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## Increase in Fluorescence Intensity of 16S rRNA In Situ Hybridization in Natural Samples Treated with Chloramphenicol

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**Despite the numerous advantages of fluorescent in situ hybridization for the identification of single prokaryotic cells with 16S rRNA probes, use of the technique with natural samples, especially those from the marine environment, is still problematic. The low percentage of fluorescently labeled cells constitutes the primary problem for in situ hybridization of natural samples, probably due to low cellular rRNA content. This study represents an attempt to improve detection of marine prokaryotes by increasing cellular rRNA content without changing the species composition. Cells from three California coastal sites were treated with chloramphenicol, an inhibitor of protein synthesis and rRNA degradation, at 100  $\mu\text{g/ml}$  and then were probed with a “universal” 16S rRNA fluorescent probe and viewed by image-intensified video microscopy. Counts of fluorescent cells increased from ca. 75% for untreated samples to ca. 93 to 99% for chloramphenicol-treated samples, compared to counts produced by DAPI (4',6-diamidino-2-phenylindole) staining, after at least 45 min of exposure to the drug (these percentages include autofluorescent cells, which averaged 6%). This suggests that most cells in these samples were active. We hypothesize that the low fluorescent-cell counts previously reported were probably often due to the fluorescence intensity of labeled cells being below the detection level rather than to high levels of dead cells in marine environments. This method may aid in the characterization of bacterioplankton with fluorescent probes.**

Fluorescent oligonucleotide probe hybridization is becoming an important tool in microbial ecology by allowing one to identify individual organisms and to quantify organisms in mixed communities without the need for isolation in pure cultures (2, 21, 25). Despite the numerous advantages of fluorescent oligonucleotide probes (4, 22), application of this technique to natural bacterial assemblages, particularly to marine bacteria, still faces many limitations (4, 16, 22).

Compared to bacteria grown in most laboratory cultures, bacteria growing in natural environments tend to grow slowly and, hence, have small amounts of rRNA (14, 18), the target molecule for the fluorescent probes. When naturally occurring bacteria are labeled with 16S rRNA fluorescent probes, the fluorescence signal produced is often dim, if not undetectable (2, 6, 13, 19), resulting in low percentages of fluorescent cells (18). Evidence suggests that the higher the ribosomal content per cell, the higher the fluorescence signal for in situ hybridization (25, 26). Attempts have been made to increase the fluorescence signal from whole-cell in situ hybridization by use of (i) a single probe type with multiple fluorochromes (3), (ii) multiple probe types (2, 18), (iii) sample enrichment (25), (iv) sample enrichment with yeast extract to enhance cellular rRNA content combined with nalidixic acid to prevent cell division (23), and (v) single probe and image-intensified video detection (7).

Among those attempts, Fuhrman et al. (7) and Lee et al. (19) reported that about 75% of marine bacterioplankton in a sample were labeled with universal probes, while Wagner et al. (25) reported that 89.3% of cells were labeled with a eubacteria probe in an activated sludge sample and Alfreider et al. (1)

reported that an average of 76.3% of cells were labeled in a mountain lake sample, also with a eubacteria probe. Nishimura et al. (23) reported an increase in the total number of visibly labeled cells growing in yeast extract-enriched seawater and nalidixic acid and labeled with an oligonucleotide probe. After 8 h, only about 10% of the total cells were labeled.

Chloramphenicol (CM), a broad-spectrum antibiotic, inhibits protein synthesis (including ribosomal proteins), arresting the growth of a wide range of gram-positive and gram-negative bacteria (10), while inhibiting degradation of rRNA in certain bacterial cells in vitro (14). As cells are unable to divide, while rRNA transcription continues and rRNA degradation decreases, the rRNA content in CM-treated cells is expected to increase over time (17), with little effect on the species composition.

Hence, the objective of this work is to attempt to boost the fluorescence signal intensity of marine bacteria labeled with fluorescent oligonucleotide probes to detectable levels by exposing those cells to CM, increasing cellular rRNA content. Furthermore, since CM inhibits cell division, the marine microbial community structure in the sample is expected to suffer insignificant changes.

### MATERIALS AND METHODS

**Sample collection.** Samples were collected from three different sites along the Southern California coast in bottles washed in 5% HCl. Experiments started within 1 h of sampling in the laboratory. A water sample from Santa Monica pier was collected on 10 April 1996 at the surface, a sample from Zuma Beach was collected on 27 April 1996 approximately 150 m from the shore and 1 meter from the surface, and a sample from San Pedro was collected on 31 May 1996 50 cm away from a rocky shore.

**Sample preparation.** Subsamples of 100 to 200 ml from each of the original samples were incubated at 16.4°C (approximate seawater temperature) 30 cm below an 18-W fluorescent light for the entire length of the experiment. Negative control subsamples were left untreated, while test subsamples were treated with CM (Sigma; crystalline no. C-0378) to a final concentration of 100  $\mu\text{g/ml}$  (dis-

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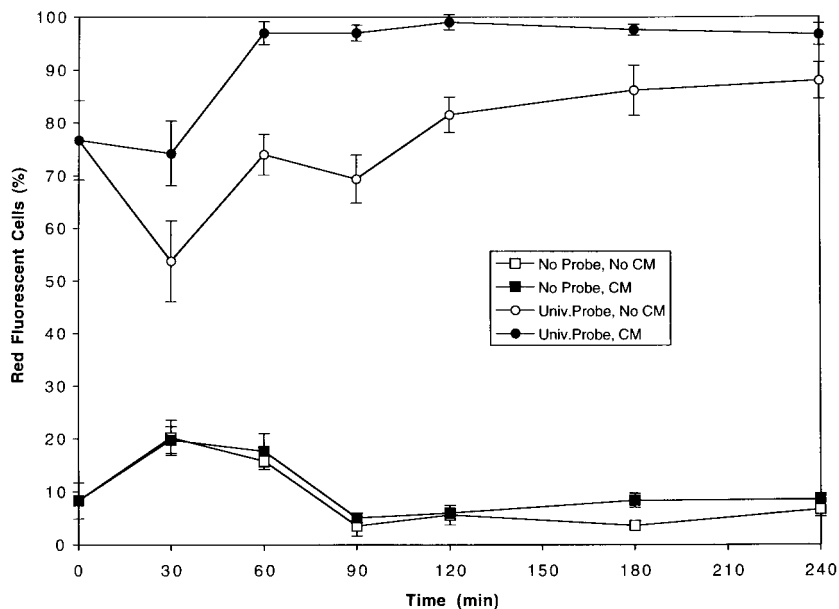


FIG. 1. Effect of CM on the RFC as a percentage of total DAPI-stained cells. Red fluorescence derives from either the probe or the natural pigments. The probe was a universal (Univ.) oligonucleotide probe conjugated to fluorochrome Cy3. The sample was from surface water of Santa Monica Beach, Calif. Bars represent standard errors.

solved in 50% ethanol). Samples from San Pedro Beach also had a control for the possible effects of ethanol at  $4.3 \times 10^{-2}$  M, the concentration of ethanol in the CM-treated samples. Five- to ten-milliliter aliquots were collected for each subsample every 30 min for the first 2 h and every hour for the next 2 h, for a total of 4 h. The aliquots were immediately fixed in 10%, 0.02- $\mu$ m-pore-size-filtered formalin and prepared for filtration.

**Whole-cell staining, filtration, and transfer to slides.** Heavy Teflon slides with 7-mm-diameter wells (Cel-Line Associates, Inc., Newfield, N.J.) were prepared by coating with gelatin (0.1% gelatin, 0.01%  $\text{CrKSO}_4$ ). Instead of coating the entire slide with gelatin solution, each well of the slide was coated separately by placing approximately 20  $\mu$ l of the gelatin at 70°C into each well and then immediately withdrawing it, leaving a thin film of gelatin on the well. Slides were allowed to dry for at least 1 h. With this treatment, Teflon areas around the wells

remained hydrophobic, preventing the hybridization solution in one well from merging with that in adjacent wells.

Four milliliters of formalin-fixed sample from each time point was stained with 70  $\mu$ l of a 0.1- $\mu$ g/ $\mu$ l 4',6-diamidino-2-phenylindole (DAPI) solution for 5 min and then filtered through a 0.2- $\mu$ m-pore-size 25-mm-diameter Nuclepore polycarbonate filter placed over a 0.8- $\mu$ m-pore-size Millipore filter type AA. Both filters were cut into four equal pieces. Cells were transferred from the filters onto slides by placing each quarter of the Nuclepore filter, cell face down, over a single coated well containing a 1- $\mu$ l drop of sterile water.

After being air dried for about 30 min, Nuclepore filters were removed from the slide, and cells were posttreated with a methanol-formalin (90:10 [vol/vol]) solution (50  $\mu$ l/well) for 15 min, rinsed with distilled water, and air dried once again.

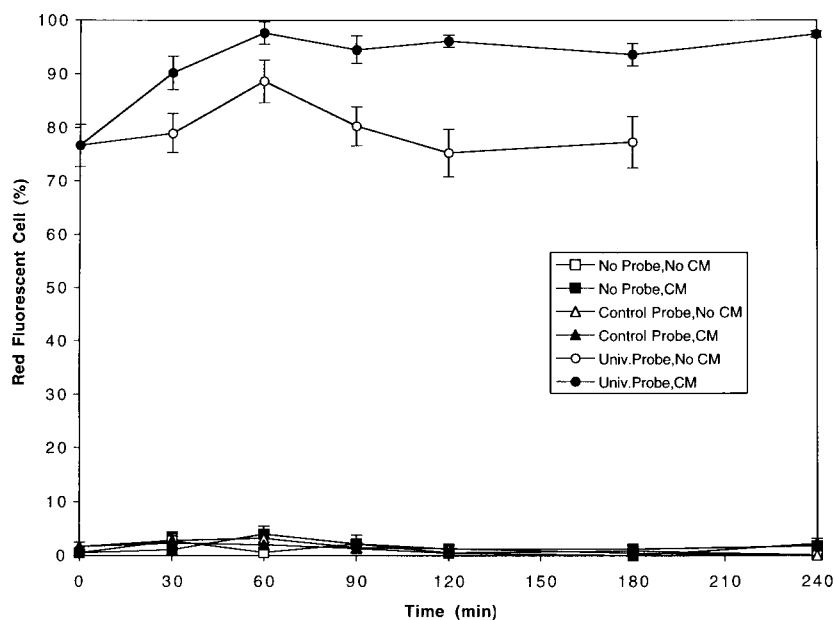


FIG. 2. Data corresponding to those shown in Fig. 1 for a sample from the surface water of Zuma Beach, Calif. Univ., universal. Bars represent standard errors.

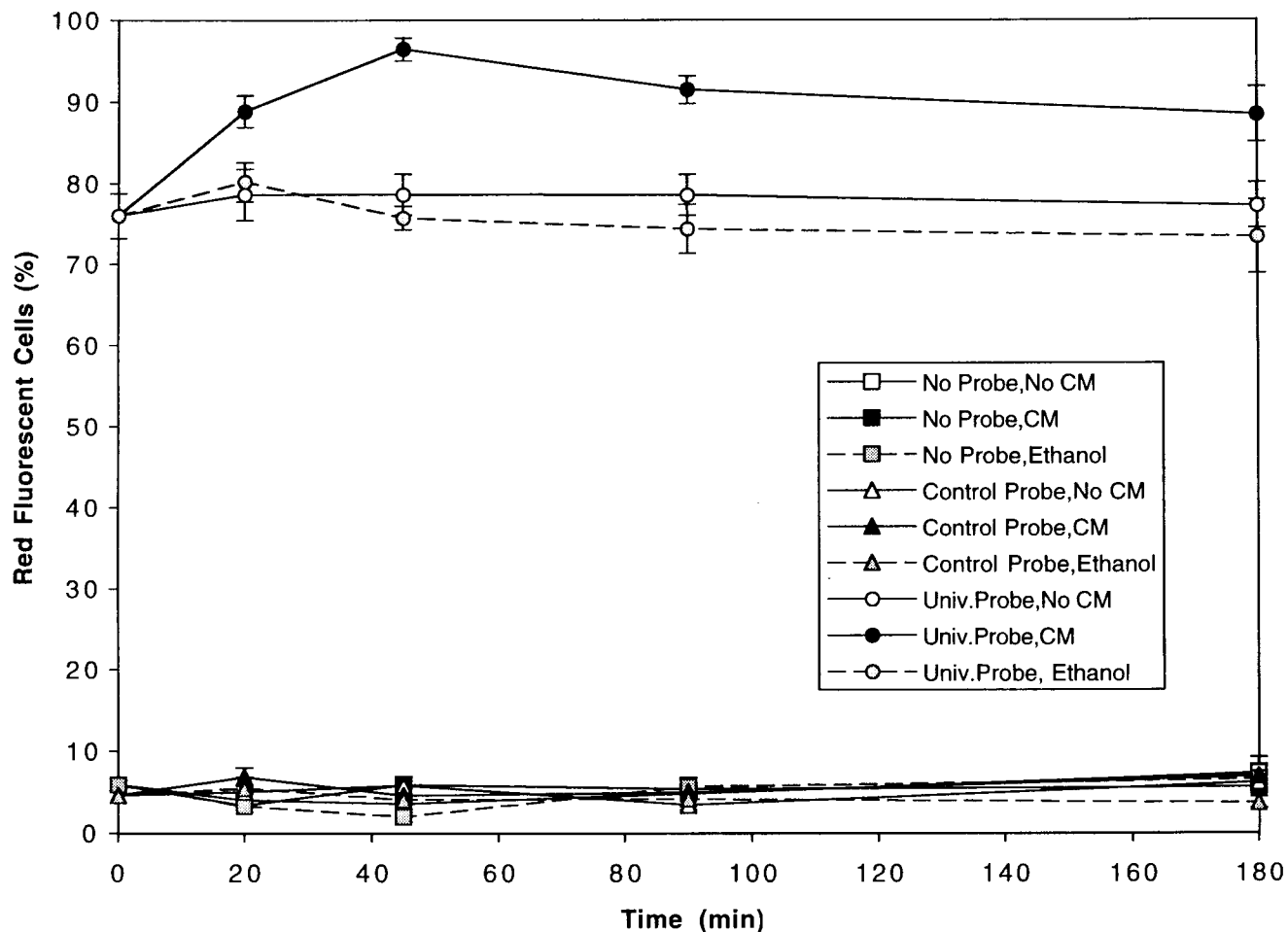


FIG. 3. Data corresponding to those shown in Fig. 1 for a sample from the surface water of San Pedro Beach, Calif. Univ., universal. Bars represent standard errors.

**Probe description and whole-cell hybridization.** Procedures for the hybridization steps were as described by Fuhrman et al. (7), as modified from DeLong et al. (6), Braun-Howland et al. (5), and Hicks et al. (13). Oligonucleotide probes were modified with a single amino linker attached at the 5' terminus (Operon Technologies, Inc., Alameda, Calif.) and labeled with cyanine Cy3 monofunctional dye (Amersham) in 0.1 M carbonate buffer, pH 8.5, overnight in the dark (12). The separation of labeled from unlabeled probes was as described by Amann et al. (3). In brief, the labeled probe purification steps consisted of separating the oligonucleotides from the unreacted Cy3 dye in an STE Select-D, G-25 spin column (5'-3' Prime, Boulder, Colo.). Labeled probes were then separated from unlabeled probes in a 20% nondenaturing polyacrylamide gel, followed by elution in low TE (low TE is 10 mM Tris-hydrochloride plus 1 mM

EDTA [pH 7.8]) at 4°C. The eluted solution was then passed through Nensorb-20 columns (Du Pont, Wilmington, Del.) as described by the manufacturer. The amount of labeled probe was determined by measuring the absorbance at 260 nm. Probes were stored dry at -80°C.

Each set of CM-treated cells and untreated cells was either labeled with the universal probe 5'GWATTACCGCGGCKGCTG3' (*Escherichia coli* positions 536 to 519) (11) or left unlabeled. For the Zuma Beach experiment, a negative control probe 5'CCTAGTGACGCCGTCGAC3', with more than three mismatches to all rRNA sequences in the Ribosomal Database Project (20), was also used.

In situ hybridization took place in buffer containing 5× SET (5× SET is 750 mM NaCl plus 100 mM Tris-Cl [pH 7.8] plus 5 mM EDTA), 0.2% bovine serum

TABLE 1. Average RFC as a percentage of total DAPI-stained cells from a universal oligonucleotide probe conjugated to a single fluorochrome Cy3 and average total cell count from DAPI-stained cells<sup>a</sup>

Sample location	Results for cells treated with:					
	No CM		CM		Ethanol (control)	
	% RFC <sup>b</sup>	Cells/ml <sup>c</sup>	% RFC	Cells/ml	% RFC	Cells/ml
Santa Monica	79.8 ± 1.8	4.7 ± 0.07	98.2 ± 1.3	4.4 ± 0.12	NA <sup>d</sup>	NA
Zuma	79.5 ± 4.2	5.6 ± 0.6	95.9 ± 1.7	5.0 ± 0.3	NA	NA
San Pedro	78.1 ± 2.6	3.6 ± 0.2	92.7 ± 2.0	3.1 ± 0.2	74.5 ± 3.0	3.6 ± 0.2

<sup>a</sup> Incubation time, ≥45 min.

<sup>b</sup> RFC as a percentage of total DAPI-stained cells ± the standard error (SE), not corrected for autofluorescence.

<sup>c</sup> Average total cell count in 10<sup>6</sup> cells ml<sup>-1</sup> over the period of treatment ± SE.

<sup>d</sup> NA, no data.

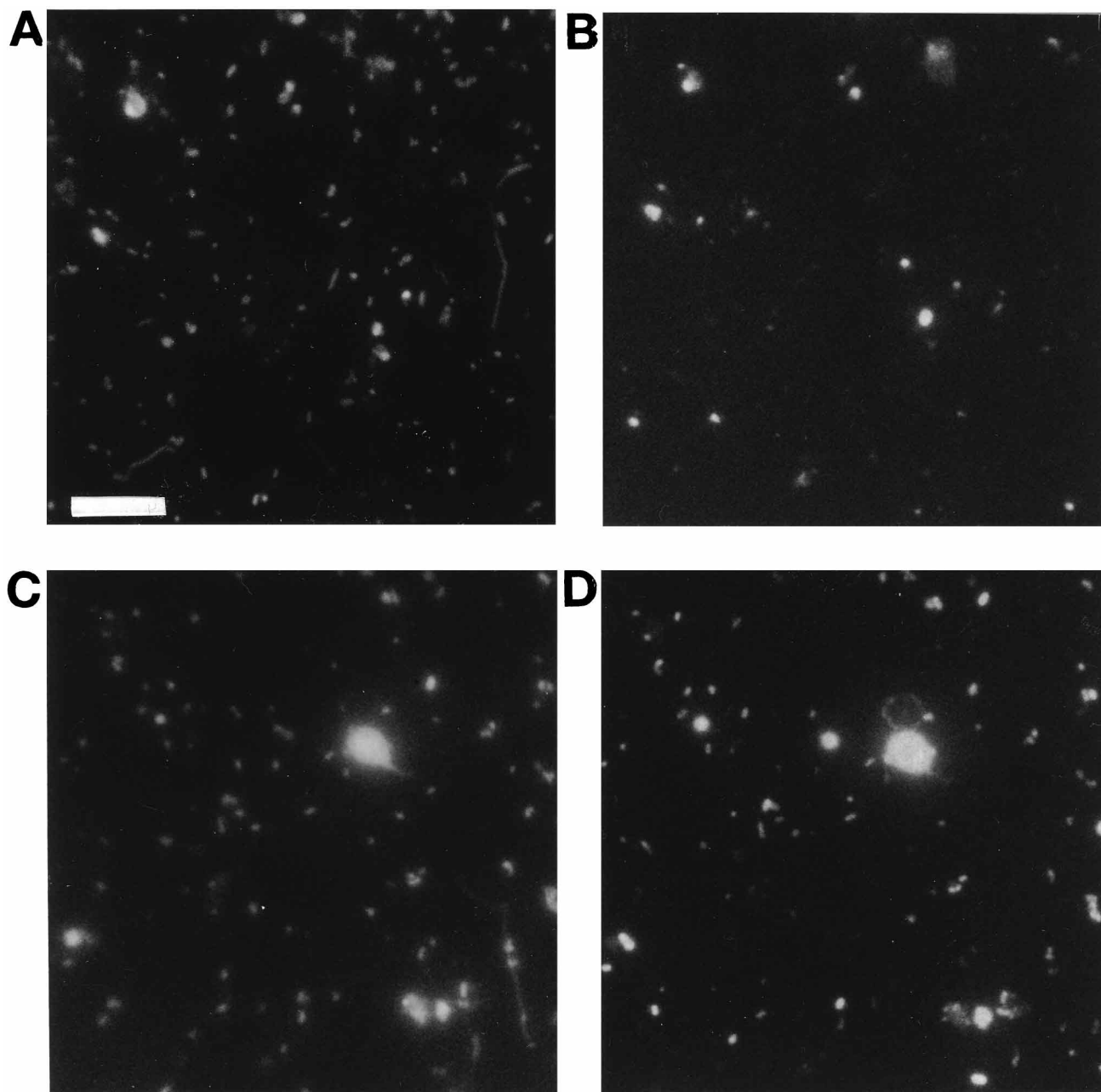


FIG. 4. Enhancement of fluorescence from 60-min CM treatment of a marine sample. (A and C) DAPI fluorescence with UV excitation; (B and D) Cy3-conjugated universal probe fluorescence and pigment autofluorescence with green excitation. (A and B) Untreated samples; (C and D) samples treated with CM (see text). All four photomicrographs were taken at  $\times 1,000$  magnification. Bar, 5  $\mu\text{m}$  (applies to all photomicrographs).

albumin (Sigma; acetylated), 10% dextran sulfate (Pharmacia; MW 500,000), 0.01% polyadenylic acid (Sigma), and 0.1% sodium dodecyl sulfate, as described in DeLong et al. (6) and Braun-Howland et al. (5), with a probe concentration of 5  $\text{ng } \mu\text{l}^{-1}$ . Slides were incubated at 43°C for 3 h for the Santa Monica Beach sample, 13 h for the Zuma Beach sample, and 11 h for the San Pedro Beach sample, then were briefly rinsed with distilled water at 43°C, and finally were immersed three times in 0.2 $\times$  SET, also at 43°C, for 10 min each time. After air drying, slides were mounted in 50% glycerol–50% 5 $\times$  SET–0.1% *p*-phenylenediamine hydrochloride solution and then observed by microscopy.

**Fluorescent-cell count.** Cells were counted within 2 days after the hybridization step with an Olympus Vanox microscope (model AH-2) with a DPlan Apo  $\times 100$  UV objective lens, an HBO 100 Hg vapor lamp, and UV (Nikon 365/20-nm excitation,  $>420$ -nm emission) and green filters (545/50-nm excitation,  $>590$ -nm emission) for fluorescence microscopy. With these filter sets, the Cy3 probe fluorescence appears red. Images were captured and intensified by a microchan-

nel plate image intensifier (Video Scope International, Ltd. Washington, D.C.), with background reduced by image averaging with a DSP-2000 image processor (Dage-MTI, Inc., Michigan City, Ind.), and visualized with a Sony Trinitron color video monitor (model PVM-1353MD). This video system can display cells with fluorescence considerably below that directly detectable by the eye.

Cell counts consisted primarily of total counts from DAPI-stained cells (UV excitation) and of counts of probe-labeled cells from red fluorescent cells (RFC; green-light excitation). Images of both DAPI and RFC could be stored separately and subsequently superimposed on the monitor screen in different colors so that each DAPI-stained cell could be examined with a possible matching RFC.

At least 400 cells were counted in each well, and the percentage of probe-labeled cells (determined from the number of RFC and total DAPI counts) was calculated for CM-treated and untreated cells, labeled with probe or unlabeled.

**Total cell count.** One to four milliliters of 10%-formalin-fixed volumes from each time point was DAPI stained for 5 min and filtered through a black 0.2- $\mu$ m-pore-size Nuclepore filter and used for total counts. Over 200 cells from each filter were counted, and the average cell concentration was determined for each time point. This separate count was done because a portion (approximately 40%) of cells are lost from the gelatin-coated slides during the transfer and hybridization processes.

**Photomicrography.** Samples were photographed on a Leica microscope fitted for epifluorescence microscopy with a PL Fluorotar  $\times$ 100 oil immersion objective lens, a high-pressure HBO 103 W/2 100-W Hg vapor lamp, and UV (390/35-nm excitation, >470-nm emission) and rhodamine (535/25-nm emission, >590-nm excitation) filters. Photomicrographs were made with Fujifilm Provia 1600 and then transferred to black-and-white prints. Exposure times for both untreated and treated cells were 0.5 s under UV excitation (DAPI-stained cells) and 2 min under green excitation (probe-labeled and autofluorescent cells).

## RESULTS

**Cell count.** Trends in the percentage of fluorescent cells showed similar results for samples from Santa Monica Beach (Fig. 1), Zuma Beach (Fig. 2), and San Pedro Beach (Fig. 3). The percentage of fluorescent cells labeled with the universal oligonucleotide probe and treated with CM was significantly higher than that of the untreated cells labeled with the same probe ( $P = 0.028$ , Wilcoxon sign rank, for all time points). The higher count remained for the duration of the experiment. Labeled cells treated with CM reached nearly 100% counts after 1 h of exposure to the drug and showed percentages of fluorescent-cell counts 10 to 30% higher than those of the untreated labeled cells (Table 1). The ethanol-treated control labeled with the universal probe was not significantly different from the untreated sample labeled with the same universal probe ( $P = 0.144$ , Wilcoxon sign rank, for all time points). The difference in percent count between treated and untreated samples decreased towards the end of the experiment for the Santa Monica Beach cells.

**Autofluorescence and control probe.** Autofluorescence counts for treated and untreated cells were generally near 5% throughout the experiments, although two early time points from the Santa Monica Beach samples showed 10 to 20% (Fig. 1 to 3). Similar percentages of fluorescence counts were also observed for CM-treated or untreated cells labeled with the negative control probe or exposed to ethanol alone.

**Total cell count.** The total cell count from DAPI-stained cells did not vary significantly ( $P \geq 0.05$ , Wilcoxon sign rank, for all time points) between treated and untreated cells for the Santa Monica, San Pedro, and Zuma Beach samples throughout the experiment (Table 1).

**Fluorescence intensity.** The intensity of fluorescence from cells labeled with oligonucleotide probes was brighter in cells treated with CM (Fig. 4). Ethanol did not seem to affect the fluorescence intensity of cells labeled with the probes (data not shown).

## DISCUSSION

The results suggest that CM treatment of marine samples can help boost the overall fluorescence signal for in situ hybridization, leading to higher counts of labeled cells. The average percentage of fluorescent cells, labeled with the universal probe, for untreated controls (75%) was similar to results reported by Fuhrman et al. (7), where the same probe was applied (although in Fuhrman et al. [7] the probe was labeled with tetramethyl rhodamine instead of Cy3). When cells were treated with CM, however, not only the overall fluorescence intensity but also the percentage of fluorescent cells increased significantly (10 to 30%) to nearly 100% compared to untreated cells. This suggests that CM affected most of the microbial types in our samples. Significant shifts in community

structure seem highly implausible given that untreated cells in these waters double only about 1 to 3 times per day (9) and given that CM slows protein synthesis about 50-fold (23a). Some marine microbes, such as archaea (8), might not be expected to be affected by CM (24), but if such were present they may have already been made visible by labeling without the CM treatment. The total percentages we report include autofluorescent cells (or those binding the control probe), which averaged 6% (Fig. 1 to 3). Because it is uncertain whether or not such cells also bind the probe (15), we have not automatically subtracted them from the total. Doing so would not alter the conclusions about the enhancement effect of CM and would reduce the total probe-labeled cell count only slightly.

The increase in the percentage of fluorescently labeled cells due to CM treatment suggests that most cells in our marine samples were alive, in contrast to previous findings suggesting that a high percentage of cells in the marine environment may be "ghosts" (27). Hence, we hypothesize that the reason for the lower fluorescence counts previously reported for in situ hybridization with 16S rRNA fluorescent probes was most probably the low level of fluorescence intensity produced per cell.

The presumed protein synthesis inhibition of CM suggests a possible mechanism for fluorescence enhancement. Continued synthesis of rRNA in the absence of ribosomal protein synthesis may lead to an accumulation of precursor forms of rRNA that may bind readily to the fluorescent probes. One might speculate that such precursor forms bind probes more easily than do intact ribosomes.

In conclusion, the results suggest that treatment with CM for 1 h prior to in situ hybridization enhances the fluorescence signal of marine bacteria labeled with fluorescent oligonucleotide probes. In addition, we would expect that CM also increases the fluorescence signal of bacteria labeled with probes made for more specific phylogenetic groups and hence could aid in the characterization of the bacterioplankton in natural samples. However, since this report is the first we are aware of to propose the application of antibiotics alone for the enhancement of the fluorescence signal for in situ hybridization with molecular probes, further investigation is necessary to better understand the advantages and limitations of this approach.

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