Comprehensive Analysis of Cell Wall-Permeabilizing Conditions for Highly Sensitive Fluorescence In Situ Hybridization

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The simultaneous detection of different bacterial strains carrying a certain functional gene is absolutely imperative in order to detect bacteria on the basis of functional gene expression products (mRNA) in situ. Functional genes are possessed by bacterial strains with different types of cell walls; therefore, the optimization of conditions for digesting different types of cell walls is significant. In this study, we defined the optimal conditions for permeabilization in eight bacterial strains belonging to different phylogenetic divisions using solutions containing different concentrations of lysozyme and/or achromopeptidase, for conventional fluorescence in situ hybridization (FISH), digoxigenin (DIG)-FISH, and catalyzed reporter deposition (CARD)-FISH with a rRNA-targeting oligonucleotide probe. Most bacterial strains were successfully detected using CARD-FISH with lysozyme 10 mg/ml pretreatment. Additionally, achromopeptidase pretreatment combined with lysozyme pretreatment was a highly effective means of permeabilizing bacterial strains that were unable to be detected using lysozyme pretreatment. However, this additional pretreatment resulted in a loss of cell morphology in some bacterial strains due to excessive permeabilization. Consequently, permeabilizing conditions for applying highly sensitive FISH to the well-defined target bacterial strains used in this study were optimized. The results of this study will contribute to the optimization of permeabilizing conditions, which is one of the most important factors for the successful application of highly sensitive FISH.

Key words: cell wall, functional gene, highly sensitive FISH, large molecule, permeabilization

One of the long-standing goals of environmental microbiology is to simultaneously ascertain the identity, activity, and biogeochemical impact of individual organisms in situ. Fluorescence in situ hybridization (FISH) with a rRNA-targeted oligonucleotide probe is a powerful tool with which to analyze the structure and dynamics of complex microbial communities in a cultivation-independent way^{4,9,26}. However, this method relies on the presence of many target sequences within an individual cell. Therefore, this approach to taxonomic identification cannot be used to detect the presence of single-copy functional genes or their products at the single-cell level. For the detection of bacteria based on a specific metabolic activity, the development

of highly sensitive methods is essential.

To detect bacterial strains on the basis of their activities, several techniques have been developed, such as catalyzed reporter deposition (CARD)-FISH^{5,12,17,18,21,22,25}, digoxigenin (DIG)-FISH²⁷, in situ loop-mediated isothermal amplification (LAMP)¹⁷, in situ PCR^{10,11,24}, in situ reverse transcription⁸, and recognition of individual genes (RING)-FISH²⁸. However, these signal amplification methods require the diffusion of large-molecular-weight molecules such as enzymes, antibodies, or (strept)avidin into fixed whole cells. Therefore, the cell walls must be permeabilized for the probe to access the target molecule, while minimizing the loss of the target molecule and cell morphology. Many strategies have been employed to permeabilize fixed cells using enzymes such as lysozyme, proteinase K, and/or achromopeptidase^{10,22}. However, this step remains problem-

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atic in that each cell wall type requires different conditions to achieve optimal results²¹). This is especially true for Gram-positive bacteria because of their thick cell walls^{20–22}). Generally, identical functional genes are possessed by bacterial strains with different types of cell walls. For example, nitrite reductase genes (*nirK* and *nirS*) have been widely found in bacterial strains belonging to almost all major physiological groups⁷). Therefore, the ability to estimate the differences in conditions required for digesting cell wall types and to define the optimal conditions for certain bacterial strains is of considerable significance. However, there are no reports on comprehensive analyses of permeabilizing conditions for highly sensitive FISH.

The purpose of the present study is to define the optimal conditions for digesting the cell walls of bacterial strains belonging to different phylogenetic divisions using different concentrations of lysozyme and/or achromopeptidase for conventional FISH, DIG-FISH, and CARD-FISH. In this study, rRNA was targeted instead of mRNA because it enables one to evaluate pretreatment conditions for detecting cell types in which rRNA can be easily detected by FISH. Consequently, the optimal conditions for the detection of the majority of bacterial strains were determined. Furthermore, the problems associated with previously developed signal amplification methods are discussed.

Materials and Methods

Bacterial strains, cultivation, and cell fixation

The bacterial strains used in this study and their phylogenetic affiliations are listed in Table 1. To estimate the permeabilizing conditions for various types of cell walls, the bacterial strains were selected from several phylogenetic groups. The media and cultivation conditions were determined in accordance with the respective catalogs of the bacterial strains. The cells were harvested at the end of their

logarithmic growth phase. Samples were fixed in 3% paraformaldehyde for 9 h at 4°C, centrifuged, and washed three times with a PBS solution (137 mM NaCl, 8.10 mM Na₂HPO₄·12H₂O, 2.68 mM KCl, and 1.47 mM KH₂PO₄; pH 7.4). Cells were then resuspended in 50% ethanol in PBS and stored at –20°C until further processing.

Permeabilization

Cell walls were permeabilized according to a method described previously¹⁰) with minor modifications. The samples were resuspended in the PBS solution. A 10-μl aliquot was spotted onto amino alkylsilane-coated slides (Applied Biosystems, Foster City, USA) and dried in an oven at 46°C. The immobilized samples were treated with a lysozyme solution (Wako Chemicals, Osaka, Japan) (0, 1, 3, 7, 10, and 20 mg of lysozyme per ml, 100 mM Tris-HCl [pH 8.0], and 50 mM EDTA) for 1 h at 37°C. As a further analysis, *Flavobacterium columnare*, *Corynebacterium glutamicum*, *Paracoccus denitrificans*, *Alcaligenes faecalis*, and *Pseudomonas stutzeri* were additionally permeabilized with an achromopeptidase solution (Sigma-Aldrich, Steinheim, Germany) (0, 25, 50, 100, and 500 units of achromopeptidase per ml, 10 mM Tris-HCl [pH 8.0], and 10 mM EDTA) for 30 min at 37°C after the lysozyme pretreatment (20 mg of lysozyme per ml), as appropriate. For CARD-FISH, the digested samples were incubated in 0.01 M HCl for 10 min at room temperature to inactivate endogenous peroxidases. Then, the HCl solution was removed by consecutive washes with distilled deionized water (ddH₂O) and the samples were dehydrated sequentially in 50, 80, and 98% ethanol.

In situ hybridization with oligonucleotide probes

The oligonucleotide probe EUB338³⁾ was used in this study. DIG- and tetramethylrhodamine isothiocyanate (TRITC)-labeled oligonucleotides were purchased from

Table 1. List of strains and their phylogenetic affiliations

Strains	Source or strain no.	Phylogenetic affiliation
<i>Paracoccus denitrificans</i>	IFO 16712 ^T	<i>Alphaproteobacteria</i>
<i>Alcaligenes faecalis</i>	IFO 13111 ^T	<i>Betaproteobacteria</i>
<i>Pseudomonas stutzeri</i>	IFO 14165 ^T	<i>Gammaproteobacteria</i>
<i>Cytophaga hutchinsonii</i>	IFO 15051 ^T	<i>Bacteroidetes</i>
<i>Flavobacterium columnare</i>	NBRC 100251 ^T	<i>Bacteroidetes</i>
<i>Enterococcus faecalis</i>	IFO 3971	<i>Firmicutes</i>
<i>Bacillus subtilis</i>	NBRC 13719 ^T	<i>Firmicutes</i>
<i>Corynebacterium glutamicum</i>	NBRC 12153	<i>Actinobacteria</i>

Takara Bio (Ohtsu, Japan) and horseradish peroxidase (HRP)-labeled probes were purchased from Greiner Japan (Tokyo, Japan). TRITC- and DIG-labeled probes were hybridized according to the method of Amann²⁾, which was followed by a stringent washing. HRP-labeled probes were hybridized at 35°C for 2 h to prevent the inactivation of HRP. Then, the slides were washed at 37°C for 20 min and kept moist until the following step.

Signal detection with tetramethylrhodamine-labeled antidigoxigenin antibody

Samples hybridized with DIG-labeled probes were detected using a tetramethylrhodamine-labeled antidigoxigenin Fab fragment (Roche Diagnostics, Mannheim, Germany). First, 10 µl of blocking buffer (5 mg of bovine serum albumin per ml of PBS solution) was spotted onto the samples and incubated for 30 min at room temperature. Then, 10 µl of tetramethylrhodamine-labeled anti-DIG Fab fragment solution (10 µg of Fab fragment per ml of blocking buffer) was spotted onto the samples and incubated for 1 h at 37°C. Next, the slides were rinsed briefly with a few milliliters of a washing buffer (0.5% Triton X-100 in PBS solution) and then immersed three times in 40 ml of the washing buffer for 15 min at room temperature. The washing buffer was removed by rinsing the slides with ddH₂O. Then, the samples were counterstained with a working solution of SYBR Green I (FMC BioProducts, Rockland, USA) for 5 min. For preparation of the working solution, SYBR Green I was diluted with the PBS solution 10,000-fold. After washing, the samples were mounted in FluoroGuard antifade reagent (Bio-Rad, Richmond, USA) for observation under a confocal laser scanning microscope (TCS4D; Leica Lasertechnik, Heidelberg, Germany).

Tyramide signal amplification

The stringently washed slides were rinsed briefly with ddH₂O and equilibrated with TNT buffer (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.3% Triton X-100) for 15 min. Excess buffer was removed without drying the slides. The tetramethylrhodamine-labeled tyramide stock solution was diluted 1:50 with amplification diluent to produce the fluorophore tyramide working solution (Dupont, NEN Research Products, Boston, USA). The samples were incubated in the tetramethylrhodamine-tyramide working solution. After 10 min at room temperature, the slides were rinsed briefly with a few milliliters of TNT buffer and then immersed three times in 40 ml of the same buffer for 5 min at room temperature. The TNT buffer was removed by rinsing the slides with ddH₂O. Then, the samples were counterstained with

SYBR Green I, and mounted in FluoroGuard antifade reagent for observation under the confocal laser scanning microscope.

Intracellular RNA staining with RiboGreen

The samples were stained with RiboGreen to evaluate the RNA content of each cell. RiboGreen (Molecular Probes, Eugene, USA) was diluted with the PBS solution 200-fold. A 10-µl aliquot of the diluted solution was spotted onto the samples and incubated for 3 min at room temperature. Subsequently, the samples were mounted in FluoroGuard antifade reagent and observed under the confocal laser scanning microscope. Signal intensities were analyzed using the public domain image processing and analysis program, NIH image software (version 1.63; the US National Institutes of Health [<http://rsb.info.nih.gov/nih-image/>]). Then, the means and standard deviations were calculated.

Microscopic evaluation and data analysis

After FISH, DIG-FISH, or CARD-FISH, images were recorded using the confocal laser scanning microscope equipped with an Ar-Kr ion laser (488, 568, and 647 nm), and identical scanning parameters were applied for each strain. Parameter settings were such that the brightest cell signals observed did not cause saturation (pixel grey values <255), while low signal intensities were still detectable. To standardize the signal intensities, images of fluorescein isothiocyanate (FITC)-labeled particles (SPHERO™ FITC Particles; Spherotech Inc., Libertyville, USA) were recorded using identical conditions for each strain. The following image analysis was performed using NIH image software. First, all color information in an image was turned into gray scale values of corresponding intensities. Then, all of the SYBR Green I-stained cells in a microscopic field (between 100 and 300 cells) were counted for each sample. In the same microscopic field, the signal intensities of the cells at an excitation wavelength of 568 nm, visualized using FISH, DIG-FISH, or CARD-FISH, were measured, and then the signal of SYBR Green I was subtracted from the measured signals. The revised signal intensities were divided by the signal intensity obtained from FITC-labeled particles; consequently, relative signal intensities were obtained for each sample. FITC-labeled particles generated a weak signal at an excitation wavelength of 568 nm; therefore, a threshold value was determined for the signal intensity of particles (relative signal intensity value = 1.0). The cells with a relative signal intensity above 1.0 were counted, and then, the percentage of positive cells was obtained.

Results

Differences in detectability using lysozyme pretreatment

The fractions of hybridized cells, which were labeled by FISH, DIG-FISH, and CARD-FISH following incubation with lysozyme at concentrations between 0 and 20 mg/ml, were evaluated (Fig. 1).

The tested representatives of the classes *Alpha*-, *Beta*-, and *Gammaproteobacteria* showed the same pattern, with the exception of *Paracoccus denitrificans* (Fig. 1A to 1C). Conventional FISH did not yield sufficient signals with cultures of *Paracoccus denitrificans* unless the lysozyme pretreatment was carried out (Fig. 1A). In contrast, *A. faecalis* and *Pseudomonas stutzeri* showed homogeneous signals without lysozyme pretreatment. In the case of DIG-FISH, more than 96% of cells were detected for each strain with lysozyme pretreatment at 7 mg/ml, and the hybridized cell fraction of only *Paracoccus denitrificans* dramatically decreased at lysozyme concentrations higher than 7 mg/ml (Fig. 1B). Following CARD-FISH without lysozyme pretreatment, only a few cells for each strain were visualized; whereas the hybridized cell fractions of the tested proteobacterial strains reached 100% with lysozyme pretreatment at 3 mg/ml (Fig. 1C).

In the case of the two *Bacteroidetes* species examined, the hybridized cell fraction of *Cytophaga hutchinsonii*

decreased from 46% without lysozyme pretreatment to 3% with lysozyme pretreatment at 20 mg/ml, whereas that of *F. columnare* increased from 3% without lysozyme pretreatment to 67% with lysozyme pretreatment at 20 mg/ml (Fig. 1D, open triangles and open squares). Neither strain was visualized using DIG-FISH (Fig. 1E, open triangles and open squares). *Cytophaga hutchinsonii* could be detected using CARD-FISH with lysozyme pretreatment, whereas *F. columnare* was not detected using this method (Fig. 1F, open triangles and open squares).

It was difficult to detect Gram-positive bacteria using FISH without lysozyme pretreatment. The maximum hybridized cell fractions were obtained by FISH with lysozyme pretreatment at 10 mg/ml for *B. subtilis*, 20 mg/ml for *Corynebacterium glutamicum*, and 10 mg/ml for *E. faecalis* (Fig. 1D, closed squares, closed diamonds, and closed triangles). Most Gram-positive bacteria could not be visualized using DIG-FISH except for *E. faecalis* with lysozyme pretreatment (Fig. 1E, closed squares, closed diamonds, and closed triangles). In contrast, *E. faecalis* and *B. subtilis* could be detected using CARD-FISH with lysozyme pretreatment at 7 mg/ml and 1 mg/ml, respectively. On the other hand, *Corynebacterium glutamicum* was not detected using CARD-FISH even with lysozyme pretreatment (Fig. 1F, closed squares, closed diamonds, and closed triangles). Thus, six of the eight bacterial strains

Fig. 1. Comparison of detection rates of each strain using FISH (A and D), DIG-FISH (B and E), and CARD-FISH (C and F). Panels A to C: detection rates of *Proteobacteria*. Symbols: open circles, *P. denitrificans*; closed circles, *A. faecalis*; open diamonds, *P. stutzeri*. Panels D to F: detection rates of *Bacteroidetes* and Gram-positive bacteria. Symbols: open triangles, *C. hutchinsonii*; open squares, *F. columnare*; closed triangles, *E. faecalis*; closed squares, *B. subtilis*; closed diamonds, *C. glutamicum*.

examined were successfully detected using CARD-FISH with 10 mg/ml of lysozyme pretreatment, suggesting that this pretreatment enables the detection of a relatively large fraction of bacterial strains.

Permeabilization with achromopeptidase

Two bacterial strains, *Corynebacterium glutamicum* and *F. columnare*, could not be detected, even using CARD-FISH. The detection of these strains was attempted following an additional permeabilization step. In the case of *Corynebacterium glutamicum*, lysozyme pretreatment (20 mg/ml) followed by incubation with achromopeptidase at 25 or 50 units/ml (30 min at 37°C) had a pronounced effect on visualization using CARD-FISH (Fig. 2, panel A and B). On the other hand, the probe signal on *A. faecalis* was completely lost using achromopeptidase at above 25 units/ml because of the destruction of the cell due to excessive permeabilization, although this strain was detected using lysozyme pretreatment (Fig. 2, panel C and D). This phenomenon was also observed in the case of the other proteobacterial strains (data not shown). *F. columnare* cells could not be detected using CARD-FISH even with lysozyme and achromopeptidase treatment (data not shown). Higher achromopeptidase concentrations (>50 units/ml) did not increase the fraction of hybridized cells, but rather caused a

visible disintegration of cells (data not shown). Achromopeptidase pretreatment followed by incubation with lysozyme and achromopeptidase pretreatment alone did not increase the fraction of hybridized cells.

Discussion

To detect bacterial strains on the basis of functional gene expression products (mRNA) in situ, the simultaneous detection of different bacterial strains carrying a certain functional gene is absolutely imperative. In this study, we analyzed different conditions for the detection of taxonomically diverse bacterial strains by using a rRNA-targeting oligonucleotide probe, particularly with highly sensitive FISH.

Explanations for why a fluorescent signal cannot be obtained using highly sensitive FISH include: (1) a low penetrating efficiency of the antibody or enzyme such as anti-DIG antibody and HRP; (2) a low rRNA content in each cell; (3) a mismatch of the oligonucleotide probes.

Cases 2 and 3 were inconceivable in this study. As for case 2, all the bacterial strains tested could be visualized using conventional FISH, which indicated that they contained a sufficient amount of the target for highly-sensitive FISH (Fig. 1A and 1D). Furthermore, the intracellular RNA content in each bacterial strain was compared using RiboGreen. As shown in Table 2, the highest signal intensities were obtained from *Cytophaga hutchinsonii* and *Corynebacterium glutamicum* while the lowest signal intensity was obtained from *E. faecalis*. The signal intensities obtained from *Cytophaga hutchinsonii* and *Corynebacterium glutamicum* were nearly 1.6 times the level for *E. faecalis*, indicating that all the bacterial strains tested contained nearly the same level of intracellular RNA. In general, the

Fig. 2. Photomicrograph of CARD-FISH-stained *C. glutamicum* (A and B) and *A. faecalis* (C and D) with lysozyme pretreatment alone (A and C) and achromopeptidase followed by lysozyme pretreatment (B and D). Yellow indicates positive cells, while green shows negative cells. Scale bar, 20 µm.

Table 2. Relative fluorescence signal intensity (%)^a of all examined strains stained with Ribo Green

Strains	% of relative signal intensity
<i>Paracoccus denitrificans</i>	109
<i>Alcaligenes faecalis</i>	127
<i>Pseudomonas stutzeri</i>	116
<i>Cytophaga hutchinsonii</i>	161
<i>Flavobacterium columnare</i>	123
<i>Enterococcus faecalis</i>	100
<i>Bacillus subtilis</i>	124
<i>Corynebacterium glutamicum</i>	161

^a Relative to signal intensity of *E. faecalis*.

RNA content of bacterial cells fluctuates more than the DNA content. Therefore, the variation in signal intensity would represent a variation in intracellular RNA content rather than DNA content although RiboGreen also binds to DNA. As for case 3, using the BLAST program¹⁾, there is no mismatch between the sequence of probe and that of any of the bacterial strains examined (data not shown). Consequently, case 1 is the only explanation of why the fluorescent signal was not visualized following highly sensitive FISH.

As shown in Table 3, we classified the bacterial strains into three types on the basis of detection results obtained from pretreatment with various lysozyme concentrations. The bacterial strains belonging to type 1 were successfully detected using both a conventional FISH procedure and highly sensitive FISH. Hence, it is evident that the oligonucleotide and antibody can easily penetrate the cell walls of these bacterial strains. In contrast, the bacterial strains belonging to type 2 could not be detected using FISH without lysozyme pretreatment. Furthermore, they were not detected using highly sensitive FISH at all. These results suggest that they possess cell walls that do not allow an antibody or enzyme to penetrate. The bacterial strains belonging to type 3 were detected using CARD-FISH, although they were poorly detected using conventional FISH, suggesting that their cell walls were more difficult to penetrate than those belonging to type 1. From these results, the classification of the detection patterns is not closely associated with that of phylogenetic affiliation.

As shown in Fig. 1, the number of bacterial strains detected using CARD-FISH was larger than that detected using DIG-FISH. This is most likely attributable to the cru-

cial difference in signal amplification efficiency between DIG-FISH and CARD-FISH. The amplification efficiency of CARD-FISH is nearly fivefold that of DIG-FISH²¹⁾. Hence, for CARD-FISH, a small number of penetrating probes would enable successful detection. Meanwhile, the hybridized cell fractions of *Cytophaga hutchinsonii* and *B. subtilis* obtained by conventional FISH decreased with 20 mg/ml of lysozyme (Fig. 1D). This is probably caused by the escape of intracellular RNA due to the excessive permeabilization. However, those obtained by CARD-FISH were maintained at the same concentration. The sensitivity of CARD-FISH is twenty times greater than that of conventional FISH²¹⁾; therefore, CARD-FISH could detect the small amount of intracellular RNA in the excessively permeabilized cells. It should be pointed out that it was difficult to quantify the target RNA in cells because the fluorescence signal was saturated by CARD-FISH.

Schönhuber *et al.*²¹⁾ have reported that CARD-FISH was unable to detect Gram-positive bacteria even with lysozyme pretreatment. However, *E. faecalis* and *B. subtilis* (low G+C Gram-positive bacteria) were detected using CARD-FISH with lysozyme pretreatment at 10 and 1 mg/ml, respectively, in our study. This was probably due to the difference in permeabilizing conditions, that is, they carried out cell wall permeabilization with 0.1 mg/ml of lysozyme for 10 min at 0°C. On the other hand, *Corynebacterium glutamicum* (high G+C Gram-positive bacteria) could not be visualized using CARD-FISH with lysozyme pretreatment in our study. This is likely because of their highly specific cell walls. The main component of their cell wall is the mycolyl-arabinogalactan peptidoglycan complex. This mycolic acid region is poor in proteins; only a small amount

Table 3. Classification of the detection types^a

Type	Strain (affiliation ^b)	Method		
		FISH	DIG-FISH	CARD-FISH
I	<i>Alcaligenes faecalis</i> (β)	++	++	(++)
	<i>Pseudomonas stutzeri</i> (γ)	++	++	(++)
II	<i>Corynebacterium glutamicum</i> (HGC)	(++)	–	–
	<i>Flavobacterium columnare</i> (CFB)	(+)	–	–
III	<i>Bacillus subtilis</i> (LGC)	(+)	–	(++)
	<i>Cytophaga hutchinsonii</i> (CFB)	+	–	(++)
	<i>Enterococcus faecalis</i> (LGC)	(++)	(++)	(++)
	<i>Paracoccus denitrificans</i> (α)	(+)	(++)	(++)

^a –, no signals; +, a proportion of all cells (10–80%) hybridized; ++, almost all cells (>80%) hybridized; sign in brackets, with lysozyme pretreatment.

^b α, Alphaproteobacteria; β, Betaproteobacteria; γ, Gammaproteobacteria; CFB, Bacteroidetes; LGC, Firmicutes (low G+C gram-positive bacteria); HGC, Actinobacteria (high G+C gram-positive bacteria).

of a pore-forming protein with weak activity has been detected, causing the low penetrability of hydrophilic substances²³). This hydrophobicity caused difficulties associated with the penetration of a hydrophilic substance such as the anti-DIG antibody or tyramide. To improve the permeabilization of mycolic acid-rich bacteria, many approaches have been examined: including reducing the fixation time¹⁹), acid hydrolysis¹⁵), and ethanol fixation²⁰). Sekar *et al.*²²) demonstrated that incubation with 10 mg/ml of lysozyme followed by 60 units/ml of achromopeptidase can successfully permeabilize the cell walls of this group of bacterial strains for subsequent CARD-FISH. We were also able to detect *Corynebacterium glutamicum* by using this procedure (Fig. 2, panel A and B); however, this procedure resulted in the loss of the target molecule and cell morphology for other bacterial strains (Fig. 2, panel C and D). Lan *et al.*¹³) reported that microwave oven heating can be used as a substitute for enzymatic cell wall digestion for a wide variety of eukaryotic cells, and the hybridization signal obtained after microwave pretreatment was very uniform in six different tissues. We tried to apply this pretreatment to *Corynebacterium glutamicum*; however, we were unable to obtain signals using CARD-FISH (data not shown). This is most likely due to the differences in cell structure between prokaryotic cells and eukaryotic cells. These results imply that it is difficult to find a universal method for permeabilizing all kinds of cell walls.

Surprisingly, for most bacterial strains, except for the proteobacterial strains *A. faecalis* and *Pseudomonas stutzeri*, conventional FISH without lysozyme pretreatment could not generate a sufficient hybridization signal (Fig. 1A and 1D). These results suggest that well-suited cell wall permeabilization techniques are required for quantitative FISH, especially when *Bacteroidetes* and/or Gram-positive bacteria are targeted. Luxmy *et al.*¹⁴) have described that pretreatment with 10 mg/ml of lysozyme was essential to increase the permeability of the oligonucleotide probes for *Microthrix parvicella* (high G+C Gram-positive bacteria). This result agrees with our results because high G+C Gram-positive bacteria (*Corynebacterium glutamicum*) were sufficiently detected with 10 mg/ml of lysozyme. It must be noted that all images were obtained under identical conditions in this study; therefore, these results might be improved if photomicroscopic conditions such as laser voltage settings were optimized for each strain. Meanwhile, we must take into account the possibility of underestimating the number of positive cells when using universal probes such as the eubacterial probe EUB338. Bouvier and Giorgio⁶) have reported that for cases in which the results are not

affected by the contribution of *Archea*, the percentage of cells detected using EUB338 varies from 1 to 100% of the total bacterial count in different published reports, with an average of 56%. Provided that bacterial strains that have an unusual cell wall structure, such as a mycolic acid-rich cell wall, are not dominant species, a more refined quantification could be carried out using CARD-FISH with 10 mg/ml lysozyme pretreatment.

In this study, most bacterial strains were successfully detected using CARD-FISH with 10 mg/ml lysozyme pretreatment. Additionally, achromopeptidase pretreatment was a highly effective means of permeabilizing the bacterial strains that were unable to be detected with lysozyme pretreatment, while it was considered impossible to visualize all bacterial strains used in this study. If highly sensitive FISH methods are applied to well-defined target bacterial strains, permeabilizing conditions should be optimized according to our results. It should be noted that, for environmental samples, there are many variable factors such as the type of ecosystem, bacterial growth rate, and growth phase. Nevertheless, our results will contribute to the optimization of permeabilizing conditions, which is one of the most important factors for the successful application of highly sensitive FISH. It is anticipated that in future studies, a method that is not affected by cell wall structures will be developed, and consequently, this method will enable us to universalize the highly sensitive FISH procedure.

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