APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1998, p. 4729–4735 Vol. 64, No. 12
0099-2240/98/$04.00+0

An Ecological Study of Lactococci Isolated from Raw Milk in the Camembert Cheese Registered Designation of Origin Area

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Received 19 June 1998/Accepted 17 September 1998

The genetic diversity of lactococci isolated from raw milk in the Camembert cheese Registered Designation of Origin area was studied. Two seasonal samples (winter and summer) of raw milk were obtained from six farms in two areas (Bessin and Bocage Falaisien) of Normandy. All of the strains analyzed had a *Lactococcus lactis* subsp. *lactis* phenotype, whereas the randomly amplified polymorphic DNA (RAPD) technique genotypically identified the strains as members of *L. lactis* subsp. *lactis* or *L. lactis* subsp. *cremoris*. The genotypes were confirmed by performing standard PCR with primers corresponding to a region of the histidine biosynthesis operon. The geographic distribution of each subspecies of *L. lactis* was determined; 80% of the Bocage Falaisien strains were members of *L. lactis* subsp. *lactis*, and 30.5% of the Bessin strains were members of *L. lactis* subsp. *lactis*. A dendrogram was produced from a computer analysis of the RAPD profiles in order to evaluate the diversity of the lactococci below the subspecies level. The coefficient of similarity for 117 of the 139 strains identified as members of *L. lactis* subsp. *cremoris* was as high as 66%. The *L. lactis* subsp. *lactis* strains were more heterogeneous and formed 10 separate clusters (the level of similarity among the clusters was 18%). Reference strains of *L. lactis* subsp. *lactis* fell into 2 of these 10 clusters, demonstrating that lactococcal isolates are clearly different. As determined by the RAPD profiles, some *L. lactis* subsp. *lactis* strains were specific to the farms from which they originated and were recovered throughout the year (in both summer and winter). Therefore, the typicality of *L. lactis* subsp. *lactis* strains was linked to the farm of origin rather than the area. These findings emphasize the significance of designation of origin and the specificity of "Camembert de Normandie" cheese.

The specificity of Registered Designation of Origin (RDO) cheeses implies that these cheeses lose their specific characteristics when they are produced in other areas. This specificity is related to the race and nutrition of dairy cows, which determine the physical and chemical properties of the raw milk, and to basic traditional cheese-making practices. The microbiological aspect is also important because RDO cheeses are often made with raw milk which contains adventitious microflora, including lactococci. Most members of the dairy industry use starter cultures for rapid acidification because the small amounts of lactic acid bacteria in raw milk do not significantly acidify the milk. These starter cultures are selected and maintained by subculturing them in milk, which reduces the number of strains present. The use of such cultures is necessary and is partially responsible for the uniformity of the products, particularly pasteurized fermented milk products and cheeses. Raw milk microfloras, particularly the nonstarter lactic acid bacterial floras, increase the diversity of the flavors of RDO cheeses and therefore have been extensively studied (10, 17, 25). They may also be involved in producing the typical organoleptic characteristics of cheeses during ripening. Understanding the importance of the microfloras in the production of traditional cheeses requires discrimination of strains in a mixed population. Classical identification methods, such as physiological and biochemical tests, cannot differentiate organisms at the species and subspecies levels. New approaches, such as molecular characterization, have been developed in the last decade. Pulsed-field gel electrophoresis has been successfully used for lactococcal strain discrimination (16, 28), as has rRNA restriction gene analysis (14). In recent years, randomly amplified polymorphic DNA (RAPD) tests (34, 35) have been used for rapid typing of lactococcal strains (3, 4, 27). Unlike the other techniques, which are labor-intensive when they are used for rapid identification of organisms in multiple microbiological samples, the RAPD technique is fast and reliable, although its taxonomic efficiency is a matter of debate.

In a previous study (9), lactococcal microflora samples obtained at two dairy farms in the "Camembert de Normandie" area were characterized both phenotypically and genotypically. The specificity of the strains was correlated with their origins and led to further investigation. The effect of the geographic origin of such microorganisms on the manufacture and ripening of traditional cheeses is unknown. In soft ripened cheeses, lactococci are initially involved in lactic acid production, which lowers the pH. They are then involved in proteolysis (15) and, through their endo- and exocellular enzyme activities, in the production of aroma compounds during ripening.

The aims of this work were (i) to evaluate the genetic diversity of lactococcal strains isolated from raw milk in the RDO Camembert cheese area by using both phenotypic criteria (physiological and biochemical tests) and genotypic (RAPD) criteria, (ii) to assess the relatedness of the strains to reference strains of *Lactococcus* species, and (iii) to study the potential correlation of the organisms with their geographic origins.

MATERIALS AND METHODS

Reference strains. Sixteen reference strains were obtained from the Unité de Recherche Laitières et de Génétique Appliquée, Laboratoire de Génétique Microbienne (CNRZ and IL strains) (Institut National de la Recherche Agronomique, Jouy-en-Josas, France) and from the Reading Laboratory (NCDO strain) (Agriculture and Food Research Laboratory, Reading, England). On the basis of genetic criteria, these strains included *Lactococcus lactis* subsp. *lactis*

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TABLE 1. Distribution and phenotypes of lactococcal isolates obtained from raw milk from six dairy farms

Area	Farm	Season	No. of *Lactococcus*-like CFU/ml on selective medium[a]	Dilution plate	No. of colonies selected in 48 h[b]				No. of strains with *Lactococcus* phenotype	% of colonies selected with *Lactococcus* phenotype
					Total	Type 1	Type 2	Type 3		
Bessin	B	Winter	150	1/1	104	46	22	36	46	44
		Summer	120	1/1	59	39	20	0	38	64
	D	Winter	130	1/1	86	8	39	39	13	15
		Summer	650	1/5	59	59	0	0	50	85
	M	Winter	120	1/1	82	28	52	2	27	33
		Summer	100	1/1	60	40	20	0	10	17
Bocage Falaisien	G	Winter	500	1/10	87	22	65	1	18	21
		Summer	200	1/1	72	66	6	0	28	39
	C	Winter	500	1/5	122	50	30	42	50	41
		Summer	250	1/5	41	41	0	0	29	71
	Z	Winter	600	1/5	118	67	17	34	60	51
		Summer	1,000	1/10	87	87	0	0	20	23

[a] Selective medium is described in the text.
[b] Type 1 colonies were oval and ochre, had diameters of ≥1 mm, and had yellow halos that were more than 3 mm in diameter; type 2 colonies were oval and ochre, had diameters of ≥0.5 mm, and had yellow halos that were less than 3 mm in diameter; and type 3 colonies were irregular and had pale yellow halos that were less than 3 mm in diameter.

CNRZ142^T (= NCDO604^T = ATCC 19435^T) (T = type strain), CNRZ157, IL1403, NCDO176^T (previously the type strain of *Streptococcus diacetylactis*), CNRZ124 (= NCDO1007 = DRC1), CNRZ1337 (= Bu2-60), CNRZ194, and CNRZ365 and *L. lactis* subsp. *cremoris* CNRZ105^T (= NCDO607^T = ATCC 19257^T = HP^T), CNRZ359, CNRZ379 (= NCDO1991 = AM1), CNRZ123, CNRZ109 (= NCDO508 = C7), CNRZ144 (= C2), CNRZ156, and ML3.

Selection of dairy farms. Six dairy farms in two dairy areas of the RDO Camembert cheese zone were selected based on their high-quality raw milk (which contained less than 5×10^4 microorganisms per ml). Three of these farms (farms B, D, and M) were on the English Channel coast in the Bessin region and were 100 km away from the other three farms, (farms C, G, and Z), which were inland in the Bocage Falaisien region (Table 1). At each farm, raw milk samples (two milkings) were collected in the winter (when the dairy cows were housed) and in the summer (when the cattle were on pasture).

Isolation of lactococci. Raw milk samples were kept refrigerated (4°C) until analysis. They were plated as previously described (8) on plate count agar (Biokar Diagnostics, Beauvais, France) containing 10% (vol/vol) sterile skim milk, 20 mg of bromcresol purple per liter, 40 mg of nalidixic acid per liter, and 20 mg of Delvocid (Gist Brocades, Seclin, France) per liter, which corresponded to 10 mg of natamycin per liter. The plates were incubated for 48 h at 30°C, and 40 to more than 100 acidifying colonies per plate were recovered at the appropriate dilution. Isolates were purified by subculturing them on M17 agar supplemented with 5 g of lactose per liter (29). A final subculture was prepared by using fast-slow differential agar (12) to test the lactose use and proteinase activity of the strains. Cells were frozen and stored at −80°C in M17 medium containing 5 g of lactose per liter and 15% glycerol.

Phenotypic characterization. Gram-positive and catalase-negative isolates were first analyzed at the genus level. We assessed growth in brain heart infusion broth (AES Laboratories, Combourg, France) at 10°C for 1 week, at 45°C, at pH 9.6, and in the presence of 6.5% NaCl for 96 h, as well as growth in litmus milk.

The isolates identified as members of the genus *Lactococcus* were further characterized at the subspecies level by performing the following tests: arginine hydrolysis (32), growth in the presence of 4% NaCl and at 40°C in brain heart infusion broth, and ability to ferment maltose in red phenol broth (9). Strains with a *L. lactis* subsp. *lactis* phenotype were also tested for acetoin production by the Voges-Proskauer reaction.

DNA extraction. Bacterial cells were grown overnight at 30°C in M17 broth containing 5 g of glucose per liter. Genomic DNA was isolated by phenol-chloroform extraction as previously described (18), except that protoplasts were obtained by incubation with a 100-mg liter^{-1} lysozyme solution for 1 h at 37°C.

PCR amplification. Random DNA fragments were amplified with an Amplitron II thermocycler (Bioblock Scientific, Illkirch, France) by using a single primer having an arbitrary nucleotide sequence and a G+C content of 70% (5′ TGCTCTGCCC 3′) (Isoprim, Toulouse, France). The reaction mixture was treated as described elsewhere (26). *L. lactis* subspecies-specific bands were amplified by using primers specific for a region of the histidine biosynthesis operon of *L. lactis* (forward primer 5′ CTTCGTTATGATTTTACA 3′ and reverse primer 5′ CAATATCAACAATTCCAT 3′) (Isoprim). The corresponding base positions were positions 671 to 688 and 1587 to 1604 (*L. lactis* subsp. *lactis* NCDO 2118 numbering [7]). The amplification reaction involved initial denaturation at 94°C for 5 min, 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 45°C for 2 min, and extension at 72°C for 2 min, and a final extension step consisting of 72°C for 5 min. The RAPD and PCR products were stored at 4°C until analysis.

DNA analysis. RAPD and PCR products were loaded onto a 1% SeaKem GTG agarose gel (Tebu, Seclin, France) and electrophoresed in TBE (Tris-borate-EDTA [pH 8]) buffer. DNA molecular weight markers (123-bp DNA ladder; Life Technologies, Cergy Pontoise, France) were used as size standards. The DNA fragments were stained with ethidium bromide, viewed under UV (254-nm) light, and photographed on Polaroid 665 film (PolyLabo, Strasbourg, France).

Computer analysis of RAPD profiles. The photographic negatives were scanned (ScanJet IIcx/T; Hewlett Packard, Evry, France), and the data were recorded with Desk Scan 2 software (Hewlett Packard). The band patterns were then normalized and processed by using the GelCompar 3.1 program (Applied Maths, Kortrijk, Belgium). The densitometric traces were analyzed by using Pearson's product moment correlation coefficient (33) and were clustered by using the unweighted pair group method with arithmetic averages (UPGMA) (24). Variance analysis was performed with the StatView software (Abacus Concepts, Inc., Berkeley, Calif.). The reproducibility of the RAPD technique was evaluated by studying three strains in duplicate (separate cell preparations and PCR were used for individual strains).

RESULTS

Phenotypic characterization of dairy lactococci. There were significant differences in the *Lactococcus*-like microfloras of the farms, as determined on plate count agar plates; the sizes of the populations ranged from 100 to 1,000 CFU per ml of raw milk, and the average was 350 CFU · ml^{-1}) (Table 1). The seasonal variations in the population were minor on each farm except farm D. Phenotypic characterization of the isolates revealed changes in the quantitative composition of the lactococci in raw milk; 34% of the isolates recovered in winter were lactococci, and more than 50% of the isolates recovered in summer were lactococci. These differences correlated with the type of colony rather than with the sampling season. The winter isolates were classified into three categories on the basis of the diameters of the colonies and the yellow halos of acidification detected with bromcresol purple. The phenotype and genotype analysis of strains collected in the winter (the first sampling period) identified mainly large colonies with good acidification (type 1) (Table 1) as lactococci. Therefore, only strains with these characteristics were selected from summer samples, which resulted in the higher percentage of lactococci recovered. One exception was the summer sample from farm M; most of the colonies obtained from this sample were small and slightly acidifying, and 17% of the colonies selected had a *Lactococcus* phenotype. A few samples were contaminated with enterococci, which grow at 45°C (lactococci do not grow at this temperature). This was confirmed by an API 50CHS identification analysis (data not shown). Raw milk from the farm D winter sample contained more than 50% enterococci. Unlike the colonies of the more slowly growing lactobacilli and *Leuco-*

TABLE 2. Geographic distribution of *L. lactis* subspecies isolates classified by the RAPD method

Area	Farm	Season	No. of isolates with:		G/P (%)[a]	% of isolates with:		*L. lactis* subsp. *lactis* genotype/*L. lactis* subsp. *cremoris* genotype (%)
			Lactococcus phenotype	*Lactococcus* genotype		*L. lactis* subsp. *lactis* genotype	*L. lactis* subsp. *cremoris* genotype	
Bessin	B	Winter	46	38	83	61	39	39.5/60.5[b]
		Summer	38	34	89	74	26	
	D	Winter	13	11	85	27	73	
		Summer	50	49	98	35	65	
	M	Winter	27	15	56	7	93	
		Summer	10	9	90	33	67	
Bocage Falaisien	G	Winter	18	16	89	94	6	78/22[b]
		Summer	28	26	93	46	54	
	C	Winter	50	48	96	94	6	
		Summer	29	25	86	100	0	
	Z	Winter	60	58	97	59	41	
		Summer	20	20	100	75	25	

[a] (Number of isolates with *Lactococcus* genotype/number of isolates with *Lactococcus* phenotype) × 100.
[b] The difference between the two areas was significant at $P < 0.05$, as determined by variance analysis.

nostoc species, colonies of lactococci and enterococci had similar characteristics on selection broth and therefore could not be distinguished at this stage. An inability to grow at pH 9.6 or in the presence of 6.5% NaCl was not considered specific for lactococcal identification because too few strains would have been characterized. Electron microscopy of some of the strains showed that they were not contaminated, particularly by enterococci (data not shown).

A total of 389 isolates had a *Lactococcus* phenotype, and most grew at pH 9.6 and in the presence of 6.5% NaCl. Most strains reduced, acidified, and coagulated litmus milk, but all of the strains identified at the subspecies level had an *L. lactis* subsp. *lactis* phenotype. Thus, all of the strains grew at 40°C and in the presence of 4% NaCl, hydrolyzed arginine, and produced acid from maltose. Five strains produced acetoin and were classified as members of biovar diacetylactis of *L. lactis* subsp. *lactis*. The phenotypes of 16 reference strains of lactococci were confirmed. Nevertheless, unlike the wild type, the reference strains had typical phenotypes, as most of them failed to grow in the presence of 6.5% NaCl. In addition, four of eight *L. lactis* subsp. *cremoris* strains were identified both phenotypically and genotypically as members of this subspecies; these four strains were CNRZ105^T, CNRZ379, CNRZ123, and CNRZ109.

Genotypic characterization of *Lactococcus* isolates. The genotypes of the 389 strains that had an *L. lactis* subsp. *lactis* phenotype were analyzed by the RAPD method. The results are presented in Table 2 and Fig. 1. All of the RAPD patterns were digitized with GelCompar software and were normalized by comparison with reference bands at 123 to 2,460 bp. The RAPD profiles that had bands in this size range were then analyzed by calculating Pearson's correlation coefficient and were clustered by using the UPGMA method. The resulting dendrogram grouped strains in clusters and subclusters; 40 strains were discarded because they produced atypical patterns. Therefore, on the basis of the RAPD profiles of the 16 reference strains, the wild-type lactococci were grouped into two main clusters. A total of 226 strains (8 reference strains and 218 wild-type strains) were identified as members of *L. lactis* subsp. *lactis*, and 139 strains (8 reference strains and 131 wild-type strains) were identified as members of *L. lactis* subsp. *cremoris*. The percentages of agreement between phenotype and genotype assignments were more than 80% except for the winter sample from farm M (percentage of agreement, 56%). This high percentage reconciles phenotypic and genotypic identifications at the genus level. The distributions of the two subspecies of *L. lactis* in the two dairy areas for the two sampling periods are shown in Table 2. A variance analysis showed that *L. lactis* subsp. *lactis* strains were the predominant strains ($P < 0.05$ [0.0266]), accounting for 60.5% of the strains in all raw milk samples, in the Bocage Falaisien area, whereas *L. lactis* subsp. *cremoris* strains were the predominant strains, accounting for 78% of the strains, in the Bessin area.

Genetic diversity of lactococcal strains. The genetic diversity of lactococcal strains was assessed by examining the clustering on the two dendrograms. Figure 1A and Table 3 show that the *L. lactis* subsp. *lactis* strains grouped into 10 clusters with coefficients of similarity ranging from 47 to 75%. The distribution of the strains could not be correlated with the area in which the samples were obtained because no cluster contained only strains isolated from the three dairy farms in one area. In addition, the samples from the Bocage Falaisien area, described above, contained a higher proportion of *L. lactis* subsp. *lactis* strains than samples from the Bessin area, which made area comparisons more difficult. Nevertheless, similar profiles of strains were obtained for winter and summer samples from some dairy farms (Table 3). In cluster L2, similar strains were obtained from farms B and C during the two sampling periods. Some strains from farm Z and other strains from farm C belonging to clusters L8 and L6, respectively, were present in both the winter and the summer. The farm Z strains in cluster L6 were typical of their farm of origin and were not isolated from samples from any other farm. All of the cluster L10 strains were also specific but not well-established because they originated from the winter sample from only one farm, farm G. Finally, the eight reference strains clustered in clusters L4 and L5, which contained only 21 of the 226 strains genotypically identified as *L. lactis* subsp. *lactis*.

Strains having the *L. lactis* subsp. *cremoris* genotype formed three groups and produced a single pattern, corresponding to the pattern produced by reference strain CNRZ123 (Fig. 1B). The main cluster (cluster C1) contained 84% (117 of 139) of the *L. lactis* subsp. *cremoris* strains and had a coefficient of similarity of 66% (Table 3). The four strains in cluster C3 were isolated in the winter from samples from farm M. Their identical RAPD profiles and common origin suggest that these organisms are multiple isolates of the same strain. Reference strains of *L. lactis* subsp. *cremoris* were found in all clusters, but, surprisingly, strains CNRZ144 (= C2) and ML3, which were both derived from commonly used strain NCDO712, pro-

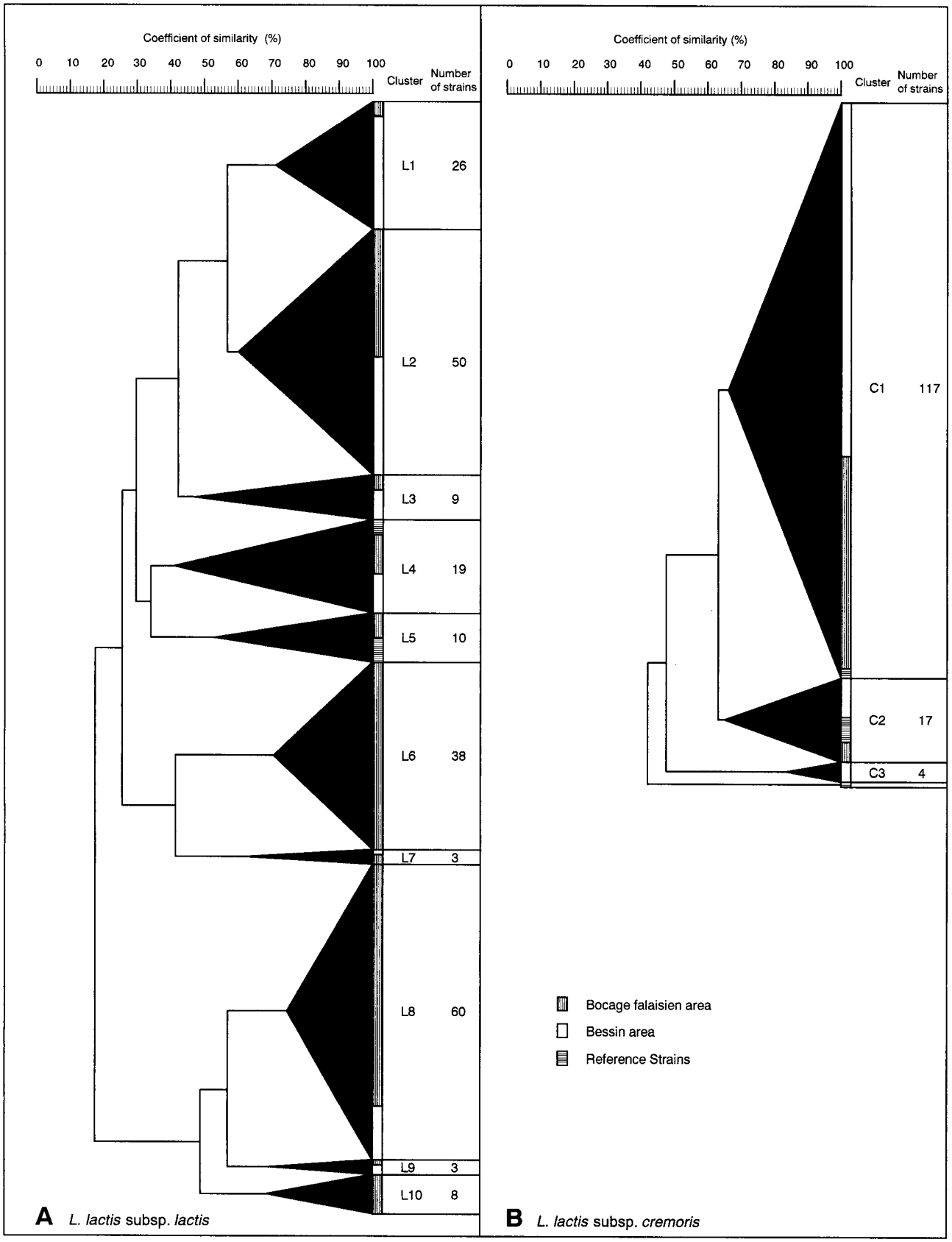

FIG. 1. Dendrogram clustering wild and reference *Lactococcus* strains. Normalized RAPD patterns were analyzed by using Pearson's product moment correlation coefficient and were clustered by the UPGMA method. (A) *L. lactis* subsp. *lactis* strains. (B) *L. lactis* subsp. *cremoris* strains.

TABLE 3. Distribution of wild and reference strains in the clusters of the two *L. lactis* subspecies (strains were analyzed by standard PCR targeting the *his* operon)

Subspecies	Cluster	Coefficient of similarity (%)	Total no. of strains	No. of strains from:														
				Bessin area							Bocage Falaisien area							No. of collection strains
				Total	Farm B		Farm M		Farm D		Total	Farm C		Farm G		Farm Z		
					Winter	Summer	Winter	Summer	Winter	Summer		Winter	Summer	Winter	Summer	Winter	Summer	
L. lactis subsp. *lactis*	L1	71	26	23	2	12[a]				9	3					3		0
	L2	60	50	21	10	11					29	20[b]	6		3			0
	L3	47	9	6						6	3	1			2[c]			0
	L4	41	19	8	3	2	1		1	1[c]	8	2[c]		2	2	2		3[d]
	L5	53	10	0							5			3[c]			2	5[e]
	L6	70	38	0							38	20[a]	18[a]					0
	L7	62	3	1	1						2	2						0
	L8	75	60	11	5			3[c]	2	1	49			2	5	29[a]	13[c]	0
	L9	69	3	2	2						1		1					0
	L10	68	8	0							8			8[a]				0
	Total		226	72	23	25	1	3	3	17	146	45	25	15	12	34	15	8
L. lactis subsp. *cremoris*	C1	66	117	72	15[a]	9[c]	10[c]	6[a]	8[c]	24[a]	43	3[c]		1	11[c]	24	4	2[f]
	C2	65	17	8						8[c]	4				3		1	5[g]
	C3	83	4	4			4[c]				0							0
	C4	100	1	0							0							1[h]
	Total		139	84	15	9	14	6	8	32	47	3	0	1	14	24	5	8

[a] Two wild-type strains.
[b] Three wild-type strains.
[c] One wild-type strain.
[d] Three reference strains (strains IL1403, CNRZ194, and CNRZ142^T).
[e] Five reference strains (strains CNRZ365, CNRZ124, CNRZ157, CNRZ1337, and NCDO176).
[f] Two reference strains (strains NRZ156 and ML3).
[g] Five refernece strains (strains CNRZ359, CNRZ109, CNRZ144, CNRZ379, and CNRZ105^T).
[h] One reference strain (strain NRZ123).

duced different RAPD profiles. In addition to minor modifications (short deletions or insertions), these two strains differed in that the conjugative element of CNRZ144 (= C2) has been excised (−55 kb), whereas ML3 has a large chromosome inversion that is 1,300 kb long (5a). Moreover, ML3 has a different lysogenic status because, unlike strains NCDO712 and CNRZ144 (= C2), treatment with UV light does not result in lysis of this organism (6). Therefore, strain ML3 may have been cured of its prophage, φT712. The lower level of heterogeneity among *L. lactis* subsp. *cremoris* patterns was due to the 10-bp primer used, which was not discriminating enough for this subspecies; this prevented estimation of the diversity among strains.

Confirmation of RAPD classification by standard PCR. The use of the RAPD technique for bacterial typing is controversial, so the identities of the genotypes of strains determined by this method were confirmed by a standard PCR assay. A primer pair corresponding to a region of the histidine biosynthesis operon was selected because of its *L. lactis* species specificity. Based on its size, the resulting fragment was subspecies specific (7). *L. lactis* subsp. *cremoris* strains have a DNA sequence of about 200 bp between the *orf3* and *hisC* genes. This sequence consists of various numbers of 59-bp repeats, which are not present in *L. lactis* subsp. *lactis* strains. We corroborated the classification obtained with the RAPD analysis by testing at least one strain belonging to each cluster on the *L. lactis* subsp. *lactis* dendrogram and up to four strains belonging to groups containing more than 30 strains by PCR. *L. lactis* subsp. *cremoris* strains that occurred in the three clusters on the *L. lactis* subsp. *cremoris* dendrogram were analyzed in the same way. All of the reference strains and 32 wild-type lactococcal strains (19 *L. lactis* subsp. *lactis* strains and 13 *L. lactis* subsp. *cremoris* strains) were analyzed by this standard PCR assay (the strains tested are listed in Table 3). The results confirmed the original genotype assignments made by the RAPD technique. For *L. lactis* subsp. *lactis* strains, the expected 933-bp fragment was amplified, whereas the size of the amplified band for *L. lactis* subsp. *cremoris* strains was 1,100 to 1,150 bp, depending on the number of 59-bp repeats in the insertion sequence (5).

DISCUSSION

A nonspecific medium, plate count agar enriched with milk, which is commonly used for counting all microorganisms, was used to isolate wild-type lactococci from a complex microflora. This medium was made specific for screening lactococci by adding two antibiotics, nalidixic acid (to inhibit the development of gram-negative bacteria) and natamycin (to inhibit the growth of yeasts and molds) (8). At the concentrations used, these antibiotics had no negative effects on the growth of lactococci. The recovery of isolates of lactococci from natural sources depends on the selective medium used. Tornadijo et al. (31) compared the selectivity of various media, including M17, Rogosa, MSE, and KAA, for isolating lactic acid bacteria from raw milk. These authors showed that M17 and MSE were the most suitable media for recovery of lactococci; 50 and 45% of all isolates were recovered with M17 and MSE, respectively. Colony hybridization with ribosomal DNA probes (1, 21) is an efficient technique for screening *Lactococcus* species but is time-consuming.

The standard methods used for phenotype characterization can give ambiguous responses with lactococcal strains. Growth of natural lactococci at pH 9.6 or in the presence of 6.5% salt is not surprising because these microorganisms survive in hostile conditions and are commonly confronted by many stresses. Thus, phenotypic characterization of environmental lactococci is unclear, and many strains are misclassified at both the genus and species levels. In this investigation, the only reliable criterion for differentiating enterococci from lactococci (which were indistinguishable on the selective medium) was the inability of *Lactococcus* strains to grow at 45°C. All of the strains had an *L. lactis* subsp. *lactis* phenotype, but genotype analysis showed that they could be grouped into two main clusters. One cluster included the *L. lactis* subsp. *lactis* strains, whereas the other was composed of *L. lactis* subsp. *cremoris* strains. It is known that there is divergence between the phenotypic and genotypic assignments of the two *L. lactis* subspecies (11, 20). Although most strains with an *L. lactis* subsp. *lactis* genotype have the same phenotype, strains with an *L. lactis* subsp. *cremoris* genotype can be grouped into two phenotypes, the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* phenotypes, on the basis of the following criteria: growth at pH 9.2, at 40°C, and in the presence of 4% salt, ability to ferment maltose, and ability to hydrolyze arginine. *L. lactis* subsp. *lactis* strains are commonly isolated from environmental sources, whereas strains with an *L. lactis* subsp. *cremoris* phenotype are isolated only from dairy environments, especially from fermented milk products in which starters are often used. Salama et al. (20) isolated strains from plant material, such as corn samples, and these strains had an *L. lactis* subsp. *lactis* phenotype but an *L. lactis* subsp. *cremoris* genotype, as determined by hybridization with a 16S rRNA-targeted *L. lactis* subsp. *cremoris*-specific probe (probe 68Rca). The most recently isolated natural strains with an *L. lactis* subsp. *cremoris* phenotype and genotype were obtained from Moroccan, Chinese, Yugoslavian, and Ukrainian raw milk samples by the same investigators (22). These milk samples, unlike other samples, spontaneously fermented because they were naturally enriched. The habitat of the organisms remains uncertain because strains which have this particular *L. lactis* subsp. *cremoris* phenotype may not survive in nature and are exclusively confined to dairy environments (13). The use of *L. lactis* subsp. *cremoris* to improve industrial performance may have resulted in a loss of functions that are vital for the survival of the organisms outside a dairy environment.

In a previous study in which the same primer was used (9), the RAPD method was shown to be reliable for identifying lactococcal strains because rRNA restriction gene analysis confirmed the RAPD classification. This method recognizes duplicates that have the same RAPD profile, similar phenotypic characteristics, and a common origin. The peculiar distribution of *L. lactis* subspecies observed in this study seems to be linked to areas but restricted to limited zones, because the three farms in each area were close together (less than 10 km apart). No significant differences in the breed of cows (each area had farms with Normandy and Holstein milking cows) or feeding practices could account for the distribution; a possible exception is the types of grass in the different areas. The Bessin area is located near the coast and has inundated plains with high salinity in which *L. lactis* subsp. *cremoris* strains are surprisingly common despite the fact that this subspecies is known to have a higher salt sensitivity than *L. lactis* subsp. *lactis*. Some *L. lactis* subsp. *lactis* strains were typical of the farm from which they originated and were consistently present (in both the winter and the summer) in raw milk samples. Therefore, the specificity of lactococcal strains, especially those with an *L. lactis* subsp. *lactis* genotype, was associated with farm practices rather than with the area of origin. The RAPD technique, performed with a 10-bp arbitrary primer, gave better discrimination within *L. lactis* subsp. *lactis* that within *L. lactis* subsp. *cremoris*. The clustering of the eight *L. lactis* subsp. *lactis* reference strains in only 2 of the 10 groups shows the typicality and diversity of lactococci isolated in the RDO Camembert cheese area. Indeed, many *L. lactis* subsp. *lactis* strains from

each producer were widely distributed in the various clusters on the dendrogram; examples are the strains which were isolated in the winter from samples from farm B), which were dispersed in 6 of the 10 clusters.

Only a few large ecological studies of lactococci have been undertaken (22, 23), and no investigation of the genetic diversity and establishment of strains in well-defined regions has been reported. Our results suggest that wild-type lactococci isolated from raw milk are a potential source of new strains with particular properties. It is well-known that traditional cheeses made with raw milk ripen faster and develop a more intense flavor than cheeses made with pasteurized or microfiltered milk. McSweeney et al. (19) and Bouton and Grappin (2) showed that the adventitious microflora was responsible for this flavor enhancement and therefore makes a significant and positive contribution to cheese quality. In addition, Thomas (30) showed that nonstarter lactic acid bacteria, especially pediococci and lactobacilli, grow on products released during autolysis of starter cells, which supports the idea that the raw milk microflora is important for ripening. The specificity and the consistent presence of wild-type lactococcal strains scientifically confirm the dairy significance of the RDO Camembert cheese area. Reference strains had RAPD profiles different from those of wild-type lactococci, but investigations to evaluate whether such typical strains are recovered in other areas must be undertaken to definitively show that the more geographically specific the strains are, the more typical is the Camembert cheese produced.

ACKNOWLEDGMENTS

We thank Jocelyne Perreux for technical assistance and Patrick Tailliez for collaboration in the RAPD pattern analysis performed with the Gel Compar program.

This work was supported by a grant from the Regional Council of Basse Normandie, by the Syndicat Normand des Fabricants de Camembert, (SNFC) and by European Funds for Regional Development (FEDER).

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