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Polymorphism of *Listeria monocytogenes* and *Listeria innocua* strains isolated from short-ripened cheeses

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A. MARGOLLES, B. MAYO AND C. G. DE LOS REYES-GAVILÁN. 1998. Thirty isolates of *Listeria monocytogenes* and 18 of *L. innocua* obtained from different short-ripened cheeses manufactured in Asturias (northern Spain), were compared with each other and with reference strains using serotype, phage type and pulsed-field restriction endonuclease digestion profiles analysis of the total DNA. Restriction enzymes *Apa*I and *Sma*I defined five clusters in *L. monocytogenes* (*m1* to *m5*) and two main clusters in *L. innocua* (*i1* and *i2*). Cluster *i2* was further arranged into three subclusters (*i2a*, *i2b* and *i2c*) based on the different *Eco*52I (*Xma*III) and *Crf*42I (*Sac*II) patterns of its isolates. Clusters of *L. innocua* were clearly different whereas those of *L. monocytogenes* were more closely related to each other. In this latter species, serotype 4b isolates (*m4* and *m5*) constituted a more homogeneous group than serogroup 1 isolates (*m1*, *m2* and *m3*). Cluster *m3* contained two strains of serotype 1/2a whereas *m1* and *m2* harboured strains of both serotypes, 1/2a and 1/2b. Therefore, the combined use of restriction patterns and serotype may be useful to differentiate *L. monocytogenes* strains showing identical restriction profiles but differing in serotype. The cheese source of *Listeria* strains proved that isolates from cluster *m1* were repeatedly detected as a contaminant in the same type of cheese. Comparison of *L. monocytogenes Apa*I profiles showed a genetic proximity of *m4* and *m5* to the recognized pathogenic strains ATCC 13932 and NCTC 11994, responsible for meningitis cases in other countries. Finally, bacteriophage typing data indicated that *m4*, the sole phage typable group, had a phage type resembling that of strains causing the Auckland (New Zealand) outbreak of listeriosis in 1969. These data suggest a wide distribution of closely related types which might cause, under several circumstances, sporadic cases of listeriosis.

INTRODUCTION

Listeria monocytogenes has been involved in the past decade in several outbreaks and sporadic cases of listeriosis associated with the consumption of pasteurized milk, soft cheeses and other dairy products in North America and Europe (Fleming *et al.* 1985; Bannister 1987; Linnan *et al.* 1988; Azadian *et al.* 1989; Bille 1990; Farber *et al.* 1990; Goulet *et al.* 1995; Proctor *et al.* 1995). The ubiquity of *L. monocytogenes* makes typing methods essential for searching the source of food contamination and for the study of listeriosis epidemiology. Several typing techniques that include serotyping and phage typing have usually been successfully applied in *L. monocytogenes* but they also have several limitations. Serotyping lacks discriminatory power when used as the only typing tool, because three serotypes (1/2a, 1/2b and 4b) prevail in foods (Farber and Peterkin 1991). Phage typing allows further strain subdivision although not all the isolates are typable with the existing set of phages (Rocourt *et al.* 1985; van der Mee and Audurier 1995). Molecular techniques such as multilocus enzyme electrophoresis (MEE), restriction enzyme analysis of the DNA (DNA-REA), restriction enzyme digestion profiles analysis by pulsed field gel electrophoresis (REDP), ribotyping and randomly amplified polymorphic DNA (RAPD) have also been applied for typing *Listeria* strains (Howard *et al.* 1992; Nocera *et al.* 1993; Brosch *et al.* 1994; Graves *et al.* 1994; Niederhauser *et al.* 1994; Erics-

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son *et al.* 1995). These methods are more discriminating than serotyping or phage typing but they are also expensive and time consuming. On the other hand, due to its clinical relevance, most typing studies have focused on *L. monocytogenes* and relatively few data are available from other *Listeria* species. Recently, the WHO food safety unit sponsored a multicentre study on *L. monocytogenes* subtyping methods with the aim to select and standardize the most appropriate procedures (Bille and Rocourt 1996). Serotyping appears as a very useful preliminary first step typing method (Schönberg *et al.* 1996) and phage typing was the most valuable for mass screening (McLauchlin *et al.* 1996). REDP was highly discriminatory and reproducible and looks very promising, particularly if an analysis of the results is used in association with a strict standardization of the protocols (Brosch *et al.* 1996).

In recent work (Margolles *et al.* 1996) we described the incidence of *L. monocytogenes* and *L. innocua* in several short-ripened cheeses manufactured in Asturias (northern Spain). The present study deals with the analysis of these isolates by REDP and serotyping and phage typing for *L. monocytogenes.* It has shown the enhanced discriminatory power of the combined use of REDP and serotyping to survey *L. monocytogenes* contaminations in cheeses. Genetic proximity among *L. monocytogenes* isolates and their relation with several reference strains, some of them with recognized pathogenicity, was also evaluated.

MATERIALS AND METHODS

Bacterial strains

Forty-eight isolates of *L. monocytogenes* (Lm) and *L. innocua* (Li) obtained from short-ripened cheeses manufactured in Asturias (northern Spain) (Margolles *et al.* 1996) were analysed (Table 1). The *L. monocytogenes* reference strains used in this work are listed in Table 2. Strains Scott A and Ohio were provided by P. Hernández (Departamento de Nutrición y Bromatología III. Facultad de Veterinaria, Universidad Complutense, Madrid, Spain) and M. Medina (Area de Tecnología de Alimentos, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain). *Listeria* was cultured on Tryptone Soya agar and subcultured on Tryptone Soya broth (TSB) (Adsa-Micro, Barcelona, Spain) at 30 °C overnight. Pure cultures of *Listeria* strains were maintained in TSB plus 30% or 50% glycerol at – 20 or – 80 °C, respectively.

Serotyping and phage typing

Strains of *L. monocytogenes* were first serogrouped using specific antisera 1 and 4 (Difco Laboratories, Detroit, MI, USA) according to the manufacturer's instructions, and they were further serotyped by J. Vázquez (Centro Nacional de Microbiología, Virología e Inmunología Sanitarias, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain) according to the Seeliger and Höhne criteria (1979). Phage types were determined by J. Rocourt (Unité d'Ecologie Bacterienne, Institut Pasteur, Paris, France) with a defined set of phages (Rocourt *et al.* 1985).

REDP analysis

Intact high molecular weight genomic DNA was isolated and digested in agarose plugs essentially as described by Howard *et al.* (1992) and Michel and Cossart (1992) with minor modifications. Briefly, overnight cultures of *Listeria* spp. were used to inoculate 10 ml of pre-warmed TSB tubes at 5% and cells were cultured for 4 h at 37 °C without shaking ($O.D._{600\,nm}$ 0·2). Then, chloramphenicol (200 g ml^{-1}) was added and the incubation continued for 1 h. The present study used 8×10^8 cells (250 μl) mixed with an equal volume of a 2% solution of InCert agarose (FMC BioProducts, Rockland, ME, USA) in TBE buffer (Sambrook *et al.* 1989). After cell lysis and deproteinization with proteinase K (Boehringer Mannheim, Mannheim, Germany), DNA within the agarose plugs was digested with 20 U of the restriction enzyme *Apa*I (Boehringer Mannheim), *Sma*I, *Eco*52I (*Xma*III) or *Cfr*42I (*Sac*II) (Fermentas MBI, Vilnius, Lithuania) for 24 h at 37 °C (25 °C for *Sma*I) in 200 μl of the buffer recommended by the supplier. DNA digests were submitted to a contour-clamped homogeneous electric field (CHEF) in a CHEF-DR II apparatus (Bio-Rad, Richmond, CA, USA). Electrophoresis was carried out in 1% FastLane agarose gels (FMC BioProducts) with 0·5 × TBE buffer (Sambrook *et al.* 1989) for 20–24 h at 6 V cm^{-1} and 14 °C, with pulse times ranging from 0·5 to 50 s. Phage lambda DNA concatemers were used as molecular size standards (Bio-Rad).

Similarity of REDP groups

REDP of *Listeria* strains were compared with Dice's index (Dice 1945). Where appropriate, a combined similarity matrix of various enzymes was constructed, calculating the average of their respective Dice's indices. Subsequent cluster analysis and similarity between clusters (S_j) was obtained using the unweighted pair groups average linkage analysis (UPGMA) (Priest and Austin 1993).

RESULTS

Typing *L. monocytogenes* and *L. innocua* isolates

Thirty isolates of *L. monocytogenes and* 18 of *L. innocua* obtained from different short-ripened cheeses (Margolles *et al.* 1996) were compared using REDP analysis, and sero-

Table 1 Serotype and pulsed-field restriction endonuclease digestion profiles (REDP) of *Listeria monocytogenes* (Lm) and *Listeria innocua* (Li) isolates obtained from several short-ripened cheeses at different seasons of the year

Cheese sample	Type of cheese	Season	Isolates	REDP cluster*	Serotype†
1	Afuega'l Pitu	Fall 92	Lm1	*m1*	1/2b
			Lm3	*m1*	1/2a
			Lm2	*m2*	1/2a
			Lm4	*m2*	1/2b
2	Afuega'l Pitu	Fall 92	Lm6, Lm9	*m2*	1/2a
			Lm5, Lm7, Lm8, Lm10	*m2*	1/2b
3	Afuega'l Pitu	Fall 92	Lm11	*m2*	1/2a
4	Oscos	Fall 92	Li12, Li13, Li14, Li15, Li16	*i1*	—
5	Peñamellera 2	Winter 92–93	Li17, Li18, Li19, Li20, Li21	*i2a*	—
6	Vidiago	Winter 92–93	Lm22, Lm23, Lm26	*m4*	4b
			Li24, Li25	*i2b*	
7	Vidiago	Winter 92–93	Li27, Li28, Li29, Li30	*i2c*	—
8	Afuega'l Pitu	Spring 93	Lm31	*m2*	1/2a
			Lm32	*m1*	1/2a
9	Afuega'l Pitu	Spring 93	Lm33	*m1*	1/2a
10	Afuega'l Pitu	Spring 93	Lm34, Lm35, Lm36	*m2*	1/2b
			Lm37	*m3*	1/2a
11	Peñamellera 1	Spring 93	Li38, Li40	*i1*	—
			Lm39	*m4*	4b
12	Afuega'l Pitu	Summer 93	Lm41, Lm42, Lm43, Lm44, Lm46	*m5*	4b
			Lm45	*m3*	1/2a
13	Afuega'l Pitu	Winter 94	Lm47, Lm48	*m1*	ND‡

*Clusters were arranged according to the *Apa*I REDP for *L. monocytogenes* and to the *Sma*I/*Xma*III/*Sac*II REDP for *L. innocua*.
†Only for *L. monocytogenes* strains.
‡ND, not determined.

Table 2 Serotypes and origins of *Listeria monocytogenes* reference strains used in this study. Abbreviations and sources of strains: ATCC, American Type Culture Collection, Rockville, USA; NCTC, National Collection of Type Cultures, Colindale, London, UK

Strain	Serotype	Origin
ATCC 35152	1/2a	Guinea pig
ATCC 13932	4b	Child (meningitis)
NCTC 11994	4b	Soft cheese (associated with a case of meningitis after eating cheese)
Scott A	4b	Human. Massachusetts outbreak 1983
Ohio	4b	Liederkranz cheese

typing, and phage typing in the case of *L. monocytogenes*. The REDP of the same isolate generated by each of the enzymes used was found to be stable and reproducible on at least two or three separate occasions (data not shown).

Visual comparison of macrorestriction patterns of the *L. monocytogenes* isolates revealed five different *Apa*I REDP (Fig. 1). Although the digestion patterns generated by *Sma*I were obviously different from those of *Apa*I, both enzymes established the same groups in this species (data not shown). *Listeria monocytogenes* isolates were then classed into five clusters (Table 1) that showed neighbouring profiles. Clusters *m1* and *m2* were proximate (S_i 0·84) and differed in the electrophoretic migration of the upper band and of several low molecular size bands. According to the *Apa*I restriction

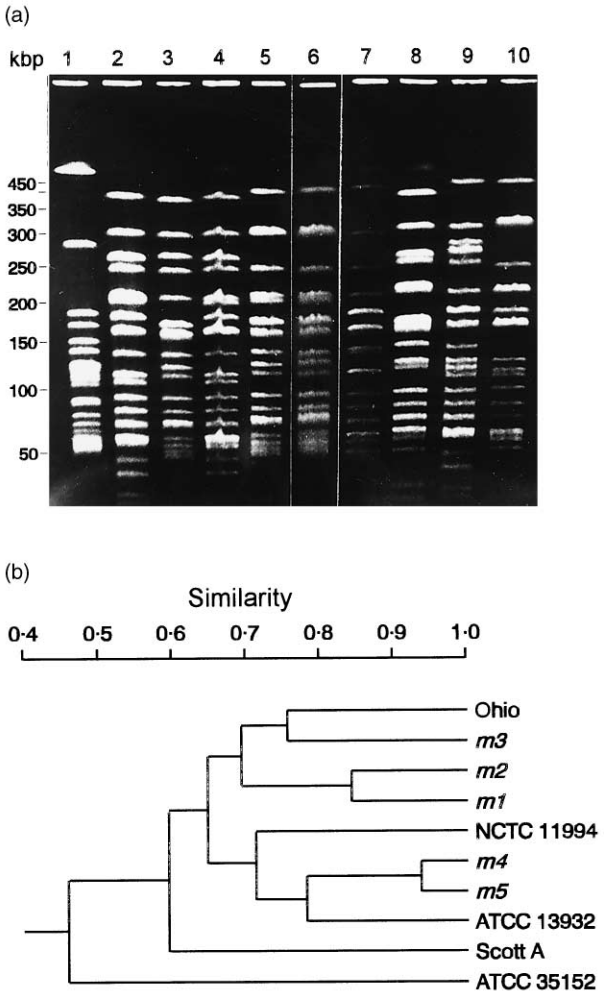

Fig. 1 (a) Representative pulsed-field restriction endonuclease digestion profiles (REDP) of *Listeria monocytogenes* DNA with *Apa*I. Lanes: 1 to 10, *L. monocytogenes* ATCC 35152, cluster *m1*, ATCC 13932, cluster *m2*, cluster *m4*, cluster *m5*, NCTC 11994, Scott A, cluster *m3*, and Ohio. (b) The similarity matrix of *Apa*I REDP was subjected to cluster analysis as unweighted pair groups average linkage analysis clustering method (using the Dice's index). Vertical lines of the dendrogram represent the degree of similarity shared by the groups connected by the lines

profiles, isolates Lm47 and Lm48 were included into the cluster *m1* although the restriction patterns obtained with *Cfr*42I (*Sac*II) and *Eco*52I (*Xma*III) displayed minor differences with respect to the other isolates of the same group obtained one or two years before (Lm1, Lm3, Lm32 and Lm33) (data not shown). Clusters *m4* and *m5* were the most closely related (S_i 0·94) and differed in the electrophoretic migration of three *Apa*I bands sized between 100 and 150 kbp. On the other hand, 19 out of 28 *L. monocytogenes* isolates belonged to the serotypes 1/2a or 1/2b, and the remaining nine isolates were classed as serotype 4b (Table 1). Three out of the five *L. monocytogenes* clusters contained strains with identical serotype so that isolates of clusters *m4* and *m5* were serotype 4b and the two isolates of *m3* were serotype 1/2a. However, clusters *m1* and *m2* harboured strains of both serotypes, 1/2a and 1/2b. This fact allowed the internal division of clusters *m1* and *m2* on the basis of the different serotypes displayed by their isolates. Remarkably, the S_i values obtained between *L. monocytogenes* clusters indicated that they are relatively close in this species (S_i between serogroup 1 and 4 was 0·65), being isolates of serogroup 4 genetically more homogeneous than those of serogroup 1. Finally, bacteriophage typing data of *L. monocytogenes* showed that only isolates of cluster *m4* were typable, sharing the phage type 3552: 1444: 1317: 3274: 52: 340: 312.

In *L. innocua* species, both enzymes, *Apa*I and *Sma*I, defined two clusters (i*1 and i2*) (Fig. 2). However, the DNA of isolates included into cluster *i2* was not digested by *Apa*I, whereas a characteristic banding pattern was obtained with the DNA of isolates classed into cluster *i1*. On the contrary, the enzyme *Sma*I produced a clearly different restriction pattern in each group. Other restriction enzymes did not generate further divisions into cluster *i1*. However, cluster *i2* was divided into three subclusters (*i2a*, *i2b* and *i2c*) on the basis of the different electrophoretic migration of the major bands generated by DNA digestion with *Eco*52I (*Xma*III) and *Cfr*42I (*Sac*II) (Fig. 2a). Then, S_i in *L. innocua* was obtained from the combined analysis of restriction profiles generated by the enzymes *Sma*I, *Cfr*42I (*Sac*II) and *Eco*52I (*Xma*III). Unlike what happened in *L. monocytogenes,* main clusters *i1* and *i2* of *L. innocua* seemed to be clearly different (S_i 0·29). However, the three clusters *i2a*, *i2b* and *i2c* are closely related ($S_i > 0{\cdot}88$).

Environmental source of *Listeria* isolates

Table 1 shows the distribution of *Listeria* REDP clusters and serotypes according to the type of cheese and the season of the year in which they were isolated. Some interesting features can be seen concerning the distribution of clusters and serotypes.

(i) *Listeria innocua* strains appeared as sporadic contamination in different cheeses and seasons.

(ii) *Listeria monocytogenes* isolates of clusters *m1* and *m2* were obtained from different samples of the same type of cheese (Afuega'l Pitu) along an extensive period of time, indicating that a repeated contamination probably occurred from the same source during that time.

(iii) On the contrary, isolates of cluster *m4* of *L. monocytogenes* were obtained from two different types of cheeses (Vidiago and Peñamellera 1) manufactured at different and geographically dispersed farms. This fact may indicate a widespread distribution of several strains.

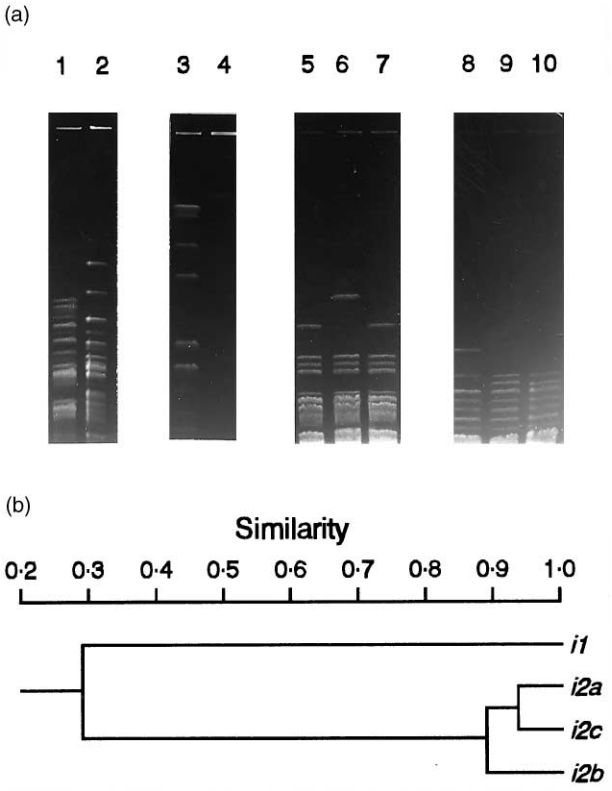

Fig. 2 (a) Representative pulsed-field restriction endonuclease digestion profiles (REDP) of *Listeria innocua* DNA with *Apa*I, *Sma*I, *Cfr*42I (*Sac*II) and *Eco*52I (*Xma*III). Lanes 1 and 2: cluster *i2* and cluster *i1* with *Sma*I; lanes 3 and 4: cluster *i1* and main cluster *i2* with *Apa*I; lanes 5 to 7: cluster *i2a*, cluster *i2b*, and cluster *i2c* with *Eco*52I (*Xma*III); lanes 8 to 10: cluster *i2a*, cluster *i2b*, and cluster *i2c* with *Cfr*42I (*Sac*II). (b) The similarity matrix of the combined *Sma*I/*Crf*42I/*Eco*52I REDP was subjected to cluster analysis as unweighted pair groups average linkage analysis clustering method (using the Dice's index). Vertical lines of the dendrogram represent the degree of similarity shared by the groups connected by the lines

(iv) In some cases (samples 1, 8, 10 and 12), the same sample was contaminated with two *L. monocytogenes* strains displaying different REDP. Moreover, in two cases (samples 1 and 2), we isolated from the same cheese sample (Afuega'l Pitu) strains with identical REDP (clusters *m1* or *m2*) that were only distinguished on the basis of its different serotype (1/2a or 1/2b). These results pointed to an enhanced discriminatory power by means of the combined use of REDP and serotype.

Similarity between REDP clusters and reference strains of *L. monocytogenes*

As there were not *L. monocytogenes* isolates available associated with human or bovine listeriosis in the area in which the cheeses under study were manufactured (listeriosis is not a reportable disease in Spain), the REDP of *L. monocytogenes* isolates were compared with those of several reference strains (Table 2). As shown in Fig. 1, the strains more closely related to clusters *m4* and *m5* were ATCC 13932 and NCTC 11994 (S_i 0·78 and 0·71, respectively), both of them associated with sporadic cases of listeriosis in other countries. On the other hand, clusters *m4* and *m5* were relatively separated from other reference strains of serotype 4b (Scott A and Ohio, S_i 0·60 and 0·65, respectively) as well as from clusters *m1*, *m2* and *m3* (S_i 0·65).

DISCUSSION

In the present work, several strains of *L. monocytogenes* and *L. innocua* isolated during a two year period from different short-ripened cheeses made in Asturias (northern Spain), were submitted to REDP analysis and serotyping, and phage typing for *L. monocytogenes*. With respect to the serotype, most of our *L. monocytogenes* isolates belonged to serogroup 1 (serotypes 1/2a and 1/2b). In general, a major incidence of this serogroup in foods has been found (Schönberg *et al.* 1989; Greenwood *et al.* 1991) although several researchers have also indicated in certain cases a greater incidence of serogroup 4 (Ibrahim and McRae 1991; Gaya *et al.* 1996).

REDP analysis of our isolates was carried out using the enzymes *Apa*I, *SmaI*, *Cfr*42I (*Sac*II) and *Eco*52I (*Xma*III). With *Apa*I or *Sma*I, *L. monocytogenes* isolates were classed into five clusters and *L. innocua* strains into two main clusters, one of which was subdivided into three subgroups by digestion with the enzymes *Cfr*42I (*Sac*II) and *Eco*52I (*Xma*III). At this point, it is interesting to take into account several findings. Danielsson-Tham *et al.* (1993) reported that some strains of *L. innocua* were not sensitive to *Apa*I but were digested by *Sma*I, as was the case for our isolates of cluster *i2*. Similar observations were made by Brosch *et al.* (1994) with the enzyme *Apa*I on the strain *L. monocytogenes* ATCC 19116. These results suggest a modification on the DNA recognition sequence of *Apa*I in certain *Listeria* strains. Several studies indicated that REDP analysis is at present the most discriminatory and powerful technique for typing *Listeria* (Baloga and Harlander 1991; Carrière *et al.* 1991; Howard *et al.* 1992; Moore and Datta 1994; Jacquet *et al.* 1995). However, nowadays it is generally admitted that for typing *L. monocytogenes* and *L. innocua,* better results may be obtained with a set of typing systems (Slade 1992). In accordance with that is the further division of *L. monocytogenes* clusters *m1* and *m2* that we accomplished by serotypes. The combined use of REDP and serotype enhanced the discriminatory power of each technique and may be a useful tool to distinguish different strains of *L. monocytogenes* that were identical by a single method. These findings are also in agreement with results already published by several

authors who found different serotypes yielding identical PFGE DNA fingerprints (Nocera *et al.* 1990; Szabo and Desmarchelier 1990; Louie *et al.* 1996) or RAPD profiles (Mazurier and Wernars 1992; Niederhauser *et al.* 1994; Lawrence and Gilmour 1995; Louie *et al.* 1996). On the other hand, Brosch *et al.* (1994, 1996), based on the profile analysis of the low molecular weight bands generated by *Apa*I and *Asc*I in *L. monocytogenes*, were able to establish genomic divisions that were coincident with groups of flagellar antigens (serotype). It is also interesting to note that the use of monoclonal antibodies (Kathariou *et al.* 1994) and the restriction of PCR products (Ericsson *et al.* 1995) have recently been reported as useful tools for subtyping *L. monocytogenes* serotype 4b.

The combined use of REDP analysis and serotyping allowed us to follow the *Listeria* contamination of several types of cheeses. In certain cases, the same cheese sample contained *L. monocytogenes* strains with different REDP. In turn, from a given cheese sample, there were isolated strains of identical REDP but with a different serotype. On the other hand, *L. innocua* appeared as sporadic contamination in different types of cheese and seasons whereas strains of clusters *m1* and *m2* of *L. monocytogenes* were present as a continuous contamination being isolated from the same type of cheese at several times. This fact indicated that a contaminating source may be present in the manufacturing environment and caused a repeated contamination of the product. In this respect it is also interesting to note the slight differences displayed by the *Cfr*42I (*Sac*II) and *Eco*52I (*Xma*III) REDP of winter 1994 isolates belonging to the cluster *m1* (Lm47 and Lm48) and the isolates of the same cluster obtained two years before from the same type of cheese (Lm1, Lm3, Lm32 and Lm33). This fact suggests that genomic reorganizations have probably occurred with time from a single and common ancestor that originated from closely related variants in the *L. monocytogenes* population contaminating this cheese. On the other hand, strains belonging to the cluster *m4* were isolated from unrelated types of cheese in different seasons, suggesting that several strains may be widespread in natural environments and that they could contaminate these cheeses in preference to other strains.

Different S_i were obtained among *Listeria* REDP groups. In *L. innocua* species, two clearly distinct clusters were differentiated whereas the five clusters of *L. monocytogenes* were more closely related. In agreement with other authors' results, *L. monocytogenes* isolates belonging to serotypes 1/2a and 1/2b displayed greater variability than those of serotype 4b which constitutes a more homogeneous group (Carrière *et al.* 1991; Farber and Addison 1994; Moore and Datta 1994). It is worth noting that it was proved that there is a certain genetic proximity between *L. monocytogenes* clusters *m4* and *m5* (serotype 4b) and the reference strains ATCC 13932 and NCTC 11994, each of them responsible for a case of meningitis. It is also interesting to note that *L. monocytogenes* cluster *m4*, although not displaying any known epidemic phage type, has a phage susceptibility profile (3552: 1444: 1317: 3274: 52: 340: 312) that shows a certain proximity to the phage type of strains isolated during the Auckland (New Zealand) outbreak which occurred in 1969 (2389: 3552: 1444: 1317: 3274: 2671: 47: 108: 340: 312) (Le Souëf and Walters 1981). These data pointed to a wide distribution of several closely related *L. monocytogenes* types and suggests that several food products might constitute a reservoir of strains which might be responsible, under several circumstances, for the appearance of sporadic cases of listeriosis.

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