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Molecular Fingerprinting of Dairy Microbial Ecosystems by Use of Temporal Temperature and Denaturing Gradient Gel Electrophoresis

J.-C. Ogier,[1]* V. Lafarge,[2] V. Girard,[1,2] A. Rault,[2] V. Maladen,[2] A. Gruss,[1] J.-Y. Leveau,[3] and A. Delacroix-Buchet[1]

Unité de Recherches Laitières et Génétique Appliquée, Institut National de la Recherche Agronomique, Jouy-en-Josas,[1] *Unité Composition et Hygiène des Produits Laitiers, Agence Française de Sécurité Sanitaire des Aliments, Maisons-Alfort,*[2] *and Département de Microbiologie, Ecole Nationale Supérieure des Industries Alimentaires, Massy,*[3] *France*

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Numerous microorganisms, including bacteria, yeasts, and molds, constitute the complex ecosystem present in milk and fermented dairy products. Our aim was to describe the bacterial ecosystem of various cheeses that differ by production technology and therefore by their bacterial content. For this purpose, we developed a rapid, semisystematic approach based on genetic profiling by temporal temperature gradient electrophoresis (TTGE) for bacteria with low-G+C-content genomes and denaturing gradient gel electrophoresis (DGGE) for those with medium- and high-G+C-content genomes. Bacteria in the unknown ecosystems were assigned an identity by comparison with a comprehensive bacterial reference database of ~150 species that included useful dairy microorganisms (lactic acid bacteria), spoilage bacteria (e.g., *Pseudomonas* and *Enterobacteriaceae*), and pathogenic bacteria (e.g., *Listeria monocytogenes* and *Staphylococcus aureus*). Our analyses provide a high resolution of bacteria comprising the ecosystems of different commercial cheeses and identify species that could not be discerned by conventional methods; at least two species, belonging to the *Halomonas* and *Pseudoalteromonas* genera, are identified for the first time in a dairy ecosystem. Our analyses also reveal a surprising difference in ecosystems of the cheese surface versus those of the interior; the aerobic surface bacteria are generally G+C rich and represent diverse species, while the cheese interior comprises fewer species that are generally low in G+C content. TTGE and DGGE have proven here to be powerful methods to rapidly identify a broad range of bacterial species within dairy products.

Numerous dairy products are home to a complex microbial ecosystem, which is responsible for the broad diversity of tastes, aromas, and textures that are associated with them. Many bacteria make a positive contribution to the organoleptic qualities of cheeses or fermented milk, while others may have adverse effects or may even constitute a health risk. Cheese processing is largely based on fermentation by lactic acid bacteria (LAB), which are both deliberately added as starter cultures or adventitiously present in the biotope and selected during the fermentation process. Furthermore, raw milk bacteria, including nonstarter LAB, reportedly enhance cheese flavor and diversity (30, 32, 42). Ripened cheeses are characterized by a succession of largely undefined microbial communities on their surface (6, 59). These aerobic microorganisms have a strong impact on the appearance, odor, flavor, and texture development of the respective cheese products (6). Nondesirable microorganisms, such as the psychrotrophic *Pseudomonas fluorescens* (52) or certain proteolytic LAB, may cause flavor defects (e.g., bitterness and putrid flavors) in milks and cheeses (7, 8, 51). The presence of *Escherichia coli*, *Listeria monocytogenes*, and *Staphylococcus aureus* in raw milks and cheeses constitutes a health risk (3, 10, 12, 33, 41, 49). The above descriptions illustrate the present indeterminate state of the relatively complex cheese ecosystem. Enumeration of dairy microorganisms was previously based on bacterial cultivation, followed by identification of the dominating microorganisms by phenotypic methods (11, 18, 53). These approaches are tedious, restricted to cultivatable bacteria, and liable to introduce serious biases to community analyses (4, 50). Recent advances in molecular biology and phylogeny analysis techniques have opened the field of microbial ecology and have replaced the less-accurate bacteriological tests. Molecular approaches based on 16S rRNA genes (rDNAs) have facilitated a culture-independent approach for analysis of complex ecosystems (1, 2, 23, 40). In particular, single-strand conformation polymorphism, temporal temperature gradient gel electrophoresis (TTGE), and related denaturing gradient gel electrophoresis (DGGE) methods of PCR-amplified rDNA fragment separation have been applied to a variety of environmental studies for analyzing microbial communities (13, 17, 19, 20, 35, 36, 39, 45, 60). In both TTGE and DGGE, DNA fragments of the same length but with different sequences are separated, based on decreased electrophoretic mobility of partially melted double-strand DNA molecules. Separation is performed with polylacrylamide gels containing a linear gradient of chemical denaturant gradient (DGGE) or a linear temperature gradient (TTGE). TTGE and DGGE are now frequently

* Corresponding author. Mailing address: Unité de Recherches Laitières et Génétique Appliquée, Institut National de la Recherche Agronomique, Domaine de Vilvert, 78352 Jouy-en-Josas, France. Phone: (33) 1 34 65 20 97. Fax: (33) 1 34 65 20 65. E-mail: ogier@jouy.inra.fr.

applied in microbial ecology to compare the compositions of complex microbial communities and study their dynamics.

We recently applied TTGE to describe the diversity of LAB in commercial dairy products by setting up a bacterial database that allows rapid identification of the unknown bands (39). This database essentially included bacteria with a low-G+C-content genome, i.e., numerous LAB and a few dairy *Staphylococcus* species. In the present study, we modified our approach to expand the bacterial database to other species of dairy interest, including psychrotrophic and spoilage bacteria, pathogens, and bacteria present on the cheese surface. One limitation of TTGE is the poor resolution of species having high-G+C-content genomes. We therefore made combined use of TTGE and DGGE, which is more suitable for these bacterial species. Using this combined approach, new strains were identified, and the profiles of commercial cheeses were described and compared. Our results confirm the usefulness of these approaches for identifying different cheese ecosystems and have revealed some unexpected features of the flora that distinguish the cheese surface from the interior.

MATERIALS AND METHODS

Choice of bacterial strains. The bacterial strains used in this study for the construction of the database species are listed in Table 1. They originate from culture collections or correspond to bacteria isolated from raw milk and dairy environments (see below for the species identification). We selected 150 bacterial species of dairy interest, including useful dairy microorganisms (LAB), spoilage bacteria (e.g., *Pseudomonas* and *Enterobacteriaceae*) and a few pathogenic bacteria (e.g., *Listeria* species and *Staphylococcus aureus*). Several strains from each species group were generally selected, except in cases where only one strain was available. Genomic DNA was prepared as previously described (14).

Bacterial species identification from raw milk isolates. The strains isolated from different medium plates were purified and characterized by biochemical tests (Gram stain coloration, mobility, and catalase and oxidase tests) and the API system (BioMérieux, Marcy-l'Etoile, France) to identify the species level (Table 1).

PCR amplification. TTGE samples were prepared by performing two successive PCRs with the GenAmp system, model 2400 (Perkin-Elmer, Courtaboeuf, France). First, a 700-bp fragment of the 16S rDNA including the V3 region was amplified with primers W01 and W012 as previously described (39). Second, the 700-bp fragment was used to amplify the V3 region with primers HDA1-GC and HDA2 as previously described (39). PCR mixtures and the amplification program were the same as described by Ogier et al. (39). Sizes and quantities of PCR products were determined by 2% agarose gel electrophoresis (Seakem CTG agarose; TEBU, Le Perray-en-Yvelines, France).

TTGE analysis. PCR products obtained from V3 region amplification were submitted for TTGE analyses. TTGE was performed with the DCode universal mutation detection system (Bio-Rad, Marnes-la-Coquette, France) on 16 cm × 16 cm × 1 mm gels. Polyacrylamide gels (8%) were prepared and run with 1× TAE buffer diluted from 50× TAE buffer (2 M Tris base, 1 M glacial acetic acid, and 50 mM EDTA). Gels were prepared with 8% (wt/vol) acrylamide stock solutions (37.5:1) and a final urea concentration of 6 M. Five-microliter samples of PCR products (~100 ng of DNA) were deposited in wells, under previously described running conditions (39). After runs, gels were stained for 15 min with an ethidium bromide solution (0.5 μg/ml of 1× TAE buffer), rinsed for 20 min in 1× TAE buffer, and photographed on a UV transillumination table.

DGGE analysis. Denaturing gradient gel electrophoresis analysis of the V3 amplicons was performed with the Bio-Rad DCode universal mutation detection system. The denaturing gradient gel contained a 40 to 70% gradient of urea and formamide increasing in the direction of electrophoresis. A 100% denaturing solution consisted of 7 M urea and 40% (vol/vol) deionized formamide. Electrophoresis was conducted with 1× TAE buffer (92 V at 60°C for 16 h for one gel). DNA bands were visualized as for TTGE analysis.

Gel analysis and reference database setup. TTGE and DGGE gels were standardized by including an identification ladder made up of reference species. The ladder consisted of four bacterial strains for the low-G+C-content (TTGE) conditions (39) and six bacterial strains for the high-G+C-content (DGGE) conditions (*Kytococcus sedentarius* CNRZ880, *Arthrobacter citreus* CNRZ928^T, *Micrococcus kristinae* CNRZ872, *Bacillus pumilus* ATCC7725, *Propionibacterium jensenii* Z87). An ordered data set was generated with GelCompar software (Applied-Maths, Sint-Martens-Latem, Belgium), a data-processing tool. For this purpose, the photographed gels were converted into a file image, which was then analyzed by GelCompar. The software standardizes TTGE and DGGE profiles to minimize migration differences between gels (39). The molecular fingerprints of each bacterial species were integrated into the GelCompar database. We also used WinMelt software (Bio-Rad), which calculates the melting temperature (T_m) of PCR-amplified fragments (25) to predict their migration positions on TTGE and DGGE gels.

Commercial cheeses. (i) Identification of bacterial species in commercial cheeses by culture-dependent and culture-independent methods. We chose four commercial cheeses that were made according to different technologies. We analyzed two raw milk cheeses: Morbier (France), a semihard cheese, and Munster (France), a red smear cheese. We also analyzed two pasteurized milk cheeses: Epoisses (France), a red smear cheese, and Leerdamer (The Netherlands), a Swiss-type cheese.

(ii) Comparison of bacterial microflora present in the core versus the cheese surface. Analyses were performed with six different commercial cheeses (sample cheeses different than those used above): two Swiss-type cheeses, Comté (France) and Beaufort (France); two semihard cheeses, Saint Nectaire (France) and Morbier (France); and two red-smear cheeses, Epoisses (France) and Langres (France). For each type of commercial cheese, we analyzed samples from two different producers. Four samples were tested per cheese; two originated from the cheese core, and two originated from the cheese surface.

Determination of bacterial counts. Cheese samples (each, 10 g of nonfractionated cheese) were emulsified in 100 ml of sterile 2% (wt/vol) trisodium citrate (Merck Eurolab, Fontenay-sous-Bois, France) and homogenized with an Ultra-Turrax mechanical blender at 19,000 rpm for 45 s (T-25 IKA; Labo Moderne, Paris, France) to disrupt lactococcal chains (29). Serial dilutions were prepared in sterile 1% (wt/vol) peptone (Merck Eurolab) and plated on selective agar medium with a spiral plater (Spiral System, Cincinnati, Ohio). Lactococci and streptococci on M17 agar (Difco, Elancourt, France) were counted after 48 h of incubation at 30 or 42°C (56). Lactobacilli were counted on modified MRS agar plates (Difco) (pH adjusted to 5.2) after incubation for 72 h in anaerobic conditions, either at 30°C for mesophilic lactobacilli or at 42°C for thermophilic lactobacilli (15). The *Leuconostoc* population was estimated on MSE agar (Difco) after 48 h of incubation at 30°C (31).

Coryneforms were isolated on brain heart infusion (BHI) medium (Difco) supplemented with 5% (wt/vol) NaCl, and plates were incubated at 25°C for 3 days, following an exposure to daylight for 4 days at room temperature to enhance pigment production (J. J. Gratadoux, URLGA, personal communication). Staphylococci were isolated on mannitol salt agar medium (Difco) supplemented with 1% (wt/vol) calcium bicarbonate, and plates were incubated at 37°C for 2 days (47). Enterococci were numerated on bile esculin azide agar medium (Difco) after incubation at 37°C for 3 days under anaerobic conditions (46). Propionibacteria were isolated on yeast extract-sodium lactate medium, and plates were incubated at 25°C for 7 days under anaerobic conditions (58). Gram-negative bacteria were isolated on violet red bile agar medium (Difco), by incubation for 2 days at 30°C (48).

Genomic DNA extraction in cheeses. Cheese samples for Morbier, Epoisses, Leerdamer, and Munster cheeses (each sample, 5 g of nonfractionated cheese) and for Beaufort, Comté, Saint Nectaire, Morbier, Epoisses, and Langres cheeses (each sample, 3 g of surface cheese or 3 g of core cheese) were dissolved in 40 ml of sterile 2% (wt/vol) trisodium citrate and homogenized (19,000 rpm/min) with an Ultra-Turrax blender until solutions were opaque. Fifty milligrams of pronase (Boerhinger, Mannheim, Germany) and 100 μl of β-mercaptoethanol were added to each sample, followed by 3 h of incubation at 52°C. Bacteria were washed twice by centrifugation at 13,000 × *g* for 15 min. Pellets were first resuspended in sterile water and then in 10 ml of Tris-EDTA-saccharose (TES) buffer (25 mM Tris HCl, 0.1 M EDTA, 25% [wt/vol] saccharose [pH 8]). Cells were recentrifuged, resuspended in 500 μl of TES buffer, transferred into Eppendorf tubes, and cooled in ice for 10 min.

Cell lysis was performed by glass bead (150 to 200 μm) (Sigma, Saint Quentin Fallavier, France) treatment in the presence of TES buffer with the Fast Prep apparatus (FP120; Bio101 Savant; Ozyme, Saint-Quentin-en-Yvelines, France) (two cycles of 40 s each of shaking and 3 min of storage in ice). After settling, the supernatant (2 × 200 μl) was stored for 10 min in ice. DNA was then extracted by the phenol-chloroform method as previously described (14). The DNA pellet was dissolved in 100 μl of Tris-EDTA buffer plus RNase (Sigma, Saint Quentin Fallavier, France) and then examined by 0.8% agarose gel electrophoresis.

TABLE 1. Bacterial strains included in the reference set

Species or subspecies	Strain(s) (% identification by API)[a]
Hafnia alvei	AF15 (99.8), AF16 (99.9), AF17 (99.7), AF22 (99.7), AF24 (99.9), AF26 (99.8)
Bacillus licheniformis	AF5 (99.9), AF7 (99.9), AF8 (99.9), AF10 (99.9), CIP52.71^T (99.9)
Bacillus pumilus	CIP77.25 (99.9)
Bacillus subtilis	AF6 (95.6), CIP52.65^T (93.4)
Bacillus megaterium	AF4 (99.9)
Bacillus sphaericus	AF44 (89.1), AF45 (89.9), AF46 (89.9)
Bacillus lentus	AF9 (98.3)
Bacillus circulans	12 (99% homology with V3 of *B. circulans*; GenBank AY043084)
Streptococcus uberis	Clone C4 (99% homology with V3 of *S. uberis*; GenBank AB002527)
Streptococcus agalactiae	CIP103227^T (99.1), CIP82.41 (99.9)
Streptococcus dysgalactiae subsp.	CIP102914^T (99.9), CIP55.119 (99.3)
Streptococcus equinus	CIP103232 (99.9)
Streptococcus bovis	CIP102302^T (99.7)
Staphylococcus epidermidis	AF42 (94.5), CNRZ478
Staphylococcus aureus	AF43 (92.7), CNRZ740
Staphylococcus haemolyticus	AF49 (99.9), AF50 (99.9), CIP81.56^T (99.9), AF69
Staphylococcus chromogenes	AF41, CIP81.59^T (74.2)
Staphylococcus simulans	CIP81.64^T (99.9)
Staphylococcus lentus	CIP81.63^T (99.9)
Staphylococcus warneri	CIP103960 (99.5)
Staphylococcus sciuri	CIP105826 (99.9), URLGA2, URLGA13
Staphylococcus equorum	CIP103502^T
Staphylococcus cohnii	CIP81.54^T (91.9)
Staphylococcus capitis	CIP81.53^T (99.9)
Pseudomonas alcaligenes	AF19 (95), AF25 (95.5)
Pseudomonas fluorescens	AF16 (99.9), AF17 (99.9), AF23 (99.9), AF24 (99.9), CIP69.13^T (99.9), CIP63.47 (98.7)
Pseudomonas fragi	CIP55.4^T (99.9)
Pseudomonas putida	CIP55.5 (99.3)
Pseudomonas stutzeri	AF51 (99.9)
Pseudomonas aeruginosa	CIP100720^T (99.9), CIP104060 (99.9)
Enterobacter cloacae	AF27 (99.9), AF98 (99.8), AF99 (96.7), CIP60.85^T (99.9)
Enterobacter sakazakii	AF20 (95.1), AF21 (98), CIP5733 (99.9)
Enterobacter amnigenus	CIP103169^T (88.5)
Enterobacter intermedius	AF92 (99.4), AF94 (99.4), AF95 (99)
Aeromonas hydrophila	AF28 (99.9), AF29 (99.9)
Aeromonas sobria	AF66 (99.2), AF71 (99.2)
Alcaligenes tolerans	CIP55.94 (94.2), CIP55.95
Alcaligenes faecalis	CIP62.32 (92.9), CIP60.80^T (92.9)
Klebsiella pneumoniae subsp. *pneumoniae*	AF65 (99.9), AF97 (99.9), CIP82.91^T (99.9) CIP52.145 (99.9)
Klebsiella oxytoca	AF70 (99.7), AF90 (99.9), AF11 (99.9), CIP103434^T (99.9), AF82 (99.9)
Klebsiella terrigena	AF56 (99.9), AF57 (99.9), AF77 (99.9), AF87 (99.9), AF88 (99.9), AF89 (99.9), AF91 (99.7)
Acinetobacter baumannii	AF12 (99), CIP70.34^T (99.9)
Acinetobacter johnsonii	AF47 (83), AF48 (87.4)
Acinetobacter species	CIP104272 (84.9)
Acinetobacter lwoffii	AF63 (99.5)
Listeria innocua	AF1 (99.8), AF2 (99.8), AF3 (99.8)
Escherichia coli	AF13 (99.9), AF14 (99.9), AF18 (99.9), AF76 (99.5), AF80 (99.9)
Stenotrophomonas maltophilia	AF40 (99.9)
Buttiauxella agrestis	AF30 (99.9), AF31 (99.9), AF32 (99.9), AF33 (98.7), AF15 (99.8)
Citrobacter freundii	AF53 (99.9), AF37 (99.9), AF38 (99.9), AF39 (99.9), AF54 (99.9), AF55 (99.9), AF58 (99.9)
Serratia liquefaciens	AF61 (99.9), AF62 (99.9), AF64 (99.9), AF67 (99.9), AF68 (99.9), AF34 (99.9), AF35 (99.9), AF36 (99.9), AF81, CIP103238^T (99.9), CIP60.85 (99.9)
Serratia marcescens	AF52 (99.9), AF59 (99.9), AF78 (99.9), AF79 (99.9), AF93 (99.9)
Serratia fonticola	CIP78.64^T (99.9), CIP52.191 (99.9)
Chryseobacterium species	clone 2 (91% homology with V3 of *Chryseobacterium*; GenBank AF207077); CIP104270 (99)
Clostridium butyricum	CIP60.51 (99.3)
Clostridium sporogenes	CIP100651
Comamonas species	AF96 (97.3)
Raoultella planticola	CIP81.36 (98.6), AF60 (98.6)
Pantoea spp.	AF72 (99.9), AF73 (99.9), AF74 (99.9)
Kluyvera ascorbata	AF85 (99.9), AF86 (99.9)
Kluyvera cryocrescens	AF83 (94.1), AF84 (94.1)
Microbacterium lacticum	CIP101097
Geobacillus stearothermophilus	CIP67.5
Moraxella bovis	CIP70.40
Lactobacillus buchneri	CNRZ36R, CNRZ214
Aerococcus viridans	URLGA23ag, URLGA23ap

Continued on facing page

TABLE 1—*Continued*

Species or subspecies	Strain(s) (% identification by API)[a]
Brevibacterium linens	CNRZ910, CNRZ940G, CIP101125O^T, CNRZ915, CNRZ929O, CNRZ931
Brevibacterium casei	CNRZ912, CIP102111^T
Brevibacterium epidermidis	CIP102110B^T
Brevibacterium iodenum	LMG2201B^T
Brevibacterium species	CNRZ971, CNRZ938; CNRZ909
Arthrobacter sulfureus	LMG16694^T
Arthrobacter citreus	CNRZ928^T
Arthrobacter nicotianae	LMG16305J2^T, LMG16305B^T
Arthrobacter protophormiae	LMG16324^T
Arthrobacter uratoxydans	LMG16220-2^T
Arthrobacter globiformis	CNRZ907, CNRZ908
Arthrobacter spp. RAPD group I	CNRZ2057, CNRZ900, CNRZ2052
Arthrobacter spp. RAPD group II	CNRZ2075, CNRZ983, CNRZ2062
Corynebacterium variabile	CIP102112G^T, CNRZ2076, CNRZ923b
Corynebacterium vitaeruminis	CIP82.7p^T, CNRZ929J2^T
Corynebacterium ammoniagenes	CIP101283^T, CNRZ931^T, CNRZ922G
Corynebacterium spp.	CNRZ2069, CNRZ921, CNRZ978, CNRZ2065
Brachybacterium alimentarium	CNRZ929J4, CNRZ911J, CNRZ925^T
Brachybacterium tyrofermentans	CNRZ926^T
Micrococcus lilae	CNRZ882
Micrococcus luteus	CNRZ881
Kytococcus sedentarius	CNRZ880
Kocuria rosea	AF79 (99)
Kocuria varians	CIP81.73
Kocuria kristinae	CNRZ872
Propionibacterium thoenii	ATCC4874, CNRZ924, CNRZ83, CNRZ732, ATCC4872, CNRZ724
Propionibacterium freudenreichii	CNRZ81^T, CNRZ82, CNRZ88, CNRZ729, CNRZ288, ATCC 13678, ATCC 9616
Propionibacterium acidipropionici	CNRZ733, ATCC 965, CNRZ287, NCIMB8895, CIP3025^T, ATCC 9616
Propionibacterium jensenii	ATCC4870, CNRZ85R, CNRZ8728, NCIMB8904, NCIMB8069

[a] Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); CIP, Collection of the Institute Pasteur (Paris, France); CNRZ, Collection of the Centre National de la Recherche Zootechnique (INRA de Jouy-en-Josas, Jouy-en-Josas, France); LMG, Collection of the Laboratorium voor Microbiologie (University of Gand, Gand, Belgium); NCIMB, National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland); AF, isolated strains from raw milk (AFSSA, Maisons-Alfort, France), URLGA, isolated strains from dairy environment (INRA de Jouy-en-Josas); RAPD, randomly amplified polymorphic DNA. See Ogier et al. (39) for the LAB strains and *Staphylococcus* strains of dairy interest included in the low-G+C-content species database.

Specific primer tests. Specific primers based on previously published sequences were synthesized by MWG Biotech AG (Ebersberg, Germany) and the analyses were carried out with DNA extracted directly from the sample cheeses. Primers were prepared at a final concentration of 60 μM in deionized autoclaved water. PCR was performed with the Perkin-Elmer GenAmp system, model 2400, and all reactions were carried out following previously published conditions (see Table 4). Sizes of PCR products were determined by 2% agarose gel electrophoresis (TEBU Seakem CTG agarose).

Sequencing of TTGE and DGGE fragments. Bands obtained by TTGE and DGGE analyses of commercial cheeses were excised from the denaturing gels, purified, cloned, and sequenced as previously described (39). Sequences were compared to sequences in the Ribosomal Database Project (27) to determine the closest known relative species of the V3 16S rDNA fragment.

RESULTS

The bacterial species database. The molecular fingerprints of bacteria that correspond to common dairy species were determined by amplification of the V3 fragment with the HDA1 and HDA2 primers, followed by denaturing gel analysis of the resulting DNA products (Fig. 1). We tested different running conditions in order to determine the optimal parameters for separating the bacteria under study. For the low-G+C-content species bacteria (calculated T_m of the V3 sequence, <75°C with the Win Melt software), optimal resolution was achieved by TTGE; for the medium- and high-G+C-content species bacteria (calculated T_m of the V3 sequence, >75°C), optimal resolution was achieved by DGGE. The gel running parameters were optimized for each method (see Materials and Methods). All the strains described in Table 1 (including culture collection strains and isolates originating from microbial analyses of dairy samples) were run under TTGE or DGGE conditions to determine their position migrations. Note that some species bacteria (with calculated T_m values of the V3 sequence that were ±75°C) were run under both TTGE and DGGE conditions, i.e., *Escherichia coli* or *Lactobacillus reuteri*; their molecular fingerprints were integrated into the two-species database (Fig. 1). As a rule, bands generated by strains of the same species migrated identically (data not shown). We observed a few exceptions with *P. fluorescens*, *Staphylococcus haemolyticus*, *Klebsiella oxytoca*, and *Brevibacterium linens* species (Fig. 2). Strains of *Pseudomonas fluorescens* isolated from dairy products showed the same migration distance, whereas strain CIP69.13^T, which originated from a water tank, and strain CIP6347, isolated from soil, migrated at a different position. Migration anomalies were also observed for species of *Staphylococcus haemolyticus* and *Klebsiella oxytoca*, which originated from different biotopes (Fig. 2). The heterogeneity of the V3 sequence within a species could be explained here by differences in ecological origins (as described for *Pseudomonas fluorescens*) (5, 16, 34). Only the strains originating from dairy environments are conserved in the database species. However, *Brevibacterium linens* strains exhibited significant variability in V3 sequence migration (Fig. 2), although all strains used were isolated from dairy products. This may be explained by genetic strain variability, as revealed by heterogeneity of the randomly amplified polymorphic DNA

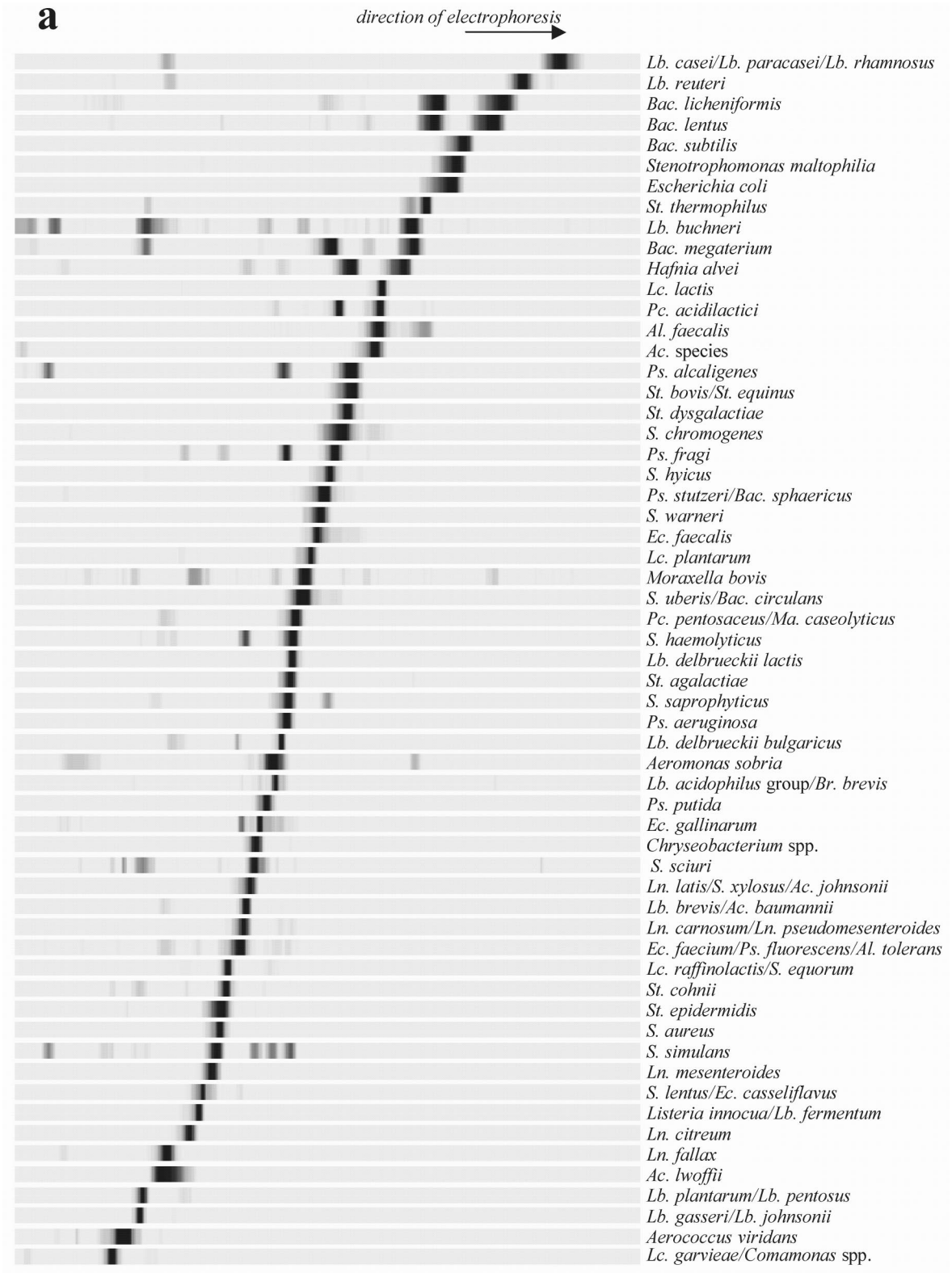

FIG. 1. Low-G+C-content (TTGE) and high-G+C-content (DGGE) species database. The V3 fragments originated from pure bacterial strains are separated by TTGE electrophoresis (a) or DGGE electrophoresis (b) according to their respective T_m values. The gels are then standardized using the GelCompar software (Applied-Maths) and transferred into the PowerPoint software. The profiles are then gathered according to their migration positions. Each species (indicated on the right of the profile) is characterized by a specific TTGE or DGGE fingerprint. The resolution is discussed in the text. Abbreviations: *Ac., Acinetobacter*; *Al*., *Alcaligenes*; *Ar.*, *Arthrobacter*; *B.*, *Brevibacterium*; *Bac.*, *Bacillus; Br.*, *Brevibacillus*; *C*., *Corynebacterium*; *Cl*., *Clostridium*; *Ec.*, *Enterococcus*; *Ent.*, *Enterobacter*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *Leuconostoc*; *M.*, *Micrococcus*; *Ma.*, *Macrococcus*; *Mc., Microbacterium*; *Pc.*, *Pediococcus*; *Ps.*, *Pseudomonas*; *Pr.*, *Propionibacterium*; *S.*, *Staphylococcus*; *St.*, *Streptococcus.*

FIG. 1—*Continued*.

profiles of numerous strains of *Brevibacterium linens* (E. Lepage, personal communication).

In some cases, the close phylogenetic relationships between species belonging to the same genus made it impossible to differentiate them, as described for the *Lactobacillus acidophilus* group or *Enterococcus faecium* group (39). For example, the members of the *Listeria* genus could not be differentiated using primers HDA1 and HDA2; the V3 sequences of *Listeria*

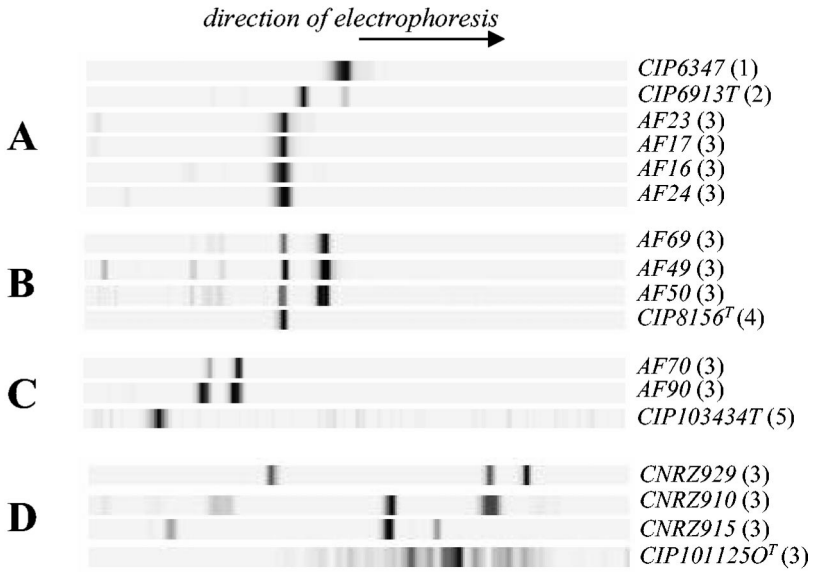

FIG. 2. Heterogeneity of the V3 fingerprints between strains belonging to the same species: *Pseudomonas fluorescens* (A), *Staphylococcus haemolyticus* (B), *Klebsiella oxytoca* (C), *Brevibacterium linens* (D). The V3 fragments originated from pure bacterial strains are separated by TTGE electrophoresis (*Pseudomonas fluorescens* and *Staphylococcus haemolyticus*) or DGGE electrophoresis (*Brevibacterium linens* and *Klebsiella oxytoca*). The gels are then standardized with GelCompar software (Applied-Maths) and transferred into the PowerPoint software. Strain numbers are indicated on the right of the profile. The numbers in parentheses correspond to biotope origins of the strains: 1, water tank; 2, soil; 3, dairy origin; 4, pharynx; 5, human skin. Strains differing by their ecologic origin generally showed a different migration behavior, as discussed in the text.

monocytogenes, *Listeria ivanovii*, and *Listeria innocua* were similar. The different species of the *Arthrobacter sulfureus* group (*Arthrobacter sulfureus*, *Arthrobacter nicotianae*, *Arthrobacter urotoxydans*, and *Arthrobacter globiformis*) or the *Micrococcus luteus* group (*Micrococcus lylae*, *Kytococcus sedentarius*, *Micrococcus luteus*, and *Microbacterium lacticum*) could not be separated by DGGE electrophoresis (see Fig. 1b).

In a few cases, species belonging to different genera could not be differentiated, i.e., "*Alcaligenes tolerans*," *Pseudomonas fluorescens*, and *Enterococcus faecium* (Fig. 1a) or *Buttiauxella agrestis* and *Enterobacter intermedius* (Fig. 1b). Despite sequence differences, the T_m values of comigrated fragments from these bacteria were similar (data not shown). Some strains were characterized by two bands of similar intensities (e.g., *Hafnia alvei* and *Bacillus licheniformis*) (Fig. 1a). The presence of two amplified V3 segments is probably due to the heterogeneity of the 16S rDNA operon (38).

The band position of each bacterial species was stored in a GelCompar database (Applied-Maths). Our reference database was then used to identify the bacterial species present in commercial cheeses. The potential of TTGE and DGGE for ecosystem analyses was compared to the culture-dependent analysis approach.

Identification of bacterial species. Analyses of commercial cheeses were carried out by (i) culture-dependent and (ii) culture-independent methods.

(i) Culture-dependent analysis. Microbial analyses of cheeses were performed by plating samples on different media to select different groups of bacteria (Table 2). For Munster cheese, plate counts revealed a high level of presumed LAB (lactococci, streptococci, and mesophilic and thermophilic lactobacilli) and salt-tolerant bacteria (as determined by growth on BHI medium). Epoisses cheese was characterized by low numbers of bacteria that could grow on MRS medium. We could not enumerate colonies on M17 plates, because they were overgrown by gram-negative bacteria that originated from the cheese.

Morbier cheese was characterized as having a diverse bacterial content, including presumed lactococci, streptococci, mesophilic and thermophilic lactobacilli, and salt-tolerant bacteria. The dominant bacteria of Leerdamer cheese consisted of salt-tolerant bacteria and presumed lactococci and propionibacteria. With all four cheeses, we enumerated a high-salt-tolerant bacterial population, which represents a major group of bacteria inhabiting the cheese surface. A wide variety of bacterial colonies, differing in their morphology and color, grew on BHI medium supplemented with 5% NaCl, indicative of the great biodiversity of bacteria species among the salt-tolerant microorganisms. However, we were unable to differentiate the salt-tolerant species on plates. After a few days at room temperature, orange colonies appeared and may have corresponded to *Brevibacterium linens*. Gram-negative bacteria were present at high levels in the different cheeses (except for the Leerdamer cheese); enumeration of colonies growing on violet red bile agar medium revealed bacterial counts higher than 10^6 UFC/g.

(ii) Culture-independent analysis. To investigate microbial diversity of cheeses with molecular tools, genomic bacterial DNA was directly extracted from the cheeses, followed by amplification of the V3 regions of 16S rDNA. Separation of resulting amplicons was performed by TTGE and DGGE electrophoresis (Fig. 3). The molecular fingerprint was identified by comparing the migration position of a band to that of a species in the database or by DNA sequencing of the unknown band. Confirmation was achieved by specific PCR analysis of DNA extracted directly from the sample.

TABLE 2. Microbial enumeration of commercial cheese samples on different media

Sample	Bacterial count (log_{10} of sample)[a]									
	VRBA, 30°C (total coliforms)	M17, 30°C (*Lactococcus* spp.)	M17, 42°C (*Streptococcus* spp.)	MRS, 30°C (mesophilic *Lactobacillus*)	MRS, 42°C (thermophilic *Lactobacillus*)	BHI + 5% NaCl, 25°C (salt-tolerant flora)	MSA, 37°C (*Staphylococcus* spp.)	MSE, 30°C (*Leuconostoc* spp.)	YEL, 25°C (*Propionibacterium* spp.)	BEA, 37°C (*Enterococcus* spp.)
Leerdamer	3.6	7.62	6	5.5	4.9	8.9	7.4	<2.2	7.4	6.1
Munster	6.5	9.1	9	7.2	7.3	8.4 (7.1)[c]	5.8	<2.2		6.1
Morbier	6.1	9	8.9	8	7.6	8.9 (8)[c]	7.4	<2.2		6.1
Epoisses	6.3	—[b]	—[b]	6	5	9.3 (6.5)[c]	6.2	<2.2		4.7

[a] Bacterial counts under different culture conditions (medium and temperature). VRBA, violet red bile agar; YEl, yeast extract-sodium lactate; BEA, bile esculin azide agar.
[b] —, overgown by gram-negative bacteria.
[c] Numbers in parentheses indicate bacterial counts of orange colonies.

(a) Identification of the low-G+C-content bacteria species by TTGE (Fig. 3A). The Morbier cheese profile includes nine bands, seven of which were assigned by using the species database (Fig. 3A, lane 2). Two bands were directly (unambiguously) assigned to *Lactococcus lactis* (band f) and *Streptococcus thermophilus* (band h). The presence of these two species was confirmed by cloning and sequencing of the two corresponding bands (Table 3). The other bands were assigned to several possible comigrating species, and we therefore performed further analyses using specific species primers to identify them (Table 4). For example, the presence of *Lactobacillus delbrueckii* subsp. *lactis* in the Morbier cheese (Fig. 3A, lane 2, band e) was confirmed by specific PCR, whereas the comigrating species *Staphylococcus saprophyticus* was not found (Table 4). Using combination of different specific PCR tests (Table 4), the bands a, b, and d (Fig. 3A, lane 2) were presumably identified as *Staphylococcus lentus*, *Staphylococcus equorum*, and *Lactobacillus acidophilus*. The unassigned band g (Fig. 3A, lane 2) was excised from the gel, cloned, sequenced, and identified as *Lactobacillus buchneri* (Table 3); its migration profile was confirmed by running two pure strains on TTGE, and then it was added to the reference database. We were unable to identify band c in the data shown in Fig. 3A. The strong intensity of the band corresponding to *Lactococcus lactis* indicates that it is the dominant species in Morbier cheese. Note that band intensities seem to reflect the relative proportion of each species in the total bacterial population (35).

The Munster cheese (Fig. 3A, lane 3) sample was characterized by three intense bands, identified as *Enterococcus faecalis* (band c), *Lactococcus lactis* (band d), and *Streptococcus thermophilus* (band e), and two bands of lower intensity, respectively, assigned to *Lactobacillus plantarum* (band a) and *Lactobacillus acidophilus* group (band b). We confirmed the assignations to *Lactococcus lactis* and *Streptococcus thermophilus* by cloning sequences of the corresponding bands (Table 3). With specific primer tests, *Lactobacillus plantarum* was found, but the assignation of band c shown in Fig. 3A to *Enterococcus faecalis* was not confirmed (Table 4).

The Epoisses cheese profile (Fig. 3A, lane 4) includes two intense bands, a and e (*Lactobacillus plantarum* and *Lactococcus lactis*), and three bands, b, c, and d, of lower intensity (*Leuconostoc mesenteroides*, *Lactococcus raffinolactis-Staphylococcus equorum*, and *Pseudomonas fluorescens-Enterococcus faecium* group-"*Alcaligenes tolerans*"). The presence of *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, *Lactococcus raffinolactis*, and *Pseudomonas* species in the Epoisses cheese was confirmed with specific primers; members of the *Enterococcus faecium* group were not found (Table 4).

In pasteurized Leerdamer cheese (Fig. 3A, lane 1), the TTGE analysis revealed the presence of only two species: *Leuconostoc mesenteroides* (band a), confirmed using specific primer tests (Table 4), and *Lactococcus lactis* (band b).

TTGE band identities for each sample cheese are listed in Table 5.

(b) Identification of the medium- and high-G+C-content % bacteria by DGGE (Fig. 3B). The DGGE pattern of Leerdamer (Fig. 3B, lane 4) cheese was very simple and was confined to one main band (*Propionibacterium freudenreichii*). The presence of *Propionibacterium freudenreichii* in the Leerdamer

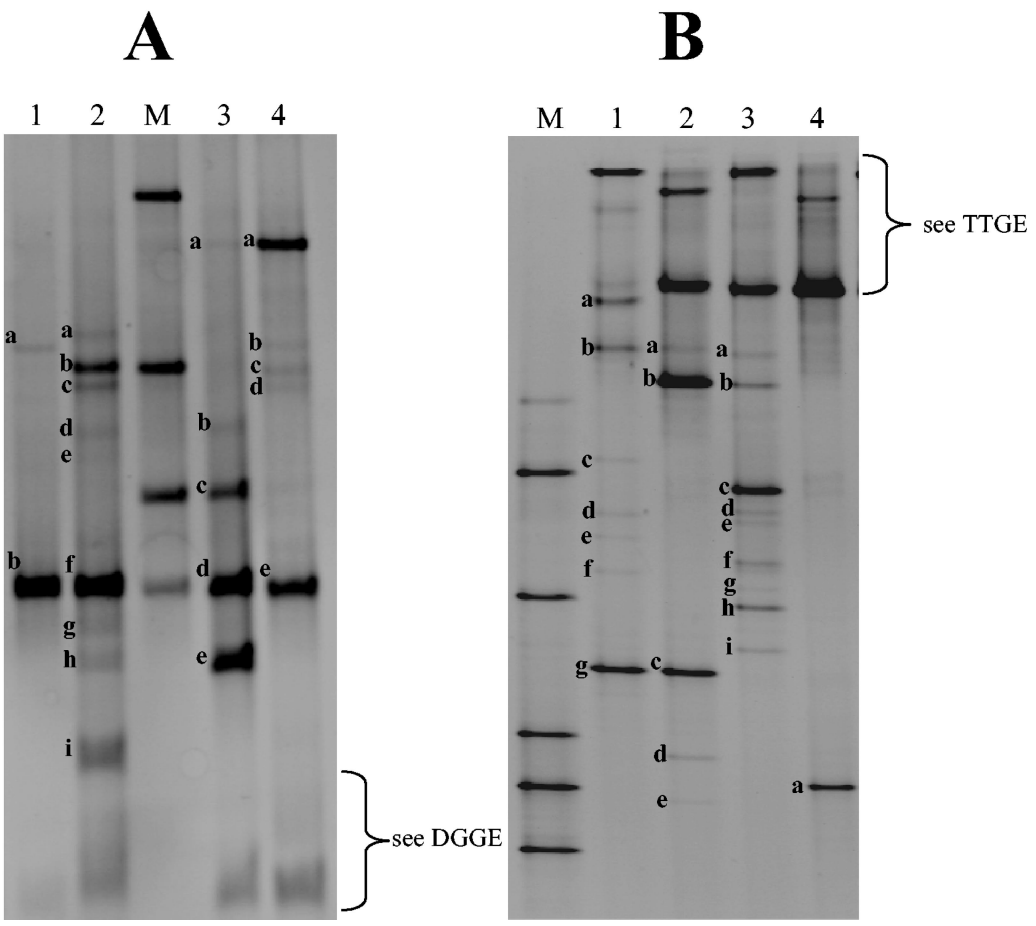

FIG. 3. Photographed gel (ethidium bromide revelation) after TTGE (A) and DGGE (B) electrophoresis of V3 16S rDNA fragments from various commercial dairy products differing by their technology processes. Genomic DNA was extracted from nonfractionated cheese sample, followed by amplification of the V3 16S rDNA region and separation of the amplicons by gel electrophoresis. After standardization of the gel with GelCompar software, the bands were identified by comparison with the species database. We have indicated the position of the respective assigned bands by lowercase letters. See Table 5 for a list of band identities corresponding to each labeled band. (A) Lane M, standardization ladder; lane 1, Leerdamer cheese; lane 2, Morbier cheese; lane 3, Munster cheese; lane 4, Epoisses cheese. (B) Lane M, standardization ladder; lane 1, Epoisses cheese; lane 2, Munster cheese; lane 3, Morbier cheese; lane 4, Leerdamer cheese.

cheese is consistent with its process technology, as propionibacteria are added to pasteurized milk.

With the Munster cheese (Fig. 3B, lane 2), we detected five bands; three bands, a, b, and c, were respectively assigned to *Kluyvera cryocrescens*, *Streptococcus thermophilus*, and the *Arthrobacter sulfureus* group; two bands, d and e, were assigned to *Brevibacterium linens*. However, we did not confirm the presence of *Brevibacterium linens* in the Munster cheese with specific primers designed by S. Furlan et al. (unpublished data) (Table 4).

With Morbier cheese, the DGGE method revealed 9 bands. The species database allowed us to identify six of them (Fig. 3B, lane 3) as corresponding to *Lactobacillus buchneri* (band a), *Streptococcus thermophilus* (band b), *Lactobacillus reuteri-Enterobacter sakazakii* (band d), *Lactobacillus casei* (band f), the *Micrococcus luteus* group (band h), and *Brevibacterium linens* (band i). The presence of *Brevibacterium linens* in the Morbier cheese was confirmed by specific PCR testing (Table 4). However, a very faint band (not visible on the gel photograph) was assigned to *E. coli*. Three of the unassigned bands were cloned and sequenced; sequence comparison against the GenBank database allowed us to identify them as *Corynebacterium variabile* (band c), *Arthrobacter* species (band e), and *Corynebacterium casei* (band g). We also identified the *Corynebacterium mastitidis* species bacteria by sequencing a clone originating from band h (Fig. 3B, lane 3). Each new species fingerprint was added to the database after validation with pure strains.

We detected seven bands in the DGGE pattern of the Epoisses cheese (Fig. 3B, lane 1); four bands were assigned to *Kluyvera cryocrescens* (band b), *Buttiauxella agrestis-Enterobacter intermedius* (band c), *Brevibacterium linens* (band d), and the *Arthrobacter sulfureus* group (band g). The presence of *Brevibacterium linens* was confirmed by a specific PCR test (Table 4). Sequencing of the unassigned bands a and f (Fig. 3B, lane 1) revealed the presence of *Pseudoalteromonas* and *Halomonas*, although the habitat of these two genera is generally salt water (57). We did not succeed in sequencing the unknown band e (Fig. 3B, lane 1). We were also unable to determine the precise identity of band c (Fig. 3B, lane 1)

TABLE 3. Identification by cloning sequencing of V3 fragments excised from TTGE and DGGE patterns of total microbial community cheeses

Sample cheese	Fragments (method[s])	Clone(s) no.	Closest sequence relative (species)[a]	% Identity	GenBank accession no.
Morbier	f (TTGE)	45	*Lc. lactis* sup sp. *lactis*	99	AJ419572
Munster	d (TTGE)	41, 42	*Lc. lactis lactis*	99	AF530330.1
Morbier	g (TTGE)	9, 21	*Lb. buchneri*	99	AY026751.1
Epoisses	a (DGGE)	9, 18, 19	*Pse. agarovorans*	100	AY100680.1
Morbier	b (DGGE) and h (TTGE)	2, 10, 19	Uncultured *St.*	100	AF408263.1
Munster	b (DGGE) and e (TTGE)	47	*St. thermophilus*	99	AY188354
Morbier	c (DGGE)	24	*C. variabile*	100	AJ222816.1
Morbier	e (DGGE)	19	*Ar.* species	91	AB017540.1
Epoisses	f (DGGE)	7	*H. variabilis*	96	AF173968
Morbier	g (DGGE)	22	*C. casei*	94	AF267152.1
Morbier	h (DGGE)	29	*C. mastitidis*	97	Y09806.1
Epoisses	g (DGGE)	22, 25	*Ar.* species	100	AF487785.1
			Ar. woluwensis	100	AY112986.1
			Ar. sulfonivorans	100	AF235091
			Ar. nicotiane	100	AJ315492.1
			Ar. globiformis	100	M23411.1
			Ar. sulfureus	100	AB046358.1

[a] Abbreviations: *Lc.*, *Lactococcus*; *Lb.*, *Lactobacillus*; *Pse.*, *Pseudoalteromonas*; *C.*, *Corynebacterium*; *Ar.*, *Arthrobacter*; *H.*, *Halomonas.*

corresponding to comigrating bacterial species *Buttiauxella agrestis* and *Enterobacter intermedius*. Identification of some bands corresponding to comigrating species awaits the use of specific primers. DGGE band identities for sample cheeses are listed in Table 5.

We noted that the intensity of bands corresponding to *Brevibacterium linens* was very low in Epoisses, and its presence was not confirmed in Munster cheeses. This was unexpected, as these cheeses belong to the red smear cheese type, in which *Brevibacterium linen*s is a common colonizer of the cheese surface and produces red pigment.

Comparison of the bacterial microflora between surface and core cheese samples. We used (i) TTGE and (ii) DGGE to separately analyze core or surface fractions of six commercial cheeses, some of which originate from two different commercial producers. Two samplings of each cheese fraction were analyzed.

(i) TTGE analysis. We performed TTGE analysis of core and surface samples of Langres, Epoisses, Morbier, Beaufort, Comté, and Saint Nectaire cheeses, each coming from one producer (Fig. 4). Two samples of each cheese fraction were analyzed (Fig. 4). TTGE profiles of core Beaufort cheeses (data not shown) are nearly identical to those of core Comté cheeses. No differences were seen in TTGE patterns between samples originating from the same part of the cheese. However, TTGE patterns revealed differences in bacterial composition between the cheese core and cheese surface. The cheese core was generally inhabited by various LAB (*Lactococcus lactis*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Streptococcus thermophilus*), whereas the cheese surface was generally inhabited by staphylococci and gram-negative bacteria (*Moraxella bovis* and *Pseudomonas* sp.).

We observed homogeneity in bacterial composition and cheese technology. For example, we identified *Lactococcus lactis*, *Leuconostoc mesenteroides*, and *Lactobacillus plantarum* as major components of the red smear cheeses (Langres and Epoisses cheese cores) or *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *lactis* in Swiss-type cheeses (Comté and Beaufort cheese cores). The LAB species found in the cheese cores probably originated from the starters added by manufacturers.

The differences between bacterial compositions of the cheese core and cheese surface coincide with difference of pH between these fractions (Table 6). The increase in pH of the cheese surface allowed the growth of nonstarter bacterial species, such as staphylococci or gram-negative bacteria.

(ii) DGGE analysis. We performed DGGE analysis of core and surface samples of Beaufort, Comté and Saint Nectaire, each coming from two different producers (Fig. 5). As with TTGE, the DGGE patterns of two samples originating from the same part of the cheese were identical (data not shown). Cheeses of the same type but from different producers displayed similarities in bacterial composition, as seen by numerous common bands in DGGE profiles. In view of the complexity of the surface profiles, it appears that bacteria of the cheese surface are generally far more diversified than those in the core. Indeed, the total number of bands is significantly higher in the surface than in core samples. As described above, more-permissive surface conditions (due to higher pH levels) may favor the outgrowth of a greater variety of bacteria. Presumed identification of some of the bands (Table 7) suggests that many of the surface bacteria belong to the coryneform group. Several DGGE bands were unassigned and may correspond to undescribed bacterial species (numerous coryneform species remain poorly described in the literature). Thus, full characterization of the cheese surface awaits completion of the reference database.

These studies revealed an interesting difference between surface and core profiles: the essentially anaerobic core samples are dominated by low-G+C-content bacterial species but are devoid of high-G+C-content bacterial species (Fig. 5). In contrast, aerobic surface cheese samples produced complex

TABLE 4. Primers used in this study for the specific PCR tests

Primer	Sequence (5′–3′) of reverse primer	Specificity of target[a]	Source or reference[b]	PCR response for cheese			
				Morbier	Epoisses	Munster	Leerdamer
Ec1 Ec2	Unpublished data	*Ec. casseliflavus*	Firmesse	−	−	−	
Efm1 Efm2	Unpublished data	*Ec. faecium*	Firmesse	−	−	−	
Ed1 Ed2	Unpublished data	*Ec. durans*	Firmesse	−	−		
Eh1 Eh2	Unpublished data	*Ec. hirae*	Firmesse	−	−		
Efs1 Efs2	Unpublished data	*Ec. faecalis*	Firmesse		−	−	
fPs16S rPs16S	ACT GAC ACT GAG GTG CGA AAG CG ACC GTA TGC GCT TCT TCA CTT GAC C	*Pseudomonas*	26	+	+		
Laci 01 Laci 02	GAC CGC ATG ATC AGC TTA TA AGT CTC TCA ACT CGG CTA TG	*Lb. acidophilus*	22	+	−	−	
SLH19857S LH29860	CTA GAC AAT CAA TTG CAC CG TAC CAG TTC TTC TTG AAG CC	*Lb. helveticus*	43	−	−	−	−
Ldel01 Ldel02	ACA TGC ATC GCA TGA TTC AAG AAC TCG GCT ACG CAT CAT TG	*Lb. delbrueckii*	22	+	−	−	
fSt sap rSt sap	TCA AAA AGT TTT CTA AAA AAT TTA C ACG GGC GTC CAC AAA ATC AAT AGG A	*S. saprophyticus*	28	−			
Lfpr16S-23S PlanII	GCCGCCTAAGGTGGGACAGAT TTACCTAACGGTAAATGCGA	*Lb. plantarum*	61		+	+	
IRL PipLraR	TTTGAGAGTTTGATCCTGG CGTCACTGAGGGCTGGAT	*Lc. raffinolactis*	44	−	+	−	
STAE-EpI STAE-EpII	TCTACGAAGATGAGGGATA TTTCCACCATATTTTGAATTGT	*S. epidermidis*	21	−	−	−	
STAA-AuI STAA-AuII	TCTTCAGAAGATGCGGAATA TAAGTCAAACGTTAACATACG	*S. aureus*	21	−	−	−	
Lnm1 Lnm2	TGTCGCATGACACAAAGTTA ATCATTTCCTATTCTAGCTG	*Ln. mesenteroides*	9	−	+	−	+
Brevib Blin	Unpublished data	*B. linens*	Furlan	+	+	−	−
Cvar Corb	Unpublished data	*C. variabile*	Furlan	+	−	−	−

[a] Abbreviations: *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *Leuconostoc*; *Ec.*, *Enterococcus*; *S.*, *Staphylococus*; *St.*, *Streptococcus*; *B.*, *Brevibacterium*; *C.*, *Corynebacterium*.

[b] Firmesse, O. Firmesse et al., unpublished data; Furlan, S. Furlan et al., unpublished data.

DGGE patterns, revealing the presence of a great diversity of bacteria with a high-G+C-content genome (more than 10 bands per sample). These results indicate a strong bias in G+C content between the bacterial flora present at the surface versus that present in the core. These observations are in agreement with the study of Naya et al. (37), suggesting that aerobiosis selects for bacteria having high G+C content in their genomes.

DISCUSSION

We combined TTGE and DGGE methods to provide a thorough description of bacterial diversity in dairy products. This project was realized by first setting up a comprehensive bacterial reference database, which now comprises more than 150 species. Identification of species in dairy samples is identified by alignment with one of the species in the database. When a new band cannot be assigned through the reference set, it is directly excised and sequenced. The new species assignment is then used to enrich the reference set. By setting up this system, we have a rapid means of describing the flora in a variety of dairy ecosystems. This strategy is much less time consuming and more specific than conventional analysis by cloning and sequencing. In addition, ambiguities in species assignments are readily resolved by either (i) cloning and sequencing the V3 bands or (ii) modifying the PCR primers to

narrow down the species assignments. The latter approach was also used to validate some of the species assignments made using the reference database.

The potential of the molecular approach to describe bacterial composition of different cheeses was compared to the culture-dependent method. Results obtained by the molecular approach reflected, to a large extent, those obtained by microbial enumerations on different media, but the molecular approach was advantageous in its rapidity and specificity. Indeed, some media are not very selective (2, 20, 45). For example, Ercolini et al. (20) analyzed cells harvested from a variety of viable count media and identified staphylococci on M17 and MRS agar, enterococci on mannitol salt agar, and *Leuconostoc* on M17 agar plates. The molecular approach was thus more informative and generally allowed bacterial identification at the species level within 2 days.

In molecular studies of microbial communities, DNA extraction is the most critical step. Isolated DNA should reflect the existing genetic diversity, but microorganisms, particularly the gram-positive bacteria, may not all lyse equally well. An effective and reproducible method for DNA isolation from dairy products was developed in this study (Materials and Methods) to assure representative extraction of microbial members of cheese ecosystems. Using TTGE and DGGE, we identified about 17 different bacterial species in Morbier cheeses manufactured with raw milk but only 3 bacterial species in Leerdamer cheese manufactured with pasteurized milk. This microbial diversity reflected the dominant bacterial species of the ecosystem (39).

The molecular approach allowed us to identify some species that are of particular interest: *Lactobacillus buchneri*, present in Morbier cheese, is a heterofermentative lactobacillus responsible for off-flavor defects caused by biogenic amine production (24, 54, 55). Specific detection of this microorganism could be of interest to the dairy industry. We also note that *Propionibacterium freudenreichii* was rapidly identified in Leerdamer cheese by DGGE; this microorganism is particularly difficult to cultivate on artificial medium and requires an incubation time of at least 7 days. Unexpectedly, we identified *Pseudoalteromonas* species and *Halomonas* species by sequencing two unassigned bands found in the Epoisses cheese profiles. This is the first time that these two salt-tolerant bacterial species are found in dairy products; their identification was enabled by molecular methods. The presence of these bacteria may be explained by surface cheese treatments performed during the cheese ripening process that includes washes in saline water. It will be of interest to know whether these unexpected bacterial species play a significant role in the cheese ripening process, or in aroma formation.

The combination approach using TTGE and DGGE has proved valuable in describing bacterial diversity at the cheese surface. The microbial communities present on Morbier, Epoisses, and Munster cheese surfaces were largely undefined (59). Most of the bacteria were described as coryneform, but species level classification to date has proved unsuccessful. Identification of coryneforms has been mainly based on determination of the types of peptidoglycan, menaquinones, and fatty acids in the cell wall (6). Some of these analyses are labor intensive, which means that only small numbers of isolates can

TABLE 5. Approaches used to identify bands of TTGE and DGGE gels[a]

Cheese	Band and presumed species in gel		Band and identification by sequencing		Band and species revealed by specific PCR	
	TGGE	DGGE	TGGE	DGGE	TGGE	DGGE
Morbier	a, *Ec. casseliflavus-S. lentus*; b, *Lc. raffinolactis-S. equorum*; c, unknown band; d, *Lb. acidophilus* group; e, *Lb. delb.* subsp. *lactis-S. saproplyticus*; f, *Lc. lactis*; g, *Lb. buchneri*; h, *St. thermophilus*; i, *Escherichia coli*	a, *Lb. buchneri*; b, *St. thermophilus*; c, *C. variabile*; d, *Lb. reuteri-Enterobacter sakazakii*; e, *Ar.* species; f, *Lb. casei*; g, *C. casei*; h, *M. luteus* group-*Corynebacterium* sp.; i, *B. linens*	f, *Lc. lactis*; g, *Lb. buchneri*; h, *St. thermophilus*	b, Uncultured, *St.*; c, *C. variabile*; e, *Ar.* species; g, *C. casei*; h, *Corynebacterium mastidis*	d, *Lb. acidophilus*; e, *Lb. delb.* subsp. *lactis*	c, *C. variabile*; i, *B. linens*
Munster	a, *Lb. plantarum-Lb. pentosus*; b, *Lb. acidophilus* group; c, *Ec. faecalis*; d, *Lc. lactis*; e, *St. thermophilus*	a, *Klayvera cryocrescens*; b, *St. thermophilus*; c, *Ar. sulfureus group*; d, *B. linens*; e, *B. linens*	d, *Lc. Lactis*; e, *St. thermophilus*	b, *St. thermophilus*; c, *Ar. sulfureus*	a, *Lb. plantarum*; b, *Lb. acidophilus*	
Epoisses	a, *Lb. plantarum-Lb. pentosus*; b, *Ln. mesenteroides*; c, *Lc. raffinolactis-S. equorum*; d, *Ec. faecium* group-*Ps. fluorescens-Al. tolerans*; e, *Lc. lactis*	a, *Pseudoalteromonas agarivorans*; b, *Klayvera cryocrescens*; c, *Buttiauxella agrestis-Enterobacter intermedius*; d, *B. linens*; e, unknown band; f, *Halomonas variabilis*; g, *Ar. sulfureus* group		a, *Pseudoalteromonas agarivorans*; f, *Halomonas variabilis*; g, *Ar. sulfureus*	a, *Lb. plantarum*; b, *Ln. mesenteroides*; c, *Lc. raffinolactis*; d, *Ps.* genus	d, *B. linens*
Leerdamer	a, *Ln. mesenteroides*; b, *Lc. lactis*	a, *Propionibacterium freudenreichii*			a, *Ln. mesenteroides*	

[a] Presumed bacterial species were assigned with the reference database. Bands are shown in Fig. 3; see the legend to Fig. 3 for details of analyses and lanes. Bands are identified by lowercase letters. Abbreviations: *Al.*, *Alcaligenes*; *Ar.*, *Arthrobacter*; *B.*, *Brevibacterium*; *C.*, *Coynebacterium*; *Ec.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *Leuconostoc*; *M.*, *Micrococcus*; *Ps.*, *Pseudomonas*; *S.*, *Staphylococcus*; *St.*, *Streptococcus*; *delb.*, *delbrueckii*.

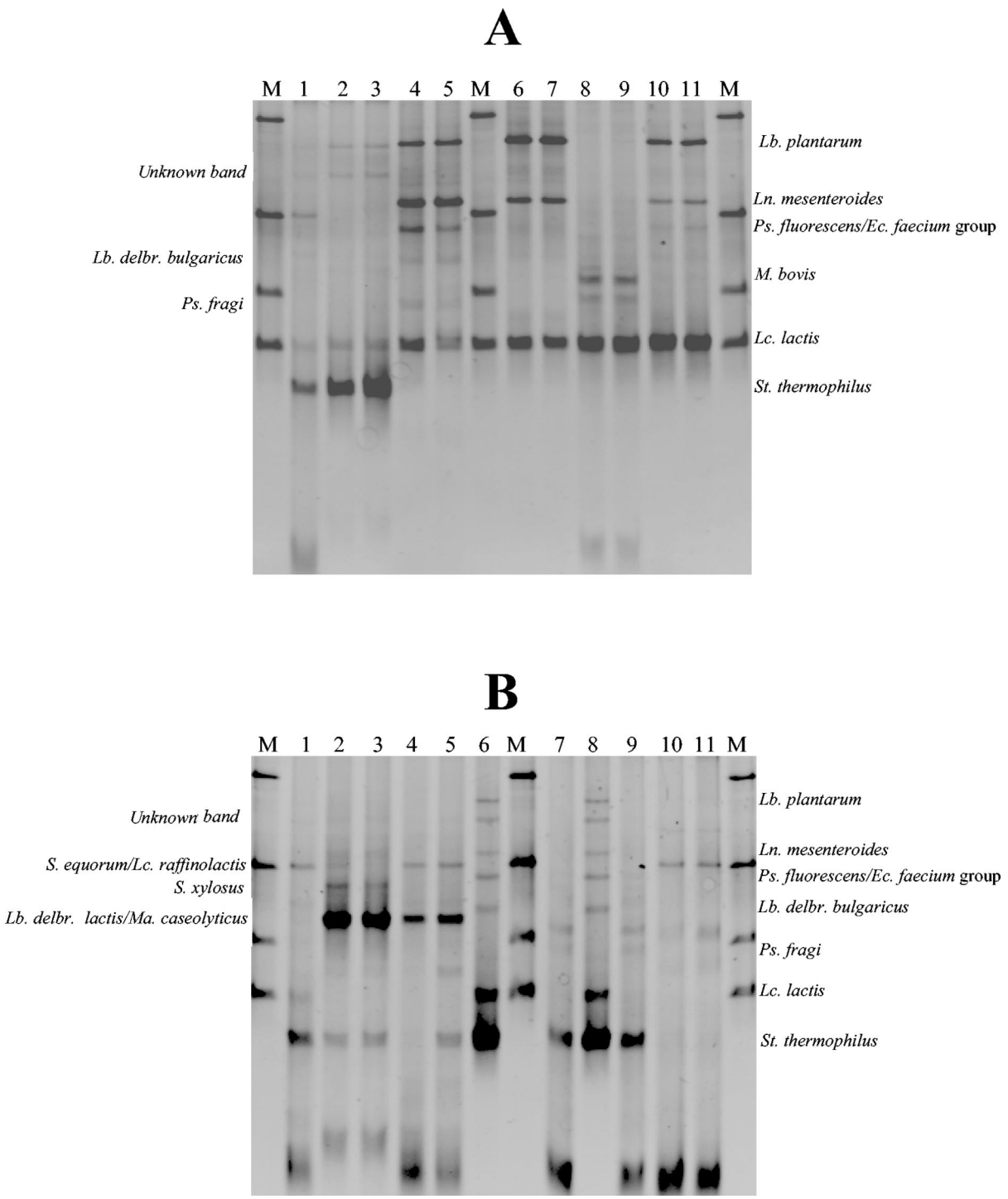

FIG. 4. Comparison of bacterial microflora present in surface versus core cheeses samples by TTGE electrophoresis of V3 amplified fragments. For each sample cheese, genomic DNA extraction was performed either with two surface cheese samples (indicated by S1 or S2) or with two core cheese samples (indicated by C1 or C2), followed by amplification of the V3 16S rDNA region and separation of the amplicons by TTGE. The bands are compared with the reference strain fingerprints of the data bank; assignments of major bands are indicated on the right. Analysis of the profile is discussed in the text. (A) Lanes: M, identification ladder; 1, Morbier cheese S1; 2, Morbier cheese C1; 3, Morbier cheese C2; 4, Langres cheese S1; 5, Langres cheese S2; 6, Langres cheese C1; 7, Langres cheese C2; 8, Epoisses cheese S1; 9, Epoisses cheese S2; 10, Epoisses cheese C1; 11, Epoisses cheese C2. (B) Lanes: M, identification ladder; 1, Morbier cheese S2; 2, Comté cheese C1; 3, Comté cheese C2; 4, Comté cheese S1; 5, Comté cheese S2; 6, Saint Nectaire cheese C1; 7, Saint Nectaire cheese S1; 8, Saint Nectaire cheese C2; 9, Saint Nectaire cheese S2; 10, Beaufort cheese S1; 11, Beaufort cheese S2. Abbreviations: *Ec.*, *Enterococcus*; *Lb.*, *Lactobacillus*; *Lc.*, *Lactococcus*; *Ln.*, *Leuconostoc*; *Ma.*, *Macrococcus*; *Ps.*, *Pseudomonas*; *S.*, *Staphylococcus*; *St.*, *Streptococcus*.

TABLE 6. pH of core and surface commercial cheeses samples

Sample site	pH					
	Epoisses	Langres	Morbier	Saint Nectaire	Comté	Beaufort
Core	6.21	4.96	5.28	6.21	5.88	5.90
Surface	6.61	6.18	6.83	7.49	7.06	6.91

be analyzed. Using the DGGE method, we identified numerous coryneform species in cheese samples, including *Corynebacterium variabile*, *Corynebacterium mastitidis*, *Corynebacterium casei*, *Arthrobacter* species, and *Brevibacterium linens*. Unexpectedly, *Brevibacterium linens* was not the major bacterial species in Epoisses and Munster cheeses, although it is often inoculated onto the surface of these cheeses during the early ripening process. Our results are in agreement with those of Brennan et al. (6), who also reported that *Brevibacterium linens* is not recovered from the surface of cheeses that were deliberately smeared with *Brevibacterium linens* BL2. Moreover, the taxonomy of the *Brevibacterium linens* group is still unclear; using genotypic methods, E. Lepage et al. (personal communication) reclassified numerous strains of *Brevibacterium linens* as members of the *Arthrobacter* genus. In fact, coryneform strains were often classified as part of the *Brevibacterium linens* group because they formed orange colonies on plates.

Information concerning the natural bacterial species composition of cheese ecosystems, as described in this work, should enable industrial dairy producers to design ripening cultures by selecting appropriate species. For example, this approach would provide a positive alternative to the use of traditional methods of "old-young smearing" which is considered the source of undesirable microorganisms like *Listeria monocytogenes* or *Staphylococcus aureus* (59). Until now, attempts to design a well-defined red smear culture failed; the resulting cheeses were of low quality, due to the lack of knowledge concerning the natural composition of red smear (59).

Analyses of the cheese core and surface as separate fractions revealed two more fundamental characteristics of the cheese ecosystems. First, microbial composition of a cheese is nonuniform, and the diversity of surface microflora appears to be richer than that of the core microflora. This is in agreement with the more-selective conditions occurring in the core of the cheese where the pH is lower than on the surface. Ercolini et al. (20) also observed a spatial distribution of bacterial species in Stilton cheese, based on metabolite availability and competition between bacteria. Second, we observed that bacteria of the cheese surface generally correspond to high-G+C-content genomes, whereas bacteria present in the core generally have

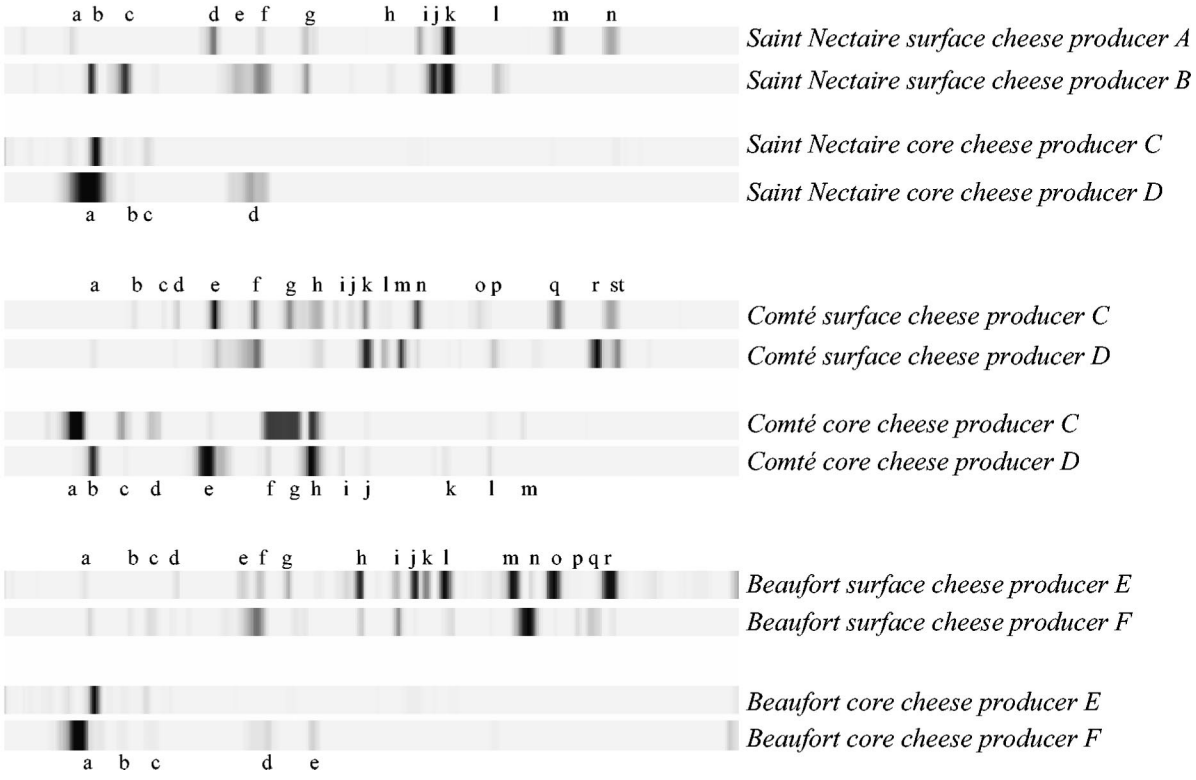

FIG. 5. Comparison of bacterial microflora present in surface cheese samples versus core cheeses samples by DGGE of V3 amplified fragments. For each sample cheese, genomic DNA extraction was performed either from surface cheese samples or from core cheese samples, followed by amplification of the V3 16S rDNA region and separation of the amplicons by DGGE. Analyses were performed with sample cheeses originating from different producers (A to F). After standardization of band migrations was carried out with GelCompar software, bands (indicated by lowercase letters) were identified by comparison with known species in the reference database. Profile analyses are discussed in the text; Table 7 recapitulates the presumed species bacteria corresponding to each labeled band.

TABLE 7. Presumed species bacteria present in core and surface commercial cheeses by assignations of the DGGE bands to the database species

Cheese	Sample site	Band and presumed bacterial species[a]
Saint Nectaire	Core	a, *St. thermophilus*; b, *E. coli*; c, *unknown band*; d, *B. linens*
	Surface	a and b, *St. thermophilus*; c, *E. coli*; d, *Ent. cloacae-Kl. oxytoca*; e, *Ent. sakazakii*; f, unknown *band*; g, *Lb. casei*; h, *K. rosea*; i, *B. linens*; j, *Ar.* species; k, *Ar. sulfureus* group; l, *B. species*; m, *B. linens*; n, unknown band
Comté	Core	a and b, *St. thermophilus*; c, *E. coli*; d, unknown band; e, *Ent. cloacae-Kl. oxytoca*; f, *S. fonticola*; g, *S. marscesens*; h, *Lb. casei*; i, *Cl. sporogenes*; j, *Br. tyrofermentans*; k, *Ar. sulfureus* group; l, *Ar. protophormiae*; m, unknown band
	Surface	a, *St. thermophilus*; b, *E. coli*; c, unknown band; d, *Kl. oxytoca*; e, *Ent. cloacae-Kl. oxytoca*; f, *B. linens*; g, *S. marscesens*; h, *C. vitaerumints*; i, unknown band; j, *M. luteus* group-*Mc. lacticum*; k, *Br. tyrofermentans*; l, unknown band; m, unknown band; n, *B. linens*; o, *Br. alimentarium-B. species*; p, unknown band; q, *B. linens*; r, *K. kristinae*; s, unknown band; t, unknown band
Beaufort	Core	a, *St. thermophilus*; b, *E. coli*; c, unknown band; d, *S. fonticola*; e, *Lb. casei*
	Surface	a, *St. thermophilus*; b, *E. coli*; c, unknown band; d, *Kl. oxytoca*; e, *Ent. sakazakii*; f, *Ar.* species-*B. linens*; g, *S. marcescens*; h, *M. luteus group-Mc. lacticum*; i, *K. rosea*; j, *C.* species; k, unknown band; l, *Ar. sulfureus* group; m, *B. linens*; n, *Ar. citreus*; o, *B. linens*; p, *B. casei*; q, *K. kristinae*; r, unknown band

[a] Abbreviations: *Ar.*, *Arthrobacter*; *B.*, *Brevibacterium*; *Br.*, *Brachybacterium*; *C.*, *Corynebacterium*; *Cl.*, *Clostridium*; *E.*, *Escherichia*, *Ent.*, *Enterobacter*; *K.*, *Kocuria*; *Kl.*, *Klebsiella*; *Lb.*, *Lactobacillus*; *M.*, *Micrococcus*; *Mc.*, *Microbacterium*; *S.*, *Serratia*, *St., Streptococcus*.

low-G+C-content genomes. These results are consistent with a recent study (37), in which a correlation was made between bacteria having a high-G+C-content genome and the capacity to live under aerobic conditions.

In this study, TTGE and DGGE have proven to be powerful methods to describe the bacterial diversity of various cheeses differing in their technology and microbial composition. Improved knowledge about the composition and location of different bacterial species present in cheese may prove valuable in controlling development of specific microflora during the ripening process.

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