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Evaluation of the Fluorescence *In Situ* Hybridization Technique for Detection of Eubacteria and Sulfate-Reducing Bacteria from Samples of Water in Oil Fields

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The detection, identification and confirmation of microorganisms from cultures and environmental samples of waters from oil fields representing the population of its habitat can be achieved with greater confidence with FISH than with other techniques. In this work we describe our experience in the use of FISH to evaluate the microbial composition of sulfate-reducing bacteria (SRB) cultures and direct samples of waters from oil fields. Composition of the microbial community by DAPI and Fluorescent *in situ* hybridization (FISH) for 16S rRNA with probes EUB338, for general Eubacteria, SRB-385, group specific for SRB and major species of δ-Proteobacteria, DSV-698, *Desulfovibrio*-specific and, DSB-129 (SRB-129), *Desulfobacteriaceae*-specific revealed that 98% of total cells stained with 4', 6-diamidino-2-phenylindole (DAPI), within these 61, 51 and 0 % respectively bound to the probes. This indicated that SRB and, especially *Desulfovibrio,* were the dominant Eubacteria in the whole community of water samples from oil fields. Modifications made to the formamide, NaCl and probe concentrations for hybridization, represented refinements of the experimental conditions utilized by previous investigators.

Introduction

Fluorescent in situ hybridization (FISH) with rRNA targeted oligonucleotide probes permits the characterization of bacterial population in environmental samples. In bacterial work, FISH probes are usually designed to recognize DNA coding for 16S rRNA (Bidinenko et al., 1998), providing reliable information in phylogenetic studies (Amann et al., 1995). FISH analysis is extremely important for microbial ecology to solve the problem of impossibility in cultivating most of the cells present at a habitat (Head et al, 1998), and to allow *in situ* identification of microorganisms (Amann et al., 1992).

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The identification of bacteria from both pure and mixed cultures representing the population of an environmental sample can be established with FISH (Amann, et al., 1991). The techniques have been used widely for the detection and quantification of sulfate-reducing bacteria (SRB) in different environmental samples such as soils, sediments, aquatic atmospheres, besides samples cultivated at laboratory, showing to be a fast and specific methodology for identification of individual microbial cells, getting to describe the temporary and space distribution of these organisms, and the specific taxes of microorganisms in the cycles biogeochemical and in the dynamics of the alimentary chain (Lobert-Brossa et al., 1998; Icgen and Harrison, 2006).

Material and Methods

DAPI and fluorescent in situ hybridization were applied to evaluate these techniques for detecting and enumeration of sulfate-reducing bacteria directly in water of oil fields or after culture in Postage's medium. Microbial samples from culture medium and water from oil fields were processed according to the protocol of Amman et al. (1990) with some modifications. While the culture was still in the exponential growth phase, 1 mL of the suspension was removed and centrifuged in a 2 mL microcentrifuge tube at 9000 rpm for 2 minutes. For the water samples, we centrifuged 450ml of water at 9000 rpm for 10 minutes. The supernatant of both (from culture and form water) was then removed and the pellet was resuspended in 1 mL of PBS (1X). Samples were washed twice in this manner. After the second wash, the cells were resuspended in 200 µL of PBS (1X) and then fixed (4ºC for 16 hours) with 600 µL of 4% paraformaldehyde prepared in PBS (1X). Fixed cells were washed two times in PBS (1X) and resuspended in a 1 ml solution of 1:1, PBS:ethanol. For the fixation and permeabilization of both samples it was used the methodologies described by Sarti (2007) and Glöckner et al. (1996).

Hybridization with oligonucleotide probes. The methodology for samples hybridization from culture medium and also from water samples was adapted from Sarti (2007). In summary, 1 µL of each fixed sample for culture and 10 µL of fixed sample for water was applied into each site of a multi-well slide, and then dried at 45ºC for 20 minutes. Dry slide/samples were dehydrated by sequential washes of 50%, 80%, and 100% ethanol (3 minutes each), and then followed by drying at room temperature. For hybridization, 9 µL of solution [0.9M NaCl, 20mM Tris/HCl, 10mM EDTA, 0,01% SDS] was distributed to each well of the slide along with formamide (as specified in Table 1), and 1 µL of specific probe. Hybridization was performed in humidified chambers made from a capped, 50 mL polypropylene tube, wrapped in aluminum foil, and containing a wet filter paper inside. Hybridization time and temperature were adjusted for each probe. After hybridization, the slides were quickly washed with a preheated solution [20mM Tris/HCl, 10mM EDTA, 0.01% SDS plus NaCl] according to the probe used. The final wash of the slides was done with Milli-Q water. At this point the samples were left to dry at room temperature. Once dry, samples were incubated with 10 µL of DAPI (4',6-diamino-2-phenyl indole) [200 ng/µL] for 20 minutes in the dark. Following DAPI staining, the slides were again washed with Milli-Q water and then allowed to dry at room temperature. Both samples were covered with 4 µL of Gel/Mount and Hybridization Coverslipe.

Microscopic evaluation. The slides were visualized using an Olympus BX51 microscope with an Olympus Q-Color camera for image capture. Image analysis was carried out using the software Image Pro-Plus version 5.1. Cells stained with DAPI,

and either Cy3 or Alexa 555-labeled probes were visualized using two sets of filters (U-MWU2 - 330/420nm and U-MSWG2 - 480/590nm), respectively.

Table 1: Oligonucleotide probes used for FISH of SRB.

PROBE	SEQUENCE (5'-3')	SPECIFICITY	% FORMAMIDE	REFERENCE
NON-338	ACTCCTACGGGAGGCAGC	-	20	Manz et al., 1992
EUB-338	GCTGCCTCCCGTAGGAGT	Eubacteria	20	Amann el al., 1990
SRB-385	CGGCGTCGCTGCGTCAGG	Sulfate-Reducing Bacteria (SRB) and major Species of δ-Proteobacteria	30	Amann et al., 1990
DSV-698	GTTCCTCCAGATATCTACGG	Family *Desulfovibrionaceae*	20	Manz et al., 1998
SRB-129	CAGGCTTGAAGGCAGATT	Genus *Desulfobacter*	10	Devereux et al., 1992

Results and Discussion

SRB are a highly diverse group, both metabolically and phylogenetically (Mubmann et al, 2005). The physiological diversity in the dissimilative reduction of sulfate of the SRB group, allows these microorganisms to occupy a great variety of environmental niches where they are important in the sulfur and carbon biochemical cycles (Lucker et al., 2007). According to Kleikemper (2003), SRB arise from four different phylogenetic ancestries. Because of this diversity, several FISH probes are necessary to detect all SRB subgroups.

Cell concentrations of samples from culture medium were estimated in 4.8 x 10^8 cells/mL, as determined by DAPI staining. This number was indicative of total sample population, because DAPI is a non specific fluorescence staining that permits the enumeration of total bacteria. For a more precise quantification, we used manual counting; a procedure that afforded us the advantage of being able to differentiate between positively stained cells and non specific fluorescence in the sample. Manual counting also allowed us to identify individual cells in the captured images. Figure 1 shows the different microorganisms identified through FISH analysis in our samples from culture medium.

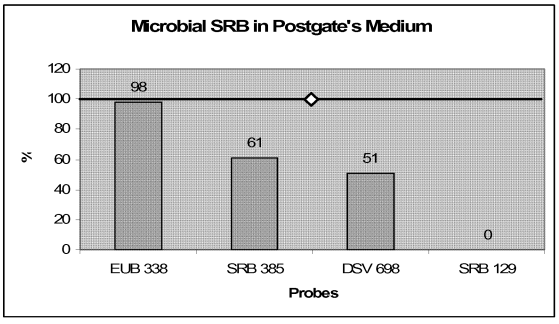

Figure 1. Percent composition of the SRB community identified by specific probes for 16S rRNA. EUB-338, general probe; SRB-385, specific for sulfate-reducing bacteria and major species of δ-Proteobacteria ; DSV-698, Desulfovibrio-specific; SRB-129, Desulfobacter-specific. —◇— DAPI.

Figure 2 shows the results for DAPI staining (A-total population) and hybridization with probe SRB-385 (B-population that hybridizes with the probe). The circles and arrows indicate cells that were positively stained for DAPI, but that did not hybridize with the SRB-385 probe. This was indicative of the different microbial groups in the sample.

We found that the same cells stained with DAPI hybridized to probe EUB-338, as all the cells from our culture samples were from the Eubacteria group. From those, probe SRB-385 hybridized to 61% of all the cells (stained with DAPI and hybridized to probe EUB-338). Using this same probe Icgen, and Harrison (2006) found a higher percentage of positively hybridized cells (86%) than we observed. However, in such study, microbial cells were cultured in a bioreactor, in contrast to our field samples. In the study by Ito (2001), the percentage of SRB detected in a biofilm sample coming from a sewer was 4.8%.

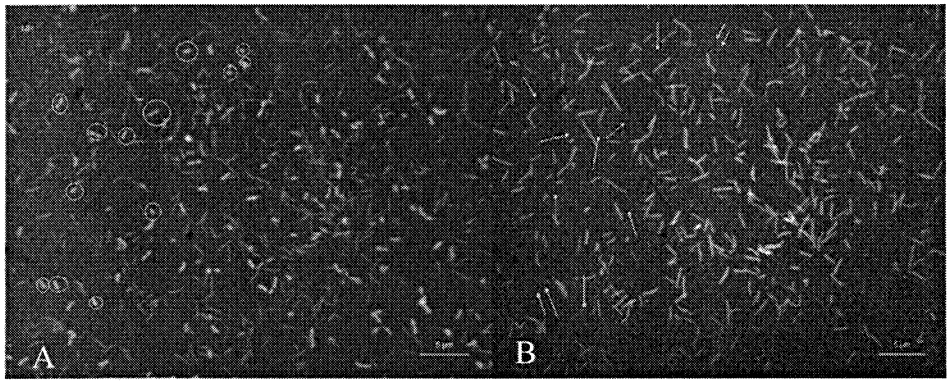

Figure 2. FISH analysis for identification of SRB in mixed culture. A) Mixed SRB stained with DAPI. B) Same viewing field using a filter for Cy3/Alexa555 fluorescence in samples hybridized with probe SRB-385. The arrows and circles indicate morphological types that are stained with DAPI, but were not hybridized with probe SRB-385, respectively.

Figure 3 shows morphological types of total microbial cells and those of SRB that have come from the sample of water from oil fields. Figure 3A shows DAPI staining for the total population in that environmental sample, while in figure 3B there are only cells of SRB hybridized with probe SRB-385. By comparison, the SRB represent only 29% of the total population (figure 3A and 3B). Density ($9{,}2 \times 10^3$ cells/ml of water) and diversity are much more intense and visible in figure 3A.

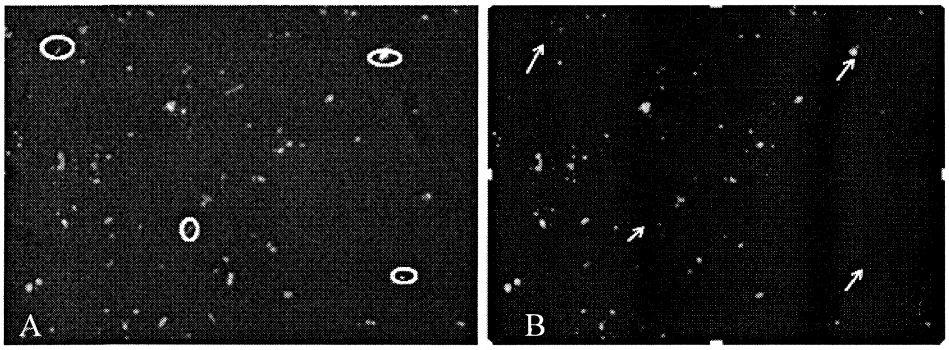

Figure 3. FISH analysis for identification of SRB directly from samples of water from oil fields. A) Total microbiota present in the sample of water from oil fields stained with DAPI. B) Same viewing using a filter for Cy3/Alexa555 fluorescence in samples hybridized with probe SRB-385. The arrows and circles indicate morphological types that are stained with DAPI, but were not hybridized with probe SRB-385.

DAPI was used to enumerate total population of microorganisms and also to distinguish the fluorescent signals of hybridized cells compared to that originated from the debris which are very common in environmental samples. This is due to the ability of DAPI to bind only to nucleic acids (DNA – preferentially AT-rich regions in the minor groove of B-DNA in solution) and not the material artifacts present in the environmental water samples (Porter et al., 1980; Manzini et al., 1983).

The advantage in using water samples from oil fields it is the possibility to detect spatial distribution of microorganisms at the environment (Amann et al, 2001). When working with culture samples, it is possible to detect only cultivable species, the minority of microorganisms present in that environment. Moreover, FISH technique makes possible to visualize the morphology of the cells *in situ* and supply important information about identification of bacterial groups of cells without culturing.

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