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Detection and identification of lactic acid bacteria in milk and industrial starter culture with fluorescently labeled rRNA-targeted peptide nucleic acid probes

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Abstract — A fast and simple method for whole-cell hybridization using fluorescently labeled rRNA-targeted peptide nucleic acid (PNA) probes was developed for use in detection and identification of thermophillic lactobacilli cells growing in milk or present in industrial starter cultures. The protocol uses a filtration technique of the samples and epifluorescence microscopy as a detection system, and is completed within 1.5 h. Seven oligonucleotide probes with different ranges of specificity have been tested in in situ hybridization experiments against a number of collection and industrial strains including those of the species *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Leuconostoc* sp. and *Streptococcus thermophilus*. Certain limitations in the use of DNA probes due to non-specific hybridization were encountered and taken into account. Depending on the probe used, a specific and simultaneous detection of the different species present can be achieved. The equipment used was able to detect between 10^4 and 10^6 cells per mL. The physiological state of a starter cultures of thermophilic lactobacilli can also be evaluated by in situ hybridization. Thus, this fast method can be used for starter cultures and industrial fermentation quality control. This work constitutes the first reported use of PNA molecules for in situ detection and identification of bacteria in milk or in an industrial medium.

PNA / fluorescent in situ hybridization / lactic acid bacteria / rRNA / industrial starter culture

Résumé — Détection et identification des bactéries lactiques dans le lait et les levains industriels par des sondes « *peptide nucleic acids* » fluorescentes ciblant l'ARNr. Une méthode rapide et simple pour détecter et identifier les bactéries lactiques en croissance, au niveau cellulaire, dans le lait ou les levains industriels a été développée. Elle repose sur la technique d'hybridation de sondes fluorescentes à l'ARN ribosomique présent dans les cellules entières. Les « *peptide nucleic acids* »

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(PNA), analogues de l'ADN, ont été utilisés pour la synthèse de certaines de ces sondes. La méthode utilise aussi une technique de filtration des échantillons et la microscopie à épifluorescence. La détection et l'identification des cellules sont réalisées en une heure et demie. Sept sondes oligonucléotidiques présentant des spécificités différentes ont été testées dans des expériences d'hybridation in situ pour détecter des souches industrielles et d'autres provenant de collections de micro-organismes. Les espèces suivantes étaient représentées : *Lactobacillus delbrueckii*, *Lactobacillus helveticus*, *Lactobacillus pentosus*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Leuconostoc* sp. et *Streptococcus thermophilus*. Des limites dans l'utilisation de certaines sondes ont été rencontrées à cause d'hybridations non spécifiques observées. En fonction de la sonde utilisée, une détection spécifique et simultanée des différentes espèces en présence peut être réalisée. L'équipement utilisé permet la détection d'un nombre de cellules compris entre 10^4 et 10^6 par mL de milieu. L'état physiologique de levains thermophiles a pu aussi être évalué par hybridation in situ. Ainsi, cette technique rapide est tout à fait adaptée pour le contrôle de la qualité des levains et des fermentations industrielles. Ce travail constitue une première description de l'utilisation de molécules de PNA pour la détection et l'identification des bactéries dans le lait et les milieux industriels.

PNA / hybridation in situ / bactérie lactique / ARNr / levain industriel

1. INTRODUCTION

Direct detection and identification of bacterial species at the single-cell level constitute an important approach that can lead to important applications in several areas of microbiology, including public health, quality of environment and depollution, and quality of water, food and other manufactured products sensitive to microbial contamination. Rapid and accurate detection of industrial micro-organisms such as lactic acid bacteria directly in milk, starter culture or fermented food allows a better control of the fermentation process leading to a better quality of final products.

Recent advances result in improved sensitivity and reliability of nucleic-acid-based methods for species-specific detection of microorganisms. 16S rDNA (and to some extent, 23S rDNA) sequences are now routinely used for the identification of bacteria [18, 24, 29]. Conserved and variable regions of the molecule can be used as potential targeted regions for hybridization and allow classification at the species, genus or family level [1, 4]. The huge sequence database that is currently available [6, 19] provides the necessary information: (i) to define probes from sequence alignments, and (ii) to evaluate their specificity and thus confirm the choice of the probe by comparing sequences with those of the databank before hybridization experiments are performed.

The large amounts of rRNA in exponential-phase bacteria assures the excellent sensitivities of in situ hybridization assays and facilitates whole-cell detection. In *E. coli*, a direct correlation is observed between the growth rate of the cells, the average ribosome content, and the fluorescence conferred by hybridization probes [9].

In this study, we developed a protocol for rapid whole-cell hybridization of fluorescently labeled rRNA probes to detect and identify lactic acid bacteria cells in milk or in industrial starter cultures. The protocol uses peptide nucleic acid (PNA; [7, 8]) probes in conjunction with a rapid membrane filtration-epifluorescent microscopy technique (Biocom, Les Ulis, France) [21]. PNA molecules are DNA analogues in which the sugar-phosphate backbone is replaced with a neutral backbone consisting of N-(2-aminoethyl) glycine units. The PNA-RNA duplex exhibits higher thermal stability than the corresponding DNA-RNA

duplex, in part due to the lack of electrostatic repulsion between the target site and the PNA strand and to hydrophobic interactions [11]. Moreover, hybridization kinetics of duplex formation is faster for PNA-RNA than for DNA-RNA [11]. The hydrophobicity of PNA molecules improves their ability to penetrate more easily through the bacterial cell membrane and to hybridize rapidly on rRNA targeted molecules, and thereby makes of PNA a feasible tool for the development of fast protocols for in situ hybridization [22, 27].

In the present study, we describe new PNA- and DNA-based protocols for in situ rRNA probing of lactic acid bacteria in milk or in industrial starter cultures. An application is proposed to control the quality of an industrial thermophilic lactic acid starter culture.

2. MATERIALS AND METHODS

2.1. Organisms and growth conditions

Lactococcus lactis and *Streptococcus thermophilus* strains were grown in M17 broth [26] at 30 °C and 42 °C, respectively. *Leuconostoc*, *Weissella*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus paraplantarum*, *Lactobacillus pentosus* and *Lactobacillus rhamnosus* strains were grown at 30 °C in MRS broth [10]. *Lactobacillus delbrueckii* subspecies *bulgaricus* and subspecies *lactis*, *Lactobacillus fermentum* and *Lactobacillus helveticus* strains were grown at 42 °C in MRS broth. *Enterococcus faecalis* strains were grown at 42 °C in brain heart infusion broth (Difco, Paris, France). Strains of these species were also grown in co-culture in milk or in an industrial broth (thermophilic lactobacilli) of undefined composition (Phagex, Standa-Industrie, Caen, France). Ready-to-use freeze-dried co-cultures of *Lb. helveticus* strain LH56 and *Lb. delbrueckii* subspecies *lactis* strain LL57 (commercial culture PAL LH56-LL57) were supplied by Standa-Industrie.

2.2. Acid stress test

Lb. helveticus strain CNRZ32 was grown overnight in MRS broth at 42 °C. A 500 µL-aliquot was resuspended in 10 mL of fresh MRS broth. The culture was incubated for 1 h at 42 °C. Two mL-aliquots were centrifuged and the cells were resuspended either in 2 mL fresh MRS broth (control) or in 2 mL fresh MRS broth acidified up to 3.5–3.6 with HCl (acid stress test). After a 2 h incubation at 42 °C, the cultures were treated by in situ hybridization as described below (see Sects. 2.4 and 2.5).

2.3. Labeling rRNA targeted probes

The general probe, Eub338a (5'-GCTGCCTCCCGTAGG-3'), is a 15-mer derivative of the previously published Eub338 [2]. Probes P1 to P6 are 18-mer oligonucleotides, specific for different species (P1, P2, P4) or group of species (P3, P5, P6) of lactic acid bacteria, as presented in Tables Ia and Ib. Fluorescent DNA probes, 5'-labeled with either carboxymethylrhodamine or carboxyfluorescein and purified by HPLC were supplied by Cybergene S.A. (Saint-Malo, France). Fluorescent PNA probes P1, P2 and Eub338a, 5'-labeled with the fluorochromes cited above and purified by HPLC were synthesized by Dako A/S (Glostrup, Denmark) and supplied by Biocom S.A. (Les Ulis, France). The purity of the fluorescent PNA molecules was checked using mass-spectrophotometry (Dako). PNA and DNA fluorescent probes reported here are commercially available at Biocom (http://www.biocom.fr) and at INRA (URLGA, Jouy-en-Josas, France), respectively.

2.4. Sample preparation

Bacterial mono-cellular layers were obtained by using a filtration technique of samples. This technique was used to count bacterial cells stained with acridine orange in milk [21]. Appropriate dilutions of bacterial cultures grown in laboratory broths,

in milk or in Phagex medium were prepared in phosphate buffer saline (PBS: Na_2HPO_4, 2 mmol·L^{-1}; Na_2HPO_4-$7H_20$, 8 mmol·L^{-1}; NaCl, 130 mmol·L^{-1}; pH 7.00–7.20), to obtain concentrations in the range of 10^4 to 10^6 cells per mL. For direct detection of bacterial cells in milk, a 100 µL aliquot containing cells and 800 µL of a destabilizing solution (0.5% Triton X–100 and 1.32 g·L^{-1} trypsin in 0.16 × PBS) were mixed in a 2 mL Eppendorf tube and incubated in a water bath at 50 °C for 10 min. Bacterial cells were harvested by filtration on a polycarbonate membrane filter (37 mm × 50 mm; pore size, 0.6 µm) [13] using a 12-well membrane filter tower (diameter, 7 mm; Biocom, Les Ulis, France). The tower was held in a manifold connected to a vacuum line, with a system for pressurizing solutions at a maximum of 2 bars, which facilitates the filtration operation. The filter tower was used in the same way for all treatments. Samples were washed by filtration with 1 mL of PBS. Bacterial cells were fixed by addition of 400 µL of a 4% cold paraformaldehyde solution [1]. The system was then incubated at +4 °C for 20 min (PNA probes) or 1 h (DNA probes). The fixative was removed and the samples were washed twice by filtration of 1 mL of PBS. Lactic acid bacteria fixed cells were permeabilized by adding 400 µL of a 10 g·L^{-1} lysozyme solution. The system was then incubated at +42 °C for 10 min. The samples were washed twice by filtration of 1 mL of PBS. Cells were further dehydrated by a series of additions of 500 µL of 50, 80 and 100% ethanol in each well (3 min each).

2.5. In situ hybridization and fluorescence microscopy

The working solutions of PNA and DNA probes were 2.5 µmol·L^{-1} and 8 µmol·L^{-1}, respectively. DNA and PNA hybridization solutions contained 15% and 30% formamide respectively, as described [1, 27]. Each well of the filter tower received 40 µL composed of 36 µL of hybridization solution and 4 µL of PNA or DNA probe working solution. Generally, two probes labeled with two different fluorochromes were used simultaneously; 2 µL of each working solution was added. The tower was then incubated at 55 °C for 20 min (PNA/rRNA hybridization) or at 46 °C for 2.5 h (DNA/rRNA hybridization). Following hybridization, filters were removed from the tower and transferred to: (i) pre-warmed buffer (Tris, 0.3 mol·L^{-1}, pH 10.0; NaCl, 0.9 mol·L^{-1}) in a water bath at 55 °C and washed for 25 min with gentle shaking when PNA probes were used, and to (ii) pre-warmed buffer (Tris-HCl, 20 mmol·L^{-1}, pH 8.00; NaCl, 200 mmol·L^{-1}; EDTA, 5 mmol·L^{-1}. SDS, 0.01%) in a water bath at 48 °C and washed for 10 min with gentle shaking when DNA probes were used. After a post-hybridization wash, filters were washed in pure water, air-dried and mounted with Citifluor antibleaching agent AF1 (Citifluor Ltd., Canterbury, UK). Organisms were viewed under a 100 W mercury vapour lamp attached to a Nikon Optiphot-2 epifluorescence microscope (Nikon, Champigny-sur-Marne, France) using a 40× dry immersion objective. Two excitation filters (diameter 25 series), one for fluorescein (Nikon EX 465–495) and the other for rhodamine (Nikon EX 540/25) and a triple band filter block (Nikon DAPI, fluorescein, rhodamine) were used. The microscopic fields were captured using a black and white CCD camera 4 980 series (COHU Inc., San Diego, USA) connected to a computer. The fluorescent cells were detected using the Visiolab 1 000 software (Biocom, Les Ulis, France).

3. RESULTS

3.1. Fluorescent in situ hybridization conditions for the direct identification of lactic acid bacteria in milk and industrial starter cultures

The established protocols for whole-cell hybridization of fluorescently labeled 16S or

23S rRNA-targeted probes for Gram-positive bacterial cells involve a fixation step, a permeabilization step, a dehydration step, a hybridization step and a wash step [1, 5]. We optimized fixation and hybridization steps. With a longer fixation time, cell morphology is better conserved, however the permeability of the cell envelope to the probe is reduced. Using fluorescent DNA probes, we found that a fixation time of 1 h was sufficient to keep the integrity of Gram-positive cells and to obtain an optimized hybridization signal. The hybridization time was kept to a minimum of 2.5 h. A shorter hybridization time leads to a reduction of the signal. Using the optimized protocol, identification of Gram-positive bacterial cells using fluorescent DNA probes can be completed within 4 h. If fluorescent PNA probes are used, the fixation and hybridization times are both reduced to 20 min. The greater hydrophobicity of PNA as well as its higher affinity for DNA or RNA molecules than their nucleic acid counterparts may contribute to its easy cell penetration and to the shorter hybridization time. However, to avoid false positive hybridization signals, we found it important to perform the post-hybridization wash for at least 25 min (instead of 10 min with DNA probes). The hydrophobic character of the PNA molecule slows down the dispersion of the unfixed molecules in the aqueous wash buffer. Nevertheless, the identification of lactic acid bacteria cells using fluorescent PNA probes can be achieved within 1.5 h. The dehydration step composed of a series of three baths of different ethanol concentrations, 3 min each, cannot be omitted without decreasing the hybridization signal. The use of a single bath at 100% ethanol causes damage to the cell integrity. The permeability for oligonucleotide probes is hindered in fixed cells of Gram-positive bacteria. Therefore, the lysozyme treatment is necessary to achieve optimal whole cell hybridization of lactic acid bacteria and cannot be omitted.

3.2. Probe specificity

The RDP database sequences were used to confirm the specificity of the seven designed probes. The results (Tabs. Ia and Ib) confirm that they exhibit different specificities. PNA and DNA probe specificity was then evaluated using in situ hybridization experiments and the optimized protocols. All species tested were detected using the eubacterial probe Eub338 [2] thus confirming the interest of this sequence for the evaluation of the total bacterial count. *Lb. helveticus*, *Lb. delbrueckii*, and *L. lactis* cells were specifically detected at the species level by probes P1, P2 and P4, respectively. *Leuconostoc* cells, except *Ln. fallax*, were specifically detected at the genus level by probe P5. Based on the comparison of 16S rRNA sequences, *Ln. fallax* represents a peripherical line in the *Leuconostoc* genus [20]. The absence of the hybridization signal is explained by the presence of three mismatches out of eighteen bases between the *Ln. fallax* rRNA-target and the probe P5 sequences. Probe P3 detects *S. thermophilus* cells. However, false positive hybridization signals are also obtained in the conditions used, with cells of certain strains of *Lb. paraplantarum* (CNRZ1885^T) and *Lb. pentosus* (CNRZ1858^T) despite three mismatches out of eighteen bases between the targeted and the probe P3 sequences. Likewise, probe P6 detects cells of the *Lb. plantarum–Lb. pentosus* group and those of certain strains of *S. thermophilus* (CNRZ408 and CNRZ1580). Moreover, probe P6 should also detect *Lb. fermentum* cells as only one mismatch out of eighteen differs between probe and target. Thus, probe P6 shows a limited interest in the identification of *Lb. plantarum-Lb. pentosus* cells in natural starter cultures. Except for these few examples cited above, results obtained using either DNA or PNA probes are in agreement with the prediction (Tabs. Ia and Ib). This is due to the fact that, in most cases, the differences between the rRNA-targeted variable regions and the probe sequences

Table Ia. Results of in situ hybridization experiments on thermophilic lactic acid bacteria using 3 PNA-probes and 4 DNA-probes, and comparison with results predicted from RDP databank sequence assignments [19].

Name of the probe rRNA targeted molecule		Eub338 16S rRNA			P1 23S rRNA			P2 23S rRNA			P3 23S rRNA		
		1	2	3	1	2	3	1	2	3	1	2	–
Species	strains												
Lb. helveticus	CNRZ32	+	+	+	+	+	+	–	–	–	–	ND	
Lb. helveticus	CNRZ223^T	+	+	+	+	+	+	–	–	–	–	–	
Lb. helveticus	CNRZ303	+	+	+	+	+	+	–	–	–	–	ND	
Lb. helveticus	CNRZ891	+	+	+	+	+	+	–	–	–	–	–	
Lb. helveticus	CNRZ1078	+	+	+	+	+	+	–	–	–	–	ND	
Lb. helveticus	CNRZ1102	+	+	+	+	+	+	–	–	–	–	ND	
Lb. helveticus	*LH56	+	ND	+	+	ND	+	–	ND	–	–	ND	
Lb. delbrueckii lactis	CNRZ207^T	+	+	+	–	–	–	+	+	+	–	–	
Lb. delbrueckii lactis	CNRZ327	+	+	+	–	–	–	+	+	+	–	ND	
Lb. delbrueckii lactis	*LL57	+	ND	+	–	ND	–	+	ND	+	–	ND	
Lb. delbrueckii bulgaricus	CNRZ208^T	+	+	+	–	–	–	+	+	+	–	–	
S. thermophilus	CNRZ7	+	+	+	–	–	–	–	–	–	+	+	
S. thermophilus	CNRZ22	+	+	+	–	–	–	–	–	–	+	+	
S. thermophilus	CNRZ1446^T	+	+	+	–	–	–	–	–	–	+	+	
S. thermophilus	CNRZ1580	+	+	+	–	ND	–	–	ND	–	+	+	
S. thermophilus	**1T	+	+	+	–	–	–	–	–	–	+	+	

+ (in column 1): Identity between the probe sequence and the rRNA-targeted sequence.
+ (in column 2): Cells are detected by the DNA-probe.
+ (in column 3): Cells are detected by the PNA-probe.
– (in column 1): At least five mismatches between the probe sequence and the rRNA-targeted sequence.
– (in column 2): Cells are not detected by the DNA-probe.
– (in column 3): Cells are not detected by the PNA-probe.
CNRZ: Collection of lactic acid bacteria and propionic acid bacteria, INRA, Jouy-en-Josas, France. * Strains from Institut Technique Français des Fromages, la Roche-sur-Foron, France, supplied by STANDA-INDUSTRIE. ** Strains from Universidad del Litoral, Santa Fe, Argentina. Type strains are indicated by a T. ND: not determined.

Table Ib. Results of in situ hybridization experiments on mesophilic lactic acid bacteria using four DNA-probes and comparison with results predicted from RDP databank sequence assignments [19].

Name of the probe rRNA targeted molecule		Eub338 16S rRNA		P4 16S rRNA		P5 16S rRNA		P6 16S rRNA	
		1	2	1	2	1	2	1	2
Species	strains								
Lc. lactis cremoris	CNRZ105^T	+	+	+	+	–	–	–	–
Lc. lactis lactis	CNRZ142^T	+	+	+	+	–	–	–	–
Ln. carnosum	LMG11498^T	+	+	–	–	+	+	–	ND
Ln. citreum	*22R	+	+	–	–	+	+	–	–
Ln. fallax	*17D	+	+	–	–	–	–	–	ND
Ln. gelidum	LMG18279^T	+	+	–	–	+	+	–	ND
Ln. mesenteroides	CNRZ749^T	+	+	–	–	+	+	–	–
Ln. lactis	LMG7940	+	+	–	–	+	+	–	ND
Ln. pseudomesenteroides	LMG11482^T	+	+	–	–	+	+	–	ND
W. paramesenteroides	LMG9852^T	+	+	–	–	–	–	–	ND
Lb. casei	*22M	+	+	–	–	–	–	–	ND
Lb. rhamnosus	*5	+	+	–	–	–	–	–	ND
Lb. fermentum	*26	+	+	–	–	–	–	~	ND
Lb. plantarum	CNRZ211^T	+	+	–	–	–	–	+	+
Lb. paraplantarum	CNRZ1885^T	+	+	–	–	–	–	+	+
Lb. pentosus	CNRZ1858^T	+	+	–	–	–	–	+	+
Ec. faecalis	*17	+	+	–	–	–	–	–	ND

+ (in columns 1): Identity between the probe sequence and the rRNA-targeted sequence.
~ (in columns 1): One mismatch between the probe sequence and the rRNA-targeted sequence.
+ (in columns 2): Cells are detected by the DNA-probe.
– (in columns 1): At least three mismatches between the probe sequence and the rRNA-targeted sequence.
– (in columns 2): Cells are not detected by the DNA-probe.
CNRZ: Collection of lactic acid bacteria and propionic acid bacteria, INRA, Jouy-en-Josas, France. LMG: Collection of the Laboratorium voor Microbiologie, Gent, Belgium.
* Isolates identified by their 16S rDNA sequence. Type strains are indicated by a T. ND: not determined.

are at least of five mismatches out of eighteen bases. These results allow the development of multiple applications by combining the different probe specificity as shown in Figures 1a, 1b, 1c and 1d.

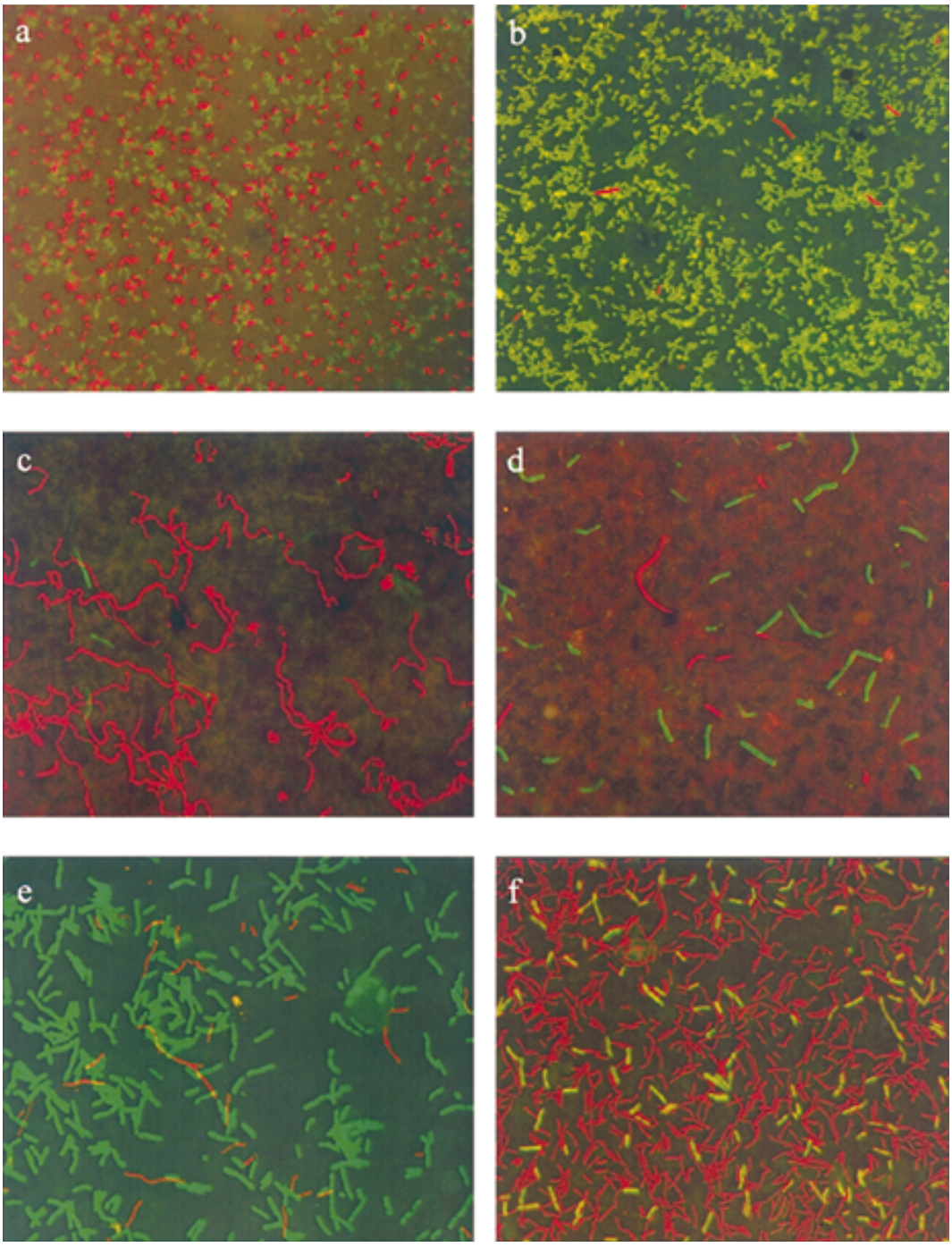
3.3. Specific identification of *Lb. helveticus* and *Lb. delbrueckii* subspecies *lactis* cells in industrial starters and determination of the detection threshold

Differential detection of *Lb. helveticus* strains LH56 and *Lb. delbrueckii* subspecies *lactis* LL56 cells in the Phagex medium using PNA probes are shown in Figures 1e and 1f. The minimal detection threshold allowed with our equipment was of 10^4 cells·mL^{-1} of culture equivalent to less than 10 fluorescent cells detected by microscopic field. The maximum concentration that can be counted with our system was 10^6 cells per mL. However, the thermophilic lactobacilli cells are able to produce long chains when they are in exponential growth phase. This is generally the case when a starter culture is prepared. Thus, in these conditions, the cells cannot be accurately counted by automatic image analysis because they get mixed up. The counting is approximative but the two species proportion can be evaluated within the limit of 1 to 100.

3.4. Evaluation of the state of the lactobacilli cells by in situ hybridization

Long rods in chains are characteristic traits for growing thermophilic bacteria such as *Lb. helveticus* and *Lb. delbrueckii*. The growing cells give a strong and homogenous hybridization signal (Figs. 2a and 2b). In contrast, slow growing cells or cells subjected to certain stress treatment show short rods. Certain cells have a weak hybridization signal (Figs. 2c and 2d). Bacteriophage attacks in the starter culture can also be detected by this method. The cells are partially or totally lysed and a bad or no hybridization signal is observed in these conditions (data not shown). Thus, microscopic examination of the culture after whole cell hybridization should give information on the physiological state of the cell population. The cheese-maker is then able to choose the better starter preparation to inoculate the milk which will be transformed

Figure 1. Differential detection of lactic acid bacteria cells with fluorescently labeled rRNA-targeted probes. Separate images of cells stained simultaneously with fluorescein and rhodamine labeled probes were taken. The two images of the same microscopic field were then overlaid to produce a third image presented here. (**a**) *Lactococcus lactis* CNRZ105 and *Leuconostoc citreum* 22R cells in milk. *L. lactis* and *Ln. citreum* cells were specifically detected using fluoresceine labeled DNA probe P4 and rhodamine labeled DNA probe P5, respectively. (**b**) Detection of bacilli contaminant cells in a culture of *Leuconostoc* in MRS broth. All the bacterial cells were detected using rhodamine labeled DNA probe Eub338. *Leuconostoc* cells were specifically detected using fluoresceine labeled DNA probe P5. (**c**) Co-culture of *Streptococcus thermophilus* CNRZ7 and *Lactobacillus delbrueckii* subsp. *lactis* CNRZ327 in milk. *S. thermophilus* and *Lb. delbrueckii* subsp. *lactis* cells were specifically detected using rhodamine labeled DNA probe P3 and fluoresceine labeled DNA probe P2, respectively. (**d**) Co-culture of *Lactobacillus helveticus* CNRZ32 and *Lactobacillus delbrueckii* subsp. *lactis* CNRZ327 in milk. *Lb. helveticus* and *Lb. delbrueckii* subsp. *lactis* cells were specifically detected using fluoresceine labeled DNA probe P1 and rhodamine labeled DNA probe P2, respectively. (**e**) and (**f**) Co-cultures of *Lactobacillus helveticus* LH56 and *Lactobacillus delbrueckii* subsp. *lactis* LL57 in Phagex medium. *Lb. helveticus* and *Lb. delbrueckii* cells were specifically detected using fluoresceine labeled PNA probe P1 and rhodamine labeled PNA probe P2, respectively in (e). All the bacterial cells were detected using rhodamine labeled PNA probe Eub338a. *Lb. helveticus* cells were specifically detected using fluorescein labeled probe P1 in (f).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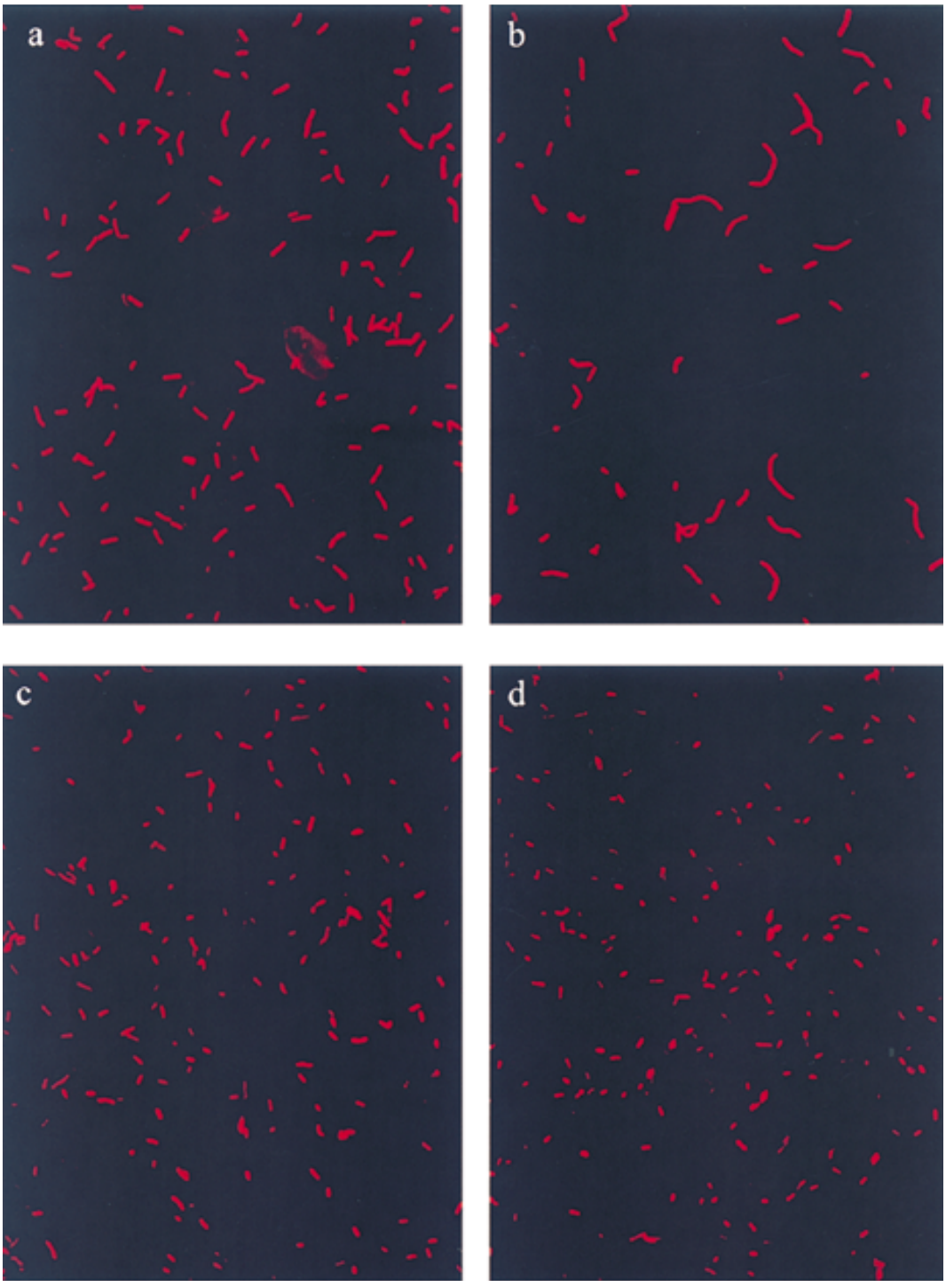
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into cheese or yoghourt. This technique should contribute to the quality of the final products.

4. DISCUSSION

We developed new protocols for detecting and identifying growing lactic acid bacteria cells in milk or in industrial starter cultures using in situ hybridization. A better quality control for cheese or yoghourt production can be achieved by: (i) the use of PNA molecules as probes allowing the direct identification and following of bacterial cells in milk or starter cultures within 1.5 h, and (ii) the possibility to evaluate the physiological state of the thermophilic lactobacilli in starter cultures.

The possibility of combining probes of different specificity to detect a particular species or groups of species or genera is another interesting feature of this technique. For example, *Listeria monocytogenes* and *Staphylococcus aureus* cells could be detected together as contaminants using two specific probes (one for each species) labeled with one fluorochrome (rhodamine) and lactic acid bacteria could be detected in the same sample using a combination of probes P2 to P6 labeled with another fluorochrome (fluoresceine).

However, the application of this technique is limited to growing bacterial cells. Slow growing and dormant cells [3, 16, 30] or cells subjected to certain stress treatments [28] are difficult to detect by this approach because of their low cellular rRNA content. In situ approaches either based on the amplification of the targeted molecule [14, 15, 25] or on the amplification of the in situ hybridization signal [12, 17, 23] have been developed. However, these techniques are generally too time consuming to be used in routine laboratory tests. A combination of PNA probes and amplification systems should constitute an alternative approach to overcome this problem [22].

In this study, we show for the first time that the PNA fluorescent in situ hybridization constitutes a fast and efficient approach to perform the bacteriological control of starter cultures made with milk or industrial complex medium. We are also developing this approach for the quality control of dairy starter cultures or raw milk samples containing Gram-negative bacterial cells or Gram-positive pathogens such as *S. aureus* and *L. monocytogenes*.

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Figure 2. Evaluation of the physiological state of *Lactobacillus helveticus* CNRZ32 in different conditions using in situ hybridization. (**a**) Cells at the beginning of the exponential growth phase, incubated in MRS broth for 1 h at 42 °C. (**b**) Cells in exponential growth phase, incubated in MRS broth for 3 h at 42 °C (control). (**c**) Cells submitted to a stress treatment in acid MRS broth (pH 3.5–3.6) for 2 h at 42 °C after growth in optimal conditions for 1 h at 42 °C. (**d**) Cells incubated for 24 h in MRS broth at 42 °C.

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