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MINI-REVIEW

Application of FISH technology for microbiological analysis: current state and prospects

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Abstract In order to identify and quantify the microorganisms present in a certain ecosystem, it has become necessary to develop molecular methods avoiding cultivation, which allows to characterize only the countable part of the microorganisms in the sample, therefore losing the information related to the microbial component which presents a vitality condition, although it cannot duplicate in culture medium. In this context, one of the most used techniques is fluorescence in situ hybridization (FISH) with ribosomal RNA targeted oligonucleotide probes. Owing to its speed and sensitivity, this technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology. Through the use of species-specific probes, it is possible to identify different microorganisms in complex microbial communities, thus providing a solid support to the understanding of interspecies interaction. The knowledge of the composition and distribution of microorganisms in natural habitats can be interesting for ecological reasons in microbial ecology, and for safety and technological aspects in food microbiology. Methodological aspects, use of different probes and applications of FISH to microbial ecosystems are presented in this review.

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Introduction

The lack of morphological details in many bacteria and microorganisms has usually hampered their identification, making the use of cultivation necessary for identification. This has proven difficult for many environmental or medically important microorganisms (Amann and Kuhl 1998).

Traditional methods to determine the composition of microflora require cultivation on selective media, which is laborious, time-consuming and prone to statistical and methodological errors (Moter and Gobel 2000). According to classical microbiology, in order to study a microorganism, it is necessary to isolate it from the original matrix, and this isolation can be done only on the appropriate medium, from the plates showing separately grown colonies, i.e. from the plates corresponding to the so-called countable dilutions. Moreover, it has been demonstrated how any environmental modification during cultivation could affect the structure of microbial communities, thus preventing a complete view of the ecosystem considered. Therefore, isolation and cultivation in agar medium allow to characterize only those microorganisms capable of growing, multiplying and forming colonies in the selected medium and condition of growth, with the loss of the information relative to the microbial component which presents a vitality condition despite not being able to duplicate in culture medium.

Although new microorganisms are continuously isolated, it is estimated that only a small fraction of the existent microorganisms have been grown in pure culture and characterized. The lack of knowledge is most severe for complex multi-species microbial communities. Even when all bacteria could ultimately be cultured (which is quite unlikely), progress in the understanding of the ecology of complex microbial communities would require studies on

the activity and distribution of microbes directly in minimally disturbed samples (Amann and Kuhl 1998).

Therefore, in order to identify and quantify the microorganisms occurring in a certain ecosystem, the development of molecular methods avoiding cultivation has become necessary. In this context, one of most used techniques is fluorescence in situ hybridization (FISH) with ribosomal RNA (rRNA)-targeted oligonucleotide probes (Langendijk et al. 1995; Amann et al. 1990). This method is based on the hybridization of synthetic oligonucleotide probes to specific regions within the bacterial ribosome and does not require cultivation.

FISH originated in medicine and developmental biology for the localization of particular DNA sequences in mammalian chromosomes and subsequently has been applied primarily in environmental bacteriology (Amann et al. 1995) and to a lesser extent in protist ecology (Lim et al. 1996). Owing to its speed and sensitivity, this technique is considered a powerful tool for phylogenetic, ecological, diagnostic and environmental studies in microbiology.

FISH has so far been applied to the study of microbial symbiosis and microbial diversity in environmental samples and wastewater treatment (Amann et al. 2001). It is also routinely used in medicine as a diagnostic tool for the identification of bacteria in complex communities colonising the oral cavity and the respiratory and gastro-intestinal tracts, as well as for the detection of pathogens within human and animal tissues (Moter and Gobel 2000).

FISH combines the precision of molecular genetics with the visual information from microscopy, allowing visualization and identification of individual cells within their natural microhabitat or diseased tissue, so that nucleic acid sequences can be examined inside cells without altering the cell's morphology or the integrity of its various compartments (Moter and Gobel 2000). FISH techniques for detecting RNAs have been introduced into living cells using either fluorophores that can be 'uncaged' in vivo or probes that fluoresce only when hybridized.

One drawback of live-cell in situ hybridization is that FISH requires mechanical disturbance of the cell in order to introduce probes. In situ identification of individual microbial cells with fluorescently labelled, rRNA-targeted oligonucleotide probes, the so-called phylogenetic stains, is based on the high cellular content of ribosomes, which can be found in all living organisms, and consequently, as many 16S and 23S rRNA molecules (Amann and Kuhl 1998). rRNAs are the main target molecules because they are relatively stable and they include both variable and highly conserved sequence domains (Amann et al. 2001). The selection of particular regions of the rRNA molecule then enables phylogenetic specificity to be varied from the universal to the subspecies level (DeLong et al. 1989; Amann et al. 1990), even if because of the relatively slow mutation rate of rRNA, this molecule generally possesses no target sites that differentiate between strains of prokaryotic species (Wagner et al. 2003). Under appropriate reaction conditions, complementary sequences in the probe and target cell anneal, and the site of probe hybridization is detected by fluorescence microscopy (DeLong et al. 1989; Amann et al. 1990). FISH allowed significant advances in resolution, speed and safety, and later paved the way for the development of simultaneous detection of multiple targets, quantitative analysis and live-cell imaging (Levsky and Singer 2003). These techniques allow a deeper study of live gene expression in a minimally disturbed context, but must be interpreted taking into consideration the possible artefacts that may result as physiological ramifications of hybridization (Levsky and Singer 2003).

Methodological aspects

FISH with rRNA target probes has been developed for the in situ identification of single microbial cells and is the most commonly applied among the 'non-PCR [polymerase chain reaction]-based' molecular techniques (Amann et al. 1990, 2001; Moter and Gobel 2000).

A typical FISH protocol (Fig. 1) includes four steps: fixation and permeabilization of the sample, hybridization, washing steps to remove unbound probe and detection of labelled cells by microscopy or flow cytometry (Amann et al. 2001).

Prior to hybridization, bacteria must be fixed and permeabilized in order to allow penetration of the fluorescent probes into the cell and protect the RNA from degradation by endogenous RNAses (Moter and Gobel 2000). The sample is either settled on membrane filters and covered with the fixing agent (Glockner et al. 1999), or mixed with the fixing agent, incubated, sedimented by

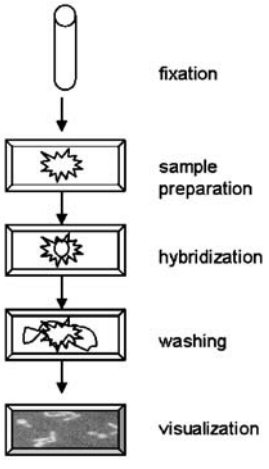

Fig. 1 Flow chart of a typical FISH. The procedure includes the following steps: (1) fixation of the specimen directly in sample tubes; (2) transfer of the sample on a glass slide and preparation of the sample, including specific pretreatment steps; (3) hybridization with the respective probes for detecting the respective target sequences; (4) washing steps to remove unbound probes; (5) mounting, visualization and documentation of results

centrifugation, resuspended, transferred to glass slides and dried (Amann et al. 1990). For better attachment of specimens to glass slides, some authors suggested first treating the surfaces with coating agents such as gelatin (Amann et al. 1990), poly-L-lysine (Lee et al. 1999) or silanating agents (Moter et al. 1998). If cell suspensions are investigated, bacteria are fixed in suspension, spotted onto microscopic slides, air-dried and dehydrated in an ethanol series. In some cases, e.g. for Gram-positive cells, an additional enzymatic treatment with lysozyme (Schonhuber et al. 1999; Wagner et al. 1998), lysostaphin or an enzyme mixture (Krimmer et al. 1999) may be necessary to open the peptidoglycan layer.

Hybridization must be carried out under stringent conditions for proper annealing of the probe to the target sequence. For this crucial step of the FISH procedure, a preheated hybridization buffer is applied to the sample containing fluorescently labelled probes complementary to the target RNA. The hybridization takes place in a dark humid chamber, usually at temperatures between 37 and 50°C. Hybridization time varies between 30 min and several hours.

The slides are then briefly rinsed with distilled water to remove unbound probe. Finally, the slides are rinsed with water again, dried, possibly mounted in anti-fading agents to prevent fluorescence 'bleaching' (Moter and Gobel 2000), then the results are visualized and documented.

The oligonucleotide probes used in FISH are generally between 15 and 30 nucleotides long and covalently linked to a single fluorescent dye. Common fluorophores are reported in Table 1, and include fluorescein, tetramethylrhodamine, Texas red and, increasingly, carbocyanine dyes like Cy3 or Cy5 (Amann et al. 2001). The number of target cells detected, for example, with the universal oligonucleotide probe Eub338 ranges from 1 to 100% of the total bacterial count in enriched culture, even depending on the physiological state of the cells (Bouvier and Del Giorgio 2003). This wide range implies that FISH is extremely sensitive not only to variations in the methodological aspects of the protocol but even to environmental conditions. De Vries et al. (2004) observed that the growth phase influenced both the amount of the rRNA present per cell and the fluorescence intensity of FISH experiments. They noticed that even if the amount of rRNA is maximum at the start of the stationary growth phase as a reflection of adaptation to new conditions representative for the growth phase, FISH can be used to estimate the in situ activity of *Lactobacillus plantarum* only during the first exponential growth phase. These contrasting results are probably due to changes taking place in the cell envelope during the second exponential growth phase which prepare cells for survival under stationary growth phase and which cause difficulties in the permeabilization of the cells.

Table 1 Most commonly employed fluorescent dyes to label oligonucleotides for FISH analysis

Fluorochrome	Colour	Max. excitation λ (nm)	Max. emission λ (nm)
Alexa488	Green	493	517
AMCA	Blue	399	446
CY3	Red	552	565
CY5	Red	649	670
CY7	Violet	743	767
DAPI	Blue	350	456
Fluorescein	Green	494	523
Rodamine	Red	555	580
TAMRA	Red	543	575
Texas red	Red	590	615
TRITC	Red-orange	550	580

AMCA methyl cumarinic acetic acid, *CY* carbocyanine, *DAPI* 4′6-diamidino-2-phenylindole dihydrochloride, *TAMRA* tertramethyl rhodamine, *TRITC* tetramethylrodamine-isotiocianate

Probes labelling

The earliest in situ hybridizations, performed in the late 1960s, were not fluorescent at all, but rather utilized probes labelled with radioisotopes. The first application of fluorescent in situ detection came in 1980, when RNA was directly labelled on the 3′ end with a fluorescent molecule (Bauman et al. 1980). Enzymatic incorporation of fluorophore-modified bases throughout the length of the probe has been widely used for the preparation of fluorescent probes (Wiegant et al. 1991). In the early 1990s, improved labelling of synthetic, single-stranded DNA probes allowed the chemical preparation of hybridization probes carrying enough fluorescent molecules to allow direct detection (Kislauskis et al. 1993). Nowadays, there are different ways of labelling (Fig. 2).

Direct fluorescent labelling is most commonly used and is also the fastest, easiest and cheapest method because it does not require any further detection steps after hybridization (Moter and Gobel 2000). With direct labelling, the probe DNA is synthesized with one or more modified dNTPs (the building block molecules of DNA) that have a fluorochrome chemical side group. By incorporating the fluorochrome directly into the probe, the fluorescent signal can be bound to the target in a single hybridization step (Jain 2004). Given the need to simultaneously identify several bacterial species, the contemporaneous labelling with different fluorophores could be useful: employing two or three dyes together, a colour combination can be obtained which allows to delineate multiple targets by varying the relative contribution of each colour to the total signal (Levsky and Singer 2003). It is essential to use fluorochromes with fluorescent spectra that can be clearly

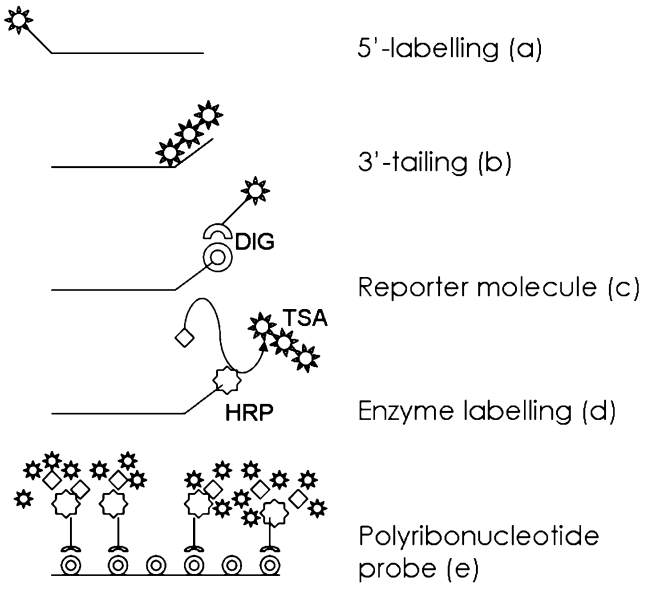

Fig. 2 Direct (**a**, **b**) and indirect (**c**–**e**) labelling of probes using a reporter molecule like digoxygenin (*DIG*) that is then detected by fluorescent antibody, horseradish peroxidase (*HRP*) that used fluorescein–tyramide as substrate for the enzymatic signal amplification [tyramide signal amplification (*TSA*)], or with the combined use of polyribonucleotide probes, internally labelled with digoxygenin, with the tyramide signal amplification systems

differentiated to create a unique spectral signature for each species in images after multi-colour staining. A multi-colour FISH technique was developed for example for the analysis of seven *Bifidobacterium* species in human faeces (Takada et al. 2004).

Probe size and targets

Early probes produced from clones had to be large because they were sparsely labelled in order to allow specific hybridization and because of the methods used for their synthesis and purification, and for this reason, they caused high background fluorescence, problem which could be overcome by pretreatment with unlabelled nucleic acids to compete for non-specific sites of binding.

An improved signal-to-noise ratio and thus single-copy detection of genes could also be obtained with a reduction in probe size (Landegent et al. 1987; Lawrence et al. 1988). The utilization of these fluorescent oligonucleotide probes that specifically bind to the ribosomal RNA is nowadays a very useful tool for the detection, identification and enumeration of bacteria.

In the vast majority of applications, FISH probes target 16S rRNA. In fact, comparative analysis of 16S (and 23S) rRNA sequences is today the most commonly used method for studying the phylogeny of microorganisms, and such rRNA sequences can be obtained from environmental or medical samples without cultivation. The public databases now include 16S rRNA sequences, which can be used with specific programme packages for probe design (Amann and Kuhl 1998).

The choice of probes for FISH must consider specificity, sensitivity and ease of tissue penetration. A typical oligonucleotide probe is between 15 and 30 base pairs (bp) in length and is generated on an automated synthesizer. Short probes have easier access to their target, but they might carry fewer labels. However, not all bacterial and archaeal cells can be permeabilized by oligonucleotide probes using standard fixation protocols. The accessibility of selected target sites for oligonucleotide probes can be increased by adding unlabelled oligonucleotide probes that bind adjacent to the probe target site. The aim is to unfold the nucleic acid and thus facilitate probe hybridization. These so-called helper probes need to be designed carefully because of their specificity to the respective probe and must have a differentiation temperature (Td) at least as high as the Td of the probe to prevent dissociation of the helper at stringent hybridization conditions (Fuchs et al. 2000a,b).

Fluorescently labelled oligonucleotide and polynucleotide probes

Fluorescently labelled, rRNA-targeted oligonucleotides are used to specifically stain different members of microbial communities. The specificity of the probes ranges from the phylo-type to the kingdom, depending on the targeted region on the rRNA. Such probes can be readily developed and tested to detect lineages of uncultured microbes in environmental samples, and the signal intensity of cells hybridized with oligonucleotide probes is directly related to the cellular rRNA content. Polynucleotide probes consisting of nearly full-length 16S and/or 23S rRNA genes, each labelled with several fluorochrome molecules, were shown to detect almost all cells present in environmental samples (DeLong et al. 1999). Shorter polynucleotide probes that target a defined variable region of approximately 250 nucleotides of the 23S rRNA have been shown to allow differentiation among genera (Trebesius et al. 1994). Oligonucleotide probes can be labelled at both 5′ and 3′ ends, and/or several probes applied simultaneously, thereby targeting each rRNA molecule with several fluorophores (Lee et al. 1993).

Poly(ribo)nucleotide probes to discriminate marine bacteria and uncultured marine archea have been successfully applied in the open ocean (Karner et al. 2001). Such probes are RNA transcripts from PCR amplicons of 16S and 23S rRNA genes from either environmental DNA or fosmid clones (DeLong et al. 1999). The use of rRNA-targeted oligonucleotide probes, which are covalently mono-labelled

with fluorescent dye molecules, limits the sensitivity of the method and aggravates the use of FISH for identification of prokaryotes with low ribosome content per cell (Wagner et al. 2003). The fluorescently labelled, rRNA-targeted polynucleotide probes have been reported to yield higher signal intensities than oligonucleotide probes and may thus represent a better means of detecting microbes with a low ribosome content (Trebesius et al. 1994). Due to their big dimensions, these probes could lead to a reduction in hybridization efficiency because of the inaccessibility of both the cell wall and the target site to the longer probes.

FISH with peptide nucleic acid probes

Ribosomal RNA molecules are key functional and structural elements of cells and are highly conserved between closely related species (Perry-O'Keefe et al. 2001). Comparative study of rRNA sequences has allowed evolutionary microbiologists to define the phylogeny and taxonomy of bacteria, yeasts and fungi.

In addition, as rRNAs are present at a high copy number in bacteria, they have often been the target of choice for oligonucleotide probe assays (Amann et al. 1995). Various groups have noted that the degree of fluorescent signal is dependent on the choice of the probe target region within the 16S rRNA molecule, and this variability has been ascribed to poor accessibility of DNA probes to the rRNA target because of the highly stable secondary structures in the rRNA (Amann et al. 1995; Frischer et al. 1996; Fuchs et al. 1998). Furthermore, DNA probes are often limited in their capability to distinguish single nucleotide changes. As a result, it is often difficult to design DNA probes that hybridize efficiently within a given stretch of the rRNA dictated by the nucleotide differences found between closely related species (Perry-O'Keefe et al. 2001). The use of peptide nucleic acids (PNAs) has been considered useful in overcoming the variable and sometimes insufficient penetration of probes into bacteria depending on their cell wall characteristics, which is one of the major drawbacks of FISH using oligonucleotide probes, particularly in Gram-positive bacteria (Stender et al. 2001).

Peptide nucleic acid molecules are uncharged DNA analogues that bind to nucleic acids much more strongly than oligonucleotides because there is no electrostatic repulsion between the PNA probe and the negatively charged sugar-phosphate backbone of the target molecule (Ray and Norden 2000).

The unique character of PNA allows these probes to hybridise to target nucleic acid molecules more rapidly and with higher affinity and specificity compared with DNA probes (Jain 2004). This highly sensitive approach detects rRNA in bacterial cells long after cell death (Moter and Gobel 2000). Moreover, PNA probes hybridize virtually independently of the salt concentration, being ideal for targeting nucleic acids with high degree of secondary structure, like rRNA, since hybridization can be performed efficiently in a low salt buffer. Under these conditions, the stability of the secondary structures within the target rRNA is decreased, thereby allowing PNA probes to hybridize to less accessible targets. A standardized PNA FISH procedure also increases the possibility of simultaneous identification of different organisms (multiplex identification) using PNA probes labelled with different fluorophores (Perry-O'Keefe et al. 2001). At this time, high prices and specificity problems are slowing the application of PNAs to FISH (Amann et al. 2001).

Probes accessibility to rRNA

One of the main problems of FISH, besides low cellular ribosome content and impermeability of cell walls, is the inaccessibility of probe target sites (Fuchs et al. 1998). Initially, they were supposed to be hindered by rRNA–rRNA interactions, as well as by interactions of the rRNAs with ribosomal proteins, due to the densely packed three-dimensional structure of the ribosome (Beherens et al. 2003; Wimberly et al. 2000). Later, Inacio et al. (2003), after studying the effects of different hybridization and cell storage conditions, observed that, FISH being performed in a strongly denaturing environment, the three-dimensional structure of the native small ribosomal subunit is not relevant to probe accessibility, and that for the same reason, the influence of protein–RNA interactions on target site accessibility can be neglected. It was noticed that intra-helix, secondary base interactions are more important than tertiary rRNA–rRNA contacts (Inacio et al. 2003). Frequently, probes were intended to be as specific as possible, targeting particular species or genera, or even just particular 16S rDNA sequences that were retrieved from the environment. Those probes necessarily target the most variable regions of the 16S rRNA molecule which often are even the most inaccessible sites (Fuchs et al. 1998). Fuchs et al. (1998) found five regions in *Escherichia coli* where accessibility seemed to be very high: positions 38 to 108 (except for the terminal loop region of helix 6), positions 181 to 215, positions 316 to 359, positions 871 to 933, and positions 1,383 to 1,427 and 1,473 to 1,517. They found that the loop region of helix 6, the loop region and the distal 3′ side of helix 18 and the 5′ side of helix 23, which are among the most important target sites with the evolution being less conserved, are not very accessible. In this study, all probes were arbitrarily grouped according to their relative fluorescences into six classes of brightness, where the fluorescence of the probes was expressed as a

percentage of the brightest probe (Eco1482). Most probes in the first two classes are directed against five regions where accessibility for oligonucleotide probes seems to be very high, so it is possible to consider the brightest probes as the most accessible ones. In this context, probe Eub338, which is the most widely employed probe for FISH detection of *Bacteria*, has been set in the III class, with a 58% Eco1482 brightness. The fluorescent signals reported by Fuchs et al. (1998) should mainly reflect differences in 16S rRNA accessibility. Changes in accessibility are often steady along the primary structure of the 16S rRNA, but can also be rapid and are therefore quite unpredictable. Moreover, they noticed that the almost inaccessible target sites are often in the periphery of the secondary structure model, including many loops, whereas regions in the centre of this model seem to be more readily accessible. Further studies investigated the possibility of using the 23S rRNA as a probe target molecule because, despite its length of approximately 1,500 nucleotides, it is sometimes impossible to find suitable signature sites on the 16S rRNA for the identification of the organisms of interest (Fuchs et al. 2000b). The 23S rRNA, with its length of approximately 3,000 nucleotides, would be the ideal alternative as a probe target molecule (Fuchs et al. 2000b). The authors demonstrated that in *E. coli*, the 23S rRNA is more accessible to oligonucleotide probes than the 16S rRNA in the small subunit of the ribosome, and for this reason, a 23S rRNA-targeted probe is valuable in increasing the significance of in situ identification.

Transferability of *E. coli* accessibility data to other organisms

Due to the high evolutionary conservation of the rRNA, these findings on the 23S rRNA and 16S rRNA in situ accessibility for *E. coli* can be extrapolated to other microorganisms. The transferability of *E. coli* accessibility data to other organisms has been studied for organisms of all three domains of life: *Bacteria*, *Archea* and *Eucarya* (Beherens et al. 2002), and the correlations analysis clearly showed that the in situ accessibility maps are more similar for phylogenetically more related organisms. For all three prokaryotes examined by Beherens et al. (2002), regions of the 16S rRNA with high accessibility are positions 285 to 338 (helices 13 and 14), positions 871 to 925 (except helix 30 target positions) and positions 1,248 to 1,283. Seven smaller regions of good accessibility are located on helices 2, 3, 7, 9, 20, 23, 26, 27 and 31. As shown by Inacio et al. (2003), the accessibility to 26 rRNA in yeasts (*Saccharomyces cerevisiae*) follows the same general trend observed for 16S rRNA of *E. coli*: the most conserved stretches of the region studied are more accessible, and the most variable areas often show medium to low accessibility. Otherwise, the in situ accessibility does not depend exclusively on a probe target site location inside or outside the ribosome, as suggested by Beherens et al. (2003). For example, fixation and hybridization significantly increase the accessibility of 16S rRNA target sites to probe, causing massive conformational changes within the ribosome (Beherens et al. 2003). Inaccessible target sites could even be likely opened up by conformational changes introduced by hybridization of helper probes (Fuchs et al. 2000a,b).

There is even a link between the type of label and accessibility. Studies performed by Beherens et al. (2002) and by Fuchs et al. (1998) demonstrated that Cy3-labelled oligonucleotides have higher in situ accessibility in *E. coli* in comparison with the triphenilmethane derivative carboxy-fluorescein due to the more linear structure, which could reduce steric hindrance and thereby facilitate probe binding to the target.

Hybridization efficiencies of FISH probes

Although the accessibility maps from the studies cited have been used for the selection of probe target sites in FISH experiments, recent studies have demonstrated that it is not worth to eliminate rRNA target regions a priori, even if they were previously reported as seemingly inaccessible because these sites could be strongly influenced by the kinetics of the hybridization reaction (Ylmaz and Noguera 2004; Ylmaz et al. 2005). In fact, the hybridization efficiencies of FISH probes can be defined as a function of not only the accessibility of the target site, but also the thermodynamic affinity of the probe to the target site, defined by the predictable free energy change of the overall reaction ($\Delta G°_{\text{overall}}$), which describes the stability of the DNA/RNA hybrid and can be correlated with the brightness of a hybridized probe. $\Delta G°_{\text{overall}}$ is obtained from the free energy change for the individual reactions ($\Delta G°_i$): the binding of the probe to the complementary site available (reaction 1), the reversible unfolding of the target region (reaction 3) and the folding–unfolding mechanism for the DNA probe (reaction 2). $\Delta G°_i$ can be calculated as $\Delta G°_{\text{i}} = -RT\ln K_i$, where R is the ideal gas law constant, T is the hybridization temperature and K_i is the equilibrium constant for each reaction. A more negative $\Delta G°_{\text{overall}}$ represents a greater concentration of the hybrid and, thus, greater brightness from the hybridized probes (Ylmaz and Noguera 2004).

For this reason, $\Delta G°_{\text{overall}}$ is a strong predictor of hybridization efficiency, and it has been noticed that an affinity above the theoretical threshold of −13.0 Kcal/mol for maximum hybridization efficiency should be targeted for rational design of FISH probes to maximize the

possibility of satisfactory sensitivity (Ylmaz et al. 2005). Furthermore, kinetics limitations imposed by structural restraints in the ribosome could be overcome by extending the incubation period from 3 to 96 h or using formamide, so that all regions of the 16S rRNA of *E. coli* can be made accessible, thanks to the surmounting of thermodynamic and kinetic barriers (Ylmaz et al. 2005).

Use of FISH in food samples

There is an increasing need for rapid detection and enumeration of marker organisms that run to completion within a single working day in the food and drinking water industries (Ootsubo et al. 2003). A major disadvantage of culture methods is the time needed to produce results. Generally, they require incubation of culture plates for several days. Another significant disadvantage of these methods is the failure to detect viable but non-culturable organisms. Therefore, it raises doubts that culture-based methods recover sublethally injured cells that may occur in heat-treated products such as pasteurized milk. In order to monitor online sanitation practices, the industry needs rapid and reliable methods to ascertain the microbiological quality of foods and ingredients. Fluorescent oligonucleotide probe hybridization is becoming an important tool also in food microbiology to identify specific microorganisms in mixed communities without the need for isolation in pure cultures. The use of FISH allowed, for example, a rapid and accurate enumeration of *Pseudomonas*, facilitating the identification of specific contamination sources in dairy plants, the accurate validation of pasteurization treatment and the prediction of shelf life of pasteurized milk (Gunasekera et al. 2003).

Moreover, the use of group- and species-specific oligonucleotide probes for in situ hybridization has provided an insight to the microbial composition of Gruyere cheese surface (Kolloffel et al. 1999). Although the FISH analysis was performed on a cheese suspension and not directly in the food matrix , the study allowed the detection of most bacteria with bacterial probe Eub338, even in mature cheese from which only a smaller proportion of the bacteria were culturable (Kolloffel et al. 1999).

This technique was recently applied for a screening of the occurrence of *L. plantarum* on olives to be used in natural fermentations. However, due to the high detection limit of the method, the species could not be detected even though a large number of samples were screened (Ercolini et al. 2006). Moreover, technical problems arose in this study for the optimization of cell permeabilization condition due to the simultaneous occurrence of Gram-positive and Gram-negative bacteria (Ercolini et al. 2006).

FISH is useful in wine production too, where the early knowledge of the microbiological conditions allows the application of corrective measures before spoiling becomes irreparable. Therefore, rapid and accurate identification of LAB permits a better monitoring of the fermentation process, preventing the risk of alteration and the probability of the occurrence of malolactic fermentation, leading therefore to a better quality of the final wine (Blasco et al. 2003).

The use of FISH and, in particular, the application of fluorescently labelled PNA oligomers have also been reported to be a powerful method for identifying colonies of *Brettanomyces*, a well-recognized wine spoilage yeast that causes an undesirable flavour (Stender et al. 2001).

Despite the huge background of knowledge and application, the use of FISH in food microbiology has been restricted to the identification of bacteria isolated from food or in food suspension, therefore losing spatial data which might provide important information on flora development in food ecosystems.

Ercolini et al. (2003a) developed a 16S rRNA FISH method for cheese to allow the detection in situ of microorganisms within the dairy matrix. An embedding procedure using a plastic resin was applied to Stilton cheese, providing intact embedded cheese sections withstanding the hybridization reaction. The technique has the potential to study the spatial distribution of microbial population in situ in foods, especially where the matrix is too fragile to allow manipulation of cryo-sections. 16S rRNA sequences could be used for species-specific probe design for the location of specific groups of bacteria within the matrix and for the investigation of relationships within specific groups of bacteria.

The authors developed probes for the specific detection of some species of lactobacilli, lactococci and leuconostocs and demonstrated that in Stilton cheese the spatial distribution of specific colonies is not homogeneous but depends on the specific site. The availability of such information for dairy products after FISH investigation may play a key role for the detection of the sites of actual growth of certain species and can be of potential help in detecting zones of appropriate acidification, aroma development and production of antagonistic substances in food products. Moreover, the monitoring and analysis of spatial distribution of contaminant species might also be achieved by this approach, which represents a very important step forward in studying the development of microbial populations in food with the ultimate aim of process optimization and quality assurance of the final product (Ercolini et al. 2003a,b).

Applicability of FISH to biofilms

Although quantitative FISH has provided new insights into the structure and dynamics of microbial communities, it suffers from tediousness and limited accuracy for samples

containing densely aggregated cells like biofilms (Daims et al. 2001). Biofilms are formed after rapid attachment and growth of microorganisms on a broad range of surfaces in contact with natural fluids. The architecture of biofilms and the physiological status of the cells contained within these structures are of prime interest for clinical, industrial and environmental microbiology. For analyses of the spatial arrangement of bacteria in a multi-species biofilm, techniques which use specific bacterial cell markers and maintain the biofilm's natural architecture are required (Thurnheer et al. 2004).

Quantitative data on the abundance of in situ stained microorganisms in complex samples are mostly obtained by time-consuming manual microscopic counting. The accuracy of this quantification approach is relatively low in densely colonized biofilms, which probably represent the most common life form of microorganisms on earth (Wagner et al. 2003). This problem can in part be ameliorated by using confocal laser scanning microscopy (CLSM) for the detection of probe-labelled cells. However, even if optical CLSM sections are recorded, it is not feasible to manually count a sufficient number of cells in each hybridization experiment in a reasonable time period to obtain statistically reliable results. Even semi-automated digital image analysis tools are unable to efficiently count cells in dense clusters or biofilms because single-cell recognition within these structures cannot be automated. This problem can be circumvented by measuring the areas of specifically stained bacteria in randomly acquired optical CLSM sections. The abundance of a particular population is then expressed as a fraction of the area occupied by all bacteria (Daims et al. 2001).

The utilization of genus-specific, fluorescently labelled oligonucleotide probes can be a very useful tool for the study of particular kinds of biofilms, such as those formed in the gastrointestinal human tract. FISH has been applied with this aim for the hybridization of the bacterial rRNA extracted from a faecal sample or fermentation fluids (Langendijk et al. 1995) and has also been used to determine changes in populations of bifidobacteria, lactobacilli, clostridia, bacteroides, streptococci and *E. coli* in human faecal extracts (Rycroft et al. 2001). Moreover, in combining FISH with CLSM, the development of a simultaneous analysis of the spatial distribution of both Gram-negative and Gram-positive bacteria in biofilms has become possible (Thurnheer et al. 2004).

Conclusions and future perspectives

As a consequence of the continuous improvements, FISH is nowadays a very useful tool for studies in microbiology. Through the use of species-specific probes, it is possible to identify different microorganisms in complex microbial communities, giving a solid support to the understanding of inter-species interaction. The widespread use of FISH as an application directed to gaining information on the spatial distribution of microbial populations will allow the study of the contribution of each species to the organization and functioning of the multi-species bacterial communities. This is important from different points of view and in different fields of application. The knowledge of the composition and distribution of microorganisms in natural habitats can be interesting for ecological reasons in microbial ecology, and for safety and technological aspects in food microbiology. This technique will be a relatively rapid method to evaluate the presence and activity of pathogens, or other undesirable microorganisms, directly in the sample studied (food, water, wastes, soil), but it will also be a valid approach to study the activity of bacteria involved in technological processes (e.g. food fermentations) or important for human health (e.g. *Bifidobacteria*).

FISH is a very in situ technique and will keep the potential to zoom in on the composition of complex microbial associations in natural ecosystems.

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