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# In Situ PCR for Visualization of Microscale Distribution of Specific Genes and Gene Products in Prokaryotic Communities

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**Obtaining information on the genetic capabilities and phylogenetic affinities of individual prokaryotic cells within natural communities is a high priority in the fields of microbial ecology, microbial biogeochemistry, and applied microbiology, among others. A method for prokaryotic in situ PCR (PI-PCR), a technique which will allow single cells within complex mixtures to be identified and characterized genetically, is presented here. The method involves amplification of specific nucleic acid sequences inside intact prokaryotic cells followed by color or fluorescence detection of the localized PCR product via bright-field or epifluorescence microscopy. Prokaryotic DNA and mRNA were both used successfully as targets for PI-PCR. We demonstrate the use of PI-PCR to identify *nahA*-positive cells in mixtures of bacterial isolates and in model marine bacterial communities.**

Techniques currently exist to measure the distribution of various microbial processes at macroscale levels in natural systems. For example, methods for characterizing the spatial variation in such parameters as rates of degradation of individual compounds (3, 31), cycling rates of inorganic nutrients (7), and average rates of bacterial growth and respiration (8, 14) have been available for a number of years. However, at the microscale, the genetic composition of bacterial communities is virtually unknown. With few exceptions, it has been impossible to determine which individual cells in a natural bacterial assemblage possess the genetic capability to carry out a specific process or how the organisms which mediate a process are spatially associated with each other, with other organisms, or with nonliving particles in water, sediments, or soils.

Even with microscale sampling, techniques based on viable-cell counts offer little promise for providing insight into bacterial community structure, since the cultivation efficiency of bacteria from natural communities is ordinarily very low. Likewise, light and electron microscopic techniques generally identify only those cells possessing unusual morphologies, although microscopy often reveals the extraordinary complexity of the physical structure and heterogeneity of microbial communities (27). The presence of specific genes in extracts of bulk DNA from natural bacterial communities can be determined semiquantitatively by dot blot hybridization (24), but as with older techniques, such methods fail to provide information at the microscale or individual cell level.

Recently, the development of in situ (whole cell) hybridization methods for rRNA-based oligonucleotide probes has made taxonomic identification of single cells within natural bacterial communities possible for the first time (1, 2, 4, 12, 16, 30, 32). However, these methods rely on the presence of naturally occurring multiple targets within the bacterial cell to provide a detectable signal and thus are limited in utility by the small number of rRNA molecules in many slow-growing or dormant bacteria from environmental samples (11, 12). Detection of individual genes present in single copy or low copy numbers in intact bacterial cells by in situ hybridization methods is not possible.

One potential approach to characterizing the microscale genetic and taxonomic properties of natural bacterial communities would be via in situ PCR, a unique modification of PCR in which amplification and detection of specific target nucleic acid sequences are carried out inside individual cells rather than on bulk extracted nucleic acid (19). Individual genes, mRNA, and rRNA are all candidate targets for in situ PCR, so that genetic capabilities, expression of those capabilities, and taxonomic information are all potentially accessible on the individual cell level. This approach has been used successfully only in eukaryotic cells, primarily in biomedical applications (10, 15, 18, 20). Heretofore, no complementary approaches have been developed either for prokaryotic cells or for environmental or clinical samples containing prokaryotes. We report here the development of a method for prokaryotic in situ PCR (PI-PCR) and provide examples of its use in single-cell detection of a specific gene and specific gene transcripts in mixtures of bacterial isolates and in simple model communities of marine bacteria.

## MATERIALS AND METHODS

**Bacterial samples and cell fixation.** The strains of *Pseudomonas aeruginosa* and *Pseudomonas putida* used in this study are listed in Table 1. The NAH7 plasmid was mobilized to *P. aeruginosa* ATCC 19712 by triparental conjugation with pRK2013 as the helper plasmid (4). All strains used in PI-PCR were grown in Luria-Bertani (LB) broth, which contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl (17). Model marine bacterial communities were assembled from eight strains of bacteria. Five of the strains were random isolates from coastal Georgia sediments which had been cultured on half-strength YTSS agar (4 g of yeast extract, 2.5 g of tryptone, 200 ml of 2.5× sea salts solution per liter) at room temperature. The other three strains were *P. putida* AC10R-7, *P. aeruginosa* 19712, and *Escherichia coli* HB101. For development of RNA-based PI-PCR methods, a basal salt medium (BSM) as described by Schell (25) and amended with 0.5% salicylate was used for growth of strain AC10R-7 to induce the production of mRNA transcripts from the *nahA* gene.

Single colonies of each strain were taken from fresh plates, inoculated into 25 ml of broth, and grown at 30°C and 250 rpm for 6 to 12 h or until the cultures reached an optical density of 1.0. Cells were harvested by centrifugation at 2,500 × *g* for 10 min and washed twice with phosphate-buffered saline (PBS; 120 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer [pH 7.6]). Cells were resuspended in 4% fresh paraformaldehyde in PBS for 4 to 16 h, after which the cells

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TABLE 1. Bacterial strains used in PI-PCR procedures

| Strain | Species (plasmid) | Reference or source[a] |
|---|---|---|
| AC10R | *P. putida* | 28 |
| AC10R-7 | *P. putida*(NAH7) | 28 |
| 19712 | *P. aeruginosa* | ATCC |
| 19712-7 | *P. aeruginosa*(NAH7) | This work |
| HB101 | *E. coli* | ATCC |

[a] ATCC, American Type Culture Collection.

were washed twice with PBS and then resuspended in 5 ml of 50% ethanol in PBS. Fixed cells were aliquoted into 100-μl portions and stored at −20°C.

**Oligonucleotides.** The 20-mer oligonucleotide primers which were used for the amplification of the *nahA* gene on plasmid NAH7 (28) were as follows: NAH A (positions 653 to 674), TAC AAG CAT CAA GTT GAG CG; and NAH B (positions 1758 to 1779), GGA ATC AGG CTG TCA TGA GT. A third 19-mer oligonucleotide primer, NAH I, which was used for primer extension, was complementary to a region internal to the first two primers (positions 825 to 843) and had the sequence CAG TCA GCA AGA CCT CTA C. All primers were synthesized on an Oligo 1000 DNA Synthesizer (Beckman Instruments Inc., Fullerton, Calif.). The primers were cleaved and deprotected with an Ultrafast Cleavage and Deprotection Kit (Beckman Instruments Inc.), vacuum-dried in a Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, N.Y.), and resuspended in 500 μl of ultrapure water. Oligonucleotide concentrations were determined spectrophotometrically and diluted to 100 pmol $\mu l^{-1}$ before storage at −20°C.

**Cell wall permeabilization.** Fixed cells were washed twice in PBS buffer and resuspended in 200 μl of lysozyme buffer (100 mM Tris, 50 mM EDTA [pH 8.0]). Cell wall permeabilization was achieved by treatment with lysozyme at a final concentration of 0.5 mg $ml^{-1}$ for 30 min at room temperature. Lysozyme was removed from the cells by three consecutive washes with PBS. Permeabilization was furthered by treatment with either proteinase K at a final concentration of 0.10 μg $ml^{-1}$ or trypsinogen at a final concentration of 0.50 μg $ml^{-1}$ for 10 min at room temperature. Protease was removed by four consecutive washes with PBS, and cells were resuspended in 200 μl of PBS. If the amplification target was DNA, RNA was removed from the cells during the cell permeabilization steps by adding DNase-free RNase at a final concentration of 0.5 mg $ml^{-1}$ along with the lysozyme. However, if the amplification target was RNA, permeabilized cells were treated with RNase-free DNase (10 U $\mu l^{-1}$, final concentration) at room temperature for 2 to 3 h to degrade DNA.

**One-stage PI-PCR.** A two-primer PCR protocol was developed for in situ amplification inside prokaryotic cells (Fig. 1). Amplification of the *nahA* gene inside permeabilized cells was done with Thermalase Tbr (AMRESCO Inc., Solon, Ohio) and the NAH A and NAH B oligonucleotides as primers. The reaction mixture was prepared according to the manufacturer's instructions except that the $MgCl_2$ concentration was raised to 2 mM and digoxigenin (DIG)- or fluorescein (FLOUS)-labeled nucleotides were included in the nucleotide mix. For the DIG-dUTP mix, the nucleotide concentrations were 1 mM each dATP, dCTP, and dGTP; 0.65 mM dTTP; and 0.35 mM DIG-11-dUTP (DIG-DNA labeling mixture; Boehringer Mannheim, Indianapolis, Ind.). For the FLOUS-dUTP mix, the nucleotide concentrations were 1 mM each dATP, dCTP, and dGTP; 0.65 mM dTTP; and 0.35 mM fluorescein-12-dUTP. Typically, 10 μl of permeabilized cell suspension (approximately $10^6$ to $10^7$ cells) was used per 50-μl reaction mixture in Oil-Free Tubes (Barnstead Thermolyne Corp., Dubuque, Iowa). With a DNA Thermal Cycler 480 (Perkin-Elmer Corp., Norwalk, Conn.), a hot-start technique (10 min at 82°C) was used prior to the addition of the polymerase and was followed by initial denaturation at 94°C for 3 min. Amplification of the target DNA occurred during 45 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 1 min. Cells were harvested, washed with PBS, and resuspended in 10 μl of PBS. The resuspended cells were spotted onto wells of printed microscope slides (Cel-Line Associates, Inc., Newfield, N.J.) in 2- to 5-μl aliquots and allowed to air dry.

**Two-stage PI-PCR.** A two-stage PI-PCR protocol was developed based on the primed in situ DNA amplification method for eukaryotic cells described by Moss and Kaliner (18). In stage 1 (amplification stage), cell preparation and conditions for PCR amplification were the same as described above except that the nucleotides (10 mM final concentration for each) were unlabeled. Following target sequence amplification, cells were harvested, washed twice with 0.5× SSC (750 mM NaCl, 75 mM trisodium citrate [pH 7.0]), and either resuspended in 10 μl of PBS (for immediate use) or stored at 4°C in 4% paraformaldehyde overnight. In stage 2 (primer extension stage), the reaction mixture was the same as for amplification except that a labeled nucleotide mix (either DIG-dUTP mix or FLOUS-dUTP mix) was used. Forty microliters of reaction mixture was added directly to the 10 μl of resuspended cells from stage 1. NAH I at 0.2 μM was

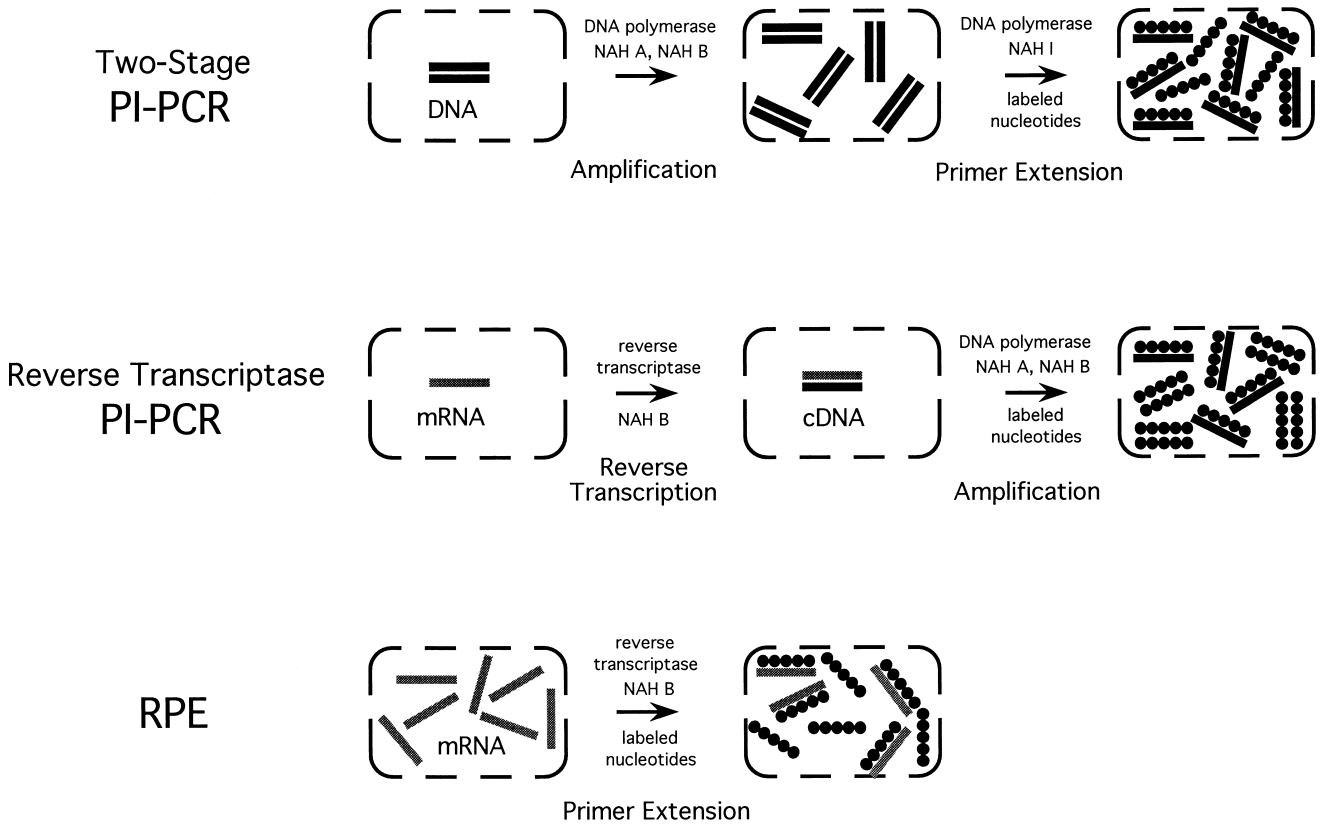


FIG. 1. Schematic of three methods for in situ detection of specific nucleic acid sequences in prokaryotic cells.

added as the primer for an internal region of the sequence amplified in stage 1. The purpose of stage 2 was to increase the specificity of detection of the amplified target sequence; amplified products of mispriming during stage 1 will not bind the NAH I primer in stage 2. The hot-start technique was again used to denature DNA duplexes. Primer extension was carried out for five cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 1 min. Cells were harvested, washed with PBS, and resuspended in 10 μl of PBS. Cells were spotted onto wells of printed glass slides in 2- to 5-μl aliquots and allowed to air dry.

**PI-PCR on microscope slides.** PI-PCR was also carried out on microscope slides placed directly on top of the block of the thermal cycler. Permeabilized cell suspensions were spotted into a 10-mm-diameter well of a printed glass slide (Cel-Line Associates, Inc.) and allowed to dry. Slides were passed through an ethanol dehydration series (50, 80, and 98%; 3 min each) to fix the cells to the slide surface. A plastic Gene Cone chamber (15-mm diameter; Gene Tech, Durham, N.C.) was mounted around the periphery of the well to serve as a reservoir for the PCR mixture, and two-stage PI-PCR was carried out as described above with the following exceptions: the concentration of NAH I was increased to 2 μM, the number of primer extension cycles was raised to 10, and a step for degradation of residual primers was added following stage 1. Primer degradation was performed by adding 50 μl of a nuclease S1 solution at a final concentration of 100 U $ml^{-1}$ in nuclease S1 buffer (200 mM NaCl, 50 μM sodium acetate [pH 4.5], 1 mM $ZnSO_4$, 0.5% glycerol) for 30 min at 37°C; nuclease S1 was inactivated by chelation in nuclease stop buffer (1 mM EDTA [pH 8.0], 10 mM sodium phosphate) followed by three consecutive washes with PBS for 10 min each at room temperature. After stage 1 amplification and before the washing and primer degradation steps, the Gene Cone ring was removed to facilitate effective treatment of the cells. A second ring was then mounted in the same place on the slide, the cover was put in place, and the primer extension step (stage 2) with labeled nucleotide mix was carried out as described above.

Another variation of in situ amplification and detection was carried out with cells immobilized on glass slides immersed in reaction mixture inside PCR tubes. Permeabilized cells were spotted onto the lower third of thin glass slides (approximately 2 by 20 mm) which had been cut from standard glass coverslips (29). The slides were inserted into standard PCR tubes so that the reaction mixture covered the cell spot. One-stage or two-stage PI-PCR was then carried out as described above, with an oil overlay used to prevent evaporation during thermal cycling. Following PCR, the slides were briefly washed with 98% ethanol to remove the oil and placed on top of standard microscope slides for detection procedures.

**Detection of amplified genes.** Following completion of the PCR procedures and spotting of cells onto printed slides, cells were dehydrated in ethanol (50, 80, and 98%; each for 3 min), dried at 50°C, and washed twice in 0.5× SSC at 45°C for 15 min to remove excess primer and unincorporated nucleotides. For PI-PCR on microscope slides, a second nuclease S1 digestion step as described above was included in addition to the 0.5× SSC washes.

For PCR procedures with the FLOUS-dUTP nucleotide mix, dehydrated and washed cell spots were viewed directly via epifluorescence microscopy. For PCR procedures with the DIG-dUTP nucleotide mix, slides were placed in blocking solution (50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 3% Radfree blocking powder [Schleicher & Schuell, Keene, N.H.] [pH 7.5]) for 1 h at 50°C and then transferred to Genius Buffer 1 (Boehringer Mannheim) containing anti-DIG Fab fragments conjugated to alkaline phosphatase (for color detection) or to fluorescein or rhodamine (for fluorescence detection) (Boehringer Mannheim) at a ratio of 1:100 for 1 h at room temperature. Nonspecifically bound antibody was removed by rinsing twice in wash solution (5 mM Tris, 15 mM NaCl, 0.1% SDS [pH 7.5]) for 15 min at room temperature followed by washing twice with Genius Buffer 1 for 15 min each at room temperature. Cells detected with alkaline phosphatase conjugate were briefly washed in Genius Buffer 3 (Boehringer Mannheim) prior to color development by the alkaline phosphatase reaction in color developing solution (10 ml of Genius Buffer 3 plus 35 μl of BCIP [5-bromo-4-chloro-3-indolylphosphate toluidinium] and 45 μl of NBT [Nitro Blue Tetrazolium] substrates [Boehringer Mannheim]). This reaction was allowed to proceed for 5 min to 1 h at 37°C before a rinse with distilled water stopped the reaction. After air drying, the slides were observed by bright-field microscopy for the presence of a blue precipitate and by phase-contrast microscopy for the presence of phase-bright cells. Photographs were taken with an Olympus OM-2 camera (Olympus America Inc., Lake Success, N.Y.) with automatic exposure settings and Kodak Ektachrome Elite 400 slide film or Kodak Enhanced Multi-Purpose 400 speed print film. Cells detected with fluorescein- or rhodamine-labeled antibody were dried for immediate examination via epifluorescence microscopy with an Olympus NIB filter cube. Photography was carried out as described above.

Dot blot hybridizations were carried out to confirm the presence of the correct PCR product by immobilizing the target (PCR mixture or intact cells) to Nytran nitrocellulose membranes (Schleicher & Schuell) with a dot blot apparatus (Schleicher & Schuell) and UV cross-linking (Fisher Scientific, Pittsburgh, Pa.). DNA was denatured with NaOH after lysis with 10% SDS (23). The oligonucleotide probe (NAH I) was labeled with [$\alpha$-$^{32}$P]dCTP by using terminal transferase (Boehringer Mannheim) as per the manufacturer's recommendations. Hybridization was carried out in 50% formamide at 39°C for 16 h, after which membranes were washed under stringent conditions (23).

**Reverse transcriptase PI-PCR.** A reverse transcriptase in situ PCR protocol based on that of Seidman et al. (26) was developed for production, amplification, and detection of cDNA copies of mRNA in prokaryotic cells. Cells with increased transcription of the *nahA* gene induced by growth on salicylate (see above) were used. cDNA was synthesized with the downstream primer (NAH B) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) or *r*Tth DNA polymerase (Perkin Elmer) as per the manufacturers' recommendations. The cells were washed once with PBS, and 2 to 5 μl of these cells was used for direct in situ PCR as described above. Amplification was carried out for 45 cycles with $MgCl_2$ at 1 mM (final concentration) and either the DIG-dUTP or FLOUS-dUTP nucleotide mix. Cells were harvested, washed with PBS, resuspended in 10 μl of PBS, and spotted onto the wells of printed glass slides. Detection was carried out as described above. Alternatively, amplification was carried out in cells spotted onto microscope slides following the reverse transcriptase protocol described above.

**RPE.** For RNA probe extension (RPE), a protocol was developed for in situ linear (nonexponential) amplification of RNA sequences by extension of a single primer with labeled nucleotides. Reactions were carried out with 5 μl of permeabilized and DNase-treated cells (induced with salicylate) in 50 μl of PCR mixture. A thermal stable reverse transcriptase (*r*Tth DNA polymerase; Perkin-Elmer) was added along with 3 mM $MgCl_2$, DIG-dUTP, and the NAH B primer to target mRNA transcribed from the *nahA* gene. Primer extension was carried out for 45 cycles, with denaturing at 94°C, annealing at 42°C, and extension at 72°C. Following primer extension, cells were harvested, washed, resuspended in 10 μl of PBS, spotted onto the wells of printed glass slides, and detected as described above. Alternatively, primer extension was carried out in cells spotted on microscope slides according to the protocols described previously.

## RESULTS

**Cell wall permeabilization.** Successful PI-PCR depends on a method which will permeabilize the bacterial cell membranes to allow entry of reagents for amplification and detection yet retard the diffusion of PCR product away from the cells while not destroying the morphology of the cells or the microscale structure of the microbial community. To optimize permeabilization procedures for *P. putida* and *P. aeruginosa* prior to in situ PCR, final lysozyme concentrations were varied between 0.03 and 0.5 mg $ml^{-1}$ and time of exposure of the cells to lysozyme was varied between 0.5 and 3 h. The effectiveness of cell permeabilization was evaluated by exposing treated cells to RNase and DNase and then staining cells with acridine orange (13) or DAPI (4′,6-diamidino-2-phenylindole) (21) to determine whether nucleic acid had been effectively degraded, indicating optimal entry of enzymes into the cell. The lysozyme concentration that permitted free entry of enzymes into the cells of the two *Pseudomonas* strains while maintaining the morphological integrity of the cell was 0.5 mg $ml^{-1}$ (final concentration) at room temperature for 30 min.

Following optimization of lysozyme treatment, protease concentrations were likewise varied to achieve optimum entry of in situ amplification reagents. Trypsinogen was used initially, but we ultimately switched to proteinase K because it had greater stability to freezing and thawing as well as a broader range of action. Cell lysis occurred at high proteinase K concentrations, but we found that cells retained integrity when treated with a concentration of 0.10 μg $ml^{-1}$ for 10 min at room temperature. The action of the protease in effecting in situ PCR may be that of either freeing nucleic acids from cross-linkages with proteins established during paraformaldehyde fixation or further degrading the cell membrane to facilitate entry of enzymes and reagents (19), or both.

**One-stage PI-PCR.** The one-stage PI-PCR approach was successful in amplifying target genes inside intact cells, as determined by the presence of strong color or fluorescence signals, although interfering levels of labeled nonspecific PCR products were produced simultaneously when this protocol was used. Amplification of specific target DNA inside *Pseudomonas* cells was confirmed by dot blot hybridizations of cell pellets (recovered from PCR mixtures) with the NAH I probe (Fig. 2). Amplification of target sequences also occurred outside the

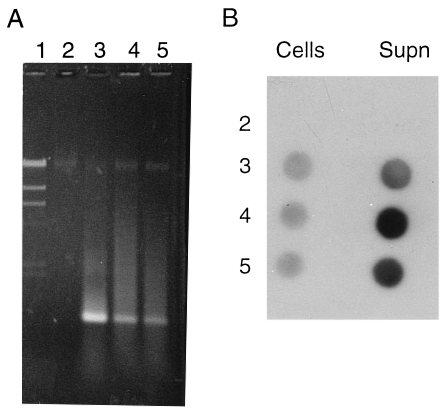


FIG. 2. Two-stage PI-PCR amplification of the *nahA* sequence in *P. putida* (NAH7) AC10R-7 carried out in solution. (A) Agarose gel electrophoresis of supernatants and (B) dot blot hybridizations of cell pellets and supernatants. Lanes: 1, ladder (0.12- to 23.1-kbp λ DNA cleaved with *Hin*dIII); 2, negative control (omission of both NAH A and NAH B primers); 3, 1.1-kb *nahA* fragment amplified in the presence of 1 mM $MgCl_2$; 4, 1.1-kb *nahA* fragment amplified in the presence of 4 mM $MgCl_2$; 5, 1.1-kb *nahA* fragment amplified in the presence of 5 mM $MgCl_2$. Dot blot numbers in panel B correspond to lane numbers.

cells in the reaction mixture, presumably with DNA released from lysed cells serving as the template. The presence of specific products outside the cells was confirmed by analysis of the cell-free reaction mixture in agarose gels and by dot blot hybridization of this mixture with the NAH I probe (Fig. 2). $MgCl_2$ concentrations in the range of 1 to 5 mM were required for successful amplification inside cells, and no signal were detected either inside or outside cells when $MgCl_2$ was omitted from the reaction mixture. The optimum concentration of $MgCl_2$ was found to be affected by a number of variables, including the density of cells in the reaction mixture as well as the batch of polymerase. For most one-stage PI-PCR procedures, we found that 2 mM $MgCl_2$ and $10^6$ to $10^7$ cells were a workable combination.

Nonspecific products of PI-PCR were recognized by two methods. First, reaction mixtures from in situ PCR amplifications analyzed on agarose gels were found to contain a range of products of various sizes along with the desired 1.1-kb product of specific amplification. Second, amplification products were detected inside cells in the absence of added primers (although never in the absence of DNA polymerase), suggesting that small pieces of DNA native to the cell were acting as PCR primers (23) or that existing replication forks were being extended. Attempts to optimize the reaction conditions for the single-stage variation of PI-PCR with direct incorporation of labeled nucleotides failed to achieve specific labeling of the target in PI-PCR.

**Two-stage PI-PCR.** To increase the specificity of PI-PCR, three approaches were used: (i) increasing the stringency of amplification conditions so that only the target DNA was amplified, (ii) increasing the specificity of detection after amplification so that only the correct PCR product was detected, and (iii) using cDNA copies of RNA as the amplification target (Fig. 1). Using the first option, we varied a number of amplification parameters, including the $MgCl_2$ concentration, number of PCR cycles, primer concentration, and annealing temperature. However, when conditions were sufficient for product detection inside prokaryotic cells, nonspecific amplification was always found to occur simultaneously. Modifying the other amplification parameters likewise did not sufficiently increase the specificity of labeled product formation inside the cells to allow us to visually distinguish between NAH7-containing cells and NAH7-free cells (although target amplification was achieved).

In the second approach, we added a second stage to the in situ PCR protocol, so that amplification and detection (by incorporation of labeled nucleotides) were temporally separated (Fig. 1). With two-stage PI-PCR, the nonspecific products of the first amplification remain undetected because they are not complementary to the internal primer used in the second stage (and thus do not get labeled). Two-stage PI-PCR was successful in differentiating between cells with and without the target gene. With this approach, *P. putida* cells containing the NAH7 plasmid gave strong signals with either color or fluorescence detection (Fig. 3A and B), while *P. aeruginosa* cells without NAH7 were only weakly visible (Fig. 3C and D). Cells of the two types could be readily distinguished in mixtures (Fig. 3E and F). To demonstrate that possible differences between the two *Pseudomonas* species (in cell wall structure, for example, or other phenotypic characteristics) were not responsible for the variations in signal intensity observed, two-stage PI-PCR was also carried out with *P. aeruginosa* 19712-7 (derived by inserting the NAH7 plasmid into *P. aeruginosa* 19712). These cells also gave a strong positive signal (Fig. 3G).

To optimize conditions for the second stage of two-stage PI-PCR, the $MgCl_2$ concentrations were varied from 1 to 5 mM, the primer concentrations were varied from 1.0 to 2.0 μM, and the DNA polymerase concentrations were varied from 0.1 to 1.0 μl per 100 μl of reaction mixture. Optimum concentrations for stringent extension of the NAH I internal primer in suspended cells were found to be 2 mM $MgCl_2$, 0.2 μM primer, and 0.2 to 0.5 μl of DNA polymerase. Optimum reagent concentrations for stringent extension of the NAH I internal primer for cells fixed to slide surfaces were found to be 2 mM $MgCl_2$, 2.0 μM primer, and 1.0 μl of DNA polymerase.

**Reverse transcriptase PI-PCR.** The third approach used to increase the specificity of PI-PCR was to first degrade cellular DNA and then make specific cDNAs from RNA via reverse transcriptase. The cDNAs then served as the target for amplification. The advantage of this technique is that the complexity of nucleic acid sequences inside the cell is greatly reduced by DNase, minimizing the possibility of mispriming and subsequent nonspecific amplification. In one variation of this approach (reverse transcriptase PI-PCR), cDNA copies of *nahA* transcripts were amplified by one-stage PI-PCR with the NAH A and NAH B primers (Fig. 1 and 4). *P. putida* cells containing the NAH7 plasmid and induced on salicylate developed a strong color signal (Fig. 4A), while *P. putida* cells containing the NAH7 plasmid and grown without inducer were only weakly visible (Fig. 4C). Cells of the two types could be readily distinguished in mixtures (Fig. 4E).

**RPE variation.** In a second variation of RNA-based PI-PCR, RPE, transcripts of the *nahA* gene were detected by repeated cycles of extension from a single primer inside intact *P. putida* cells (Fig. 1 and 4). *P. putida* cells containing the NAH7 plasmid developed a strong color signal (Fig. 4B), while *P. aeruginosa* cells without the plasmid showed little or no color (Fig. 4D). Cells of the two types could be readily distinguished in mixtures (Fig. 4F). With 45 cycles of amplification, RPE theoretically created 45 multiply labeled complementary copies from each mRNA transcript of the *nahA* gene, assuming 100% efficiency of priming.

**Model marine community.** The model marine bacterial community was treated according to the procedures optimized for permeabilization and amplification of *nahA* genes or transcripts inside *Pseudomonas* strains. We used this model community for RPE (Fig. 5) and two-stage PI-PCR. In both cases,

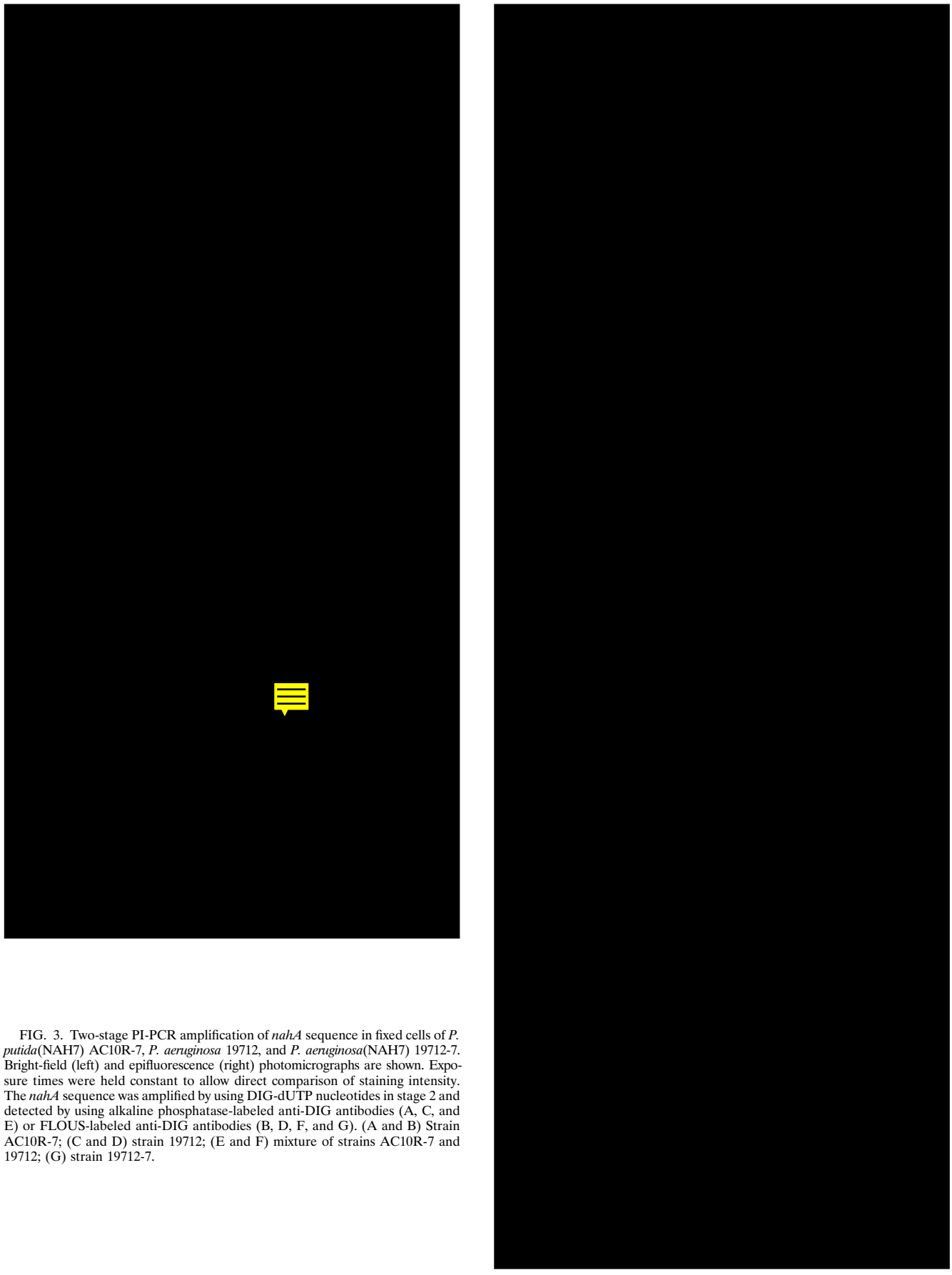

FIG. 3. Two-stage PI-PCR amplification of *nahA* sequence in fixed cells of *P. putida*(NAH7) AC10R-7, *P. aeruginosa* 19712, and *P. aeruginosa*(NAH7) 19712-7. Bright-field (left) and epifluorescence (right) photomicrographs are shown. Exposure times were held constant to allow direct comparison of staining intensity. The *nahA* sequence was amplified by using DIG-dUTP nucleotides in stage 2 and detected by using alkaline phosphatase-labeled anti-DIG antibodies (A, C, and E) or FLOUS-labeled anti-DIG antibodies (B, D, F, and G). (A and B) Strain AC10R-7; (C and D) strain 19712; (E and F) mixture of strains AC10R-7 and 19712; (G) strain 19712-7.

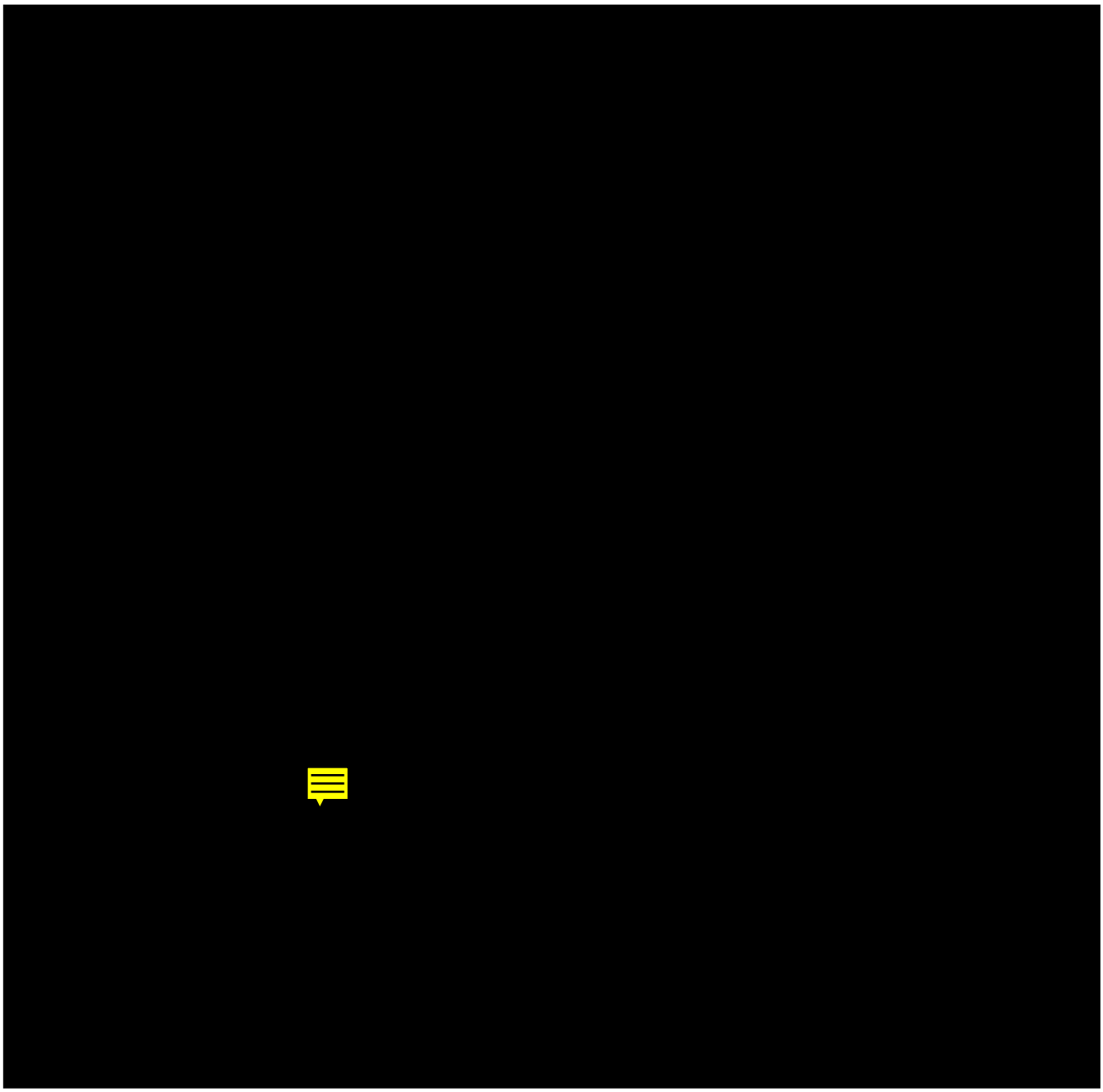

FIG. 4. Reverse transcriptase PI-PCR (left) and RPE (right) of fixed cells of *P. putida*(NAH7) AC10R-7 and *P. aeruginosa* 19712. The *nahA* sequence was amplified by using DIG-dUTP nucleotides and detected by alkaline phosphatase-labeled anti-DIG antibodies. (A and B) Strain AC10R-7 grown in the presence of an *nahA* inducer (salicylate). (C) Strain AC10R-7 grown in the absence of inducer. (D) Strain 19712 grown in the absence of inducer. (E) Mixture of cells shown in panels A and C. (F) Mixture of cells shown in panels B and D.

the results were similar. The *P. putida*(NAH7) cells were positive (darkly colored or highly fluorescent), while *P. aeruginosa* and *E. coli* cells were negative (lightly colored or minimally fluorescent), as expected. Among the five unidentified marine isolates, three appeared to be positive and two appeared to be negative. Dot blot hybridizations with $^{32}$P-labeled NAH I probe confirmed that two of the three positive marine isolates contained an *nahA*-like gene. The third strain (the large rod giving a positive signal in Fig. 5), however, did not hybridize with the NAH I probe. Neither of the visually negative strains revealed an *nahA*-like gene by dot blot hybridization.

## DISCUSSION

To date, it has not been possible to determine with any reliability which individual cells within a complex natural bacterial community possess a specific genetic trait and whether or not that trait is being expressed. Unless information on the actual numbers and the microscale patchiness and physical associations (e.g., free-living cells versus cells attached to particulate matter or other organisms) of bacterial cells is available, their functional niche cannot be described. The development of an in situ PCR protocol for prokaryotic cells makes

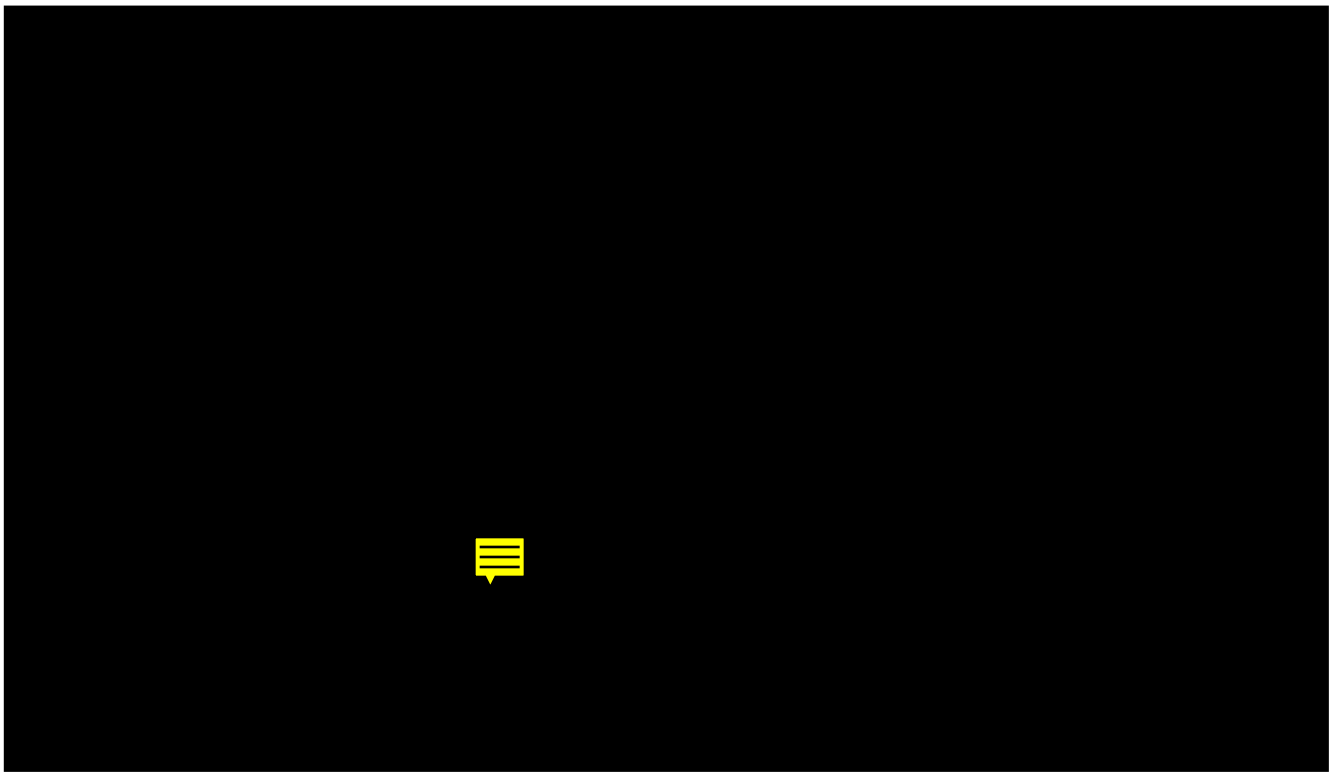

FIG. 5. Model marine bacterial community consisting of *P. putida*(NAH7) AC10R-7, *P. aeruginosa* 19712, *E. coli* HB101, and five marine sediment isolates. The *nahA* sequence was amplified by using DIG-dUTP nucleotides and detected by using alkaline phosphatase-labeled anti-DIG antibodies. Arrow 1, strain AC10R-7; arrow 2, strain 19712; arrow 3, large rod giving a possibly false-positive signal (see text).

such information available to the researcher and holds significant promise for addressing many of the deficiencies currently limiting our understanding of natural bacterial community structure and function. This approach has the potential to provide information on specific genes and gene products at the scale of the individual cell.

Permeabilization of the cell envelope was determined to be a critical step in in situ PCR, since it is necessary to modify the membrane so that enzymes and reagents freely pass into and out of the cell while the amplified PCR products remain inside and the cell retains its integrity for microscopy-based detection. We used a combination treatment with lysozyme and a mild protease to accomplish the cell permeabilization, optimizing conditions to balance product labeling and retention in positive cells with effective washing of nonincorporated labeled nucleotides from negative cells. The amplification and detection reagents used in the in situ PCR procedures included several large molecules, such as DNA polymerase (95,000 Da) and anti-DIG Fab fragments conjugated to alkaline phosphatase (>244,000 Da). Thus, even relatively large molecules were able to freely pass through the bacterial cell membrane following permeabilization. Similarly, Zarda et al. (32) reported the penetration of alkaline phosphatase-labeled anti-DIG Fab fragments into lysozyme-treated gram-negative cells.

Amplification of nontarget DNA was the most significant problem encountered during the development of the in situ PCR protocol. We found that a two-stage in situ PCR procedure involving two sequential rounds of thermal cycling greatly reduces the problem of formation of labeled nonspecific products. The first phase of two-stage PI-PCR is a conventional exponential amplification with two primers and nonlabeled nucleotides; the second phase is an arithmetic amplification of only the correct PCR products by extension (with labeled nucleotides) of a third primer complementary to a region of the product of stage 1. Similarly, a reverse transcription-PCR approach also greatly decreases the problem of false-positive signals associated with nonspecific binding of the PCR primers. The reverse transcription-PI-PCR method described herein uses pretreatment with DNase to eliminate genomic DNA to which primers might bind nonspecifically; amplification of the target sequence from the limited suite of cDNAs produced is then carried out with labeled nucleotides. An alternative method for increasing the specificity of in situ detection of target DNA, suggested by Nuovo (19) but not used in the present study, involves hybridization of a labeled oligonucleotide probe to products formed in a standard one-stage PCR.

Each of the detection methods used for PI-PCR was found to have advantages and disadvantages. Direct incorporation of fluorescent nucleotides into the PCR product was the simplest approach, and it required penetration of the smallest reagents into the cells. However, with this approach, we sometimes observed higher levels of nonspecific staining. Likewise, incorporation of DIG-labeled nucleotides followed by detection with fluorescently labeled Fab fragments sometimes yielded less distinct differences between positive and negative cells. With both fluorescence detection approaches, fluorescence from the labeled products tended to fade over time with light excitation. Ultimately, differences in fluorescence signal strength between positive and negative cells may be more easily detected by replacing direct microscopic examination with an image analysis approach (30) or flow cytometric procedure (1).

In most cases, detection with alkaline phosphatase-labeled Fab fragments (the largest of the detection reagents) gave

good subjective visual differences between positive and negative *Pseudomonas* cells, and the color precipitate was permanent. Color-based detection, however, may be less well suited for adapting PI-PCR for use with natural bacterial communities. In preliminary experiments, we found that the small size of many natural cells made distinguishing positive from negative cells difficult. Likewise, distinguishing darkly stained cells from detrital or sediment material was more problematic. In these experiments, the best results were obtained by simultaneously using DAPI as a general fluorescent stain for total counts (excitation wavelength, 330 nm; emission wavelength, 450 nm) and rhodamine-labeled anti-Fab fragments (excitation wavelength, 560 nm; emission wavelength, 610 nm) to detect PCR products in positive cells.

PI-PCR was successfully carried out with both cells suspended in a reaction mixture and cells immobilized on glass slides. With suspension PI-PCR, a significant percentage of the cells sometimes was lost during the procedure, presumably by inefficient centrifugation during intermediate washing steps. However, this approach is the simplest technically; washing steps are rapid, and the resulting intensity of signal from positive cells is uniform. When cells were immobilized on glass, washes sometimes were not as effective, as evidenced by fairly substantial formation of background signal in the negative cells. The addition of a nuclease S1 digestion step after amplification was successful in reducing the background in the negative cells while not affecting the intensity of the positive cells. When PI-PCR was carried out on microscope slides, we initially had a high failure rate of the chamber adhesive seal (about 50%) because of the high-temperature denaturation step (94°C). However, this problem was minimized by using printed microscope slides for better adherence of the chambers and by blocking the top of the chamber with melted wax. The benefits of conducting PI-PCR on microscope slides are that almost all of the bacterial cells remain at the end of the procedure and the microscale distribution of cells (e.g., association in microcolonies or attachment to detrital particles) can be preserved.

PI-PCR carried out on strips of slide coverslips suspended in reaction mixture was problematic at the wash steps because of difficulties in manipulating the small, fragile slides and interference by the mineral oil used as an overlay for thermal cycling. We are currently developing a PI-PCR method which involves collecting bacteria on filters prior to amplification (6). This approach may ultimately prove to be the most versatile for characterizing natural prokaryotic communities, since it will allow efficient concentration of cells yet maintain physical associations of bacteria with each other and with particulate matter (12).

The sensitivity of the RPE approach depends on both the natural abundance of multiple targets within the cell and the length of the labeled extension of the primer, since the amplification process is linear rather than exponential. We successfully detected mRNAs transcribed from the *nahA* gene in *Pseudomonas* cells grown in the presence of an *nahA* inducer. In addition to its usefulness for detection of expression at the individual cell level, this protocol may be particularly useful as a more sensitive alternative to the existing methods for in situ phylogenetic characterization based on detection of taxonomically important regions of 16S and 23S rRNA in prokaryotic cells (9). Although multiple rRNAs are present in these cells, the small number of ribosomes in slow-growing or dormant cells has been a limitation to in situ hybridization approaches (30). RPE linearly amplifies the signal from the rRNA target during thermal cycling and, unlike PI-PCR, requires only a single primer. In addition to the amplification of mRNA transcripts from the *nahA* gene, we also carried out successful amplification of transcripts from the *nifH* gene inside cells of *Azotobacter vinelandii* with no modification of the protocol presented here (9). Thus, there are good indications that these in situ PCR methods can be readily adapted for use with other organisms and gene systems.

Of the eight bacterial species in the model community, seven gave the expected result, as determined by colony hybridizations with the NAH I probe. The eighth species, a large gram-negative rod isolated from marine sediments, gave a positive signal during in situ PCR but a negative colony hybridization result. Possible explanations for this apparent false-positive result in the mixed community include: (i) the large rod is a true positive for the *nahA*-like gene but has mismatches with the internal NAH I probe; (ii) the large rod is a true negative for the *nahA* gene, but hybridization conditions for the in situ primer binding are not sufficiently stringent; and (iii) the permeabilization steps were not optimal for this organism, leading to insufficient degradation of DNA (for RNA-based procedures). The fact that this rod showed a positive in situ signal for both RNA- and DNA-based procedures (RPE and PI-PCR) suggests that this last hypothesis is not likely. However, false-positive results were obtained in other RNA-based procedures when species of gram-positive bacteria (i.e., *Bacillus subtilis*, *Streptococcus faecalis*, and *Staphylococcus aureus*) were used as targets. Thus, nonoptimal permeabilization may lead to false-positive signals in some in situ amplification procedures.

Optimization of in situ PCR conditions is clearly a more straightforward undertaking for a defined system, such as the *Pseudomonas* species and model communities used here, than for a complex bacterial community in an environmental sample. Several challenges remain for the continued development of PI-PCR techniques for use with natural communities and other heterogeneous samples, such as clinical tissue preparations and bacterial-eukaryotic symbioses. These include (i) identifying permeabilization and amplification conditions appropriate for a specific organism in a community (for example, when targeting expression of one gene in a single bacterial species) or appropriate for all prokaryotic species in the community, including those which are not presently culturable (for example, when targeting one gene harbored by a number of phylogenetically distinct species) and (ii) ensuring that the PI-PCR protocols are applicable to the nongrowing and slowly growing cells typical of many natural communities. The successful development of the PI-PCR method, however, promises to provide the first opportunity to link distribution and identity of prokaryotic cells in natural environments with their genetic potential and in situ activities.

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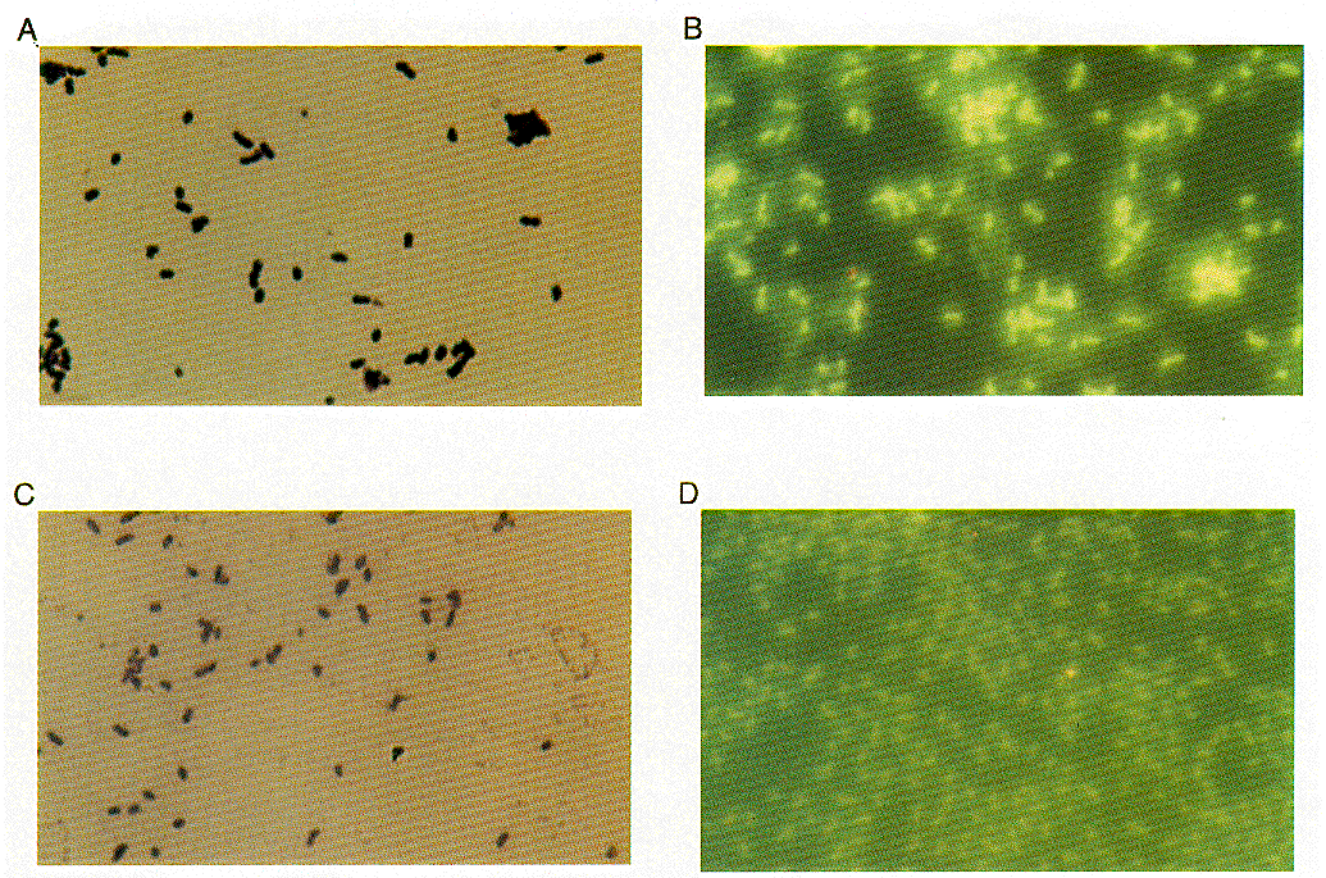
## REFERENCES

1. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisolm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.

2. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143–169.
3. **Azam, F., and R. E. Hodson.** 1977. Dissolved ATP in the sea and its utilization by marine bacteria. Nature (London) **267:**696–698.
4. **De Long, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1362.
5. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
6. **Dustman, W. A., and R. E. Hodson.** Unpublished data.
7. **Fenchel, T., and T. H. Blackburn.** 1979. Bacteria and mineral cycling. Academic Press, London.
8. **Fuhrman, J. A., and F. Azam.** 1980. Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl. Environ. Microbiol. **39:**1085–1095.
9. **Garg, R. P., W. A. Dustman, M. A. Moran, and R. E. Hodson.** Unpublished data.
10. **Gressens, P., and J. R. Martin.** 1994. *In situ* polymerase chain reaction: localization of HSV-2 DNA sequences in infections of the nervous system. J. Virol. Methods **46:**61–83.
11. **Hahn, D., R. I. Amann, W. Ludwig, A. D. L. Akkermans, and K.-H. Schleifer.** 1992. Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. J. Gen. Microbiol. **138:**879–887.
12. **Hicks, R. E., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158–2163.
13. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
14. **Kirchman, D. L., E. K'nees, and R. Hodson.** 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl. Environ. Microbiol. **49:**599–607.
15. **Komminoth, P., and A. A. Long.** 1993. *In-situ* polymerase chain reaction: an overview of methods, applications and limitations of a new molecular technique. Virchows Arch. B Cell Pathol. **64:**67–73.
16. **Manz, W., U. Szewzyk, P. Ericsson, R. Amann, K.-H. Schleifer, and T.-A. Stenström.** 1993. In situ identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. **59:**2293–2298.
17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Moss, R. B., and M. A. Kaliner.** 1994. Primed in situ DNA amplification (PIDA). J. Clin. Lab. Anal. **8:**120–122.
19. **Nuovo, G. J.** 1992. PCR *in situ* hybridization: protocols and applications. Raven Press, New York.
20. **Nuovo, G. J., F. Galley, and P. MacConnell.** 1992. Detection of amplified HPV6 and 11 DNA in vulvar lesions by hot start PCR *in situ* hybridization. Mod. Pathol. **5:**444–448.
21. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25:**943–948.
22. **Sällström, J. F., I. Zehbe, M. Alemi, and E. Wilander.** 1993. Pitfalls of *in situ* polymerase chain reaction (PCR) using direct incorporation of labelled nucleotides. Anticancer Res. **13:**1153–1154.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Sayler, G. S., and A. C. Layton.** 1990. Environmental application of nucleic acid hybridization. Annu. Rev. Microbiol. **44:**625–648.
25. **Schell, M. A.** 1985. Transciptional control of the *nah* and *sal* hydrocarbon degradation operons by the *nahR* product. Gene **36:**301–109.
26. **Seidman, R., N. S. Peress, and G. J. Nuovo.** 1994. *In situ* detection of polymerase chain reaction-amplified HIV-1 nucleic acids in skeletal muscle in patients with myopathy. Mod. Pathol. **7:**369–375.
27. **Sieburth, J. M.** 1975. Microbial seascapes: a pictorial essay on marine microorganisms and their environment. University Park Press, Baltimore.
28. **Sobecky, P. A., M. A. Schell, M. A. Moran, and R. E. Hodson.** 1992. Adaptation of model genetically engineered microorganisms to lake water: growth rate enhancements and plasmid loss. Appl. Environ. Microbiol. **58:**3630–3637.
29. **Terkelsen, C., J. Koch, S. Kølvraa, J. Hindkjaer, S. Pedersen, and L. Bolund.** 1993. Repeated primed *in situ* labeling of specific DNA sequences ("PCR-PRINS") in metaphase chromosomal spreads. Boehringer Mannheim Biochemica number 10. Boehringer, Mannheim, Germany.
30. **Trebesius, K., R. Amann, W. Lugwig, K. Mühlegger, and K.-H. Schleifer.** 1994. Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Appl. Environ. Microbiol. **60:**3228–3235.
31. **Wright, R. T., and J. E. Hobbie.** 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology **47:**447–464.
32. **Zarda, B., R. Amann, G. Wallner, and K.-H. Schleifer.** 1991. Identification of single bacterial cells using digoxigenin-labelled, rRNA-targeted oligonucleotides. J. Gen. Microbiol. **137:**2823–2830.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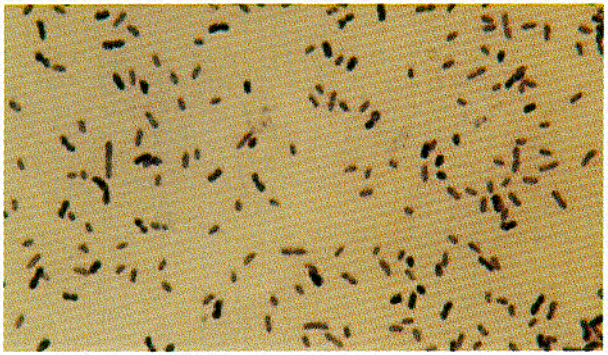

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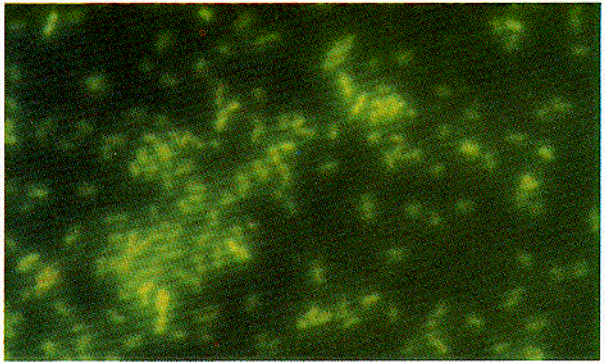

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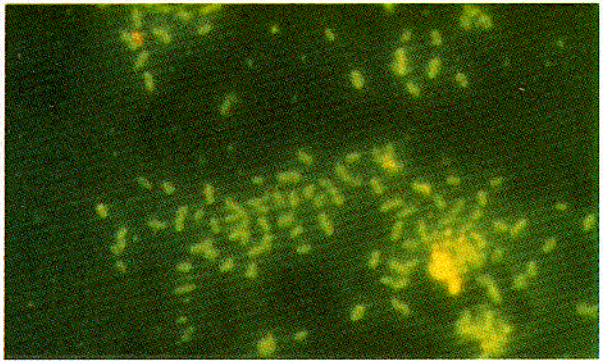

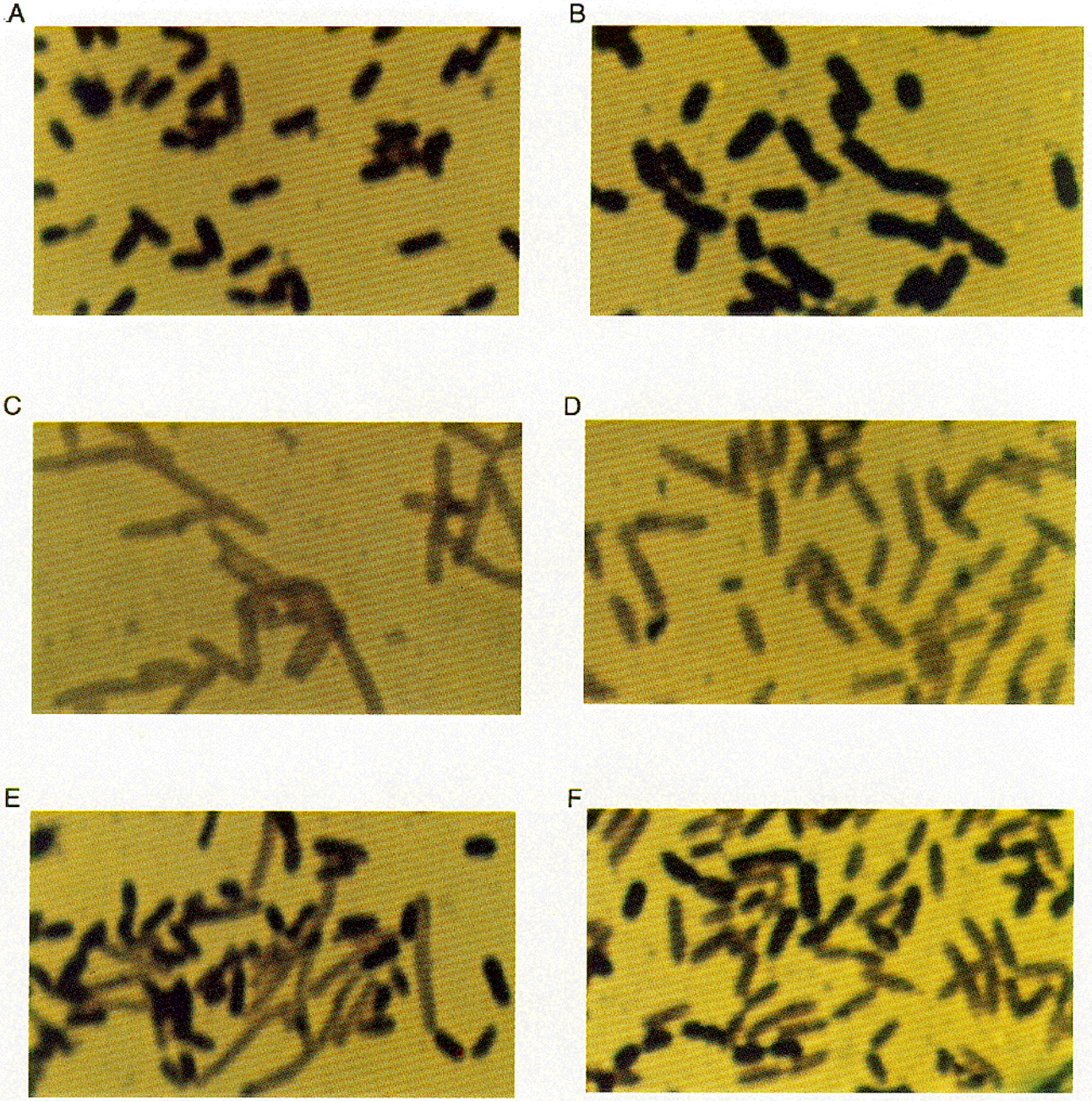
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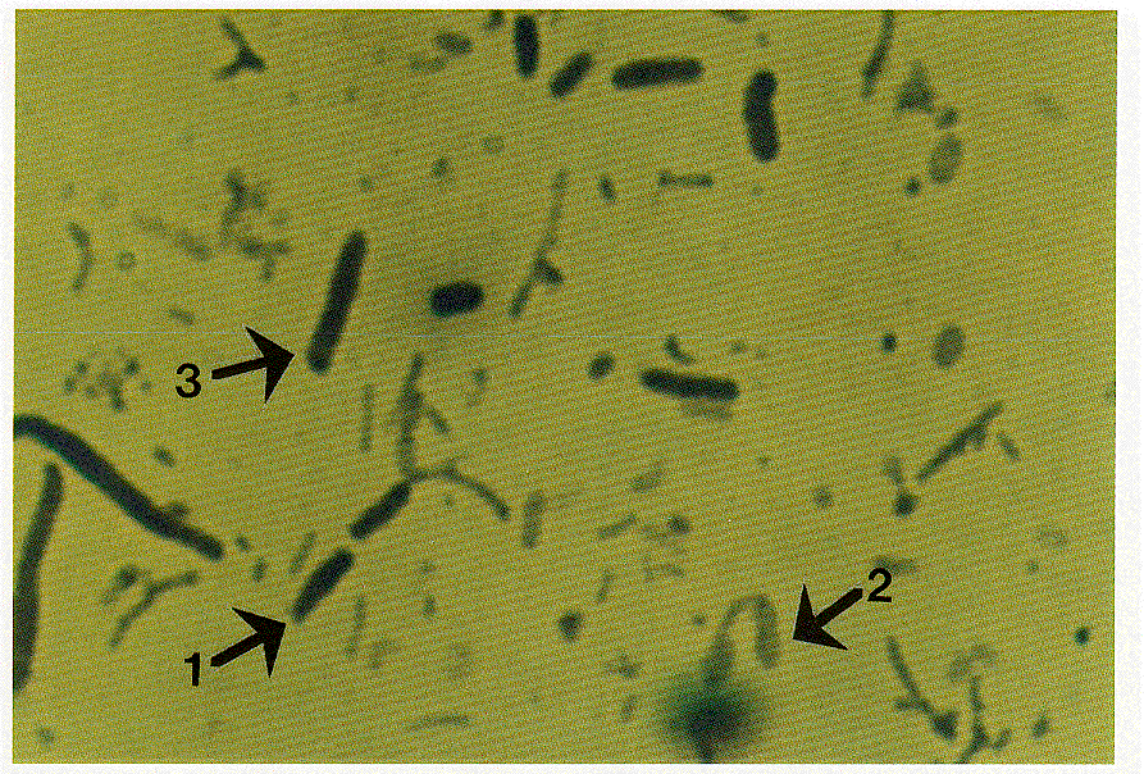
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