

Detection in coal tar waste-contaminated groundwater of mRNA transcripts related to naphthalene dioxygenase by fluorescent in situ hybridization with tyramide signal amplification

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Abstract

The ideal ecological metabolic activity assay would be applied to naturally occurring microbial populations immediately fixed in the field, and the assay would focus upon intracellular parameters indicative of a dynamic biogeochemical process. In this study, fluorescent in situ hybridization (FISH) with tyramide signal amplification (TSA) detected intracellular mRNA in bacteria. Detection sensitivity was enhanced by using a Hamamatsu color chilled CCD camera and extended exposure times. *Pseudomonas putida* NCIB 9816-4, a model naphthalene degrading bacterium, was used to refine the protocol. Probe Ac627BR was developed for detecting naphthalene dioxygenase (*nahAc*) mRNA transcripts. Only induced cells showed positive hybridization to probe Ac627BR. Results were verified by RNase A or DNase I digestion of samples prior to hybridization. When applied to field-fixed groundwater samples, the naphthalene dioxygenase mRNA probe conferred fluorescence on a subset (~1%) of the cells present in the contaminated groundwater. This methodology represents progress towards achieving one of the longstanding goals of environmental microbiology: to simultaneously ascertain the identity, activity, and biogeochemical impact of individual microorganisms in situ—in soil, water, or sediment where they dwell. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Fluorescent in situ hybridization; mRNA; Naphthalene dioxygenase; In situ; Contaminated groundwater

1. Introduction

One of the longstanding goals of environmental microbiology is to simultaneously ascertain the identity, activity, and biogeochemical impact of individual microorganisms in situ—in soil, water, or sediment

where they dwell (Amann and Kühn, 1998; Boschker et al., 1998; Madsen, 1998; Radajewski et al., 2000). The ability to simultaneously assign identity and activity to naturally occurring microorganisms has often been approached by combining several cross-disciplinary methods (Fliermans and Schmidt, 1975; Ward, 1984; Madan and Nierzwicki-Bauer, 1993; Hinrichs et al., 1999; Miskin et al., 1999; Henckel et al., 2000). Recent examples include: microautoradiography combined with fluorescent in situ hybridization (FISH) (Lee et al., 1999; Ouverney and Fuhrman, 1999); stable isotope probing (Boschker et al., 1998; Radajewski et al., 2000); 16S rRNA FISH and ¹³C isotope data (Orphan

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et al., 2001); in situ reverse transcription (Chen et al., 1997); and immunodetection of proteins (Currin et al., 1990; Lin et al., 1998). Such investigations generally sought the goal of discovering “who” was doing “what” in real-world field sites. However, estimation of biogeochemical activity often relies upon model laboratory systems that incubate substrate-amended environmental samples in sealed vessels. Yet, the ability of microorganisms in environmental samples to undergo rapid, physiological changes and population shifts during sample gathering, storage, and incubation is well-documented (Phelps et al., 1994; Madsen, 1996; Dunbar et al., 1997; Ward et al., 1997; Brockman et al., 1998). Thus, bypassing this incubation- and/or sampling-induced alteration in naturally occurring microbial communities is desirable.

Qualitatively, at least two assays linking in situ metabolic activity to individual field-derived microorganisms have been successfully implemented to date: immunolocalization of nitrogenase in field-fixed *Trichodesmium* samples (Paerl et al., 1989; Currin et al., 1990; Bergman and Carpenter, 1991; Fredriksson and Bergman, 1995) and detection of ^{13}C -depleted individual cells (Orphan et al., 2001) from methane-rich marine sediments. In these assays, the microorganisms were also identified by morphology or 16S rRNA FISH.

Fluorescent in situ hybridization with tyramide signal amplification (TSA-FISH) is routinely used to detect mRNA in eucaryotic systems (van de Corput et al., 1998a,b). TSA increases probe fluorescence 10–20 times more than a single, monolabeled probe and 2.1–3.5 times more than multiple, monolabeled probes (LeBaron et al., 1997; Schönhuber et al., 1997). Only 100–1000 copies of the hybridization target molecule per cell are needed for detection by TSA-FISH (Schönhuber et al., 1997). TSA-FISH has been applied to the detection of ribosomal RNA in marine samples from the Adriatic Sea (LeBaron et al., 1997) and microbial mats in high alpine lakes (Schönhuber et al., 1999). Although digoxigenin-labeled probes targeting a plasmid encoded thiostrepton-resistance gene have been used to detect mRNA in *Streptomyces* cells (Hahn et al., 1993), to our knowledge, FISH has never been applied to the detection of mRNA of bacteria in naturally occurring communities.

In this study, a TSA-FISH method was developed and applied to cells actively transcribing naphthalene

dioxygenase (*nahAc*) and related dioxygenase genes in environmental samples from a coal tar waste-contaminated site. Detection sensitivity was further increased by using a Hamamatsu color chilled 3CCD camera and extended exposure times. *Pseudomonas putida* NCIB 9816-4, a model naphthalene degrading bacterium, was used to refine the protocol prior to application to field-fixed groundwater samples.

2. Material and methods

2.1. Probe design

The probe EUB338, GCTGCCTCCCGTAGGAGT, targets nucleotide positions 338–355 of 16S rRNA (*Escherichia coli* numbering) (Amann et al., 1990). Probe Ac627BR, (G/C)ACGTGGTATGCATC encompassing amino acid residues 205–209 corresponding to nucleotides 627–613 of *nahAc* from *P. putida* G7, was designed to detect naphthalene dioxygenase mRNA transcripts by targeting important residues in the active site (Kauppi et al., 1998). Both probes were biotinylated at the 5' end and synthesized by the Cornell Oligonucleotide Synthesis Facility. Dot blots with genomic DNA from a variety of bacteria (see below) were used to assess the specificity of probe Ac627BR according to standard procedures (Ausubel et al., 1999). Digoxigenin-labeled probe was synthesized by Integrated DNA Technologies (Coralville, IA). Hybridization was carried out using the Genius System (Boehringer Mannheim) on nylon membranes (Micron Separations, Westboro, MA), blocked in pre-hybridization solution (probe-free hybridization solution, see below) for 30 min at 46 °C, hybridized for 2 h in hybridization solution (see below) at 46 °C, and subsequently washed for 20 min at 46 °C in TSA-FISH wash solution (see below).

2.2. Cell fixation

Cultures of *P. putida* NCIB 9816-4 were grown overnight in 50-ml glass test tubes with 5 ml 5% PTYG in a shaking water bath at 30 °C at 250 rpm. Cells were diluted into fresh media (1:5, 10 ml total volume), divided, and half received 100 μl 2% salicylic acid pH 6.0. Both treatments were incubated as above for 1 h, fixed with three volumes of freshly

prepared, cold (4 °C) 4% paraformaldehyde, and stored at 4 °C. Groundwater samples were collected from a coal tar waste-contaminated aquifer as previously described (Wilson et al., 1999; Bakermans and Madsen, 2000). Groundwater (125 ml) was pumped directly into 500-ml sample bottles containing 375 ml freshly prepared, cold 4% paraformaldehyde. Sample and fixative were mixed thoroughly by shaking and stored on ice for transport to the laboratory where they were stored at 4 °C until analysis.

2.3. TSA-FISH

The method of LeBaron et al. (1997) in combination with the TSA-Direct kit (NEN Life Sciences, Boston, MA) was used with the following modifications. Fixed samples were filtered onto 0.02- μm pore size, 25-mm diameter Anodisc membranes (Whatman International, Maidstone, England) using a glass filter tower (Kontes Glass, Vineland, NJ) and placed onto glass slides. Anodiscs were spotted with 100 μl permeabilization solution (0.1 M Tris-HCl, pH 8.2, 50 mM Na₂EDTA, 14 $\mu\text{g/ml}$ lysozyme; sometimes containing 0.5 mg/ml RNase A), incubated for 10 min at room temperature, rinsed three times with TE buffer (0.1 M Tris-HCl, 50 mM Na₂EDTA, pH 8.2) in the filter tower, and placed on fresh slides. Some samples were additionally spotted with 100 μl DNase solution (2.5 mM CaCl₂, 10 mM MgCl₂, 25 mM Tris-HCl, pH 7.5, and 0.2 U/ μl DNase I), incubated at room temperature for 1 h in a humid chamber, and rinsed with TE buffer. Hybridization was carried out in a PTC-200 thermocycler fitted with a twin tower 2 \times 16 capacity slide block (MJ Research, Watertown, MA) under 24 \times 60 mm glass cover slips and humidified with Whatman No. 1 filter paper saturated with probe-free hybridization solution. Samples were rinsed with 2 ml of 46 °C wash buffer (20 mM Tris-HCl pH 7.2, 0.01% SDS, 180 mM NaCl, and 5 mM EDTA), spotted with 100 μl wash solution, and incubated for 20 min at 46 °C under a 24 \times 60 glass cover slip in the slide block humidified with wash buffer. Samples were not air-dried. In subsequent wash steps, Anodiscs were clamped to slides with binder clips. After application of cyanine 3 tyramide (CY3T) solution, sample exposure to light was minimized. Subsequently, samples were washed twice in TNT buffer (0.1 M Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.5) for 15 min

with shaking (50 rpm) at room temperature, counterstained with 4',6-diamidino-2-phenylindole (DAPI; 2.5 $\mu\text{g/ml}$, 5 min), mounted under a 22-mm² cover slip with 30 μl Citifluor mountant medium #4 (Ted Pella, Redding, CA), sealed with vaspar (vaseline:paraffin, 1:1), and viewed immediately by epifluorescence microscopy.

2.4. Epifluorescence microscopy

All samples were examined with a Zeiss LSM-210 laser scanning microscope equipped for epifluorescence microscopy with a 50-W mercury arc lamp and for oil immersion with a 100 \times Plan-Neofluar objective lens. Cyanine 3 (CY3) imaging employed excitation, reflector, and barrier wavelengths of 510–540, 550, and 570 nm, respectively (Omega Optical, Brattleboro, VT). Likewise, DAPI imaging employed excitation, reflector, and barrier wavelengths of 365 \pm 12.5, 400, and 450 \pm 32.5 nm, respectively. A Hamamatsu color chilled 3CCD camera system was used to capture images; all samples in a given experiment were exposed for the same length of time (ranging from 1 to 20 s).

3. Results and discussion

3.1. Probe specificity

Specificity of probe Ac627BR was tested with southern hybridization to dot blots of genomic DNA from the following organisms: *P. putida* G7, *P. putida* NCIB 9816-4, *Comomonas testosteroni* GZ39, and *C. testosteroni* GZ42 represented a diversity of naphthalene dioxygenase genes; *Burkholderia* sp. JS150 and sp. DNT represented dinitrotoluene dioxygenases; *P. putida* strain F1 and strain mt2 contained toluene dioxygenase genes; and *Bacillus subtilis* ATCC 4925, *Clostridium lentocellum*, and *Dehalococcoides ethenogenes* served as negative controls. Positive hybridization was seen at equal intensity for all *Pseudomonas*, *Comomonas*, and *Burkholderia* DNA tested; *B. subtilis*, *C. lentocellum*, *D. ethenogenes*, and salmon sperm DNA did not hybridize with the probe (data not shown). No hybridization was detected at temperatures above 46.5 °C. Therefore, under conditions used here, probe Ac627BR detected a diversity of dioxygenase

genes; it was not exclusively a naphthalene dioxygenase-specific probe. Additional hybridization to the closely related dinitrotoluene dioxygenase genes was expected (3 of 15 mismatches with *dntAc*); while hybridization to toluene dioxygenase genes (6 of 15 mismatches with *todC1*) was a surprise. Because hybridization was performed against genomic DNA and PAH degraders are known to contain a variety of dioxygenase genes (Pellizari et al., 1996), probe Ac627BR could have detected alternate, uncharacterized dioxygenases in *P. putida* strain F1 and strain mt2.

3.2. Development of TSA-FISH procedure

The low levels of mRNA usually present in cells cannot be detected with traditional FISH protocols (Amann et al., 1995; Amann and Köhl, 1998). In order to increase sensitivity, TSA was used with CY3 and a CCD camera. CY3 was used because it is significantly brighter than other fluorophores (such as Texas Red, tetramethylrhodamine, or fluorescein) and shows the lowest levels of nonspecific binding (Southwick et al., 1990; Glöckner et al., 1996). FISH

is usually carried out on glass microscope slides; however, the goal was to examine groundwater samples with low cell concentrations. Therefore, the TSA-FISH procedure was developed with cells that were concentrated onto Anodisc membranes (Lemke et al., 1997). When Citifluor mountant media was used instead of DABCO (Schönhuber et al., 1997), fluorescence fading problems stopped. Preventing carryover of reagents from one step to another was essential. Only qualitative analyses were performed in this study. No attempt was made to correlate fluorescence intensity with mRNA levels. Due to the generally weak signals obtained, digital images were analyzed. Exposure times for DAPI and CY3 were kept constant within experiments. Because signal intensity varied between experiments, exposure times also varied between experiments. Positive hybridization signals were subjectively judged as those cells displaying fluorescence significantly above background and above fluorescence levels of negative controls. Cells with positive hybridization signals were counted as actively expressing 16S rRNA or *nahAc* mRNA.

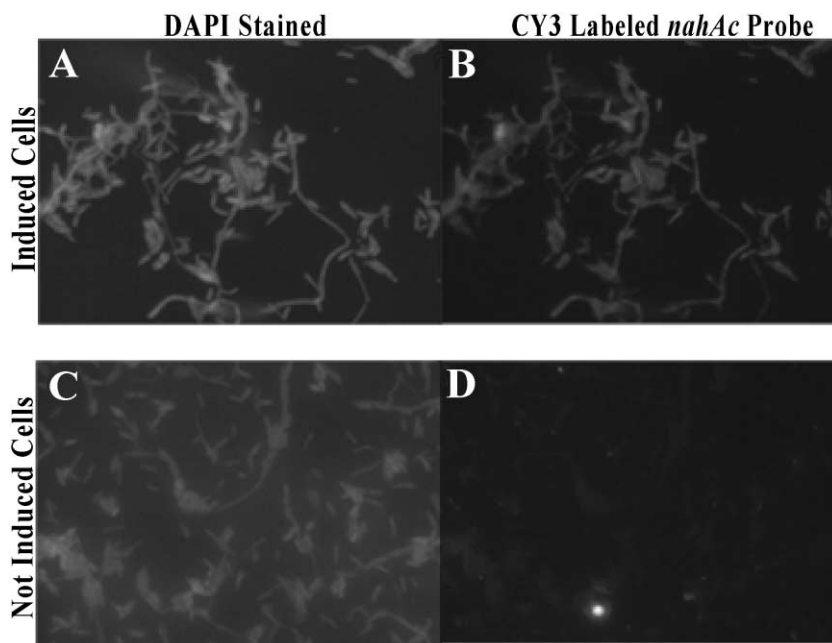


Fig. 1. Hybridization of naphthalene dioxygenase probe Ac627BR with induced and uninduced cells of *P. putida* NCIB 9816-4. Both DAPI (Panels A and C) and probe-conferred CY3 (Panels B and D) fluorescence are shown for the same field. (A, B) Induced cells. (C, D) Uninduced cells. Viewed under 100 \times magnification. Exposure time was 4 s for DAPI and 10 s for CY3.

To verify that TSA-FISH could detect target molecules expected to be present at very high levels in the cell, *P. putida* NCIB 9816-4 was hybridized with the 16S rRNA specific probe, EUB338 (data not shown). As expected, all cells (except no-probe negative controls) displayed CY3 fluorescence due to hybridization with the probe and TSA. Uniform CY3 fluorescence also confirmed that cells were adequately permeabilized and that the probe, enzyme, and fluorescent substrate had sufficient access to target molecules.

3.3. Detection of naphthalene dioxygenase (*nahAc*) mRNA in *P. putida* NCIB 9816-4

To determine if detection of *nahAc* mRNA was possible with TSA-FISH, *P. putida* NCIB 9816-4 was

hybridized with probe Ac627BR. The presence of *nahAc* transcripts in *P. putida* NCIB 9816-4 was ensured by inducing cultures with salicylate; uninduced cultures served as negative controls (Yen and Serdar, 1988). Constitutively expressed at low levels, *nahAc* expression is increased 24- to 30-fold by salicylate induction (Guerin and Boyd, 1995). As expected, CY3 fluorescence was only observed in induced cultures (Fig. 1, Panels A and B). Probe-conferred fluorescence was fairly uniform indicating adequate cell permeabilization and access to target mRNA. Because CY3 fluorescence was not observed in uninduced cells (Fig. 1, Panels C and D) and *nahAc* is constitutively expressed (Yen and Serdar, 1988), constitutive levels were deemed too low to be detected by this TSA-FISH procedure.

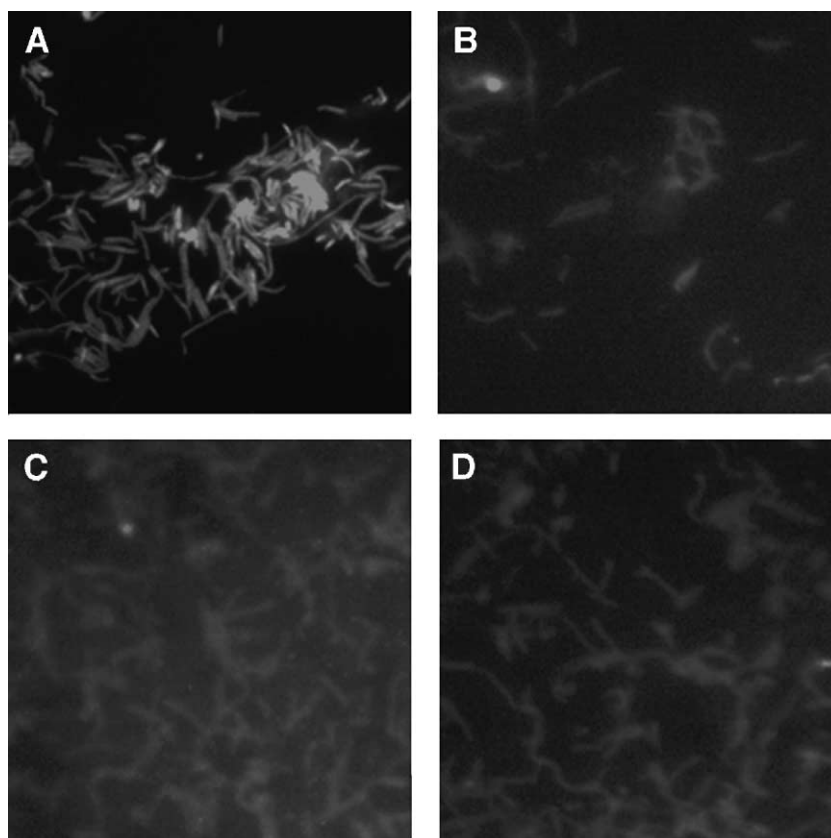


Fig. 2. RNase treatment eliminated detection of probe-conferred fluorescence in induced cells of *P. putida* NCIB 9816-4. Only probe-conferred CY3 fluorescence is shown. Both induced (Panels A and C) and uninduced (Panels B and D) cells are shown. (A, B) No treatment. (C, D) RNase treated. Viewed under 100 \times magnification. Exposure time was 1 s for all panels.

To verify that probe Ac627BR was detecting mRNA (*nahAc* transcripts), samples were digested with RNase A prior to hybridization (Fig. 2). Induced, untreated samples showed the expected probe-conferred fluorescence (Fig. 2, Panel A), while induced, treated samples showed no fluorescence presumably because RNase digestion eliminated the probe target (Fig. 2, Panel C). The lack of fluorescence (Fig. 2,

Panel C) also implies that TSA-FISH was not able to detect the copies of *nahAc* DNA present on the plasmid pDTG1. Uninduced samples served as negative controls (Fig. 2, Panels B and D).

To verify that probe Ac627BR was not detecting DNA (plasmid-borne *nahAc* genes), samples were digested with DNase I prior to hybridization (Fig. 3). Induced, undigested samples were used as positive

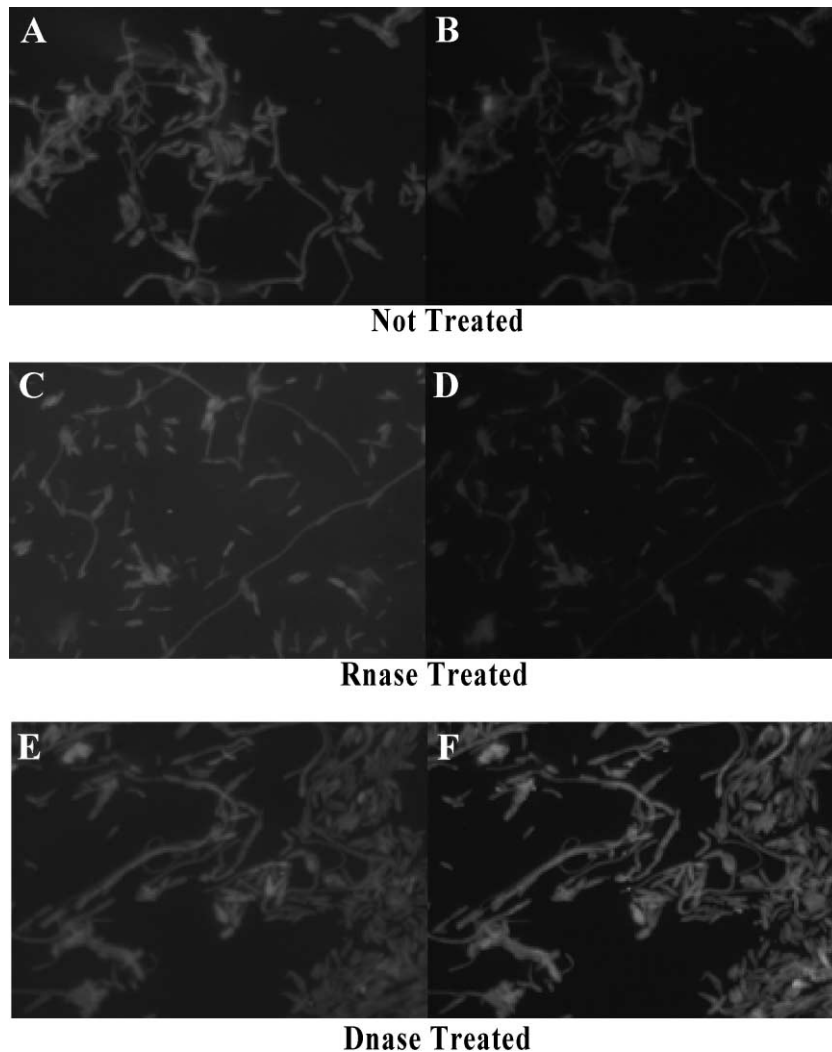


Fig. 3. DNase treatment enhanced probe-conferred fluorescence. Cultures of *P. putida* NCIB 9816-4 were induced for *nahAc* expression and hybridized with probe Ac627BR. Both DAPI (Panels A, C, and E) and probe-conferred CY3 (Panels B, D, and F) fluorescence are shown for the same field. (A, B) No treatment. (C, D) RNase treated. (E, F) DNase treated. Viewed under 100 \times magnification. Exposure time was 4 s for DAPI and 10 s for CY3.

controls (Fig. 3, Panels A and B), while induced, RNase treated cells were used as negative controls (Fig. 3, Panels C and D). DNase treated samples showed increased fluorescence (Fig. 3, Panels E and F), possibly due to increased access of the probe to the target mRNA after DNA was removed (Nuovo, 2001). Therefore, DNase treatment was incorporated into the standard TSA-FISH protocol.

3.4. Detection of dioxygenase transcripts in groundwater microorganisms

To evaluate the ability of TSA-FISH to detect bacteria containing dioxygenase transcripts within a naturally occurring community of microorganisms, cells from contaminated groundwater were gathered in June and October 1999, and hybridized with probes EUB338 and Ac627BR; the former was used to verify the adequacy of cell permeabilization procedures. As

expected, most cells (>90%) displayed EUB338 probe-conferred fluorescence (data not shown). The remaining variability in CY3 fluorescence can be attributed to differences in cell permeability (Schönhuber et al., 1997) or in probe specificity (Daims et al., 1999).

When probe Ac627BR was used in the TSA-FISH assay with groundwater samples, CY3 fluorescence varied from cell to cell (Fig. 4). These variable signals presumably reflected the presence or absence of dioxygenase transcripts within the cells. Cells were counted as having a positive hybridization signal only if the signal intensity was significantly above background levels present in the sample and if there was a corresponding cell-like structure identifiable in the DAPI image. There was no apparent correlation between cell morphology and CY3 fluorescence. The TSA-FISH assay for dioxygenase mRNA was successful on two different sampling dates (June and October 1999). Based on total acridine orange direct

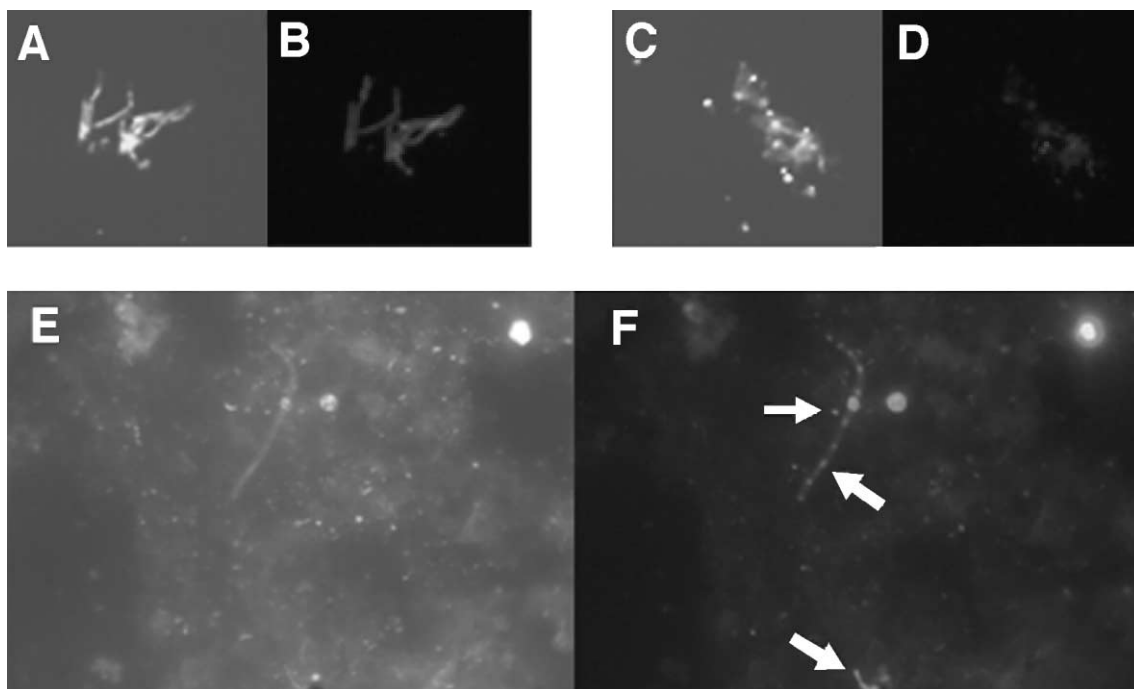


Fig. 4. Hybridization of Ac627BR with native microbial community from contaminated groundwater. Both DAPI (left panel in each pair) and probe-conferred CY3 (right panel in each pair) fluorescence are shown for the same field under $100\times$ magnification. (A, B) All cells in this field from 60 ml fixed sample hybridized with probe Ac627BR. Exposure time was 10 s. (C, D) No cells in this field from the same 60 ml fixed sample hybridized with probe Ac627BR. Exposure time was 10 s. (E, F) Coccus, rod, and a chain of cells (arrows) from 465 ml fixed sample hybridized with probe Ac627BR. Exposure time was 4 s for DAPI and 8 s for CY3.

counts and preliminary counts of positive hybridization seen during TSA-FISH analysis, we estimated that 1% of the native population from the October 1999 samples were actively transcribing dioxygenase genes. The presence of cells displaying Ac627BR-conferred CY3 fluorescence was expected because *nahAc* mRNA had been previously isolated from the same environment (Wilson et al., 1999). In addition, substrate responsive-direct viable counts from the October 1999 samples identified naphthalene degrading organisms present at approximately the same levels [0.27% of acridine orange direct counts (Bakermans and Madsen, 2000)].

4. Conclusions

The TSA-FISH procedure was capable of detecting *nahAc*-like mRNA transcripts in pure cultures of *P. putida* NCIB 9816-4 induced for *nahAc* expression and in a naturally occurring microbial community known to metabolize naphthalene. TSA-FISH can be applied to environmental samples fixed immediately in the field. Therefore, the data can be confidently interpreted as documenting the in situ expression of specific genes in individual cells. A major strength of the TSA-FISH procedure is that it is implemented with no post-sampling incubation steps that may alter the composition and activity of the naturally occurring microbial community. TSA-FISH is still limited by current databases used to design probes and by differences in cell permeabilization efficiency. Despite these limitations, TSA-FISH is a promising technique and may possibly be calibrated so that signal intensity would be indicative of in situ biogeochemical reaction rates. For example, naphthalene mineralization rates of pure cultures could be correlated with fluorescence intensity of cells hybridized with Ac627BR. Currently, the number of active cells within a population can easily be quantified by TSA-FISH via counting of positive cells. As designed, this TSA-FISH technique that examines naturally occurring microbial populations without laboratory incubation is relatively quick (5 h), and should be amenable to the use of multiple probes, such as 16S rRNA- and mRNA-targeted probes (Lee et al., 1993; Wallner et al., 1993; Herrmann et al., 1997), in order to assess the in situ activity and identity of microorganisms.

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