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Identification of Lactococci and Enterococci by Colony Hybridization with 23S rRNA-Targeted Oligonucleotide Probes

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Specific sequences of 23S rRNA of *Lactococcus lactis*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Enterococcus malodoratus/Enterococcus avium* were identified, and complementary oligonucleotide probes were synthesized. The specificity of the probes was evaluated by dot blot and colony hybridizations. The probes can be used for the specific detection and identification of colonies of the corresponding species in mixed cultures.

The identification of microorganisms is of essential importance in basic as well as applied research. In comparison with the classical techniques based on morphological, physiological, and biochemical tests, the application of specific nucleic acid hybridization probes offers the advantage that the organisms can be differentiated on the genetic level on the basis of their natural relationships. In the past few years, it has been shown that rRNAs or the corresponding genes are excellent targets for specific probes. The primary structures of these molecules represent an alternating sequence of more or less conserved regions. Specific probes directed against rRNAs or the genes that encode them (rDNA) could be designed for different levels of natural affiliations (phylogenetic groups) ranging from kingdom to species (5, 6, 11). Besides the fact that one class of molecules, the rRNAs, serves as source and target of a broad range of specificities, these components offer the additional advantage that they represent naturally amplified targets (up to 10^4 copies per cell), enhancing the sensitivity of the method. A variety of hybridization techniques can be applied in combination with specific probes (10). One of these techniques, colony hybridization (7), allows the direct analysis of mixed cultures.

While methods based on nucleic acid extracts prepared from mixed cultures such as environmental, clinical, or food samples allow only an indirect estimation, colony hybridization allows the specific enumeration of bacteria in the mixture. Colony hybridization is a well-established, standard technique for the analysis of gram-negative bacteria (7), but because of the different cell wall structure of gram-positive bacteria it cannot be applied as easily for these organisms (2).

Lactococcus lactis strains are used as starter cultures for various dairy products, whereas enterococci can occur as contaminants. There is an increasing demand for a rapid identification of these organisms. Therefore, we designed 23S rRNA-targeted species-specific probes for *L. lactis*, *Enterococcus faecalis*, and *Enterococcus faecium* and a group-specific probe for *Enterococcus malodoratus/Enterococcus avium*. The bacterial strains used to design probes and those used as reference organisms for evaluating the specificities of the probes are listed in Table 1. All probes are located in the vicinity of the 5′ terminus of the 23S rRNA. Lactococci, enterococci, *Streptococcus salivarius*, and *Lactobacillus casei* were grown anaerobically in M17 (13) broth or on M17 agar plates at 37°C. The remaining strains were cultivated aerobically in nutrient broth (containing, per liter, 10 g of peptone, 8 g of NaCl, 5 g of yeast extract, and 5 g of glucose [pH 7.0]) or on the corresponding solid medium at 30°C.

For the designing of probes, comparative partial sequence analysis of 23S rRNA of *L. lactis*, *E. faecalis*, *E. faecium*, *E. malodoratus*, and *E. avium* was performed. rRNA was extracted by the method of Kirby (9). Variable regions of 23S rRNA (8) were sequenced by using the dideoxy technique in combination with avian myeloblastosis virus reverse transcriptase (Boehringer GmbH, Mannheim, Federal Republic of Germany) and site-specific primers, as described previously (4).

The sequences of oligonucleotide probes, as well as their

TABLE 1. Strains studied and reaction of blotted nucleic acids with specific probes

Species	Strain[a]	Reaction with probe:				
		DB4	DB6	DB8	DB9	327
L. lactis subsp. *lactis*	DSM 20481[T]	+	−	−	−	+
L. lactis subsp. *cremoris*	DSM 20069[T]	+	−	−	−	+
L. lactis subsp. *diacetilactis*	DSM 20661	+	−	−	−	+
L. lactis subsp. *hordniae*	DSM 20450[T]	+	−	−	−	+
Lactococcus garvieae	DSM 20684[T]	−	−	−	−	+
Lactococcus plantarum	DSM 20686[T]	−	−	−	−	+
Lactococcus raffinolactis	DSM 20443[T]	−	−	−	−	+
E. faecalis	DSM 20478[T]	−	−	+	−	+
E. faecium	DSM 20477[T]	−	+	−	−	+
Enterococcus durans	CCM 5612[T]	−	−	−	−	+
Enterococcus hirae	DSM 20160[T]	−	−	−	−	+
Enterococcus gallinarum	DSM 20628[T]	−	−	−	−	+
Enterococcus casseliflavus	CCM 2478	−	−	−	−	+
E. avium	DSM 20063	−	−	−	+	+
E. malodoratus	DSM 20681[T]	−	−	−	+	+
Enterococcus mundtii	DSM 4838[T]	−	−	−	−	+
S. salivarius	DSM 20560[T]	−	−	−	−	+
Lactobacillus casei subsp. *rhamnosus*	DSM 20021[T]	−	−	−	−	+
Bacillus subtilis	DSM 10[T]	−	−	−	−	+
Micrococcus luteus	DSM 20030[T]	−	−	−	−	+
Staphylococcus carnosus	DSM 20501[T]	−	−	−	−	+
Pseudomonas fluorescens	DSM 50090[T]	−	−	−	−	+

[a] DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Federal Republic of Germany; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; T, type strain.

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TABLE 2. Sequences, specificities, and hybridization and washing temperatures of the probes

Probe	Sequence	Specificity	Temp (°C) for:	
			Hybridization	Washing
DB4	5'CACTCATGAATAACTGCTAG3'	*L. lactis*	42	47
DB6	5'CACACAATCGTAACATCCTA3'	*E. faecium*	42	47
DB8	5'TAGGTGTTGTTAGCATTTCG3'	*E. faecalis*	42	47
DB9	5'TAGGTGCCAGTCAAATTTTG3'	*E. avium*/*E. malodoratus*	42	47
327	5'CCTGTGTCGGTT3'	Universal	30	33

specificities, are listed in Table 2. The sequences of the probes are complementary to the corresponding 23S rRNA sequences, allowing the use of rRNAs as naturally amplified targets. The oligonucleotides were synthesized on a Cyclone DNA synthesizer (MilliGen/Biosearch, Eschborn, Federal Republic of Germany). They were 5'-end labeled by using T4 polynucleotide kinase (Boehringer GmbH) and [γ-^{32}P]ATP (NEN, Dreieich, Federal Republic of Germany) according to the procedure described by Sambrook et al. (12).

The specificities of the probes were reevaluated by dot blot hybridization to membrane-bound crude nucleic acids extracted from pure cultures of test strains (Table 1). Nucleic acids were extracted by using a rapid lysis technique, as follows. Cells from 1-ml overnight cultures were harvested by centrifugation and suspended in 400 μl of 0.01 M Tris-HCl–0.001 M EDTA, pH 8.0. After extraction in hot phenol (2 min at 100°C), the nucleic acids were precipitated with ethanol. One microgram of crude nucleic acids was denatured at 55°C for 5 min in 100 μl of a buffer containing 50% formamide, 4% formaldehyde, 0.02 M 3-(N-morpholino)propanesulfonic acid, 0.005 M sodium acetate, and 0.001 M EDTA. The denatured nucleic acids were bound to membranes (Zeta Probe; Bio-Rad, Munich, Federal Republic of Germany) in a dot blot apparatus (Minifold; Schleicher & Schuell, Dassel, Federal Republic of Germany). The nucleic acids were immobilized on the membrane by baking at 80°C in a vacuum oven for 2 h.

The membranes were incubated in 0.1× SSC (standard saline-citrate; 1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate [pH 7.0]) containing 0.5% sodium dodecyl sulfate (SDS) at 50°C for 1 h and then prehybridized in a solution containing 6× SSC, 0.1% SDS, 0.5% sodium N-lauroyl sarcosine, and 5× Denhardt solution (3) (1× Denhardt solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin) for 2 h at 50°C. Hybridization was conducted in prehybridization solution containing 5 pmol of labeled probe for 4 h. The incubation temperatures used for the particular probes are listed in Table 2. The membranes were washed in 0.1× SSC containing 0.1% SDS twice for 5 min each time at ambient temperature and subsequently once at the probe-dependent temperature as specified in Table 2. After autoradiography, the filters were prepared for further hybridization by denaturing the hybrids in 0.1× SSC containing 0.1% SDS for 1 h at 65°C.

To avoid false-negative results, all membranes used in hybridization experiments were rehybridized to a universal probe complementary to a highly conserved region of 23S rRNA. Hybridization signals obtained with this universal probe indicate the amount of target RNA present on the filter and accessible to the probe. All probes were species specific or at least group specific.

Pure and mixed cultures were analyzed by colony hybridization. Bacterial cells were grown on Zeta Probe membranes layered on M17 solid medium under the conditions described above. Then the membranes were placed on filter paper (Whatman 3) soaked in 2× SSC containing 5% SDS and heated in a microwave oven (700 W) for 1 to 2 min (1). The membranes were dried at 80°C for 10 min or at room temperature overnight and then were fixed by spraying with a colorless, fast-drying acrylic lacquer (e.g., from Wacolux, Zürich, Switzerland) and used for hybridization. Treatment with the lacquer prevented disintegration of the colonies.

A colony hybridization experiment is documented in Fig. 1. For easier differentiation, the cells were inoculated on the membrane in such a way that the colonies appeared as letters (Fig. 1a). The signals obtained by hybridization to the universal probe demonstrate that rRNAs of lactococci, enterococci, streptococci, and lactobacilli are accessible to

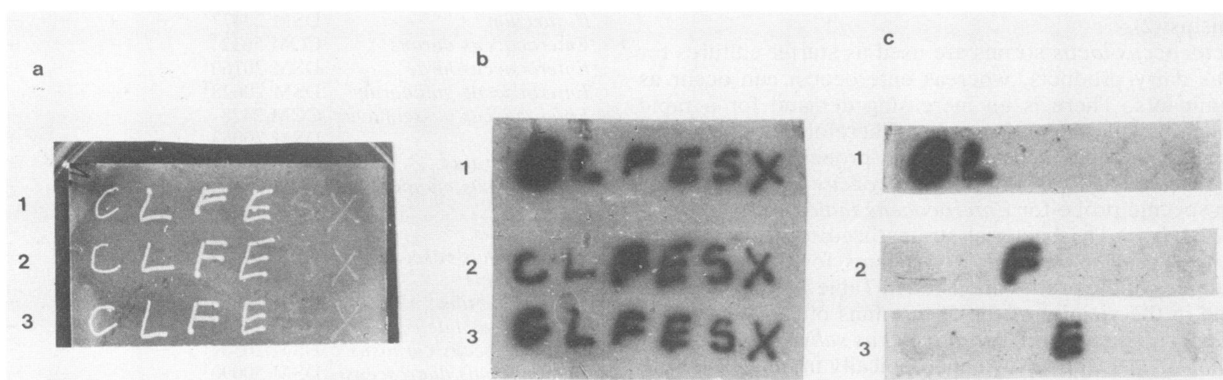

FIG. 1. Colony hybridization of *Lactococcus lactis* subsp. *cremoris* (C), *Lactococcus lactis* subsp. *lactis* (L), *E. faecalis* (F), *E. faecium* (E), *S. salivarius* (S), and *Lactobacillus casei* subsp. *rhamnosus* (X). (a) Filter-bound colonies; (b) hybridization to the universal probe 327; (c) hybridization to the specific probes DB4 (lane 1), DB6 (lane 2), and DB8 (lane 3).

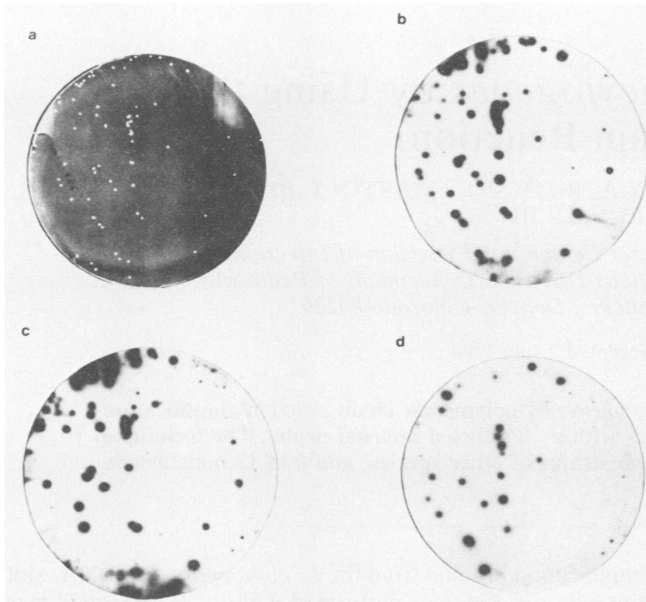

FIG. 2. Colony hybridization of a mixed culture of *Lactooccus latis* subsp. *lactis* and *E. faecalis*. The sample was plated on Zeta Probe membrane layered on M17 solid medium, cultured overnight, and analyzed by colony hybridization. (a) Filter-bound colonies; (b) hybridization to the universal probe 327; (c) hybridization to the specific probe DB8; (d) hybridization to the specific probe DB4.

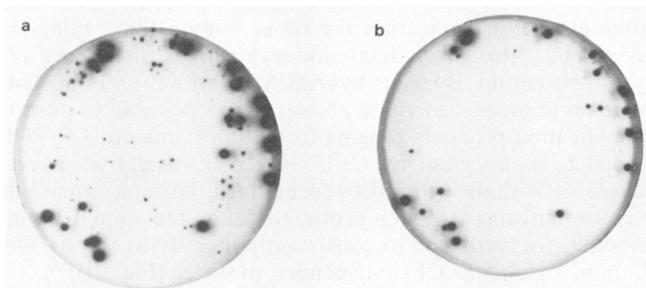

FIG. 3. Colony hybridization of the culturable flora of spontaneously fermented milk. The sample was plated on a Zeta Probe membrane layered on M17 solid medium, cultured overnight, and analyzed by colony hybridization. (a) Hybridization to a universal probe; (b) hybridization to the *Lactococcus lactis*-specific probe DB4.

oligonucleotide probes in colony hybridization experiments (Fig. 1b). Figure 1c shows the signals obtained when the membranes were rehybridized to the specific probes. The organisms can be differentiated easily. The probes presented here were also successfully used for the specific enumeration of lactococci as CFU in a mixed population of lactococci and enterococci and in spontaneously fermented milk (Fig. 2 and 3).

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