APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1997, p. 3274–3278 Vol. 63, No. 8
0099-2240/97/$04.00+0

A New Sensitive, Whole-Cell Hybridization Technique for Detection of Bacteria Involving a Biotinylated Oligonucleotide Probe Targeting rRNA and Tyramide Signal Amplification

PHILIPPE LEBARON,* PHILIPPE CATALA, CÉLINE FAJON, FABIEN JOUX, JULIA BAUDART, AND LAETITIA BERNARD

Observatoire Océanologique, Université Pierre et Marie Curie, Institut des Sciences de l'Univers, CNRS URA2071, F-66651 Banyuls-sur-Mer, France

Received 5 December 1996/Accepted 9 May 1997

A tyramide signal amplification system with biotinylated oligonucleotide probes and streptavidin-horseradish peroxidase was used to increase the sensitivity of fluorescent in situ hybridization techniques. When applied to both gram-negative and -positive bacteria immobilized on glass slides, a 7- to 12-fold amplification of the fluorescence signal was observed relative to that of cells hybridized with fluorescently monolabeled probes. A large proportion (62 to 78%) of bacteria could be detected under starvation conditions and in natural samples from the marine environment. This amplification procedure allows new investigations in marine oligotrophic ecosystems and water quality control.

The development of rapid and accurate methods for the detection and quantification of specific bacteria without cultivation is of increasing importance in several areas of microbiology, including public health, biotechnology, food technology, the water, and pharmaceutical industries and environment.

Recently, oligonucleotide probes targeting rRNA have been used to design probes with various specificities (4, 11). These probes combined with fluorescent dyes have been used successfully to detect and identify individual whole cells in mixed microbial communities (for a review, see reference 4). Although several procedures of signal amplification have been developed (3, 9, 13–15), there still remain limitations in their application to natural bacteria, which have far lower copy numbers of ribosomes than cultured bacteria.

The purpose of the present investigation was to develop a new amplification technique based on tyramide signal amplification (TSA) and compare it with techniques using single and multiple monolabeled probes for the detection of bacteria with various rRNA contents. This technique was applied to different species and to starved cells of *Salmonella typhimurium* and *Deleya aquamarina*. Its application to natural samples from the marine environment was also investigated.

Bacterial strains were provided by the Institut Pasteur Collection (CIP; Paris, France). *Pseudomonas diminuta* (CIP 63.27T) and *Chromobacterium violaceum* (CIP 103350) belong to the alpha and beta subclasses of the class *Proteobacteria*, respectively, whereas *S. typhimurium* (CIP 60.62T) and *D. aquamarina* (CIP 74.8T) belong to the gamma subclass. *Bacillus marinus* (CIP 103308T) and *Bacillus subtilis* (CIP 52.65T) were chosen as gram-positive bacteria. All of the strains were grown in liquid or solid medium as recommended by the CIP.

To obtain cells with high and low rRNA contents, 1% (vol/vol) of an overnight culture was inoculated into two fresh media from which cells were harvested (5,000 × *g*, 5 min) when the optical density at 600 nm reached 0.1 and the stationary phase, respectively. The pelleted cells were washed and fixed as reported elsewhere (13). Cells with a very low rRNA content were obtained by nutrient starvation of *Deleya* and *Salmonella* stationary-phase cells. For *Salmonella*, starved cells were prepared as reported previously (8). For *Deleya*, the procedure was similar, except that the cells were grown in marine broth (Difco Laboratories, Detroit, Mich.). After inoculation of stationary-phase cells into artificial seawater microcosms, samples were taken after different times of starvation and fixed as described below. At each sampling time, the CFU counts were determined in triplicate on marine agar (Difco). Colonies were counted after 72 h of incubation at 20°C, and the results were expressed as a percentage of total direct counts (8). For hybridization, the cells were fixed as described previously (2).

Probe sequences were taken from the literature (4) and included two universal probes (UNI-1, GWATTACCGCGGCK GCTG; UNI-2, ACGGGCGGTGTGTACAAG), three eubacterial probes (EUB-1, GCTGCCTCCCGTAGGAGT; EUB-2, CACGAGCTGACGACAGCCAT; EUB-3, GCTCGTTGCG GGACTTAACC), and one eukaryotic probe (EUK, GGGCA TCACAGACCTG). All probes were obtained from Eurogentec (Seraing, Belgium). An amino group was attached to the 5′ end of the oligonucleotide in the last stage of synthesis. Labeling was performed by linking tetramethylrhodamine isothiocyanate (TRITC) or biotin to the 5′ end of the oligonucleotide via a 6-carbon spacer arm. For indirect labeling, the TSA-Direct Red kit (TSA kit; DuPont, NEN Research Products, Boston, Mass.) was used to label cells (see below).

For slide and filter analysis, cells were treated as reported elsewhere (6, 15), except that cells on slides were permeabilized with 10 μl of a lysozyme-EDTA solution (14 μg of lysozyme [Boehringer Mannheim; 47,000 U/mg] per ml in TE buffer [100 mM Tris-HCl {pH 8.2} plus 50 mM EDTA]) at room temperature for 10 min. For flow cytometric tests, cells were treated with the lysozyme solution for 7 min at 0°C and washed and resuspended in the hybridization solution (see below).

For hybridizations with single and multiple monolabeled probes, cells were hybridized at 46°C for 2 h in 10 μl of hybridization buffer (2). Probes with very similar melting temperatures (46.3 to 48.7) were used for multiple probing to reduce possible biases of performance. The stringency of the

* Corresponding author. Mailing address: Observatoire Océanologique, Université Pierre et Marie Curie, BP44, 66651 Banyuls-sur-Mer Cedex, France. Phone: 33 4 68 88 73 53. Fax: 33 4 68 88 73 95. E-mail: lebaron@arago.obs-banyuls.fr.

conditions of the hybridization and washing buffers was sufficient since no nonspecific binding was observed, similar to the findings with more-stringent conditions (data not shown). Hybridization with the biotinylated probe was performed at 46°C with the same buffer containing 10 ng of probe. More-stringent conditions did not improve the hybridization efficiency. For TSA, we followed the manufacturer's recommendations (DuPont, NEN Research Products) except that preparations were washed in a buffer consisting of 20 mM Tris, 180 mM NaCl, 0.01% sodium dodecyl sulfate, and 5 mM EDTA for 20 min at 46°C with shaking and allowed to air dry. The cells were then incubated under a coverslip with 10 μl of horseradish peroxidase (HRP)-streptavidin (1:500 [vol/vol] in TE buffer) at room temperature for 30 min. The slide was washed by immersion in TNT buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, 0.05% Tween 20) at room temperature for 15 min, and the cells were incubated with 10 μl of tyramide-TRITC (1/50 in diluant provided by the distributor) for 6 min in the dark at room temperature. The slide was then rinsed in TNT buffer for 15 min. For all hybridization procedures, cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole; 0.1 μg/ml, 15 min) prior to microscopic examination (8).

Control experiments were performed in different ways. For monolabeled probes, the EUK probe was used to test for nonspecific binding. For the TSA system, different control experiments were performed without probe to test (i) the presence of endogenous biotin by adding TRITC-streptavidin (Eurogentec) and (ii) the presence of bacterial peroxidase activity by adding tyramide-TRITC. The nonspecific binding was determined by treating the cells with RNase (1 mg/ml) for 10 min at 4°C before hybridization.

The fluorescence of fixed cells was measured with a monochrome intensifier charge-coupled device camera (LHESA 750 LL; 50 lx) attached to the video port of an Olympus BH2 microscope. The video image of each microscopic field is converted into a numeric form. The digitized image was then subjected first to an automatic threshold procedure dividing the image into two regions, an object region and a background region. The automatic threshold procedure was used to eliminate the noise before processing (IDRA software package provided by IDES, Toulouse, France). When necessary, calibrated neutral-density filters (Kodak Gelatin) were used to decrease the luminance of bright fluorescent cells, to work with the same camera gain value. However, it was sometimes impossible to use a single gain value (when the range of fluorescence values was important, i.e., mainly for comparison between a single monolabeled probe and the TSA system). In this case, fluorescent beads (0.53 and 1.87 μm in diameter; Polysciences Europe, Eppelheim, Germany) were used as two fluorescence standards. The luminance values in object areas were automatically extracted from the image and further processed with EXCEL 4.0 (Microsoft, Redmond, Wash.). About 150 cells were measured for each slide. When detectable, the mean fluorescence value of the control (procedure used to detect nonspecific binding) was subtracted from the mean fluorescence value of the labeled cells. For flow cytometric tests, a FACSCalibur (Becton Dickinson) flow cytometer was used.

Stationary-phase cells from six different phylogenetic groups were chosen as being more representative of naturally occurring cells than log-phase cells. The fluorescent signal conferred with one monolabeled probe was compared with those of two signal amplification systems (five probes and TSA). The fluorescence signal conferred with one monolabeled probe was very low, while the TSA system resulted in a strong increase in fluorescence of target cells that was 2.1- to 3.8-fold higher than that obtained with five monolabeled probes (Table 1 and Fig. 1). The highest amplification was obtained for the marine bacteria *D. aquamarina* (Table 1). No fluorescence signal was observed during the different control experiments except for some gram-positive strains. When necessary, this signal was successfully eliminated by treating the fixed cells with neutral streptavidin (5 μg/ml in TE buffer, for 30 min, at room temperature) to inhibit endogenous biotin prior to hybridization. The control experiments suggested the absence of intrinsic peroxidase activity. Another way to reduce the nonspecific labeling due to biotin was to use HRP-labeled oligonucleotides (12).

TABLE 1. Comparison of relative fluorescence of fixed-cell preparations after hybridization with five different TRITC-labeled probes and with the TSA kit (probe EUB1)

Strain	Relative fluorescence units[a]		
	Five probes	TSA kit	Ratio[b]
Pseudomonas diminuta	61.5	153.6	2.5
Chromobacterium violaceum	76.5	159.3	2.1
Salmonella typhimurium	42.3	121.9	2.9
Deleya aquamarina	40.8	155.1	3.8
Bacillus marinus	137.2	375.0	2.7
Bacillus subtilis	85.5	182.0	2.1

[a] Relative fluorescence units were corrected by subtracting the control value (mean fluorescence recorded when hybridization was performed with no probe).
[b] Ratio of fluorescence intensity of five probes to that of the TSA kit.

Both signal amplification systems were applied to the detection of log-phase, stationary-phase, and starved cells of *S. typhimurium* (Table 2). TSA resulted in very bright signals, higher than those reported for multiple monolabeled probes. The signal amplification by the TSA system was up to 11.9-fold greater than that of single monolabeled probes and 2.1- to 3.5-fold greater than that of multiple probes for all experiments (Table 2). One hundred percent of the cells were labeled by both signal amplification procedures. Cells hybridized with a single monolabeled probe were easily detected in culture (log and stationary phase), but the fluorescence signal was not sufficient under starvation conditions to be quantified because it was very close to the fluorescence value of the control (hybridization with no probe). For each period of starvation, all the cells were detected by both amplification systems. The stability of the fluorescence signal observed at different times of the starvation period with the two amplification systems suggests the maintenance of a minimum amount of rRNA in starved cells, as already reported (7).

The efficiency of the same labeling procedures was also investigated with *D. aquamarina* cells starved in artificial seawater. With the TSA system, the fluorescence distribution within the population was very heterogeneous due to the presence of cells with low and high rRNA contents. We analyzed and compared the proportions of labeled cells for an 80-day starvation period (Table 3). During the starvation period, an increasing fraction of cells was undetectable when hybridization was performed with a monolabeled probe and no cells were detected after 42 days. With both signal amplification systems, the whole population was labeled after 80 days of starvation, including a significant fraction of nonculturable cells. The detection of *D. aquamarina* cells under starvation conditions clearly indicates that starved bacteria could be labeled by both amplification systems even when they were nonculturable. On the other hand, a large fraction of culturable cells was not detected with a single monolabeled probe, sug-

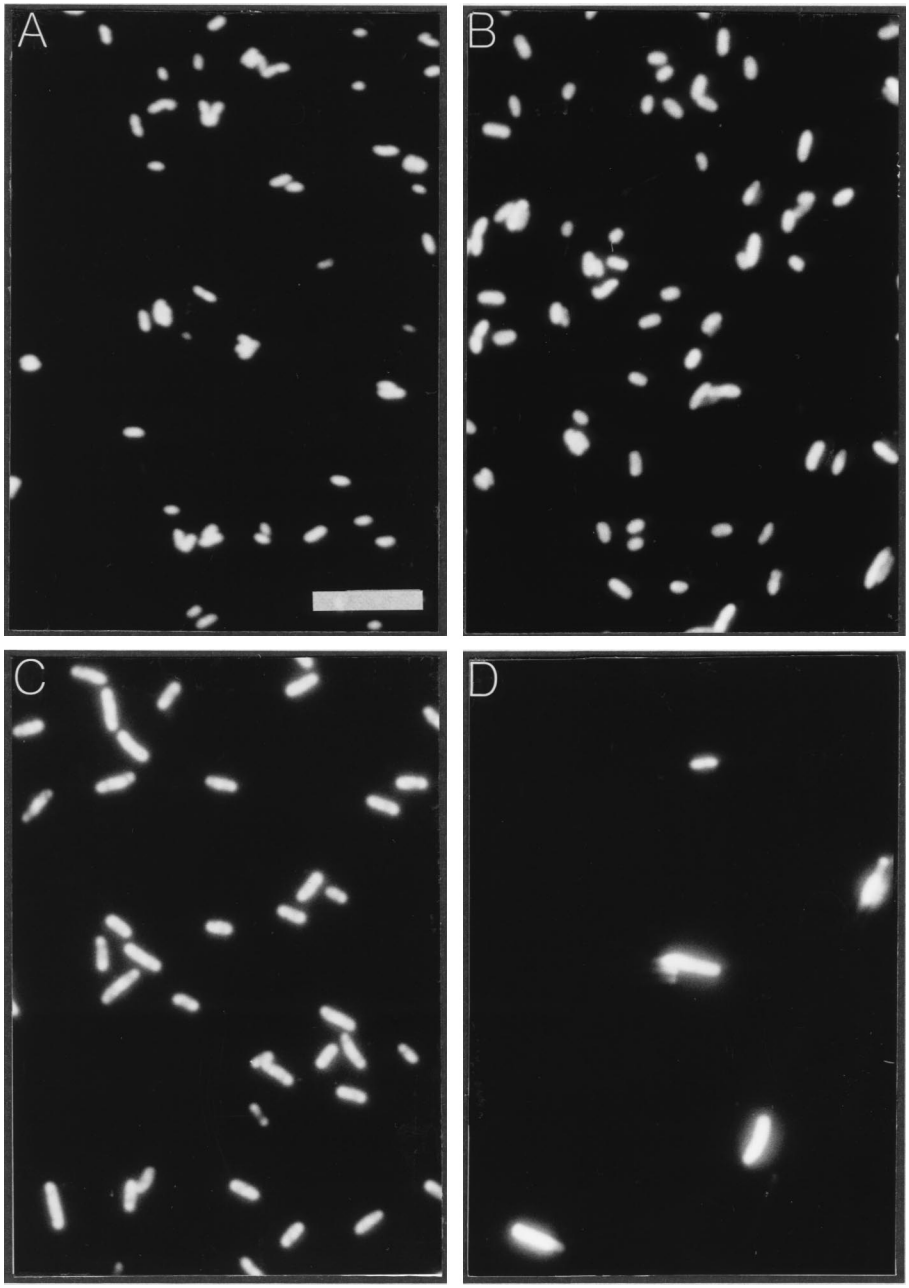

FIG. 1. Epifluorescence micrographs of *P. diminuta* (A), *C. violaceum* (B), *S. typhimurium* (C), and *B. subtilis* (D) stationary-phase cells hybridized with the *Bacteria*-specific biotinylated probe and the TSA system. Magnification, ×1,000. The bar in panel A is 10 μm and applies to panels B, C, and D.

gesting that an amplification procedure is also required for the detection of viable marine bacteria with a low rRNA content.

When applied to the detection of marine bacteria in water samples from the northern Adriatic sea (Table 3), hybridizations with one monolabeled probe resulted in a very low proportion of labeled cells (27 to 30%). This is probably due to the low RNA content of these cells, since both amplification systems resulted in a significant increase in the labeled cell fraction. The use of multiple probes resulted in a large increase of fluorescent signal, and the number of labeled cells represented up to 48% of the whole community. Similar results were reported by Lee and Kemp (10) for seawater samples from coastal areas. When hybridized by the TSA system, the proportion of labeled cells with a single rRNA sequence increased to 78% and yielded heterogeneous fluorescent signals. This heterogeneity probably results from differences in permeabilization efficiency (gram-positive strains have a lower permeabilization efficiency) since fluorescence signals were much more homogeneous when the cells were labeled with multiple probes. Unlabeled cells can be the result of some permeabilization problems, the presence of some gram-positive strains, or the existence of cells with total RNA degradation which maintain an entire cellular structure and some DNA detectable by DAPI staining.

A filtration step to concentrate the cells prior to hybridization is sometimes required (6). When the TSA system was

TABLE 2. Relative fluorescence of *S. typhimurium* cells labeled with different probes[a]

Type of sample	Relative fluorescence[b]			B/A ratio[c]	C/A ratio[c]	C/B ratio[c]
	One probe (A)	Five probes (B)	TSA (C)			
Cultures						
Log phase	35 (6.9)	120 (23)	419 (46)	3.4	11.9	3.5
Stationary phase	25 (2.3)	81 (11.6)	177 (81)	3.2	7.1	2.2
Starved cells (days)						
0	ND[d]	81 (14)	212 (86)	ND	ND	2.6
3	ND	77 (14)	198 (63)	ND	ND	2.6
7	ND	82 (13)	195 (54)	ND	ND	2.4
12	ND	87 (12)	184 (38)	ND	ND	2.1
19	ND	78 (14)	184 (59)	ND	ND	2.4

[a] Cells were from log- or stationary-phase cultures or were taken after different times of starvation in artificial seawater.
[b] For each hybridization procedure, relative fluorescence units were corrected by subtracting the control value (mean fluorescence recorded when hybridization was performed with no probe). Values in parentheses are standard deviations calculated on the mean value of triplicate slides.
[c] Ratio of fluorescence intensity of one system to that of another.
[d] ND, not detectable.

applied to *S. typhimurium* cells, the average fluorescence per cell was 121.9 ± 12.3 relative units on slides and 75.6 ± 32.4 relative units on membranes (data not shown). Our attempts to use flow cytometry with cells in suspension were also unsuccessful due to cellular lysis after permeabilization and, inversely, to low fluorescence signals when the permeabilization treatment was limited (data not shown). The tyramide deposition inside permeabilized cells may be more sensitive to the filtration pressure (even when low pressure is applied) and/or to centrifugation than covalently linked monolabeled probes are.

With slides, the TSA system was successfully applied to different phylogenetic groups and demonstrated as much as a 11.9-fold increase in fluorescence signals when compared to that of monolabeled probes. Recently, Yamaguchi et al. (14) have proposed a new amplification system based on HNPP (2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate) and fast red TR resulting in signals four to eight times more intense. Their labeling procedure was performed on gelatin-coated slides, and the amplification of fluorescence intensity was more significant with stationary-phase cells. In our study, the best amplification results were observed with log-phase cells.

To our knowledge, the sensitivity of the TSA technique is the highest of the signal amplification systems using single rRNA sequences. The high fluorescence signal amplification allows the detection of starved nonculturable cells. The TSA technique may be applied to the detection of specific bacterial groups or species for which cell permeabilization is readily achieved.

TABLE 3. Percentages of labeled cells after hybridization with different probes and signal amplification procedures[a]

Type of sample[b]	% Labeled cells[c] after hybridization with:			CFU (%)[d]
	One probe	Five probes	TSA	
Starved cells (days)				
10	38.8	100	100	96
42	ND[e]	100	100	81.9
80	ND	100	100	76.2
Natural samples[f]				
Station 1	27.9 (6.1)	46.8 (5.5)	62.5 (9.2)	0.65
Station 2	28.3 (7.3)	41.6 (4.7)	64.9 (3.0)	1.61
Station 3	27.7 (8.4)	48.4 (8.3)	69.3 (8.4)	0.24
Station 4	29.5 (6.5)	48.2 (6.8)	78.6 (7.2)	0.49

[a] For hybridization with one probe, EUB1-TRITC was used; that with five probes included five different universal and eubacterial TRITC probes. Hybridization with TSA used EUB1-biotin combined with the TSA kit.
[b] Hybridization was applied to starved *D. aquamarina* cells and to natural samples from the northern Adriatic Sea (June 1995).
[c] Percentages are relative to DAPI counts.
[d] Percentages of culturable cells (on marine agar plates).
[e] ND, not detectable.
[f] Samples were taken in situ in the water column (stations 1 and 2) and in sediment traps (stations 3 and 4). Values in parentheses represent standard deviations calculated on the mean values from triplicate slides. For each hybridization procedure, the control (hybridization with no probe) was used to determine the threshold fluorescence values.

We thank Rudolf Amann for fruitful discussions on hybridization techniques and F. Laurent, J.-L. Drocourt, and P. Cornet from Chemunex S.A., Maisons-Alfort, France, for their helpful comments. D. Purdy and C. Fricker of Thames Water Utilities Ltd., Reading, England, are also acknowledged.

This work was partly supported by a grant from Chemunex S.A. and by a grant from the French program JGOFS. The image analyzer was financed by the Conseil Régional Languedoc-Roussillon and the Programme National d'Océanologie Côtière.

REFERENCES

1. **Alfreider, A., J. Pernthaler, R. Amann, B. Sattler, F.-O. Glöckner, A. Wille, and R. Psenner.** 1996. Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization. Appl. Environ. Microbiol. **62:**2138–2144.
2. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, 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.
3. **Amann, R. I., B. Zarda, D. A. Stahl, and K.-H. Schleifer.** 1992. Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. **58:**3007–3011.
4. **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.
5. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligonucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**720–726.
6. **Heidelberg, J. F., K. R. O'Neill, D. Jacobs, and R. R. Colwell.** 1993. Enumeration of *Vibrio vulnificus* on membrane filters with a fluorescently labeled oligonucleotide probe specific for kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **59:**3474–3476.
7. **Kemp, P. F., S. Lee, and J. LaRoche.** 1993. Estimating the growth rate of slowly growing marine bacteria from RNA content. Appl. Environ. Microbiol. **59:**2594–2601.
8. **Lebaron, P., and F. Joux.** 1994. Flow cytometric analysis of the cellular DNA content of *Salmonella typhimurium* and *Alteromonas haloplanktis* during star-

vation and recovery in seawater. Appl. Environ. Microbiol. **60:**4345–4350.
9. **Lee, S., C. Malone, and P. F. Kemp.** 1993. Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Mar. Ecol. Prog. Ser. **101:**193–201.
10. **Lee, S., and P. F. Kemp.** 1994. Single-cell RNA content of natural marine planktonic bacteria measured by hybridization with multiple 16S rRNA-targeted fluorescent probes. Limnol. Oceanogr. **39:**869–879.
11. **Manz, W., U. Szewzik, P. Ericsson, R. I. Amann, K.-H. Schleifer, and T. A. Stenstrom.** 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.
12. **Schönhuber, W., B. Fuchs, S. Juretschko, and R. Amann.** 1997. Improved sensitivity of whole-cell hybridization by the combination of horseradish peroxidase-labeled oligonucleotides and tyramide signal amplification. Appl. Environ. Microbiol. **63:**3268–3273.
13. **Wallner, G., R. I. Amann, and W. Beisker.** 1993. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry **14:**136–143.
14. **Yamaguchi, N., S. Inaoka, K. Tani, T. Kenzaka, and M. Nasu.** 1996. Detection of specific bacterial cells with 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate and fast red TR in situ hybridization. Appl. Environ. Microbiol. **62:**275–278.
15. **Zarda, B., R. I. Amann, G. Wallner, and K.-H. Schleifer.** 1991. Identification of single bacteria cells using digoxigenin-labeled, rRNA-targeted oligonucleotides. J. Gen. Microbiol. **137:**2823–2830.