sequences of *C. mobile*, we detected a microheterogeneity at the *Hin*fI restriction site. In M-ISR a *Hin*fI site was located at position 267. In L-ISR, two *Hin*fI sites located at positions 393 and 419 were identified (Fig. 2). These results suggested that when the $tRNA^{Ala}$ and 23S/p10 primers were used, the amplicons corresponding to the 5′ part of 23S rDNA and the 3′ extremity of M-ISR or L-ISR contained two and three *Hin*fI sites, respectively. Digestion of M-ISR yielded three fragments (134, 180, and 469 bp), and restriction of L-ISR resulted in four fragments (27, 180, 262, and 284 bp). The small 27-bp fragment was not detected on the gel. This explains why the total length of the *Hin*fI DNA fragments from *C. mobile* is about twice the length of the ISR amplicon. *Hin*dIII restriction resulted in two or three fragments, as follows: for *C. mobile*, 465 and 235 bp; for *C. divergens*, 422 and 308 bp; for *C. gallinarum*, 460, 228, and 198 bp; and for *C. piscicola*, 460 and 408 bp.

The RFLP patterns resolved 42 isolates into the following four species: *C. mobile*, *C. divergens*, *C. gallinarum*, and *C. piscicola.* *Hin*fI and *Hin*dIII restriction produced genotypes whose sizes could be easily estimated and analyzed with Bio Profile software.

According to the spacer PCR-RFLP method, 12 isolates belonging to *C. divergens* and 14 isolates belonging to *C. piscicola* displayed monomorphic restriction patterns with all endonucleases tested and are phylogenetically related to *C. divergens* NCDO 2763^T and *C. piscicola* NCDO 2762^T, respectively.

During phenotypic identification of our *Carnobacterium* collection, some strains appeared to be incorrectly identified. This was confirmed by the 16S-23S ISR-RFLP method. A group of three isolates (INRA 508, INRA 586, and INRA 515) previously identified as *C. divergens* exhibited *C. piscicola* phenotypic and PCR-RFLP profiles. These isolates were reclassified as *C. piscicola*.

The four isolates deposited as *C. piscicola* (INRA 545, INRA 572, INRA 722, and ENSAIA 13) produced patterns that were in full agreement with the patterns obtained for *C. divergens.* These isolates were reclassified as *C. divergens*.

DISCUSSION

In this study, we sought to develop a new molecular method for differentiating the four closely related *Carnobacterium* species isolated from food. We found that our method is useful for identifying all strains at the species level accurately and reliably and is simple enough for use even in routine laboratories.

The conventional methods for identifying *Carnobacterium* strains based on phenotypic tests are time-consuming, and interpretation of their results is often ambiguous. Currently, the widely accepted strategy formulated to improve methods of *Carnobacterium* strain identification includes sequence analysis of the 16S rRNA gene and construction of genus- and/or species-specific oligonucleotide probes for use with a multiplex and multistep PCR (1, 4, 8, 32, 42). Each technique has several advantages and disadvantages. The small number of polymorphic positions in the 16S rDNA obviates the need for nucleotide sequencing. The species-specific PCR primers, designed from 16S rDNA, were not specific enough to differentiate *Carnobacterium* spp. from other bacterial strains (4). They revealed 100% matches with members of several bacterial genera, including *Vagococcus* spp., *Enterococcus* spp., and *Listeria* spp. The specific primer for *C. piscicola* (Cpg) anneals to both *C. piscicola* and *C. gallinarum* because these species exhibit >98% homology in their 16S rDNA sequences.

The principal goal of our study was to investigate the level of ISR polymorphism and thereby assess the utility of this target for *Carnobacterium* species identification. In this study we demonstrated that the ISR of the genus *Carnobacterium* exhibits considerable variation in length, which is useful for distinguishing two *Carnobacterium* groups from other LAB. Importantly, the sequence divergence includes a reasonable number of insertion and deletion sites. Besides its greater variability, two additional advantages of this target can be pointed out. Unlike a 16S rDNA-based PCR, in which the genus-specific primers are separated by a long stretch of target sequence, an ISR-based PCR should result in a smaller PCR product and in more efficient and sensitive target amplification. In addition, the ISR has the potential to be used for environmentally significant species and strain differentiation. Hence, ISR sequences have been proposed as a useful supplement when 16S rDNA shows insufficient diversity to differentiate recently diverged species (11). The ISR sequence does not code for a final product, but it has an important processing function in forming pre-RNAs. As a consequence, there is presumably some functional selective pressure for conservation of this region. This assumption is consistent with the stability of species-specific ISR signatures found with high reproducibility in different strains. As shown with other taxa, the evolutionary rate of the ISR is higher than that of 16S rRNA, and rearrangements in the central region are relatively recent. We showed that the number of tDNA genes found in carnobacterial 16S-23S ISRs varied from zero to two. It is, therefore, surprising that the *Carnobacterium* species have at least three classes of spacer regions and form three types of *rrn* operons (*rrn* S [*rrn* S], *rrn* Medium [*rrn* M], and *rrn* Large [*rrn* L]). The *rrn* S and *rrn* L operons were similar to those found in *Lactobacillus* species, which are organized as follows: one *rrn* S operon without tDNA and one *rrn* L operon with two tDNA genes (tRNA^{Ile} and tRNA^{Ala}). The *rrn* L operon was also present in a variety of bacterial taxa, including proteobacteria, cytophagas, and gram-positive bacteria with low DNA G+C contents (7, 17, 24, 25, 40). This suggests that this ISR type may be widespread among bacteria and may have been present in the common ancestor of the domain *Bacteria* (5). Two tDNAs found in carnobacterial L-ISR are homologous; i.e., they originated from the same ancestral organism. The phylogenetic relationship based on tRNA^{Ile} (GAU)-tDNA^{Ala} (UGC) sequences found in L-ISR was readily comparable to current bacterial taxonomy based on 16S rDNA sequence data. The majority of LAB, including members of the genera *Lactococcus*, *Streptococcus*, and *Leuconostoc*, have one type of *rrn* operon with a tRNA^{Ala} gene which is similar to the M-ISR of *Carnobacterium* species (21, 33, 44). *Staphylococcus aureus* (12) has three types of *rrn* operons with structures identical to those of *Carnobacterium* species. The genus *Carnobacterium* is phylogenetically related to the genus *Enterococcus* (6). In contrast, *Enterococcus* species contain two types of *rrn* operons, which correspond to the carnobacterial *rrn* S and *rrn* M operons (31). As described previously for all other bacteria, the sequences of small, medium, and large *Carnobacterium* ISRs are related to the sequences of tRNA genes inserted between the common sequences. The sequence variability of *C. divergens*, *C. mobile*, *C. gallinarum*, and *C. piscicola* can be considered an example of the higher resolution of ISR data at the species level. The resolution of the PCR-RFLP methods depended on the part of the *rrn* operon which was analyzed. The data obtained by RFLP analyses of part of the *rrn* M and *rrn* L operons showed that *C. divergens*, *C. mobile*, *C. gallinarum*, and *C. piscicola* belong to the same genomic group. Concerning the intraspecies stability of ISR-RFLP patterns, we found that the spacer-based method was successfully evaluated with expanded groups of strains belonging to *Carnobacterium* species. The closely related species in groups A and B clustered with the same or similar *Alu*I, *Rsa*I, and *Hha*I genotypes. In contrast, very different profiles were found with *Taq*I, *Hin*fI, and *Hin*dIII. The four species clustered in four genotypes. The identification of distinct *C. divergens*, *C. mobile*, *C. gallinarum*,

and *C. piscicola* groups, accurately defined by unique spacer RFLP genotypes, correlates perfectly with a previous numerical phenetic study of the genus *Carnobacterium* (19). The congruence between the results of analyses of rRNA sequences and the RFLP patterns of the 16S-23S-ISR obtained in the present study indicates that these molecular chronometers are well synchronized.

In conclusion, 16S-23S ISR PCR-RFLP analysis is a promising new method for reliable and rapid differentiation of *Carnobacterium* species.

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