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In situ Identification of Lactococci, Enterococci and Streptococci

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Summary

The genera *Lactococcus, Enterococcus,* and *Streptococcus* contain species of biotechnological and medical importance. The need for rapid and reliable identification of isolates has prompted the development of several 16S and 23S rRNA based oligonucleotide probes. Fluorescent derivatives of probes specific for *Lactococcus lactis, L. lactis* subsp. *cremoris, Enterococcus faecalis, E. faecium, Streptococcus salivarius* and *S. thermophilus* were used for whole cell hybridization. Optimal probe permeability of fixed cells required special pretreatment of these Gram-positive organisms. Fluorescent probing in combination with epifluorescence microscopy resulted in single cell identification. With this assay lactococci, enterococci, and streptococci can be identified in milk samples within one day.

Key words: Catalase-negative cocci – Whole cell hybridization – rRNA-targeted oligonucleotide – *In situ* identification – Raw milk

Introduction

The genus *Streptococcus* originally included all catalase-negative, Gram-positive cocci which are arranged in pairs and chains and are usually facultative anaerobes. The application of nucleic acid hybridization (*Kilpper-Bälz* et al., 1982) and rRNA sequencing techniques (*Ludwig* et al., 1985) revealed that this group consisted of three genetically distinct subgroups. In widely accepted taxonomic revisions the fecal streptococci were described as a new genus *Enterococcus* (*Schleifer* and *Kilpper-Bälz,* 1984), and *Streptococcus lactis* and related streptococci were transferred to the genus *Lactococcus* gen. nov. (*Schleifer* et al., 1985). The revised genus *Streptococcus sensu stricto* comprises now mainly the pyogenic and oral streptococci including the pneumococci, but not the anaerobic streptococci (*Schleifer* and *Kilpper-Bälz,* 1987).

Medical and food microbiologists have considerable interest in these three genera: Some *Streptococcus* spp. can cause serious diseases in man and animals (*Ruoff,* 1991), others (e.g. *Streptococcus thermophilus*) are beneficial and used in starter cultures (*Hardie* and *Whiley,* 1991). Enterococci are intestinal saprophytes and some species are used as indicators of fecal contamination and as probiotics preventing enteric disease (*Devriese* et al., 1991). Lactococci are important in the fermentation and ripening of dairy products (*Teuber* et al., 1991). Most members can be isolated and identified using standard techniques. Nevertheless, the need for more rapid and reliable identification has led to the development of nucleic acid probes specific for certain genera, species or subspecies within this group of bacteria (*Betzl* et al., 1990; *Salama* et al., 1991; *Colmin* et al., 1991; *Klijn* et al., 1991; *Lick* and *Teuber,* 1992; *Ehrmann* et al., 1992). Here, we tested the applicability of fluorescently labelled, rRNA-targeted oligonucleotide probes for single cell identification in a whole cell hybridization assay.

Fluorescent oligonucleotides have successfully been used to detect and identify individual bacterial cells (*DeLong* et al., 1989; *Amann* et al., 1990a; *Stahl* and *Amann,* 1991). These probes are small enough to penetrate whole fixed cells and to hybridize specifically to intracellular rRNA. Microorganisms can be identified *in situ* without prior cultivation (*Amann* et al., 1991). It has been observed that permeability for oligonucleotide probes is hindered in fixed cells of certain Gram-positive bacteria (*Schleifer* et al., 1991; *Salama* et al., 1991; *Hahn* et al., 1992). Therefore, different cell fixation and permeabiliza-

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tion protocols were compared to achieve optimal whole cell hybridization of the Gram-positive, catalase-negative cocci.

Materials and Methods

Organisms and growth conditions. Sources and strains are listed in Table 1. All lactococcal and streptococcal strains were grown anaerobically at 37 °C in M17 broth (*Terzaghi* and *Sandine,* 1975), enterococci were cultivated anaerobically at 37 °C 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].

Cell fixation and permeabilization. Cells in exponential phase ($O.D._{550\ nm}$ of 0.5–1.2) or activated sludge samples were harvested by centrifugation (2 min, 5000 × g) and washed in phosphate buffered saline (PBS: 130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4). Cells were fixed for 3 to 16 h in 3% paraformaldehyde/PBS and stored in a 1 : 1 mixture of ethanol and PBS as described (*Amann* et al., 1990b). For comparison fixations were also performed in 90% methanol/3.7% formaldehyde (*DeLong* et al., 1989) and in ethanol/PBS 1 : 1. PBS-washed or ethanol/PBS fixed cells were also heatfixed as described (*Jurtshuk* et al., 1992) followed by a dehydration in an ethanol series (50, 80, 96% for 3 min each).

Cell extraction from milk. Raw milk samples were incubated at 30 °C for 4 h to stimulate bacterial growth. 30 ml samples were centrifuged (5 min; 5000 × g) and the supernatants discarded. Particulate milk components were removed from microbial cells by two cycles of resuspension of pellets in 1 ml buffer containing 100 mM Na_2HPO_4, 150 mM NaCl, 10 mM EDTA, and 40 mM NaOH, followed by 2 min of centrifugation at 8000 × g and removal of the supernatant (Dr. *Denariaz,* pers. comm.). The resulting cell pellets were resuspended in PBS/ethanol and heatfixed (*Jurtshuk* et al., 1992).

Preparation of cell smears. Fixed-cell suspensions were spotted onto glass slides coated with gelatin (0.1% gelatin, 0.01% potassium chromium sulfate), dried at 37 °C for 30 min and finally immersed for 3 min each in 50, 80 and 96% ethanol.

Enzymatic permeabilization of fixed cells. Cell smears were covered with 20 µl of enzyme solution and incubated at 0 °C in a humidity chamber. The following enzymes were tested: (i) lysozyme (Sigma) at 0.1 to 10 mg/ml in buffer containing 100 mM Tris/HCl, 50 mM EDTA (pH 8.0); (ii) 5,000 U/ml mutanolysin (Sigma) in 100 mM potassium phosphate buffer (pH 6.2). Enzymatic treatments were stopped by rinsing the slides thoroughly with distilled water followed by a dehydration of cells in 50, 80, 96% ethanol for 3 min each.

Oligonucleotides and labelling. The following rRNA-targeted oligonucleotide probes were used: Efs specific for *E. faecalis* and Efm for *E. faecium* (*Betzl* et al., 1991; modified), Sth for *S. thermophilus* and Ssa for *S. salivarius* (*Ehrmann* et al., 1992), and Llc for *Lactococcus lactis* subsp. *cremoris* (*Salama* et al., 1991). Based on comparative analysis of 23S rRNA sequences (TUM data base; *W. Ludwig,* pers. comm.) probe Lla was designed specific for the species *Lactococcus lactis.* The target site is homologous to positions 271–289 of *Escherichia coli* 23S rRNA (*Brosius* et al., 1981). The bacterial probe EUB (*Amann* et al., 1990b) served as a positive control for permeability of fixed cells for rRNA-targeted oligonucleotides. All probe sequences are given in Table 2.

Probes were synthesized with a C6-TFA aminolink [6-(Trifluoroacetyl-amino)-hexyl-(2-cyanoethyl)-(N,N-diisopropyl)-phosphoramidite] at the 5′-end (MWG Biotech; Ebersberg, Ger-

Table 1. Listing of studied strains and results of whole cell hybridization with oligonucleotide probes

Species	Strain	Reaction with probe:						
		Efs	Efm	Ssa	Sth	Lla	Llc	Eub
L. lactis subsp. *lactis*	DSM 20481^T	−	−	−	−	+	−	+
L. lactis subsp. *lactis*	WS 1042	−	−	−	−	+	−	+
L. lactis subsp. *lactis*	WS 1683	−	−	−	−	+	+	+
L. lactis subsp. *cremoris*	DSM 20069^T	−	−	−	−	+	+	+
L. lactis subsp. *cremoris*	WS 1681	−	−	−	−	+	+	+
L. lactis subsp. *cremoris*	WS 1682	−	−	−	−	+	+	+
L. lactis subsp. *diacetilactis*	DSM 20661	−	−	−	−	+	−	+
L. lactis subsp. *diacetilactis*	WS 1677	−	−	−	−	+	−	+
L. lactis subsp. *diacetilactis*	WS 1679	−	−	−	−	+	−	+
L. lactis subsp. *diacetilactis*	WS 1685	−	−	−	−	+	−	+
L. lactis subsp. *diacetilactis*	WS 1686	−	−	−	−	+	−	+
L. raffinolactis	DSM 20443^T	−	−	−	−	−	−	+
L. garviae	WS 1029	−	−	−	−	−	−	+
L. plantarum	DSM 20686^T	−	−	−	−	−	−	+
E. faecalis	DSM 20478^T	+	−	−	−	−	−	+
E. faecium	DSM 20477^T	−	+	−	−	−	−	+
S. salivarius	DSM 20560^T	−	−	+	−	−	−	+
S. thermophilus	DSM 20479^T	−	−	−	+	−	−	+
Bacillus subtilis	DSM 10^T	−	−	−	−	−	−	+
Lactobacillus casei	DSM 20011^T	−	−	−	−	−	−	+

Abbreviations: T: type strain. DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, FRG. WS: Bakteriologisches Institut, Süddeutsche Versuchs- und Forschungsanstalt für Milchwirtschaft Weihenstephan, TU München, Freising-Weihenstephan, FRG.

Table 2. Probe sequences and optimal formamide concentration

Probe	Target organism	Sequence	% formamide
Efs	*E. faecalis*	5′-GGTGTTGTTAGCATTTCG-3′	25
Efm	*E. faecium*	5′-CACACAATCGTAACATCC-3′	20
Sth	*S. thermophilus*	5′-CATGCCTTCGCTTACGCT-3′	25
Ssa	*S. salivarius*	5′-CATACCTTCGCTATTGCT-3′	20
Llc	*L. lactis* subsp. *lactis*	5′-TGCAAGCACCAATCTTCATC-3′	10
Lla	*L. lactis* subsp. *cremoris*	5′-CTATAATGCTTAAGTCCACG-3′	20
EUB	*Bacteria*	5′-GCTGCCTCCCGTAGGAGT-3′	n.d.

many). Labelling with tetramethylrhodamine-5-isothiocyanate (TRITC, Molecular Probes, Eugene, USA) or 5(6)-carboxyfluorescein-N-hydroxysuccinimide-ester (FLUOS, Boehringer Mannheim, Mannheim, Germany) was performed as described (*Amann* et al., 1990a).

Whole cell hybridization. Samples of 8 µl hybridization solution (0.9 M NaCl, 20 mM Tris/HCl pH 7.2, 0.01% SDS, 50 ng probe, X% formamide) were applied to each well of the microscopic slide and incubated for 2 h at 46 °C in an isotonically equilibrated humid chamber. Unbound probe was removed from the slide with 2 ml of washing solution (20 mM Tris/HCl, 0.01% SDS, Y M NaCl, 5 mM EDTA). Slides were immediately transferred in 50 ml of washing solution and incubated at 48 °C for 20 min. Slides were rinsed briefly with distilled water, air-dried and mounted with Citifluor solution (Citifluor Ltd, London, UK). Fluorescence was detected with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence with a 50 W mercury high pressure bulb and Zeiss filter sets nos. 09 and 15. Photomicrographs were on Kodak Ektachrome P1600 color reversal film or on TMAX 400 black-and-white film. Exposure times were 0.01 s for phase contrast and 8–30 s for epifluorescence.

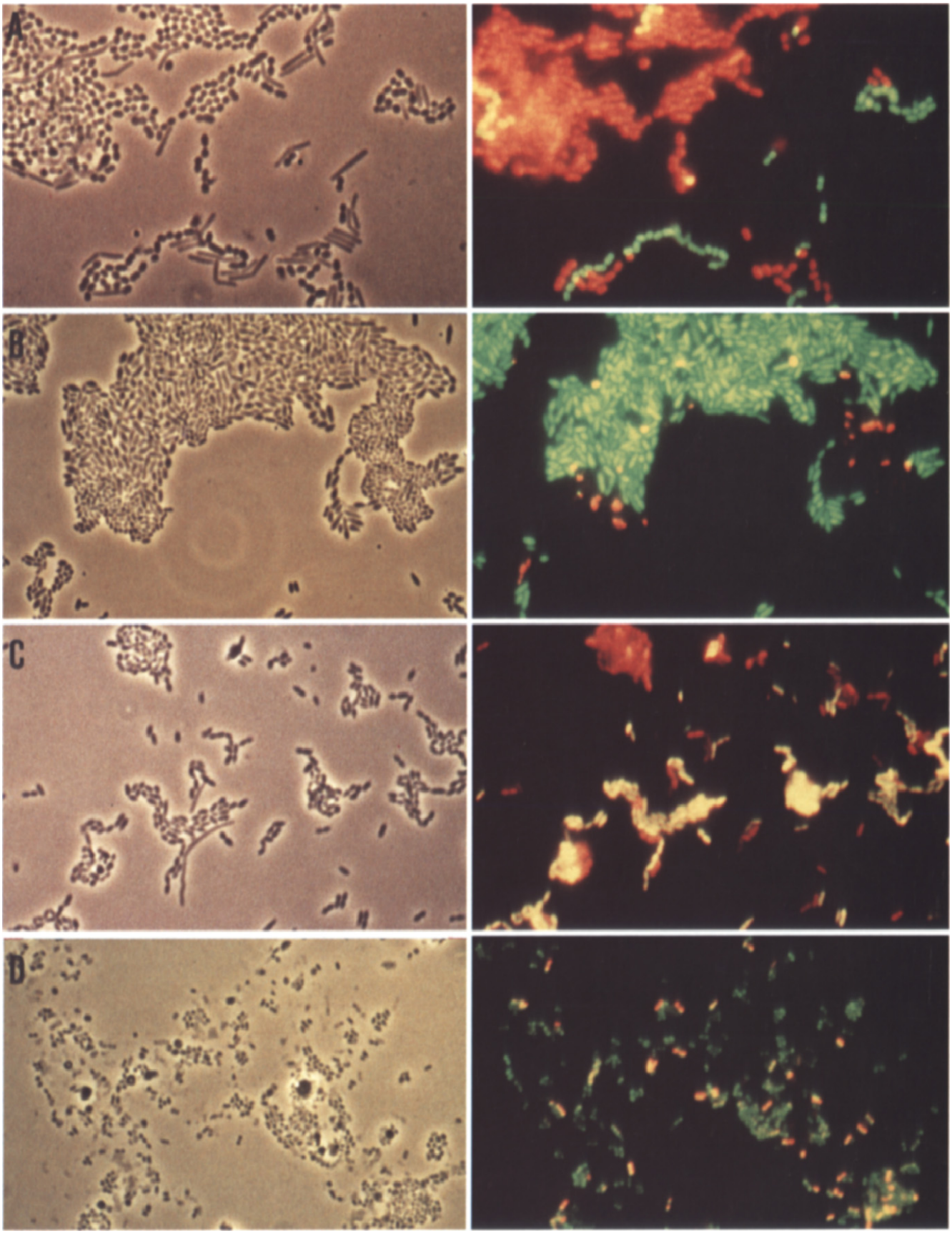
Results and Discussion

Permeabilizing Gram-positive cells for oligonucleotide probes

Hybridization of the paraformaldehyde-fixed Gram-positive cocci with fluorescent oligonucleotides resulted in hardly detectable amounts of probe-conferred signal. Since cells were taken from cultures growing in the exponential phase with generation times around one hour the most likely explanation was not a low cellular rRNA content but limited probe penetration to the intracellular targets. *Salama* et al. (1991) described limited permeability of certain lactococci strains for isotopically labeled oligonucleotides. Lysozyme pretreatments had been shown to improve permeability of *L. lactis* subsp. *cremoris* (*Schleifer* et al., 1991) and *Streptomyces scabies* (*Hahn* et al., 1992) for fluorescent oligonucleotide probes. Hybridization with probe EUB was used to evaluate the influence of treatments with different cell wall lytic enzymes on the probe permeability of various Gram-positive bacteria. An incubation with 1 mg/ml lysozyme at 0 °C for 20 min was optimal for most of the Gram-positive cocci tested. Strong and uniform whole cell hybridizations were achieved (Fig. 1). This was surprising as lactococci and enterococci are regarded as fairly resistant to lysozyme (*Kondo* and *McKay,* 1984; *Rogers* et al., 1980). Cells of *S. salivarius,* however, were only slightly permeabilized by lysozyme treatment. With a mutanolysin pretreatment, at least part of the fixed cells from a *S. salivarius* pure culture has detectable fluorescence after hybridization. Permeabilization of fixed cells with enzymes seems to be species- or strain-dependent. There is probably no universal enzymatic pre-treatment that permeabilizes all different types of Gram-positive cell walls. That Gram-negative cells were often lysed before Gram-positive cells became permeable was a disadvantage of enzymatic pretreatments of mixed samples.

Extended storage for several weeks/months in ethanol/PBS 1 : 1 gradually increased the permeability of paraformaldehyde-fixed cell preparations. The requirement for enzymatic permeabilization of Gram-positive cells was probably partly caused by a reversible crosslinking of cell

Fig. 1. Whole cell identification of enterococci, streptococci and lactococci. In each panel identical fields are viewed by phase contrast (left) and epifluorescence microscopy (right; double exposures using filter sets 09 and 15).
Panel A. *B. subtilis, E. faecium* and *E. faecalis* hybridized with the fluorescein-labelled probe Efs (specific for *E. faecalis*) and the rhodamine-labelled probe Efm (specific for *E. faecium*). In the epifluorescence micrograph cells of *E. faecalis* are visualized green and *E. faecium* red. Where cells of these two species lay on each other the resulting colour upon double exposure is yellow. *Bacillus subtilis* cells can easily be identified in phase contrast as rods and served as negative control showing no probe binding.
Panel B. *S. thermophilus* and *S. salivarius* cells were hybridized with fluorescein-labelled probe Sth (specific for *S. thermophilus*) and rhodamine-labelled probe Ssa (specific for *S. salivarius*). Only part of the *S. salivarius* cells bound detectable amounts of probe Ssa. Again yellow spots are not the result of rhodamine- and fluoresein-labelled probes binding to one cell but of overlayed cells.
Panel C. In a mixture of *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris* and *Bacillus subtilis* the rhodamine-labelled species-specific probe Lla hybridized to all cocci, the fluorescein-labelled "subspecies-cremoris" probe only to part of the cocci. Consequently, in the double exposure epifluorescence micrograph *L. lactis* subsp. *lactis* cells appear red and *L. lactis* subsp. *cremoris* appear yellow.
Panel D. Cells extracted from milk were hybridized with the fluorescein-labelled bacterial probe Eub and rhodamine-labelled probe Lla, specific for the species *Lactococcus lactis. Lactococcus lactis* cells are visualized yellow due to binding of both probes, other bacteria hybridizing only with Eub are green.


A
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wall components during paraformaldehyde fixation. Cells fixed in ethanol/PBS 1 : 1 and subsequently dehydrated in 50, 80, 96% ethanol for three min each were accessible for probes without enzymatic pretreatments as were cells fixed in methanol/formaldehyde for 20 min. Fixing by heat did not enhance further permeability of cells that had been growing in the exponential phase but increased permeability of cells harvested from stationary phase. Fluorescent signals after hybridization with all these fixation methods were slightly below the signals obtained with paraformaldehyde-fixed cells after enzyme treatment. However, since ethanol/PBS fixation followed by heat fixation permeabilized cells from various species and different growth phases, we applied this method to the characterization of cells recovered from milk.

Optimizing probe specificities

Specificities had already been determined (*Betzl* et al., 1990; *Salama* et al., 1991; *Ehrmann* et al., 1992) for all probes in this study except for the newly designed probe Lla. The composition of hybridization and wash buffers used in this study differed significantly from the buffers used in earlier studies. Furthermore, we could not apply washing temperatures above 50 °C without causing damage (often complete lysis) to the fixed cells. Stringency of hybridization at a fixed temperature of 46 °C was increased by addition of formamide to the hybridization buffer. For each probe we determined the formamide concentration that facilitated optimal discrimination between target and nontarget cells (Table 2). During the 48 °C washing step, stringencies were held at the hybridization level by lowering the concentration of sodium chloride in the buffer according to the formula of *Wahl* et al. (1987), e. g. the stringency of a 20% formamide hybridization buffer containing 0.9 M NaCl is approximately the same as in a washing buffer lacking formamide and containing only 0.18 M NaCl. For probes Ssa and Sth, addition of formamide did not only increase the specificity of probes but also the sensitivity was enhanced; more probe could bind to identical fixed cell preparations. Preliminary quantifications using image analysis showed a four-fold increase of mean fluorescence from 24.8 (buffer without formamide) to 108.4 relative units (buffer containing 25% formamide) for fluorescein-labelled probe Sth (*Trebesius* et al., unpublished data).

Whole cell hybridizations of lactococci, enterococci and streptococci resulted in clear cut identification of individual cells. *E. faecalis* and *E. faecium* could be differentiated by simultaneous hybridization with fluorescein-labeled probe Efs and rhodamine-labelled probe Efm (Fig. 1, panel A). Specific binding of probes Sth and Ssa to cells of *S. thermophilus* and *S. salivarius* is demonstrated in panel B of Fig. 1. In a mixture of *L. lactis* subsp. *lactis*, *L. lactis* subsp. *cremoris* and *B. subtilis* the "subsp.-cremoris" probe Llc bound only to part of the cocci binding the species-specific probe Lla (Fig. 1, panel C). Hybridizations of several reference strains yielded the expected results (Table 1) except for the "subsp.-cremoris" probe Llc that hybridized to one strain of *L. lactis* subsp. *lactis* (WS 1683). The presence of the probe Llc target site in this strain was also confirmed by sequencing (*Köhler* and *Ludwig*, unpublished data). The subspecies status of this strain was rechecked by conventional tests (*Teuber* et al., 1991) and the phenotype *L. lactis* subsp. *lactis* could be confirmed. Similar discrepancies between classical phenotypic and molecular identification methods have been reported before (*Godon* et al., 1992).

Rapid differentiation of lactococci and enterococci in milk

Characterization of the bacterial flora in milk with cultural methods is laborious and requires several days. We performed preliminary experiments to demonstrate the potential of fluorescent probes for more rapid identifications. Raw milk samples were transported on ice to the laboratory. Cells were concentrated from clarified milk by centrifugation and transferred to gelatine-coated glass slides for hybridization. Direct identification of bacterial cells from these samples failed, probably due to low ribosomal RNA content. A 4 h incubation at 30 °C increased cellular ribosomal RNA contents and facilitated detection of approx. 60% of the cells visualized by phase contrast microscopy. Specific identification of enterococci (data not shown) and lactococci (Fig. 1, panel D) in these cell preparations was achieved by whole cell hybridization within additional 4 h. Thus part of the bacterial flora of a milk sample can be analyzed within one day.

Future perspectives

The described method is currently far from being applicable as a routine procedure. The handling times on each sample are fairly long so that only several samples can be processed simultaneously and the final microscopic evaluation requires experienced staff. However, automatization of the procedure might be possible. It has been demonstrated that fluorescent oligonucleotide probing of whole cells can be combined with flow cytometric quantification (*Amann* et al., 1990; *Wallner* et al., 1993). Flow cytometers allow the rapid analysis of multiple samples in relatively short times.

The rRNA-targeted probes used in this study were specific to the species- or subspecies level. The method is not suitable for strain identification. Even though strain to strain differences can sometimes be found in the rRNA molecules (more likely on the 23S than the shorter 16S rRNA; *D. Betzl*, Ph.D. thesis, TU München 1990), this is by pure chance. Ribosomal RNA molecules are in general too conserved to allow strain differentiation. The fluorescent oligonucleotide probes could also be used for ecological studies on their target organisms, e. g., little is known on the natural habitats of lactococci. To demonstrate the potential of fluorescent oligonucleotide probing for *in situ* identification in complex environments activated sludge from a sewage plant receiving waste water from a dairy company was hybridized with the probe specific for *Streptococcus thermophilus*. As expected only cocci were specifically binding probe Sth (Fig. 2). Culture-independent *in*

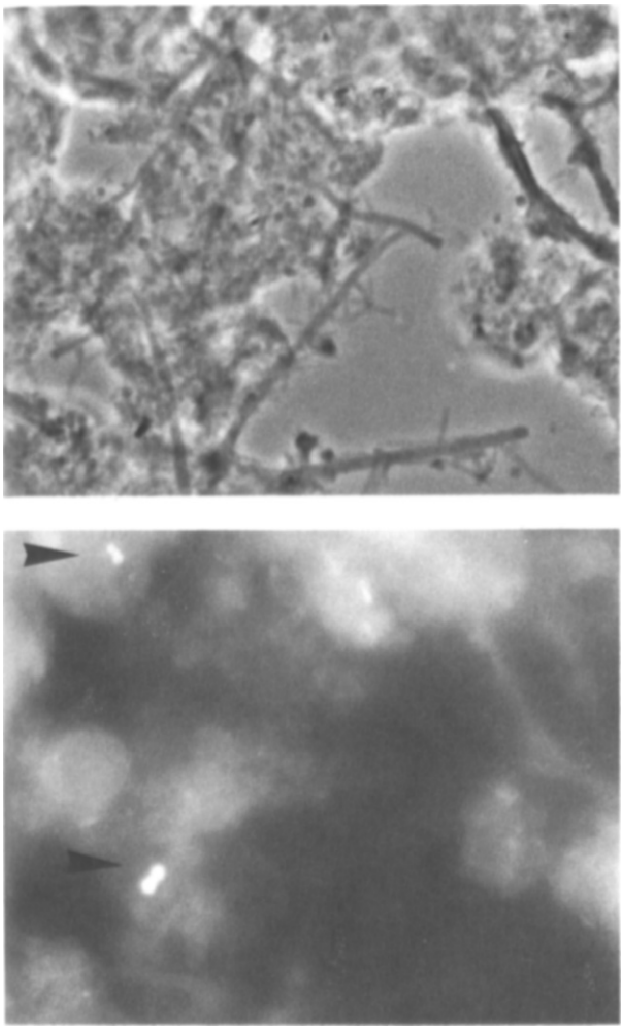

Fig. 2. *In situ* identification of *Streptococcus thermophilus* (arrow heads) in an ethanol-fixed activated sludge sample using fluorescein-labeled probe Sth. Phase contrast (upper panel) and epifluorescence micrographs (lower panel) are shown for identical microscopic fields.

situ studies could yield new insights important for both basic and applied research.

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