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In Situ Reverse Transcription, an Approach To Characterize Genetic Diversity and Activities of Prokaryotes

FENG CHEN,[1] JOSÉ M. GONZÁLEZ,[2] WENDY A. DUSTMAN,[2] MARY ANN MORAN,[1,3] AND ROBERT E. HODSON[1,2,3]*

Department of Marine Sciences,[1] Department of Microbiology,[2] and Institute of Ecology,[3] University of Georgia, Athens, Georgia 30602-2206

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Reverse transcription of RNA molecules inside intact bacterial cells was carried out by using reverse transcriptase with a single oligonucleotide complementary to specific 16S rRNA or mRNA sequences. Fluorescently labeled nucleotides were incorporated into each transcribed cDNA inside cells. This protocol is termed in situ reverse transcription (ISRT). In this study, by using species-specific primers targeting unique regions of the 16S rRNA sequences, ISRT was used successfully to detect and enumerate the two lignin-degrading bacteria *Microbulbifer hydrolyticus* IRE-31 and *Sagittula stellata* E-37 in culture mixtures and complex enrichment communities selected for lignin degradation. Image analysis revealed that *M. hydrolyticus* IRE-31 and *S. stellata* E-37 accounted for approximately 30 and 2%, respectively, of the total bacterial cells in lignin enrichment communities. Populations estimated by ISRT were comparable to those estimated by in situ hybridization (ISH) techniques and to those estimated by hybridization against extracted community DNA. ISRT was also successfully used to detect *Pseudomonas putida* F1 expressing the *todC1* gene in seawater exposed to toluene vapor. ISRT provided a higher signal intensity than ISH, especially when targeting mRNA. The calculated pixel intensities resulting from ISRT were up to 4.2 times greater than those from ISH. This suggests that multiple incorporation of fluorescently labeled nucleotides into cDNA provides a high sensitivity for phylogenetic identification of bacterial populations as well as detection of cells expressing a specific functional gene within complex bacterial communities.

Direct detection and identification of bacterial species at the single-cell level constitute an important new approach that has significantly advanced our understanding of the microscale distributions of populations and communities in aquatic environments. In situ hybridization (ISH) with radioactively or fluorescently labeled oligonucleotide probes uniquely complemented to 16S rRNA sequences has been used with epifluorescence microscopy to identify prokaryotic cells in various complex samples at phylogenetic levels from species to kingdoms (2, 3, 6, 10, 29). Most applications of monolabeled rRNA-targeted probes have been restricted to bacterial populations in relatively nutrient-rich environments, such as activated sludge (18, 32), the surfaces of large organic aggregates (lake "snow") (25, 33), and biofilms (26, 28, 30). Because ISH methods rely on multiple targets within the bacterial cell to provide a detectable signal, they are hindered by the small number of rRNA molecules in many slow-growing or dormant bacteria in natural environments (17, 20). Attempts to increase signal strength by using multiple rRNA-targeted fluorescent probes (24), polyribonucleotide probes (31), or more intensely fluorescent dyes (1, 34) have helped increase the suitability of ISH for characterization of microbial community structures in natural environments.

In situ PCR technologies have been developed to detect DNA or RNA viruses inside eukaryotic cells (13, 15, 19, 27). Recent studies have shown that specific functional genes (plasmid or chromosomal) and their expression (mRNA) in intact prokaryotic cells can be visualized by using prokaryotic in situ PCR (PI-PCR) and the combination of in situ reverse transcription and PI-PCR (PI-RT-PCR) (4, 7, 21). This approach takes advantage of the ability of PCR techniques to amplify rare gene targets inside intact bacterial cells. Thus, PI-PCR can be used to microscopically visualize which bacterial cells contain a specific gene in a complex microbial assemblage and how gene expression in prokaryotic cells responds to environmental conditions. This protocol has been successfully used to detect mRNA transcribed from the *nahA* gene in *Pseudomonas* cells and in model marine bacterial communities (21) and to detect the presence and expression of the *nifH* gene in cells of *Azotobacter vinelandii* (7) and of the *todC1* gene in cells of *Pseudomonas putida* F1 (5). A third protocol, RNA-targeted primer extension (RPE), involves multiple cycles of intracellular binding and extension of a single primer and results in arithmetic (rather than geometric) amplification of targeted sequences. In addition to its usefulness for quantitation or semiquantitative visual detection of gene expression at the individual-cell level, RPE may provide an alternative to the existing methods for in situ phylogenetic identification of prokaryotic cells based on detection of unique regions of 16S or 23S rRNA (21).

To detect RNA sequences which are present in multiple copies inside prokaryotic cells, cycles of RPE amplification may not be necessary. Transcripts (mRNA) of a functional gene inside bacterial cells can be detected by ISH if the digoxigenin (DIG)-labeled in vitro transcript (e.g., 200 bases) is used as a probe (16, 22). In this case, a signal increase was achieved by using multilabeled nucleotides in a probe rather than by amplifying the gene target. Therefore, we expect that in situ reverse transcription (ISRT) with a single primer binding to RNA and extension of the primer by using reverse transcriptase in the presence of labeled nucleotides will also be sufficient to detect low-copy-number RNA. ISRT allows multiple incorporation of labeled nucleotides (e.g., DIG-dUTP or CY3-dUTP) into a single copy of transcribed cDNA; it should pro-

* Corresponding author. Mailing address: Department of Marine Sciences, University of Georgia, Athens, GA 30602-2206. Phone: (706) 542-5952. Fax: (706) 542-5888. E-mail: rhodson@uga.cc.uga.edu.

TABLE 1. Primers used in this study

Primer	RNA target	Location	Primer sequence	T_m (°C)[c]	Specificity
IRE-31D	16S rRNA	1215–1233[b]	5′-GCACGTGTGTAGCCCAGGT	71.9	*M. hydrolyticus* IRE-31
E-37D	16S rRNA	1024–1042[b]	5′-CACTGCGTCCCGAAGGGA	74.0	*S. stellata* E-37
TOL	mRNA (*todC1*)[a]	717–738	5′-AGCAAGGTCGGCCATTTCAAGG	73.2	*P. putida* F1

[a] The *todC1* gene encodes the large subunit of the toluene dioxygenase system of *P. putida* F1.
[b] *Escherichia coli* numbering system.
[c] Temperature at which 50% of a nucleic acid is in a duplex with the complementary strand. The T_m was calculated by the percent G+C method with Oligo 4.05 primer analysis software.

vide a more intense signal than the standard ISH with a mono-labeled probe for in situ detection of RNA sequences. In this study, we demonstrate the usefulness of ISRT for detecting and quantifying the two lignin-degrading bacterial species in lignin enrichment communities and *P. putida* F1 expressing a toluene degradation gene when they were added to seawater exposed to toluene.

MATERIALS AND METHODS

Bacterial isolates and model communities. (i) Lignin-degrading systems. The bacteria *Microbulbifer hydrolyticus* IRE-31 and *Sagittula stellata* E-37 were originally isolated from a marine microbial community enriched with a lignin-rich, high (>10,000)-molecular-weight fraction of pulp mill waste (11). These two bacterial strains were abundant in the lignin enrichments; details of isolation, phenotypic and phylogenetic characterization, and in vitro hybridization assays for quantification of *M. hydrolyticus* IRE-31 and *S. stellata* E-37 are described elsewhere (11). *M. hydrolyticus* IRE-31 and *S. stellata* E-37 were grown in marine broth 2216 at 37°C, and cells were harvested at mid-logarithmic phase by centrifugation at 6,000 rpm for 10 min. *P. putida* AC10-R grown in Luria-Bertani broth was used as the negative control for probes specific for *M. hydrolyticus* IRE-31 and *S. stellata* E-37. The two lignin enrichment communities (communities A and B) were the source communities for *M. hydrolyticus* IRE-31 and *S. stellata* E-37. Experiments with radiolabeled synthetic lignins indicated that the communities could actively mineralize polymeric lignin at relatively high rates (25% per month) (12).

(ii) Toluene-degrading system. The bacterial strain *P. putida* F1 (Deutsche Sammlung von Mikroorganismen; DSM6899) has been described by Gibson et al. (8). *P. putida* F1 chromosomally encodes the toluene dioxygenase gene system, which degrades toluene via a dihydrodiol pathway to a tricarboxylic acid cycle intermediate (9). One milliliter of a mid-log-phase culture of *P. putida* F1 was washed by centrifugation and resuspended in 1 ml of phosphate-buffered saline (PBS) (120 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer, pH 7.6). An aliquot of 10 μl of washed cells was added to each of two flasks containing 100 ml of the natural seawater collected from the pier adjacent to the Skidway Marine Science Institute. Flask 1 was exposed to toluene vapor, and flask 2 remained as it was. Three milliliters of toluene was placed in a glass tube with a hole, which was then suspended in a 250-ml Erlenmeyer flask with 50 ml of BSM. Both flasks were incubated at 25°C with a shaking speed of 100 rpm. An aliquot of 2 ml was taken from each flask after 48 h of incubation and filtered on a 0.2-μm-pore-size black membrane filter. Samples on filters were used directly for ISRT detection.

Primer design. For ISRT, only a single downstream primer is needed. To achieve a sufficient incorporation of labeled nucleotides into cDNA, a primer located at least 200 bases downstream from the 5′ end of the RNA sequence is required for ISRT. By analyzing the 16S rDNA sequences, the species-specific downstream primers IRE-31D and E-37D were designed for *M. hydrolyticus* IRE-31 and *S. stellata* E-37, respectively (Table 1). For *P. putida* F1, the primer (TOL) was designed to specifically bind to the gene (*todC1*) encoding large subunit of terminal dioxygenase in the toluene dioxygenase system encoded on the chromosome of *P. putida* F1 (36) (Table 1). Sequence alignments were conducted with the Genetics Computer Group Inc. package (version 8), and oligonucleotide sequences were analyzed with Oligo 4.05 primer analysis software (National Bioscience, Inc., Plymouth, Minn.) to minimize secondary structure and dimer formation. All three probe sequences are specific for the target organisms, since there was no match with any other sequence in the data banks. Oligodeoxynucleotides were synthesized on an Oligo 1000 DNA synthesizer (Beckman Instruments Inc., Fullerton, Calif.). These three primers were also used for ISH. Oligonucleotides IRE-31D, E-37D, and TOL were 3′ end labeled with DIG-ddUTP by use of terminal transferase (Boehringer Mannheim) according to procedures described in reference 14. Alternatively, the primers were 5′ end labeled with the indocarbocyanine fluorescent dye CY3 (Genosys Biotechnologies, Inc.) to compare the direct detection system with an indirect detection system. The CY3-labeled probes were purified by cartridge. The labeled oligonucleotides were dissolved in sterile distilled water at a concentration of approximately 30 ng/μl and stored at −20°C.

Sample preparations for in situ protocols. Bacterial cell pretreatments, including cell fixation, dehydration, and permeabilization, were by the methods described by Hodson et al. (21). Water, buffer, and washing solutions were pretreated with diethyl pyrocarbonate to remove RNase activity, which may degrade cellular RNA, especially for mRNA. Briefly, cells were fixed in 4% paraformaldehyde in PBS for 3 h at room temperature, after which the cells were washed twice with PBS and then resuspended in 50% ethanol in PBS. The fixed cells were stored at −20°C at cell concentrations of approximately 10^8 to 10^9/ml. To prepare samples on the slides, a 5-μl aliquot of fixed cells was spotted onto a clean silane-coated glass slide (Perkin-Elmer Corp., Norwalk, Conn.) and air dried at room temperature. Samples on slides were treated with lysozyme (0.5 mg/ml) for 15 min at room temperature and then dehydrated sequentially in 50, 80, and 98% ethanol in PBS. For samples on filters, the same procedures for fixation and lysozyme treatment, but no alcohol dehydration, were used.

ISH. ISH mainly was by the protocol of Zarda et al. (35). Briefly, each cell smear on the slide was covered with 20 μl of hybridization solution (900 mM NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate, pH 7.2) containing 100 ng of DIG- or CY3-labeled oligonucleotide probe. Slides were incubated at 45°C overnight in a humid chamber. After incubation, the slides were washed with 0.5× SSC (750 mM NaCl plus 75 mM trisodium citrate, pH 7.0) for 10 min at 45°C. Different temperatures and salt concentrations were tested against the three primers, and more-stringent conditions did not improve the hybridization efficiency. Slides with the CY3-labeled probe are ready for microscopic examination, while slides with the DIG-labeled probe need additional steps for fluorescence detection (see "Anti-DIG recognition and signal detection" below).

ISRT. After cell fixation and permeabilization, the nonlabeled primer was hybridized to the RNA target site by the ISH method described above, except that the incubation time was 2 h instead of overnight. Unbound primers on slides were washed off with 0.5× SSC solution (10 min at 45°C). Samples on slides were dehydrated sequentially in 50, 80, and 98% ethanol in PBS before the reverse transcription mixture was added. The reverse transcription mixture consisted of 1× enzyme buffer; 200 μM each dATP, dCTP, and dGTP; 190 μM dTTP; 10 μM DIG-11-dUTP or CY3-dUTP; RNase inhibitor (0.8 U μl^{-1}; Boehringer Mannheim); 0.02% bovine serum albumin; 5 mM dithiothreitol; and reverse transcriptase (1 U μl^{-1}). Two different reverse transcriptases, avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and thermostable r*Tth* reverse transcriptase (Perkin-Elmer), were used. The cell spots were then covered with 20 μl of reverse transcription mixture, and slides were incubated at 45°C (for avian myeloblastosis virus reverse transcriptase) or 60°C (for thermostable r*Tth* reverse transcriptase) for 1 h in a humid chamber. After that, the slides were washed twice with 0.5× SSC at 45°C for 10 min. At this point, slides with CY3-dUTP are ready for microscopic examination, while slides with DIG-dUTP need further fluorescence development.

Anti-DIG recognition and signal detection. Stock anti-DIG-labeled alkaline phosphatase (Boehringer Mannheim) was diluted 100 times in blocking solution (150 mM NaCl, 100 mM Tris-HCl [pH 7.5], 0.5% bovine serum albumin, and 0.5% blocking powder [Boehringer Mannheim]). Following ISRT or ISH, each cell smear on a slide was covered with 100 μl of diluted anti-DIG-labeled alkaline phosphatase, and the slide was incubated at room temperature for 1 h.

The fluorescent signal inside target cells was detected by using (2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate (HNPP) and fast red TR (FR) (Boehringer Mannheim). HNPP (10 μl of a 10-mg/ml solution in dimethylformamide) and FR (10 μl of a 25-mg/ml solution in distilled water) were added to 1 ml of detection buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 8.0), and the mixture was filtered through a 0.2-μm-pore-size Acrodisc filter before use. Cells which had been incubated with anti-DIG-labeled alkaline phosphatase were washed twice with PBS and then incubated in 100 μl of HNPP-FR detection solution for 30 min at room temperature, followed by two washes with detection buffer.

A general DNA stain was used to visualize all cells in the sample. Cells were further counterstained by treatment with 100 μl of 4′,6′-diamidino-2-phenylindole (DAPI) at a concentration of 0.5 μg/ml for 5 min. After counterstaining, the slide was washed once with distilled water and air dried. Samples on slides were mounted in Olympus immersion oil.

Digital image analysis. Slides were examined under an epifluorescence microscope (Olympus BX-40) with a 100× high-resolution U Plan Oil objective lens (numerical aperture, 1.35 to 0.50) (C. Squared Corporation, Marietta, Calif.).

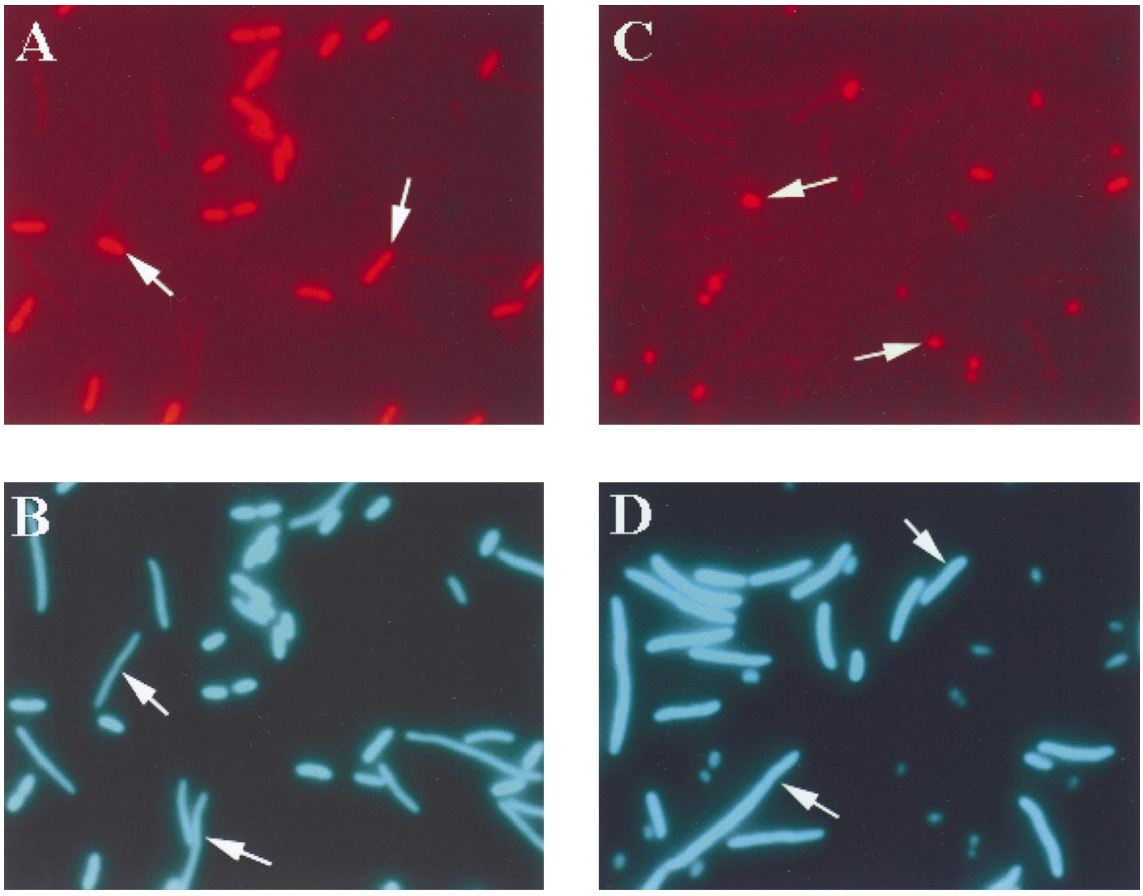

FIG. 1. Detection of lignin-degrading bacteria in culture mixtures by ISRT with species-specific probes. *M. hydrolyticus* IRE-31 and *S. stellata* E-37 cells were detected by ISRT with species-specific probes. Target cells (*M. hydrolyticus* IRE-31) show the HNP-FR fluorescent color under blue-light excitation (arrows indicate the positive cells) (A), while total mixed cells in the same field are visible by DAPI staining under UV light (arrows indicate the negative cells) (B). *S. stellata* E-37 cells detected by ISRT show the HNP-FR fluorescent color under blue-light excitation (arrows indicate the positive cells) (C), while both negative and positive cells in the same field are visible by DAPI staining under UV light (arrows indicate the negative cells) (D). *P. putida* AC10-R was used as a negative control.

Images were acquired with a cooled charge-coupled device (CCD) SenSys:1400 (1317 by 1035 image array; 6.8- by 6.8-μm pixel; 12 bit) (Photometrics, Tucson, Ariz.) and processed with the Oncor (Gaithersburg, Md.) Image software package version 2.02 on a Power Macintosh 9500. Target cells labeled with CY3 or HNP-FR were viewed under orange or blue light, respectively. Since the samples were counterstained with DAPI, the same microscopic view fields of dual-stained samples were then exposed to UV light (DAPI filter) to visualize both positive and negative cells. The two images which were acquired from the same view field under the microscope can be superimposed and saved as the PICT format for importing into Photoshop version 3.0 (Adobe, Mountain View, Calif.). To enumerate target cells in the lignin enrichment communities, at least 200 positive (HNP-FR fluorescence) cells were counted. The percentage of target cells was calculated by dividing cell counts from the HNP-FR image by cell counts from DAPI image. The fluorescence signal strength after ISRT was compared to that after ISH by measuring the pixel intensity of each target image (HNP-FR or CY3 labeled).

Dot blot hybridization. DNA extraction and hybridization conditions were as described by González et al. (11).

RESULTS

When ISRT with primer IRE-31D was used for the culture mixture of *M. hydrolyticus* IRE-31 (target cells) and *P. putida* AC10-R (negative cells), the target cells detected by ISRT emitted a strong HNP-FR fluorescent color and negative cells showed a very dim HNP-FR signal when the sample was observed under blue light excitation (Fig. 1A). However, in the same view field, both positive and negative cells counterstained by DAPI were visible when excited with UV light (Fig. 1B). The morphological difference between positive cells (short rods) and negative cells (long rods) further confirms that only the *M. hydrolyticus* cells were detected in the culture mixture. In another example, when ISRT with primer E-37D was used for the culture mixture of *S. stellata* E-37 (target cells) and *P. putida* AC10-R (negative cells), *S. stellata* E-37 cells were detected with a strong red HNP-FR color and negative cells had very dim HNP-FR fluorescence under the blue light (Fig. 1C). Both positive and negative cells counterstained by DAPI were all visible at the same view field when the sample was excited by UV light (Fig. 1D). Again, the morphological difference between positive and negative cells provided additional support for the results from ISRT.

The ISRT approach also was successfully used to detect target cells (*M. hydrolyticus* IRE-31 and *S. stellata* E-37) within complex lignin enrichment communities. Figure 2 compares the detections of *M. hydrolyticus* IRE-31 cells in lignin enrichment marine community A by the ISRT and ISH protocols. The HNP-FR fluorescent signals produced by ISRT (Fig. 2A) were brighter than those produced by conventional ISH (Fig. 2B). The calculated pixel intensities of *M. hydrolyticus* IRE-31 target cells were on average 2.4 times greater with ISRT than with ISH (Table 2).

ISRT with the *M. hydrolyticus* IRE-31-specific primer indicated that this species accounted for 24.6 and 31.5% of the

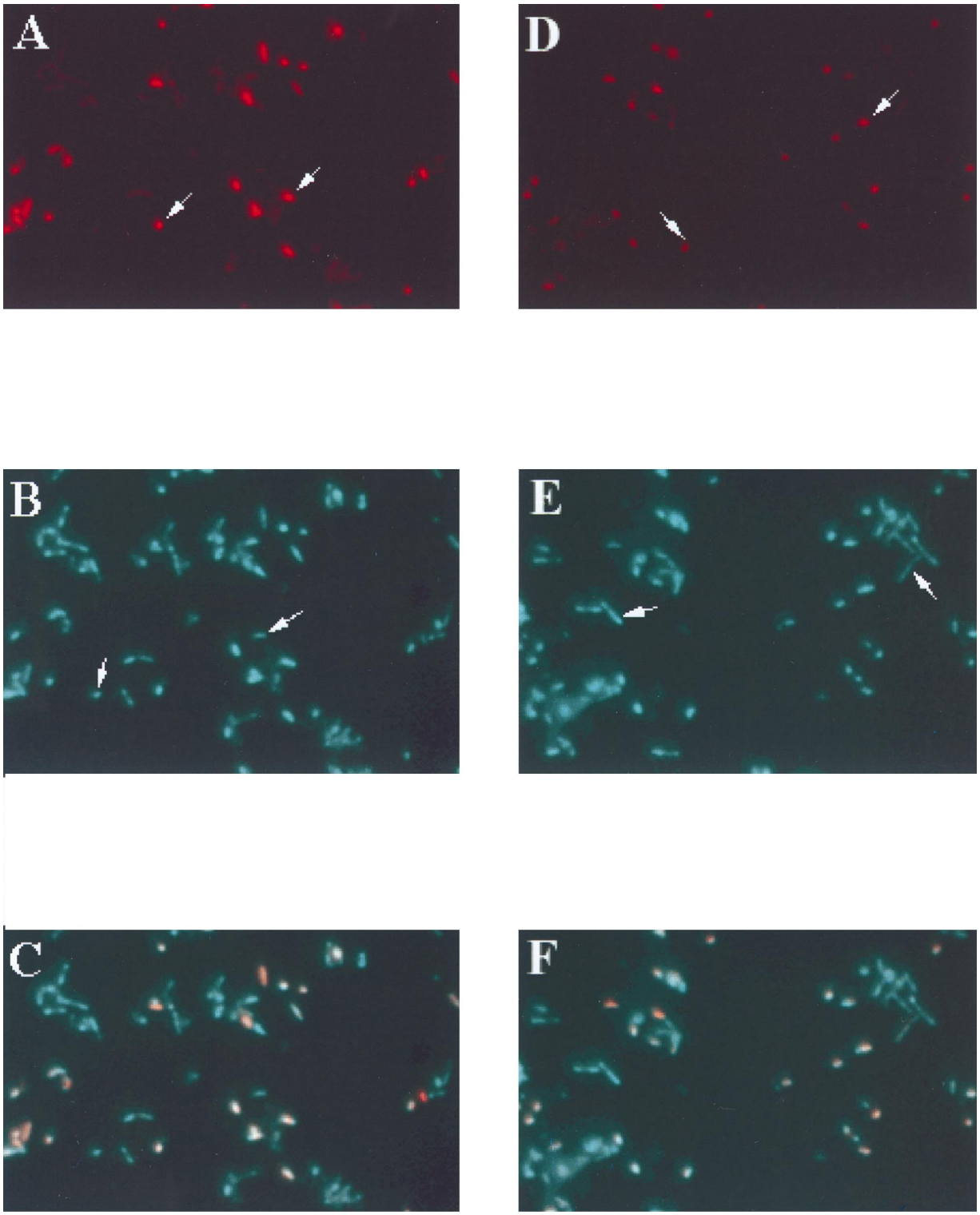

FIG. 2. *M. hydrolyticus* IRE-31 cells detected in lignin enrichment marine community A by ISRT and ISH protocols. (A and D) Target cells (HNP-FR fluorescence) in community A detected by ISRT (A) and ISH (D) under blue light (arrows indicate the positive cells). (B and E) Images viewed under UV light in the same view fields as for panels A and D, respectively (arrows indicate the negative cells). (C and F) Superimposed images of panels A and B (C) and panels D and E (F).

total cells in communities A and B, respectively. E37 accounted for only 1.2 and 2.5% of the total bacteria in communities A and B, respectively (Table 2). The target populations in each community determined by ISRT were similar to those determined by ISH, although ISH yielded slightly lower estimates (Table 2). Such a difference might result from the less intensive fluorescent signal of ISH relative to ISRT. When the percent target cells determined by ISRT and ISH were com-

TABLE 2. Percentages of target cells in communities A and B and comparison of fluorescence intensities obtained by ISRT and ISH

Community (cells/ml) and probe used to target cells	% Target cells (mean ± SD)			Pixel intensity[b] (mean ± SD)		
	ISRT	ISH	Probe hybrid[a]	ISRT	ISH	Ratio (ISRT/ISH)
A (4.4×10^7)						
IRE-31D	24.6 ± 3.2	20.6 ± 4.7	31.8	478 ± 16	211 ± 12	2.27 ± 0.21
E-37D	1.2 ± 1.1	2.3 ± 1.3		546 ± 9	158 ± 12	3.47 ± 0.21
B (2.3×10^8)						
IRE-31D	31.5 ± 5.2	26.8 ± 2.6	33.5	553 ± 24	230 ± 8	2.40 ± 0.19
E-37D	2.5 ± 1.7	1.8 ± 0.6		466 ± 15	302 ± 14	1.54 ± 0.12

[a] The percentage of IRE-31 within each community was estimated with the IRE-31-specific probe against DNA extracted from community. For details of the method, see reference 11.
[b] The pixel intensity was calculated by dividing the total pixel density value by the pixel number for a positive cell.

pared to percentages calculated from hybridization of the IRE-31-specific probe against extracted community DNA, the values were found to be very similar.

ISRT was also successfully used to detect mRNA of the *todC1* gene of *P. putida* F1. In flask 1, which was exposed to toluene vapor, ISRT with the TOL primer detected 4.2×10^8 cells ml^{-1} which expressed the *todC1* gene, accounting for about 85% of the total community estimated by DAPI counterstaining. Within 48 h, the total bacterial number in flask 1 increased from 6.5×10^5 to 4.9×10^8 cells ml^{-1}. In contrast, no cells expressing the *todC1* gene were detected with ISRT in flask 2, which was not exposed to toluene, and the total bacterial counts increased slightly, from 6.5×10^5 to 3.4×10^6 cells ml^{-1} in 48 h. In this experiment, detection of mRNA of the *todC1* gene was conducted directly against the samples on filters. Cells expressing the *todC1* gene detected by ISRT showed a red fluorescence under the orange-light excitation (Fig. 3A), and the whole community could be seen by DAPI counterstaining (Fig. 3B). When ISH with the CY3-labeled TOL probe was used to detect cells expressing the *todC1* gene, it yielded a very low fluorescent signal which was difficult to distinguish from the background noise (data not shown). The fluorescent signal generated from ISRT was 4.2 times more intensive than that from ISH.

DISCUSSION

Our study demonstrated the first successful use of ISRT for in situ detection of prokaryotic cells on the basis of their specific 16S rRNA or mRNA sequence.

When ISRT was carried out with DIG-dUTP, the detection substrate HNPP-FR reacted with anti-DIG-labeled alkaline phosphatase, yielding a bright and stable HNP-FR red fluorescent signal. This indirect detection method worked well for a bacterial culture mixture; however, ISRT with CY3-dUTP was found to work better for the small bacteria than ISRT with DIG-dUTP. Although indirect detection systems usually provide an increased sensitivity relative to direct detection systems, a potential problem for indirect detection is that it may be difficult for the anti-DIG antibody to penetrate into the small cells; therefore, the HNP-FR fluorescent signal cannot be properly detected. When the toluene-degrading bacterium *P. putida* F1 was introduced into natural seawater, we chose to use ISRT with CY3-dUTP to detect cells which express the *todC1* gene, and we compared the results with those from ISH with the CY3-labeled TOL probe. ISH with the monolabeled probe failed to detect *P. putida* F1 cells expressing the *todC1* gene, while ISRT yielded a detectable signal for the target cells.

ISRT based on 16S rRNA allows us to detect and quantify the two lignin-degrading bacteria *M. hydrolyticus* IRE-31 and *S. stellata* E-37 in the complex communities. We also demonstrated that the positive signal detected by ISRT (Fig. 2A) is stronger than that detected by ISH (Fig. 2D). Superimposition of images acquired in the same microscopic field with two

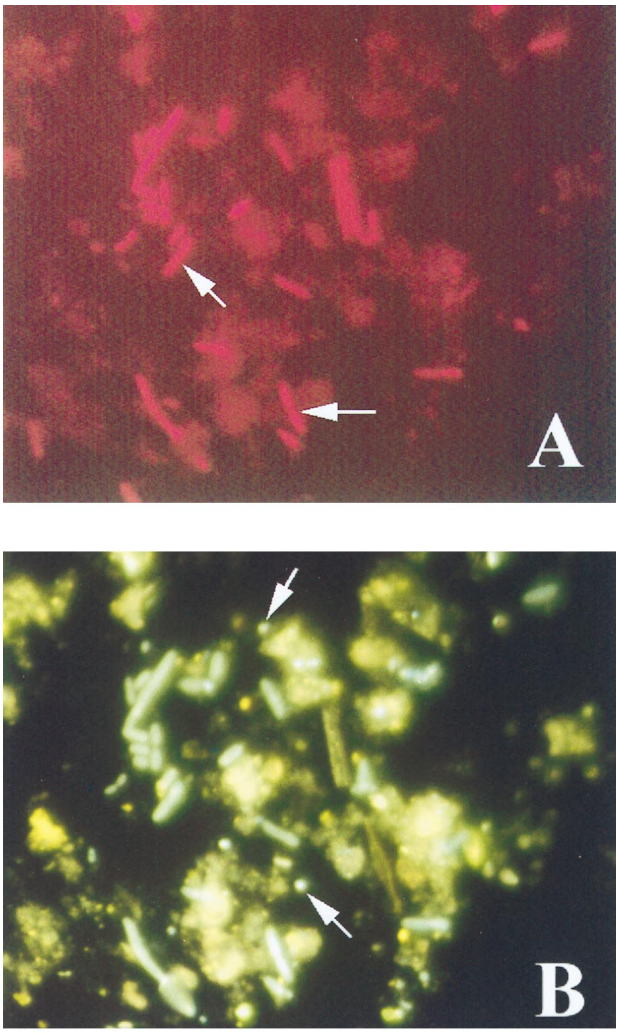

FIG. 3. ISRT detection of mRNA of the *todC1* gene of *P. putida* F1 in seawater with toluene vapor. (A) Cells with red fluorescence are *P. putida* F1 cells which express the *todC1* gene (arrows indicate the positive cells). (B) The whole community viewed by DAPI counterstaining (arrows indicate the negative cells). The two pictures were taken from the same view field but with different excitations.

different light-passing filters (Fig. 2C and F) provides a unique way to visualize both positive cells and other cells in the mixed communities. Results from digital image analysis showed that *M. hydrolyticus* IRE-31 detected by ISRT represented about 24.6 and 31.5% of communities A and B, respectively. These values were comparable to those estimated by ISH (20.6 and 26.8%, respectively) and by probe hybridization against the extracted community DNA (31.8 and 33.5%, respectively) (Table 2). *S. stellata* E-37 cells decreased significantly during the enrichment experiment; less than 3% of the communities was detected by the *S. stellata* E-37-specific probe by ISRT and ISH.

The sensitivity of in situ detection was greatly enhanced by using a cooled CCD camera. The cooled CCD camera was capable of acquiring low fluorescent signals from target cells, which were almost not visible to human eyes. The percentage of target cells detected by ISRT and ISH in each community was quantified by image analysis. In the lignin enrichment communities, we noticed that certain bacterial cells yield very weak HNP-FR signals, and it was difficult to visually decide whether they should be identified as positive cells or not. Using digital image analysis avoided possible bias due to subjective evaluations of the operator and improved the detection sensitivity. In addition, digital image analysis allows us to compare the fluorescence intensities of targeted cells obtained by the different approaches. The pixel intensities of target cells in the communities detected by ISRT with probes for rRNA averaged 2.4 times greater than those from ISH. Theoretically, ISRT with multiple uptake of DIG-dUTP should yield an even stronger signal relative to that with ISH. The reverse transcription inside bacterial cells may have much less efficiency than that with extracted RNA. It is also possible that the conditions for ISRT need further optimization. However, sometimes it is difficult to judge whether the maximum performance for enzymatically driven methods has been achieved.

The advantage of ISRT over ISH became more apparent when both protocols were used to detect cells with less-abundant RNA targets. Transcripts (mRNA) of a specific gene inside a bacterial cell are usually much less abundant than rRNA. Detection of gene expression by ISH requires synthesis of a long probe (e.g., 200 bases) prepared by using an in vitro transcription system (16, 22). ISH with long multilabeled probes (31) has been demonstrated to increase signal intensity relative to ISH with monolabeled probe. However, when many probes need to be applied, ISH with long probes can be very time-consuming. ISRT takes advantage of multiple label incorporation into multiple copies of RNA molecules inside bacterial cells. Detection of mRNA inside bacterial cells should facilitate in situ studies of genetic activity associated with a specific functional gene.

Earlier studies describing prokaryotic in situ PCR protocols have demonstrated that both RPE and RT-PCR can be used to detect the expression of a functional gene inside intact bacterial cells (21). These techniques employ either arithmetic (RPE) or exponential (RT-PCR) amplification of gene targets; about 30 thermal cycles, which normally range between 50 and 94°C, are used. The involvement of thermal cycles increases the technical complexity, and when carrying out procedures on a microscope glass slide, certain sample assembly kits or special PCR equipment is needed. Moreover, the multiple washing steps used with PCR may cause loss of cells from the slide and hence decrease the sample recovery efficiency. The ISRT protocol greatly simplifies the procedures that in situ RT-PCR requires. Similar to the ISH technique, the sample smear immersed in the reverse transcription solution (20 μl) can be incubated uncovered in a humid chamber at the required temperature. Therefore, it allows samples on filters to be detected directly and can be applied to bacteria in natural environments. Moreover, ISRT conducted on filters may provide additional information on the physical association between bacteria and other living and nonliving components of natural systems.

Recently, we have tested ISRT with a eubacterial probe (927R) against 16S rRNA sequences of natural bacterial communities collected from Georgia coastal waters. Preliminary results showed that a variable percentage (30 to 85%) of bacterial cells was detected by this method (data not shown). It is very likely that some cells in natural communities may not be able to be detected with this protocol due to their poor permeability, their low rRNA content, or nonoptimal hybridization conditions. On the other hand, the total bacterial number is usually determined by the DAPI staining method. In many cases, a certain amount of small nonbacterial particles in natural seawater samples might be stained and counted as bacterial cells. Karner and Fuhrman (23) found that the bacterial number detected by ISH with a 16S rRNA universal probe accounts for about 50% of the corresponding DAPI count. They also found that universal 16S rRNA counts correlated well with autoradiography results, indicating that the portion of bacterial cells detected by ISH probably represents a majority of viable bacterial cells in natural seawater.

In this study, we demonstrated that the ISRT protocol is sensitive enough for detection of both 16S rRNA and mRNA in complex microbial communities. Application of this approach for in situ analysis of phylogenetic diversity and genetic activities of natural microbial communities is currently under way.

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