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Detection and Differentiation of Several Food-Spoilage Lactic Acid Bacteria by Multiplex Polymerase Chain Reaction, Capillary Gel Electrophoresis, and Laser-Induced Fluorescence

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In this work, a complete analytical procedure is investigated to differentiate several food-spoilage lactic acid bacteria. To do that, a method involving multiplex Polymerase Chain Reaction (PCR), capillary gel electrophoresis (CGE), and laser-induced fluorescence (LIF) is developed. The PCR-CGE-LIF protocol allows the simultaneous detection and differentiation of the genera *Leuconostoc* and *Carnobacterium*, the nonmotile group of species within the genus *Carnobacterium*, and the three species of the group individually (*C. divergens*, *C. gallinarum*, and *C. maltaromicum*). The capability of this approach is clearly illustrated through the sensitive and efficient analysis of the two closest amplicons, with sizes equal to 397 and 412 bp, showing very different yields in all of the amplification reactions tested. These two fragments, which could not be resolved by agarose gel electrophoresis (AGE), are clearly distinguishable by CGE-LIF even when very different areas for both peaks are obtained. The PCR-CGE-LIF method also allows the sensitive detection of these bacteria, demonstrating both a significant resolution improvement compared with traditional AGE and the usefulness of this approach to solve real-life analytical challenges. Good reproducibility of the CGE-LIF procedure is shown for the analysis of multiplex PCR samples with percent relative standard deviation values for migration times and corrected peak areas as low as 0.80 and 6.50 for the same sample and three different days ($n = 12$), respectively.

KEYWORDS: CGE; LIF; *Carnobacterium*; *Leuconostoc*; foods; food spoilage; bacteria; multiplex PCR

INTRODUCTION

Lactic acid bacteria have been commonly associated with the spoilage of refrigerated meat and fish products (*1*−*3*), resulting in souring and gas, slime, and/or off-flavor formation (*1*). To better understand the spoilage processes and also to design appropriate quality assurance strategies, the origin of the different lactic acid bacteria strains should be identified and their fate monitored. This can only be accomplished by the use of reliable species-specific identification tools. However, some genera of lactic acid bacteria, including *Carnobacterium* and *Leuconostoc*, are especially challenging for species identification based on phenotypic characters (*4*). This is further complicated by the inability of some isolates of these genera to grow on media commonly used for the isolation, enumeration, and differentiation of lactic acid bacteria (*5*, *6*). The use of Polymerase Chain Reaction (PCR)-based techniques can overcome these difficulties because strain isolation becomes unnecessary for the detection or identification of the bacteria at any taxonomic level. Following this idea, a 16S rDNA-directed multiplex PCR method for the simultaneous detection of genera *Carnobacterium* and *Leuconostoc* and differentiation of the nonmotile group of *Carnobacterium* species has been recently developed for the identification of these bacteria in spoilage prevention studies (*7*). Special attention was taken in the identification of *C. divergens* and *C. maltaromicum*, the two species most frequently isolated in food products that might be related to spoilage (*3*, *4*, *8*). Differentiation among species *C. divergens*, *C. gallinarum*, and *C. maltaromicum*, the nonmotile species within the genus *Carnobacterium*, was achieved by an independent multiplex PCR method. Also in that work (*7*), it was suggested that a combination of genus-, group-, and species-specific primers could be possible, and that would help to solve some ambiguities due to the fact that motile *Carnobacterium* species give rise to an amplification product identical to the *C. maltaromicum*-specific one. However, differentiation among some of the expected amplicons was not achievable by the agarose gel electrophoresis (AGE) conditions used in that work due to poor resolution (*7*).

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Table 1. Primer Sequences Used in This Work To Perform Multiplex PCR Reactions

primer[a]	standardized probe name[b]	target group	length	sequence 5′ → 3′	t_m (min)	PCR product (bp)
LeucA	S-G-Leuc-1025-a-A	genus *Leuconostoc*	18	CAC TTT GTC TCC GAA GAG	54	
LeucS	S-G-Leuc-0430-a-S	genus *Leuconostoc*	18	AAG CAC TGT TGT ATG GGA	52	613[c]
CarnA	S-G-Carn-0454-a-A	genus *Carnobacterium*	18	GAG CAG TTA CTC TCA TCC	56	
CarnS	S-G-Carn-0148-a-S	genus *Carnobacterium*	19	ATA ACA TTC GGA AAC GGA T	52	324[d]
CnmgS	S-*-Cnmg-0060-a-S	nonmotile *Carnobacterium* spp.	18	GTC GAA CGC ACG AAG TTG	56	412[d]
CgalS	S-S-Cgal-0075-a-S	*C. gallinarum*	19	TGG AAA GCT TGC TTT CTA A	52	397[d]
CdivS	S-S-Cdiv-0190-a-S	*C. divergens*	18	GGT CGC TTG ATG AAA GGT	54	282[d]
CmalS	S-S-Cmal-0217-a-S	*C. maltaromicum*	18	CCA CTA ATG GAT GGA CCC	56	255[d]

[a] Primer used in the study. [b] Primer designation following the nomenclature of Alm et al. (*11*). [c] PCR product obtained using LeucA as antisense primer. [d] PCR product obtained using CarnA as antisense primer.

A second limitation imposed by the use of AGE for multiplex PCR analysis is its insufficient sensitivity, because frequently it becomes difficult to obtain similar amplification rates for a growing number of amplicons. As an example, the specific bands corresponding to *C. divergens* and *C. maltaromicum* were always amplified in the presence of *C. divergens*, *C. maltaromicum*, and *C. gallinarum* by combining different DNA concentrations (200, 20, 2, 0.2, and 0.02 ng each) independently of the accompanying DNA (*7*). However, in the case of *C. gallinarum*, at least 2 ng was necessary to obtain the specific band (*7*). In addition, the simultaneous amplification of the group-specific band (412 bp) together with a proper separation from the *C. gallinarum* band (397 bp) is necessary for a fast and direct identification of species in food samples.

The aim of this work was, therefore, to develop a complete analytical procedure to differentiate and detect several food-spoilage lactic acid bacteria. The new analytical method is based on the use of multiplex PCR followed by capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) to overcome the problems related to poor resolution and detection sensitivity of the DNA fragments obtained after PCR. A new multiplex PCR method is used to detect in a single PCR reaction the genera *Carnobacterium* and *Leuconostoc* and the presence of the species *C. divergens*, *C. gallinarum*, and *C. maltaromicum*. This was achieved by adequately adjusting the PCR conditions for all of the previously described primers (*7*). On the other hand, a sensitive and efficient CGE-LIF method able to resolve and to independently quantify PCR amplicons differing by only 10 bp in size was used (*9*).

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical reagent grade and used as received. Tris[hydroxymethyl]aminomethane (Tris), sodium dodecyl sulfate (SDS), guanidine hydrochloride, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), and Lambda DNA *Hin*dIII digest were from Sigma (St. Louis, MO). 2-Hydroxyethylcellulose (HEC) (M_w 90000) and poly(vinyl alcohol) (PVA) (M_w 50000) were from Aldrich (Milwaukee, WI). YOPRO1 was from Molecular Probes (Leiden, The Netherlands). Buffers were stored at 4 °C and warmed at room temperature before use. Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA).

Bacterial Strains and Growth Conditions. Reference bacterial strains used in this study were supplied by the Spanish Type Culture Collection (CECT). They were grown at 30 °C, for 2−3 days, using the recommended media (http://www.cect.org/): *C. divergens* CECT 4016^T was grown on trypticase soy yeast extract medium (TSYE), *C. gallinarum* CECT 5958^T on yeast glucose lactose peptone broth (YGLPB), *C. maltaromicum* CECT 4134^T and *C. mobile* CECT 5959^T on Man Rogosa Sharpe broth (MRS) (Oxoid CM 359), and *Leuconostoc carnosum* CECT 4024^T on MRS at pH 5.5.

DNA Extraction. DNA from reference strains was extracted following the guanidium thiocyanate method (*10*), spectrophotometrically quantified (Ultrospec 2000 spectrophotometer, Amersham Biosciences U.K. Limited, Buckinghamshire, U.K.), and adjusted to a final concentration of 200 ng/μL in ultrapure water (Sigma-Aldrich).

PCR Conditions. Multiplex PCR amplifications were carried out in a solution containing 1× PCR buffer (10 mmol L^{-1} Tris-HCl, pH 8.8; 1.5 mM $MgCl_2$, 50 mmol L^{-1} KCl, 0.1% Triton X-100), 200 μmol L^{-1} of each dNTP, 1.5 units of Taq polymerase (GenoTaq DNA polymerase, Bonsai Technologies, Madrid, Spain) using 200 ng of DNA template each strain (otherwise stated), and the corresponding primer concentrations in a final volume of 50 μL. The primers used in this work are listed in **Table 1**. Primer concentrations used were as follows: (a) species-specific reaction, 1.5 μmol L^{-1} CgalS, 0.2 μmol L^{-1} CdivS, 0.2 μmol L^{-1} CmalS, and 1.5 μmol L^{-1} CarnA; (b) genus/species-specific reaction, 2 μmol L^{-1} CarnA, 1.5 μmol L^{-1} each CgalS, LeucS, and LeucA, 0.5 μmol L^{-1} each CmalS and CarnS, and 0.2 μmol L^{-1} CdivS; (c) genus/group/species-specific reaction, 2.5 μmol L^{-1} CarnA, 1.5 μmol L^{-1} each CgalS, LeucS, and LeucA, 0.5 μmol L^{-1} each CmalS, CarnS, and CnmgS, and 0.2 μmol L^{-1} CdivS.

Amplification conditions were as follows: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 45 s at 56 °C annealing temperature and 45 s at 72 °C, and a final extension of 5 min at 72 °C. Reactions were carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems, Norwalk, CT) thermal cycler.

Slab Gel Electrophoresis. Fifteen microliters of each PCR mixture was electrophoresed through 2% agarose (Pronadisa, Hispanlab, Madrid, Spain) gel in TAE buffer (40 mM Tris−acetate, pH 7.6, and 1 mM Na_2EDTA). Gels were run at 100 V (8 V/cm) for 80 min, stained with 0.5 μg/mL ethidium bromide, and photographed under UV light. Gel images were recorded using a video camera (GelPrinter Plus, TDI, Madrid, Spain) and stored as TIFF files. A 100 bp ladder (Life Technologies) was used as DNA molecular marker.

Capillary Gel Electrophoresis. The analyses were carried out in a PACE-MDQ (Beckman Instruments, Fullerton, CA) equipped with an Ar+ laser working at 488 nm (excitation wavelength) and 520 nm (emission wavelength). Bare fused-silica capillaries with 75 μm i.d. were purchased from Composite Metal Services (Worcester, U.K.). Injections were made at the cathodic end using N_2 pressure of 1 psi for 12 s (1 psi = 6894.76 Pa). Data acquisition and integration were performed with 32 Karat software (Beckman Instruments).

Before first use, any uncoated capillary was preconditioned by rinsing with 0.1 M HCl for 30 min. Between injections, capillaries were physically coated using 0.1 M HCl for 4 min, 1% PVA for 2 min, and separation buffer for 4 min. At the end of the day, the capillary was rinsed with deionized water for 5 min and stored overnight with water inside.

The following conditions were used for CGE-LIF analysis of the PCR products: separation buffer (20 mM Tris, 10 mM phosphoric acid, 2 mM EDTA, 1.5 M urea, 500 nM YOPRO1, and 4.5% HEC at pH 7.3); temperature of separation, 45 °C; running electric field, −217 V/cm. A 100 bp ladder molecular marker (Biotools, Madrid, Spain) was used as DNA molecular marker.

RESULTS AND DISCUSSION

Differentiation of the Three Species within the Nonmotile Group of *Carnobacterium*. A complete separation of the

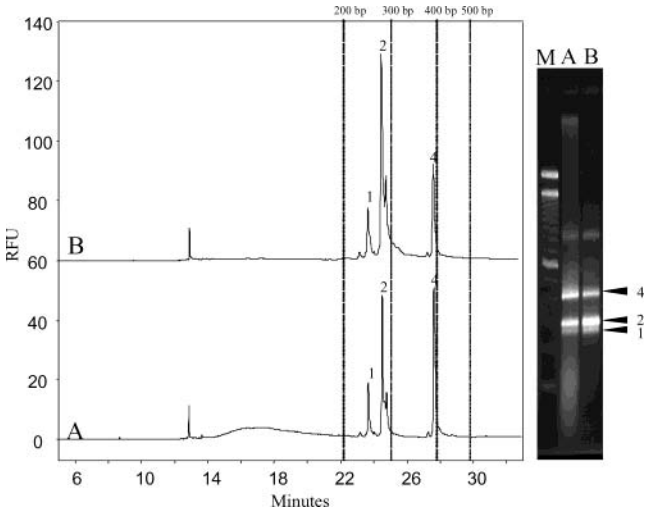

Figure 1. CGE-LIF electropherograms and slab gel electrophoresis of species-multiplex PCR reaction with direct primers CgalS, CdivS, and CmalS and the reverse primer CarnA using as template a DNA mixture containing 1350 ng of *C. gallinarum* and 200 ng each of *C. divergens* and *C. maltaromicum* (**A**) or 200 ng each of *C. divergens*, *C. maltaromicum*, and *C. gallinarum* (**B**). Numbered peaks and bands correspond to (**1**) 255 bp amplified *C. maltaromicum* DNA, (**2**) 282 bp amplified *C. divergens* DNA, and (**4**) 397 bp amplified *C. gallinarum* DNA. CGE separation conditions: uncoated fused silica capillary with 60 cm of total length, 50 cm of effective length, and 75 μm i.d.; separation voltage, –13 kV; 45 °C running temperature; running buffer, 20 mM Tris, 10 mM orthophosphoric acid, 2 mM EDTA, 1.5 M urea, 500 nM YOPRO1, 4.5% HEC at pH 7.3; injection for 12 s using N_2 pressure (1 psi). Slab gel electrophoresis conditions are described under Materials and Methods using a 100 bp ladder molecular weight standard (M).

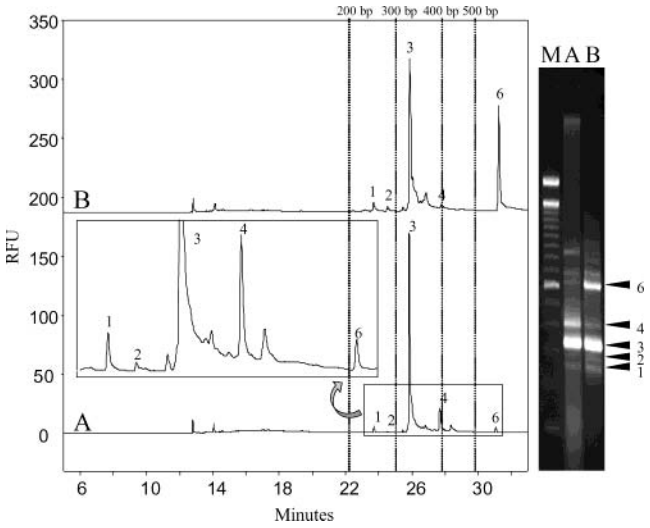

Figure 2. CGE-LIF electropherograms and slab gel electrophoresis of genus-species multiplex PCR reaction with direct primers CarnS, CgalS, CdivS, and CmalS and the reverse primer CarnA and direct primer LeucS and reverse primer LeucA using as template a DNA mixture containing 1350 ng of *C. gallinarum* and 200 ng each of *C. divergens*, *C. maltaromicum*, and *L. carnosum* (**A**) or 200 ng each of *C. divergens*, *C. maltaromicum, C. gallinarum*, and *L. carnosum* (**B**). Numbered peaks and bands correspond to (**1**) 255 bp amplified *C. maltaromicum* DNA, (**2**) 282 bp amplified *C. divergens* DNA, (**3**) 324 bp amplified *Carnobacterium* genus DNA, (**4**) 397 bp amplified *C. gallinarum* DNA, and (**6**) 613 bp amplified *Leuconostoc* genus DNA. Separation conditions were the same as indicated in **Figure 1**.

standard DNA fragments from a 100 bp ladder sample was initially performed using our CGE-LIF separation method. Thus, adequate separation of all the DNA fragments (from 80 to 1000 bp) with high efficiencies (up to 1.8×10^6 plates/m) was obtained by this CGE-LIF procedure, demonstrating its good analytical capabilities. The migration times of the known DNA fragments were used as reference to identify the size of multiplex PCR products. Considering that the expected size of the PCR products from the lactic acid bacteria will be between 200 and 600 bp, the migration times obtained for the 200, 300, 400, and 500 bp DNA standard fragments are chosen and indicated as vertical straight lines in all CGE-LIF electropherograms of this work (see **Figures 1**−**3**).

The products of species-multiplex PCR reactions for *C. divergens*, *C. gallinarum*, and *C. maltaromicum* were analyzed by both slab and capillary gel electrophoresis, and the results are shown in **Figure 1**. The three species could be detected and differentiated by both methods, but the superior resolution of CGE versus AGE allows for a better discrimination between the products derived from *C. maltaromicum* (peak 1) and *C. divergens* (peak 2) genomic DNA. Thus, considering sample B in **Figure 1**, the amount obtained after PCR amplification of *C. divergens* DNA (compound **2**) brings about a wide band in AGE that practically overlaps with the band from *C. maltaromicum* (compound **1**), which would make difficult the semiquantitation of this species. This effect is clearly overcome by the use of CGE-LIF, obtaining well-resolved peaks for this sample B.

It can be observed, also in **Figure 1**, that the higher resolution provided by CGE-LIF allows the separation of at least two different DNA molecules for peak 2 that appear comigrating as a single band in AGE. These multiple DNAs obtained for *C. divergens* could be originated by slight size differences in amplicons derived from different rRNA operons due to intragenomic 16S rRNA sequence heterogeneity (*12*). The presence of more than one operon has been suggested in *Carnobacterium* (*13*).

Differentiation of the Genera *Leuconostoc* and *Carnobacterium* and the Three Species within the Nonmotile Group. The products of genus/species-multiplex PCR reactions performed as described under Materials and Methods were analyzed in parallel by AGE and CGE-LIF. It could be observed that depending on the amount of *C. gallinarum* genomic DNA used, the yield of some PCR products made difficult their detection by AGE, due to either band overlapping or the final DNA quantity being too close (or even below) the detection limit achievable by this technique. As can be seen in **Figure 2**, the *C. divergens* (compound **2**) specific PCR product cannot be detected by AGE when the highest amount of DNA from *C. gallinarum* was used as DNA template in PCR reactions (reaction A). On the other hand, when a minor genomic DNA concentration of *C. gallinarum* was used in PCR amplifications (reaction B), the amount of *C. gallinarum* species-specific amplicon (compound **4**) falls below the detection limit of AGE. This can be partly explained by the fact that both *Carnobacterium* amplicons share the same antisense primer. Conversely, as can be seen in the electropherograms of **Figure 2**, the same amplification reactions analyzed by CGE-LIF allowed the clear differentiation and detection of all five PCR products, regardless of the amount of *C. gallinarum* genomic DNA used as target. To demonstrate this point, the signal/noise ratios were calculated from the peaks in the electropherograms of **Figure 2**. For example, the signal/noise ratios for peak 2 in the electropherograms of **Figure 2A**,**B** were 3 and 6, respectively; for peak 3 these values were 170 in **Figure 2A** and 120 in **Figure 2B**,

Table 2. CGE-LIF Reproducibility of Migration Times (t_m) and Corrected Peak Areas (*A*) for the Same Day and Three Different Days[a]

	same day ($n = 5$)	three days ($n = 12$)
t_m[b] (min)	27.80	27.90
%RSD t_m	0.48	0.80
corrected peak area (arb units)[b]	450000	444000
$\%RSD_A$	3.20	6.50

[a] All conditions as in **Figure 2.** [b] For *C. gallinarum* amplicon (peak 4).

and for peak 4 were 31 in **Figure 2A** and 3 in **Figure 2B**. These results demonstrate that CGE-LIF provides enough sensitivity to detect the PCR products obtained under these different conditions.

Moreover, under the PCR conditions assayed in **Figure 2A** (amplification performed using higher *C. gallinarum* DNA as template), a detrimental effect of DNA concentration from *C. gallinarum* (peaks 3 and 4) on the *C. maltaromicum*, *C. divergens*, and *Leuconostoc* PCR product yields (peaks 1, 2, and 6, respectively) was observed in comparison with the electrophoregram given in **Figure 2B**, obtained after PCR amplification of equal amounts of genomic DNA from each strain. This effect, which was overlooked by AGE for *C. divergens* (peak 2), could also be verified by CGE-LIF analysis.

Reproducibility of the CGE-LIF analysis procedure was demonstrated by injecting the same genus/species-multiplex PCR sample from a given multiplex PCR in the CGE instrument. **Table 2** displays the reproducibility results for migration times and corrected peak areas (calculated as peak area/analysis time). As can be seen, reproducibility of the CGE-LIF procedure was good. Thus, percent relative standard deviation (%RSD) values for migration times calculated for the peak 4 were 0.48% for the same day ($n = 5$) and 0.80% for three different days ($n = 12$). Moreover, %RSD values for corrected peak areas for peak 4 range from 3.20 to 6.50% for the same day ($n = 5$) and three different days ($n = 12$), respectively.

Differentiation of the Genera *Leuconostoc* and *Carnobacterium*, the Nonmotile Group, and Three Species within the Nonmotile Group. Products of genus/group/species-multiplex PCR reactions were analyzed by both electrophoretic methods, AGE and CGE-LIF, as above. As can be observed in the AGE separations of **Figure 3**, the high yield of the nonmotile group specific product (compound **5**), combined with its close size-similarity with the *C. gallinarum* product (compound **4**), systematically hinder the detection of *C. gallinarum* by AGE, independent of the amount of *C. gallinarum* genomic DNA used. This problem could be resolved by the use of CGE-LIF as shown in **Figure 3**. Thus, electrophoregram A in **Figure 3** shows that the higher amount of *C. gallinarum* genomic DNA (compound **5**, corresponding to nonmotile group amplicon) results in an increased signal/noise ratio for this peak. The signal/noise ratio passes from 120 in the (**A**) sample to 50 for the (**B**) sample. This decrease for compound **5** is compensated for by the increase of the signal/noise ratios of peaks 2 and 6 (corresponding to *C. divergens* and *Leuconostoc* amplicon, respectively) that could not be detected under the conditions of Figure 3A. However, after the modifications introduced in the multiplex PCR amplification of **Figure 3B** all six peaks could be detected by CGE-LIF as can be observed in the electropherogram corresponding to this sample.

In addition, the high resolution of CGE-LIF allows direct detection of *C. gallinarum* in the food sample and differentiation

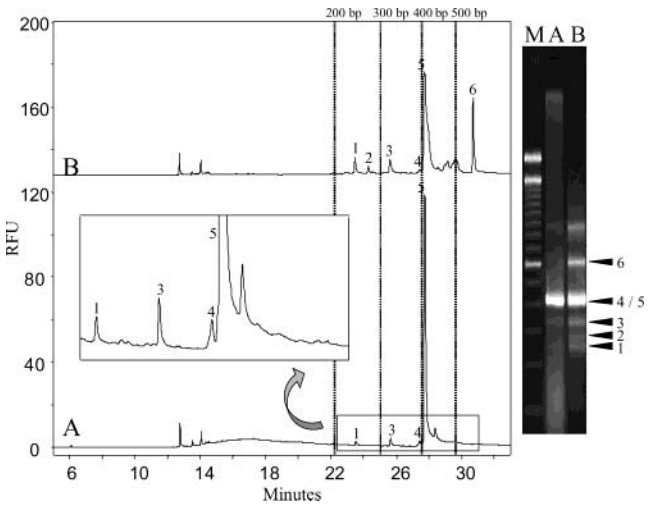

Figure 3. CGE-LIF electropherograms and slab gel electrophoresis of genus-group-species multiplex PCR reaction with direct primers CarnS, CnmgS, CgalS, CdivS, and CmalS and the reverse primer CarnA and direct primer LeucS and reverse primer LeucA using as template a DNA mixture containing 1350 ng of *C. gallinarum* and 200 ng each of *C. divergens*, *C. maltaromicum*, and *L. carnosum* (**A**) or 200 ng each of *C. divergens*, *C. maltaromicum*, *C. gallinarum*, and *L. carnosum* (**B**). Numbered peaks and bands correspond to (**1**) 255 bp amplified *C. maltaromicum* DNA, (**2**) 282 bp amplified *C. divergens* DNAI, (**3**) 324 bp amplified *Carnobacterium* genus DNA, (**4**) 397 bp amplified *C. gallinarum* DNA, (**5**) 412 bp amplified *Carnobacterium* spp. nonmotile group, and (**6**) 613 bp amplified *Leuconostoc* genus DNA. Separation conditions were the same as indicated in **Figure 1**.

of *C. maltaromicum* from the motile species. Under the multiplex PCR annealing temperature (56 °C), the band of 255 bp corresponding to *C. maltaromicum* is amplified in all species of the genus *Carnobacterium* except for *C. divergens* (*7*). Thus, the presence of the 412 bp band allows the differentiation of *C. maltaromicum* from the motile species in the genus. *C. gallinarum* is identified by the presence of the 397 bp specific band. To assess the presence of *C. maltaromicum* in the simultaneous presence of *C. gallinarum*, a second amplification would be necessary, as indicated in ref *7*.

In conclusion, in this work a multiplex Polymerase Chain Reaction assay combined with capillary gel electrophoresis and laser-induced fluorescence detection of the amplification products has been developed for the simultaneous detection and differentiation of bacteria of the genera *Leuconostoc* and *Carnobacterium*, the nonmotile group of species within the genus *Carnobacterium*, and the three species of the group individually. To a great extent this has been possible due to the high resolution and sensitivity of CGE-LIF. This is clearly illustrated in the case of the two closest amplicons, with sizes of 397 and 412 bp, showing very different yields in all of the amplification reactions tested. These two fragments were never resolved by AGE, whereas they are clearly distinguishable by CGE-LIF.

ABBREVIATIONS USED

AGE, agarose gel electrophoresis; CGE, capillary gel electrophoresis; LIF, laser-induced fluorescence; PCR, Polymerase Chain Reaction; CECT, Spanish Type Culture Collection.

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LITERATURE CITED

(1) Borch, E.; Kant-Muermans, M. L.; Blixt, Y. Bacterial spoilage of meat and cured meat products. *Int. J. Food Microbiol.* **1996**, *33*, 103−120.

(2) Gram, L.; Huss, H. Microbiological spoilage of fish products. *Int. J. Food Microbiol*. **1996**, *33*, 121−137.

(3) Barakat, R. K.; Griffiths, M. W.; Harris, L. J. Isolation and characterization of *Carnobacterium*, *Lactococcus*, and *Enterococcus* spp. from cooked, modified atmosphere packaged, refrigerated, poultry meat. *Int. J. Food Microbiol.* **2000**, *62*, 83−94.

(4) Dicks, L. M. T.; Janssen, B.; Dellaglio, F. Differentiation of *Carnobacterium divergens* and *Carnobacterium piscicola* by numerical analysis of total soluble cell protein patterns and DNA-DNA hybridization. *Curr. Microbiol*. **1995**, *31*, 77−79.

(5) Collins, M. D.; Farrow, A. E.; Phillips, B. A.; Jones, D. Classification of *Lactobacillus divergens*, *Lactobacillus piscicola*, and some catalase-negative, asporogenous rod-shaped bacteria from poultry in a new genus, *Carnobacterium*. *Int. J. Syst. Bacteriol*. **1987**, *37*, 310−316.

(6) Yost, C.; Nattress, F. M. The use of multiplex PCR reactions to characterize populations of lactic acid bacteria associated with meat spoilage. *Lett. Appl. Microbiol*. **2000**, *31*, 129−133.

(7) Macián, M. C.; Chenoll, E.; Aznar, R. Simultaneous detection of *Carnobacterium* and *Leuconostoc* in meat products by multiplex PCR. *J. Appl. Microbiol.* **2004**, in press.

(8) Samelis, J.; Kakouri, A.; Georgiadou, K. G.; Metaxopoulos, J. Evaluation of the extent and type of bacterial contamination at different stages of processing of cooked ham. *J. Appl. Microbiol.* **1998**, *84*, 649−660.

(9) García-Cañas, V.; Cifuentes, A.; González, R. Quantitation of transgenic Bt Event-176 maize using double quantitative competitive Polymerase Chain Reaction and capillary gel electrophorsesis laser-induced fluorescence. *Anal. Chem.* **2004**, *76*, 2306−2313.

(10) Pitcher, D. G.; Saunders, N. A.; Owen, R. J. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett. Appl. Microbiol.* **1989**, *8*, 151−156.

(11) Alm, E. W.; Oerther, D. B.; Larsen, N.; Stahl, D. A.; Raskin, L. The oligonucleotide probe database. *Appl. Environ. Microbiol.* **1996**, *62*, 3557−3559.

(12) Klappenbach, J. A.; Saxsman, P. R.; Cole, J. R.; Schmidt, T. M. rrndb: the ribosomal RNA operon copy number database. *Nucleic Acids Res*. **2001**, *29*, 181−184.

(13) Chenoll, E.; Macián, M. C.; Aznar, R. Identification of *Carnobacterium*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* by rDNA-based techniques. *Syst. Appl. Microbiol.* **2003**, *26*, 546−556.

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