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Title:

Detection of single-copy functional genes in prokaryotic cells by two-pass TSA-FISH with polynucleotide probes

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Abstract

In situ detection of functional genes with single-cell resolution is currently of interest to microbiologists. Here, we developed a two-pass tyramide signal amplification (TSA)–fluorescence *in situ* hybridization (FISH) protocol with PCR-derived polynucleotide probes for the detection of single-copy genes in prokaryotic cells. The *mcrA* gene and the *apsA* gene in methanogens and sulfate-reducing bacteria, respectively, were targeted. The protocol showed bright fluorescence with a good signal-to-noise ratio and achieved a high efficiency of detection (>98%). The discrimination threshold was approximately 82–89% sequence identity. Microorganisms possessing the *mcrA* or *apsA* gene in anaerobic sludge samples were successfully detected by two-pass TSA-FISH with polynucleotide probes. The developed protocol is useful for identifying single microbial cells based on functional gene sequences.

Keywords

functional genes / *in situ* whole-cell detection / polynucleotide probes / two-pass TSA-FISH

1. Introduction

In situ detection of functional genes is receiving considerable attention in microbiology as gene information is being accumulated to understand microbial physiology via metagenomic and single-cell genomic studies (Amann and Fuchs, 2008; Handelsman, 2004; Ishoey et al., 2008). However, low copy numbers of functional genes encoded on plasmid or chromosomal DNA make these studies difficult (Hoshino and Schramm, 2010; Moraru et al., 2010; Sambrook and Russell, 2001). For example, the detection limit of tyramide signal amplification (TSA)–fluorescence *in situ* hybridization (FISH) with oligonucleotide probes, which is a promising and sensitive technique, is 54 ± 7 copies per cell (practical detection limit) (Hoshino et al., 2008), and its sensitivity is still far from being sufficient for the detection of functional genes. Therefore, a sensitive technique capable of detecting functional genes is needed.

Several such techniques have been reported, and they are categorized into two groups (Moraru et al., 2010). One uses target nucleic acid amplification (Hodson et al., 1995; Hoshino and Schramm, 2010; Kenzaka et al., 2005; Maruyama et al., 2003; Maruyama et al., 2005), and the other group does not. The latter includes recognition of individual genes (RING)-FISH (Pratscher et al., 2009; Zwirglmaier et al., 2004), TSA-FISH (Moraru et al., 2010), and two-pass TSA-FISH (Kawakami et al., 2010). RING-FISH employs transcript polynucleotide probes and increases sensitivity by the formation of multiply labeled probe networks during hybridization. However, control of specificity and sensitivity can be difficult. TSA-FISH (Moraru et al., 2010) and two-pass TSA-FISH (Kawakami et al., 2010) have recently been introduced for gene FISH. Signal amplification by TSA is based on the deposition of a large number of tyramides via the enzymatic catalysis of horseradish peroxidase (HRP). The two-pass TSA technique repeats the TSA reaction combined with an immunological reaction (Kubota et al., 2006). The major problem of gene FISH using TSA

technique is its low detection efficiency (Kawakami et al., 2010; Moraru et al., 2010). Two-pass TSA-FISH with oligonucleotide probes showed only approximately 15% of the detection efficiency, although this technique is able to distinguish single-base mismatches (Kawakami et al., 2010). TSA-FISH with polynucleotide probes showed higher detection efficiency, but it still only reached approximately 40% (Moraru et al., 2010). Therefore, the development of a gene FISH technique with high detection efficiency is important to accelerate our understanding of microbiology.

Here, we describe the development of a protocol for single-copy gene detection in microorganisms with a high detection efficiency (>98%) using a two-pass TSA technique and polynucleotide probes. Two single-copy genes, one encoding the alpha subunit of the methyl-coenzyme M reductase (*mcrA*) gene in the methanogen *Methanococcus maripaludis* and the other encoding the alpha subunit of the adenosine-5'-phosphosulfate kinase (*apsA*) gene in the sulfate-reducing bacterium *Desulfobulbus propionicus*, were selected as model targets because the genes are involved in the natural nutrient cycles of carbon and sulfur, respectively. PCR-derived polynucleotide probes of different lengths were constructed and tested for their sensitivities and specificities using closely related organisms. The applicability of the technique to environmental samples was also demonstrated.

2. Materials and methods

2.1 Sample preparation

The strains used in this study were *Methanococcus maripaludis* (JCM13030), *Methanococcus vannielii* (JCM13029), *Methanothermococcus okinawensis* (JCM11175), *Methanoculleus chikugoensis (*JCM10825), *Desulfobulbus propionicus* (DSM6523), *Desulfobulbus elongatus* (DSM2908), *Desulfovibrio vulgaris* (DSM644), and *Escherichia coli* (ATCC700926). The cells were cultivated in the medium recommended by their

respective culture collections. *E. coli* was cultivated in Luria-Bertani medium at 37°C. Granular sludge was collected from mesophilic upflow anaerobic sludge blanket (UASB) reactors that treat artificial wastewater (Sekiguchi et al., 1998) or industrial wastewater containing sulfate. The samples were fixed in a 4% paraformaldehyde solution for 12 h at 4°C and stored in ethanol/phosphate-buffered saline [PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4 (pH 7.2)] at –20°C.

2.2 Preparation of polynucleotide probes

Polynucleotide probes were generated by PCR with the simultaneous incorporation of dinitrophenyl (DNP)-labeled dUTP (DNP-11-dUTP). Extracted DNA from *Mcc. maripaludis, Dsb. propionicus* or the granular sludge samples was used as a template. Three different primer sets for each gene were used to generate probes of different lengths (Table 1). For application to granular sludge samples, the ME3f/ME2r pair for the *mcrA* gene or the APS7f/APS8r pair for the *apsA* gene was used (Table 1).

PCR was performed with the following PCR mixture: 0.025 U/μl *Taq* polymerase; 0.5 pmol/μl of each primer; 1× PCR buffer; 200 μM dATP, dGTP, and dCTP (Applied Biosystems, Tokyo, Japan); 10-70 μM DNP-11-dUTP (PerkinElmer, Tokyo, Japan); 130–190 μM dTTP (Applied Biosystems); and 1.5–4 mM Mg^{2+} solution (TaKaRa, Tokyo, Japan). The appropriate amount of extracted genomic DNA was also added. For high DNP labeling and probe yield, the ratio of DNP-11-dUTP/dTTP and Mg^{2+} concentration were adjusted (Table 1). The PCR conditions were as follows: initial denaturation at 95°C for 7 min, followed by 35 cycles of 95°C for 40 s, 55°C for 30 s and 72°C for 3 min. After amplification, the PCR products were purified using a MinElute PCR purification kit (QIAGEN, Tokyo, Japan) and electrophoresed using an Agilent 2100 bioanalyzer with a DNA 1000 kit (Agilent Technology, Tokyo, Japan) to confirm the specificity of amplicons and the incorporation efficiency of the

DNP-11-dUTP. The concentrations of the generated probes were measured by UV absorption spectrometry (NanoDrop, Thermo Fisher Scientific).

2.3 TSA-FISH

TSA-FISH was performed in accordance with previous reports, with some modifications (Kawakami et al., 2010; Kubota et al., 2006; Kubota et al., 2008; Pernthaler and Amann, 2004). The samples were first dispersed by ultrasonication and then embedded in low melting point agarose in each well of a 10-well glass slide (Matsunami, Osaka, Japan), as described elsewhere (Kubota et al., 2008). Alternatively, samples were first filtered onto a 0.2 μm polycarbonate filter, and the cells were transferred onto slides by placing the filters upside down; however, not all of the cells were transferred to the slides. After drying, the filters were carefully removed, and the samples were dehydrated in 50, 80 and 96% ethanol for 3, 1 and 1 min, respectively. RNase treatment [0.5 mg/ml in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl at 37°C for 30 min] was performed to digest the RNA in cells. After the RNA digestion, the slides were washed in TNT buffer [100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.3% Tween 20] for 10 min and then in ultra-pure water for 1 min and dehydrated in 96% ethanol for 1 min. Pure cultures, except for the methanogens, were treated with lysozyme [1 mg/ml in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0)] at 37°C for 30 min (Pernthaler et al. 2002). For pure methanogen cultures, only *Mb. bryantii* was treated with recombinant pseudomurein endopeptidase (PeiW), as described elsewhere (Kubota et al., 2008; Nakamura et al., 2006). Granular samples were treated with lysozyme (the experiments for the detection of *apsA*) or PeiW (the experiments for the detection of *mcrA*).

The samples were covered with 20 μl of hybridization buffer containing 1× SSC [15 mM sodium citrate plus 150 mM sodium chloride (pH 7.5)], formamide [varying from 0 to 80% (vol/vol)], 10% (wt/vol) dextran sulfate, 1% (wt/vol) blocking reagent (Roche

Diagnostics, Mannheim, Germany; wt/vol), 0.01% (wt/vol) sodium dodecyl sulfate (SDS), 1× Denhardt's solution (Sigma-Aldrich, Steinheim, Germany), and 0.2 mg/ml sheared salmon sperm DNA (Ambion, Austin, TX). The formamide concentration was experimentally determined for each probe to achieve higher specificity and the maximum detection rate. The slides were placed in a chamber humidified with 1× SSC and formamide (the same concentrations as in the hybridization buffer) and incubated for 30 min at 46°C. Next, the hybridization buffer was replaced with 20 µl of fresh hybridization buffer containing 2.5 ng of probe (125 pg/µl). To denature the probes and chromosomes, the slides were incubated at 95°C for 20 min, and the probes were then hybridized overnight at 46°C in the humidified chamber. To remove excess probes, the slides were immersed in washing buffer 1 [1× SSC and formamide (the same concentrations as in the hybridization buffer), pH 7.5] for two times for 30 min each at 48°C and in washing buffer 2 [0.1× SSC, 0.01% SDS (pH 7.5)] for two times for 15 min each at 48°C.

For the immunochemical reaction, the slides were first immersed in TNT buffer for 10 min at room temperature. After removing excess buffer, BB buffer [1% blocking reagent (Roche), 1% BSA (Sigma Aldrich) in PBS] was applied to each well, and the slides were incubated for 1 h at room temperature. Subsequently, a mixture of 1 part anti-DNP-HRP (PerkinElmer) and 100 parts BB buffer was replaced with BB buffer in each well and incubated for 30 min at room temperature. Two washes in TNT buffer for 15 min each at room temperature with mild agitation were carried out after incubation.

For tyramide signal amplification, freshly prepared tyramide-Cy3 working solution [1 part tyramide mixed with 50 parts amplification buffer (10% dextran sulfate and 0.1% blocking reagent [Roche] in amplification diluent [PerkinElmer])] was applied to each well, and the slides were incubated for 15 min at 37°C. Afterward, the slides were immersed in TNT buffer two times for 15 min at 48°C, ultra-pure water for 1 min at room temperature, and

96% ethanol for 1 min and finally air-dried for microscopic observation.

2.4 Two-pass TSA-FISH

Two-pass TSA-FISH was performed based on previously described protocols (Kawakami et al., 2010, Kubota et al., 2006). The experimental procedure prior to the TSA reaction was the same as for TSA-FISH. However, the first amplification was performed with tyramide-DNP (PerkinElmer) instead of tyramide-Cy3. Following two washes in TNT buffer for 15 min at 48°C, a second immunochemical reaction was performed as described above. After two washes in TNT buffer for 15 min each at room temperature with mild agitation, a second TSA reaction with tyramide-Cy3 and subsequent washing were performed as described above.

2.5 Microscopic evaluation

The samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) to visualize and count the microbes. For microscopic observation and digital photography, an epifluorescence microscope (BX50F, Olympus, Tokyo, Japan) equipped with a color CCD camera (DP70, Olympus) was employed. To observe Cy3 fluorescence, a U-N41007 filter set (Chroma Technology Corp., Rockingham, VT) was used. Approximately 500 DAPI-stained cells were counted and used to determine the percent of two-pass TSA-FISH-positive cells out of DAPI-stained cells.

2.6 Sequence comparison and deposition

Sequence identity among the strains was calculated using the following data: BX950229 (*Mcc. maripaludis*), M16893 (*Mcc. vannielii*), AB353229 (*Mtc. okinawensis*), AB300779 (*Mtl. Chikugoensis*), AF418146 (*Dsb. elongatus*), Z69372 (*Dsv. vulgaris*). As

the *apsA* gene of *Dsb. propionicus* (DSM6523) was not found in the database, we sequenced and deposited it into a public database (accession number: AB638348).

3. Results and discussion

3.1 Generation of polynucleotide probes

To generate polynucleotide probes with high labeling efficiency and sufficient yield, the ratio of DNP-11-dUTP to dTTP and the concentration of Mg^{2+} in the PCR mixture were optimized. When the ratio of DNP-11-dUTP to dTTP was higher, a higher labeling efficiency, but lower yield, was observed (see supplementary Fig. S1). When a higher concentration of Mg^{2+} was included in the mixture, a higher yield was obtained. However, nonspecific amplification was also observed (data not shown). PCR conditions were optimized to amplify specifically and to generate more than 200 ng of probe (corresponding to 80 samples) from 50 µl of PCR mixture. A rough estimation of the labeling efficiency by spectrophotometry revealed that approximately every 20 bases contained one DNP-11-dUTP. The optimized parameters for each primer set are shown in Table 1.

3.2 Detection of chromosome-encoded genes in prokaryotic cells

In situ hybridization of the *mcrA* gene, a single-copy gene on the *Mcc. maripaludis* chromosome, was performed using the mcr-757 probe. Positive signals were obtained from *Mcc. maripaludis* with a high detection rate (>98%) by TSA-FISH (Fig. 1A). Some cells showed signal throughout the whole cell, and others showed spotty signals in the cells. After two-pass TSA-FISH, the signal intensity was significantly increased, and signals were obtained from whole cells (Fig. 1B). To verify the detection efficiency with shorter probes, the mcr-463 and mcr-151 probes were employed. The mcr-463 probe gave similar results as the mcr-757 probe. However, when the mcr-151 probe was used, a lower detection rate and

spotty signals were obtained even with two-pass TSA-FISH (Table 2). Next, the *apsA* gene was detected on the *Dsb. propionicus* chromosome using the aps-820 probe. Unlike the *mcrA* gene in the methanogens, most of the obtained signals were spotty in cells, and the detection rate was low (approximately 20%) with TSA-FISH (Fig. 1C). The detection rate was significantly increased (up to more than 98%) with two-pass TSA-FISH (Fig. 1D). Fluorescence was detected from whole cells or in halo-like patterns. As with the *mcrA* gene, shorter probes (aps-501 and aps-135) were tested. The detection rate decreased when the aps-501 probe was used, and a much lower detection rate was obtained when the aps-135 probe was used (Table 2). A low detection rate of the *apsA* gene in the sulfate-reducing bacterium was attributable to insufficient sensitivity of TSA-FISH, not probe hybridization, as we were able to achieve a high detection rate with two-pass TSA-FISH. Moraru et al. (2010) also reported moderate detection efficiency (approximately 40%) by TSA-FISH with 351 bp polynucleotide probes. Therefore, the use of longer probes and a highly sensitive method like two-pass TSA-FISH is important to achieve high detection efficiency. The detection rates were decreased to less than 1% under increased stringent hybridization conditions or after DNase treatment, indicating that the detection of genes on chromosomes was successful (data not shown). Note that nonspecific signals from glass slides were observed during the development of the protocol. These nonspecific signals were mostly due to nonspecific binding of the antibody to the glass surface and could be minimized by adapting and modifying the protocol described in an earlier paper (Kawakami et al., 2010). Furthermore, strong fluorescent signals achieved by two-pass TSA-FISH with polynucleotide probes resulted in an improved signal-to-noise ratio and clear discrimination from nonspecific signals.

3.3 Specificity of two-pass TSA-FISH with polynucleotide probes

The specificity of the technique was investigated using reference organisms and various probe lengths. Three *mcrA* genes from *Mcc. vannielii* (approximately 93% sequence identity to the generated probes), *Mtc. okinawensis* (approximately 82% identity), and *Mcl. chikugoensis* (approximately 59% identity) and two *apsA* genes from *Dsb. elongates* (approximately 90% sequence identity to the generated probes), and *Dsv. vulgaris* (approximately 67% identity) were used (Table 2). Strains with a sequence identity higher than 90% could not be discriminated: the detection rates were less than 20% in *Mcc. vannielii* but more than 50% in the *Dsb. elongatus*. Strains with a sequence identity less than 82% were successfully differentiated. This threshold seemed to be independent of probe length. Thus, the specificity of our technique was between 82 and 89% sequence identity, which is higher than previously reported values for transcript polynucleotide probes [70–76% (Fichtl, 2005) or 78–85% (Ludwig et al., 1994) sequence identity].

3.4 Comparison of polynucleotide probes and oligonucleotide probes for gene detection by two-pass TSA-FISH

We used polynucleotide probes for gene detection in this study and oligonucleotide probes in our previous study (Kawakami et al., 2010). In both cases, single-copy genes were successfully detected, but both showed differences in terms of specificity and detection efficiency. Oligonucleotide probes gives higher specificity than polynucleotide probes, and single-base mismatch discrimination is possible with oligonucleotide probes (Kawakami et al., 2010). Therefore, it appears that oligonucleotide probes can be used for very highly specific cell identification. Furthermore, two-pass TSA-FISH with oligonucleotide probes also can be used for comprehensive identification by targeting gene sequences of conserved regions. Nevertheless, one concern with oligonucleotide probes is low detection efficiency (discussed

below). On the contrary, the specificity of polynucleotide probes is lower than that of oligonucleotide probes. The threshold for discrimination was determined to be between 82 and 89% in this study, and it ranges from 70% to 85% in other studies as described above. Sequence identities among similar protein-coding genes are usually not very high [e.g., in the case of the *mcrA* gene, the nucleotide sequence identity within a genus is approximately 89% (Steinberg and Regan, 2008)]. Therefore, when the discrimination of alleles is not strictly required, the use of polynucleotide probes is also useful for gene detection.

When focusing on detection efficiency, a large fraction of cells (>98%) can be detected by the protocol developed in this study, but the detection efficiency of two-pass TSA-FISH with oligonucleotide probes is only approximately 15% when a single-copy gene is targeted (Kawakami et al., 2010). By comparing these two results, we conclude that probe length is likely key for obtaining higher detection efficiency. The different melting behaviors between oligonucleotides and polynucleotides are likely the reason for these differences. The melting temperature (Tm) of oligonucleotides is the point at which half the oligomers are double-stranded, whereas the Tm of polynucleotides is the point at which half of the base pairs are dissociated (Tijssen, 1993). Therefore, it is likely that not all target genes in each single cell are hybridized with the oligonucleotide probes under single-base mismatch–discriminable stringent conditions. In contrast, polynucleotides still hybridize with target molecules under stringent conditions. Thus, higher detection efficiency can be achieved with polynucleotide probes. This theory also agrees with data from other studies. *In situ* rolling circle amplification (RCA) followed by FISH showed low detection efficiency (up to 15%) for single-copy gene detection (Hoshino and Schramm, 2010). In the *in situ* RCA protocol, hybridization of two short sequences in padlock probes is essential for circularization of the probes. The hybridization behavior of two short sequences in padlock probes can be considered similar to that of oligonucleotides; therefore, detection efficiency

might be low. On the contrary, RING-FISH employs multiply labeled transcript polynucleotide probes and achieves high detection efficiency (Pratscher et al., 2009). Therefore, polynucleotide probes are superior to oligonucleotide probes in terms of detection efficiency.

3.5 Application of the protocol to anaerobic sludge samples

An artificial mixture of morphologically distinguishable pure cultures, *Mcc. maripaludis* (target microorganism) and *E. coli* (nontarget microorganism), was prepared to check the specificity of the method. Two-pass TSA-FISH with *Mcc. maripaludis*–targeted probes showed a few nonspecific signals with *E. coli* cells near *Mcc. maripaludis* cells (supplementary Fig. S2). We found that these nonspecific signals were not observed from other cells, e.g., cell aggregates without target cells or cells far from target cells. After the appropriate cell dispersion, nonspecific signals were significantly decreased (<1%). This result indicates that an appropriate cell dispersion procedure should be included in the protocol for specific detection in environmental samples. The specificity of the method was further evaluated using a paraformaldehyde-fixed granular sludge sample spiked with *Mcc. maripaludis* cells. Specific signals were obtained from almost all morphologically distinguishable *Mcc. maripaludis*–like cocci (Fig. 2A), indicating that specific detection and high detection efficiency are possible in complex microbial communities.

Gene FISH using our approach for environmental samples was conducted in two different cases. Two granular sludge samples, treated artificial wastewater or industrial wastewater containing sulfate, were employed. In this study, DNA extracted from each sample was used as a template for probe generation. The ME3f/ME2r (*mcrA*) or APS7f/APS8r (*apsA*) primer set was used to generate probes, and the formamide concentrations for the *mcrA*- and *apsA*-targeted probes were 40% and 60%, respectively.

The cloning analysis revealed that there were *Methanolinea*-relatives, *Methanobacterium*, and *Methanosaeta* in the granular sludge–treated artificial wastewater, and several sulfate reducers close to *Desulfovibrio* in the sludge-treated industrial wastewater containing sulfate (Supplementary Tables 1, 2). The PeiW and lysozyme treatments were performed before hybridizations to improve permeability (Kubota et al., 2008, Pernthaler et al., 2002). Strong signals from a variety of cells were obtained after two-pass TSA-FISH (Figs. 2B, C). To validate our protocol, we counted cells detected by two-pass TSA-FISH and calculated a detection rate. Then, we further compared the detection rate with a detection rate determined by rRNA FISH because dual staining with rRNA FISH and gene FISH by the two-pass TSA technique is still in the development stage. The detection rate of methanogens by two-pass TSA-FISH with the *mcrA*-targeted probes in the sample treated artificial wastewater was 27.5 ± 1.7%. The population of *Archaea* detected by rRNA FISH with the ARC915 probe is 34.3 ± 3.3% (Kubota et al., 2008). Likewise, the detection rate of sulfate reducers by two-pass TSA-FISH with the *apsA*-targeted probes was 20.3 ± 2.2% and that by rRNA FISH with the SRB385 probe was 24.5 ± 1.1%. In both cases, two-pass TSA-FISH showed a lower detection rate, but this may be due to differences in the probe used for detection. For example, the primer set used does not cover some alleles of the *mcrA* gene in *Methanosaeta*, which is one of major methanogens in the sample (Kubota et al., 2008) but was not very prevalent in the *mcrA* gene clone library. SRB385 matches to the most *Desulfovibrionales* but also other groups of bacteria (Loy et al., 2007). Selection of a primer set for probe generation is always an issue in gene FISH. These quantification data demonstrate the applicability of this technique to environmental samples. The use of the developed gene FISH protocol in combination with recently developed methods (e.g., cell sorting or laser capture microdissection followed by metagenomic analysis and isotope probing coupled with secondary ion mass spectrometry analysis) will provide new insight into

our understanding of environmental microorganisms.

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Figure legends

Fig. 1. Detection of the *mcrA* gene (A & B) or *apsA* gene (C & D) by TSA-FISH (A & C) or two-pass TSA-FISH (B & D). A & B: a pure culture of *Mcc. maripaludis*. C & D: a pure culture of *Dsb. propionicus*. Photomicrographs of phase contrast (left) and epifluorescence (right) showing identical fields. The exposure times are indicated at the upper right of the epifluorescent micrographs. Scale bar represents 5 μm.

Fig. 2. Two-pass TSA-FISH for the detection of the *mcrA* gene or *apsA* gene. A: Detection of the *mcrA* gene of *Mcc. maripaludis* in a *Mcc. maripaludis*-spiked granular sludge sample. B: Detection of the *mcrA* gene in a granular sludge sample taken from a UASB reactor treating artificial wastewater. C: Detection of the *apsA* gene in a granular sludge sample taken from a UASB reactor treating industrial wastewater containing sulfate. Probes were generated from the *Mcc. maripaludis* genome DNA (A) or DNA extracted from each sample (B & C). Photomicrographs of phase contrast (left), DAPI-stained cells (center) and epifluorescence (right) showing identical fields. Scale bar represents 5 μm.

Table1
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Table 1 Primers used for probe generation in this study.

Target gene	Probe name	Length (bp)	Primer name	Sequence (5'-3') [a]	DNP-11-dUTP conc. (μM) [b]	Mg^{2+} conc. (mM) [c]
mcrA gene	mcr-757	757	ME1f [d]	GCM ATG CAR ATH GGW ATG TC	60	3.5
			ME2r [d]	TCA TKG CRT AGT TDG GRT AGT		
	mcr-463	463	ME3f [d]	GGT GGH GTM GGW TTC ACA CA	50	3.5
			ME2r	TCA TKG CRT AGT TDG GRT AGT		
	mcr-151	151	MES2_150 [e]	CAA ATC TTA CAC AAA GAA TAC CAC	60	4
			ME2r	TCA TKG CRT AGT TDG GRT AGT		
apsA gene	aps-960	960	APS7f [f]	GGG YCT KTC CGC YAT CAA YAC	50	3.5
			APS8r [f]	GCA CAT GTC GAG GAA GTC TTC		
	aps-820	820	RH3 [g]	CTG TTY GAR GAG TGG GG	60	4
			APS8r	GCA CAT GTC GAG GAA GTC TTC		
	aps-501	501	RH3	CTG TTY GAR GAG TGG GG	50	3.5
			APS-RV [h]	GGG CCG TAW CCG TCY TTG AA		
	aps-135	135	RH3	CTG TTY GAR GAG TGG GG	60	4
			RH2 [g]	CCG TTG ATC ATG ATC TGC CA		

[a] M; A or C, R; A or G, H; not G, W; A or T, K; G or T, D; not C.

[b] Recommended DNP-11-dUTP concentration in PCR mixture. [c] Recommended Mg^{2+} concentration in PCR mixture.

[d] Hales et al., 1996; [e] this study; [f] Friedrich, 2002; [g] Ben-Dov et al., 2007; [h] Deplancke et al., 2000.

Table 2 Mismatch discrimination and detection efficiency by two-pass TSA-FISH.

Target gene	Microorganism	Identity to the probe sequence (%) [a]	Detection efficiency with each probe [b]		
			mcr-757	mcr-463	mcr-151
mcrA gene	*Mcc. maripaludis*	100	+++	+++	++
	Mcc. vanneilii	93	+	+	+
	Mtc. okinawensis	82	-	-	-
	Mcl. chikugoensis	59	-	-	-
	E. coli	<<59	-	-	-
			aps-820	aps-501	aps-135
apsA gene	*Dsb. propionicus*	100	+++	++	+
	Dsb. elongatus	90	++	+	+
	Dsv. vulgaris	67	-	-	-
	E. coli	<<60	-	-	-

[a] Sequence identities were calculated using the mcr-463 probe for *mcrA* gene and the aps-820 probe for *apsA* gene, respectively.

[b] +++; >98%, ++; >50%, +; <20%, -; <1%.

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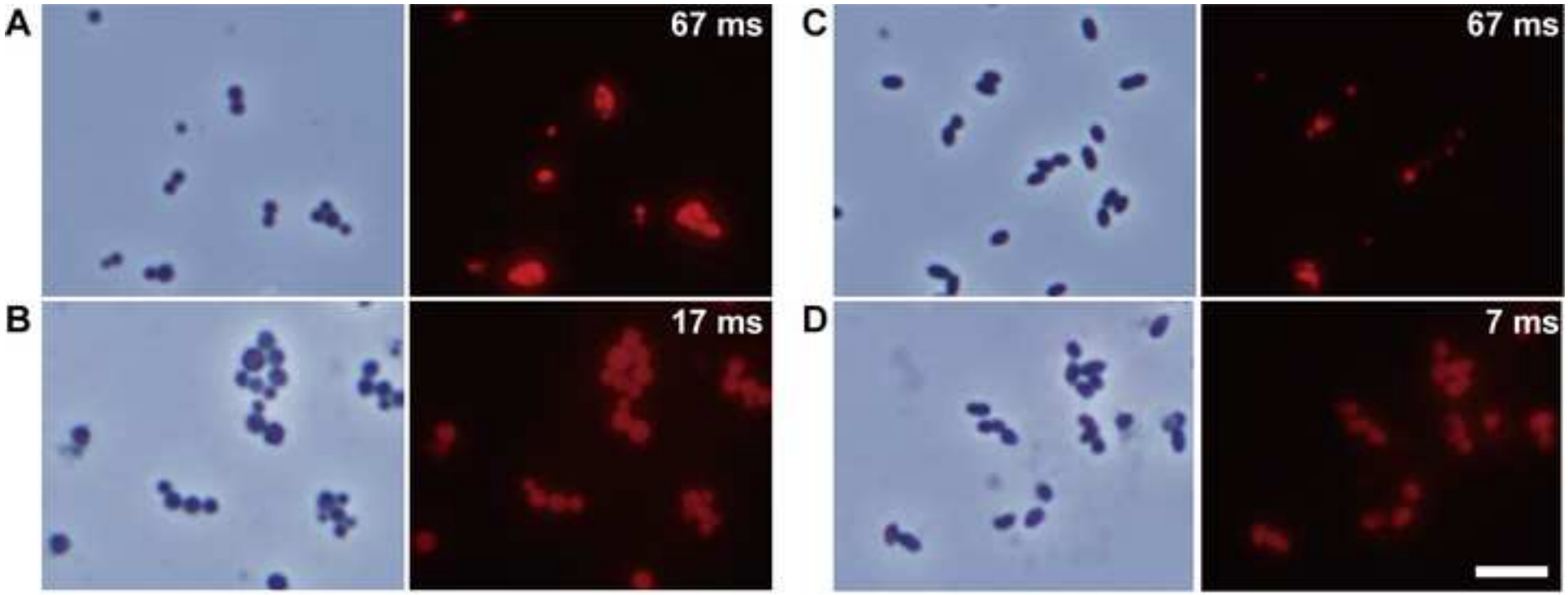

Fig.1. Kawakami et al.

Figure(s)

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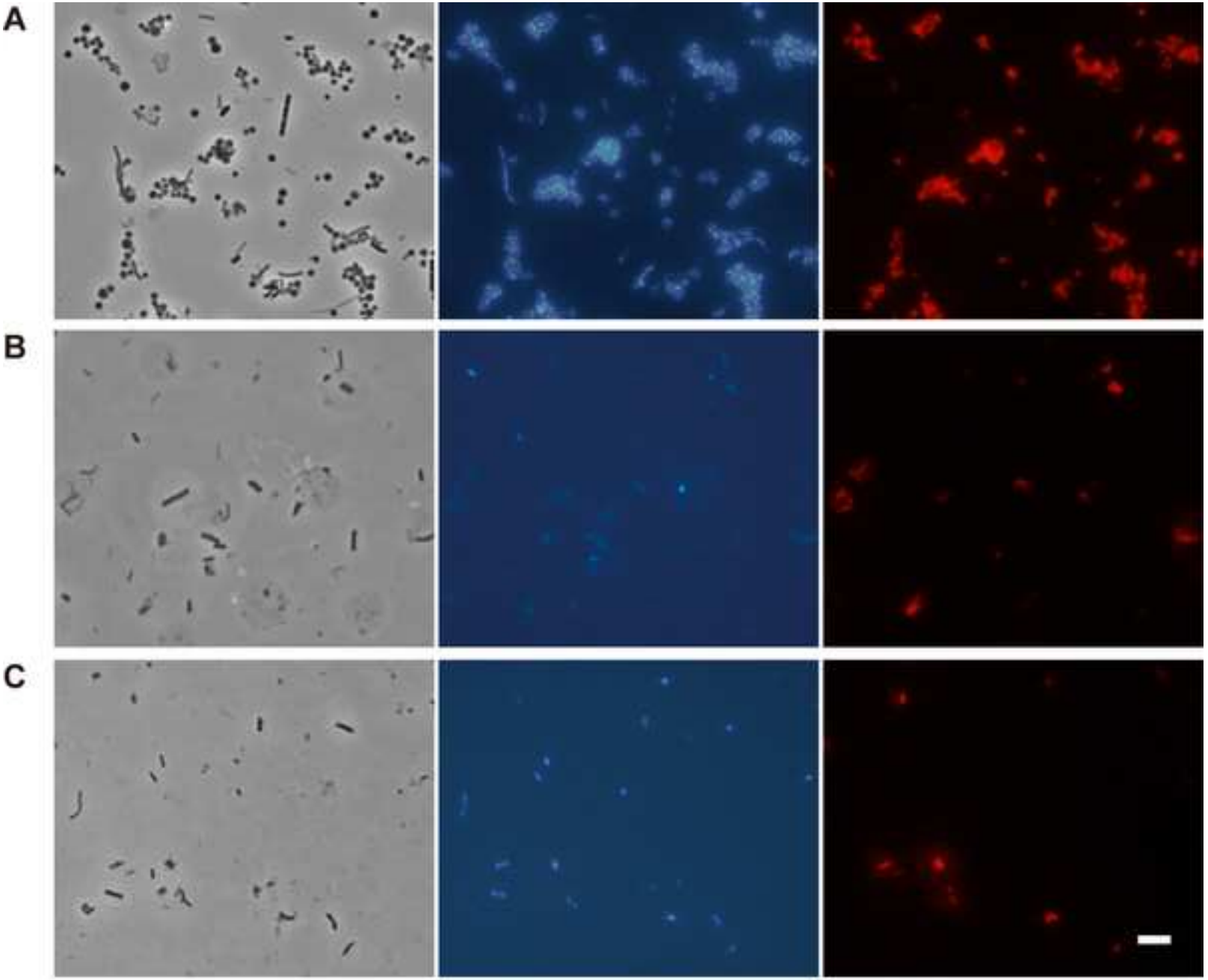

Fig.2. Kawakami et al.

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