Detection of Single Copy Genes by Two-Pass Tyramide Signal Amplification Fluorescence in situ Hybridization (Two-Pass TSA-FISH) with Single Oligonucleotide Probes

SHUJI KAWAKAMI1,2, KENGO KUBOTA*, HIROYUKI IMACHI1, TAKASHI YAMAGUCHI1, HIDEKI HARADA2, and AKIYOSHI OHASHI3

1Department of Environmental Systems Engineering, Nagoya University of Technology, 1603–1 Kamitomioka, Nagoya, Niigata 940–2188, Japan; 2Department of Civil and Environmental Engineering, Tohoku University, 6–6–06 Aoba, Arahata, Aoba-ku, Sendai, Miyagi 980–8579, Japan; 3Subsurface Geobiology Advanced Research (SUGAR) Team, Extremobiosphere Research Program, Institute of Biogeosciences, Japan Agency for Marine-Earth Science & Technology (JAMSTEC), 2–15 Natatsushima, Yokosuka, Kanagawa 237–0061, Japan; and 4Department of Social and Environmental Systems Engineering, Hiroshima University, 1–4–1 Kagamiyama, Higashihiroshima, Hiroshima 739–8527, Japan

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In situ detection of functional genes is informative for understanding microbial physiology. Most methods of detecting functional genes employ multiple oligonucleotides or polynucleotide probes. However, single oligonucleotide probes are superior in terms of specificity and flexibility in probe design. Here we describe the detection of a single copy functional gene, the methyl coenzyme M reductase gene, in a methanogen by two-pass tyramide signal amplification (TSA-FISH) with single oligonucleotide probes. Although problems associated with non-removable non-specific binding of the antibody could not be overcome completely, single copy gene detection was carried out with single mismatch discriminatable specificity; however, only around 15% of cells were detected. The detection rate increased when a multiple copy gene like rrrn in Escherichia coli was targeted, indicating that a certain number of target molecules are necessary to achieve a high detection rate. Although possible applications of this technique to environmental samples remain restricted, the results presented the potential of gene detection by FISH with single oligonucleotide probes.

**Key words:** oligonucleotide probes, functional genes, two-pass TSA-FISH, locked nucleic acid (LNA), methyl coenzyme M reductase (mcr) gene

Fluorescence in situ hybridization (FISH) has been widely applied to the in situ detection, identification and enumeration of prokaryotes in various environments (1, 2). The signal intensity obtained by FISH with fluorophore-labeled oligonucleotide probes (approximately 20 bases) depends on the number of target molecules in a cell. In general, rRNA is chosen for probing due to its abundant availability in addition to its evolutional conservation and variability. On the other hand, mRNAs and functional genes encoded on chromosomes or plasmids are usually of low abundance in a cell, resulting in insufficient signal intensities for detection by microscopic observation. To overcome this problem, the application of polynucleotide probes (several hundred bases; e.g., recognition of individual genes [RING]-FISH (26, 40), enzymatic signal amplification (e.g., tyramide signal amplification [TSA]-FISH, two-pass TSA-FISH) (9, 16, 24, 29), enzymatic target nucleic acid amplification (e.g., in situ PCR, in situ loop-mediated isothermal amplification [LAMP], in situ rolling circle amplification [RCA], cycle primed in situ amplification [cPRINS]-FISH) (12, 15, 22, 23) and the use of new dyes and new fluorescent materials have been tried (6, 37). These methods have been successfully applied to the detection of mRNAs and genes to understand metabolic activities, functional potential, and horizontal gene transfer among bacteria. However, most of them employ polynucleotide probes or multiple oligonucleotides. The application of polynucleotide probes is known to intensify signals through multiple labeling, but the mismatch discrimination power of polynucleotides is lower than that of oligonucleotides (34, 39). Methods requiring multiple oligonucleotides (primers and/or probes) usually involve in situ target nucleic acid amplification, resulting in high sensitivity and also high specificity due to multiple priming and/or probing. Nevertheless, primer and probe designs are usually complex, and it is especially difficult to design a set of primers and probes targeting a broad range of prokaryotes. Methods employing only single oligonucleotide probes would be superior in terms of design flexibility and probe specificity, but not sensitivity. To our knowledge, in situ detection with single oligonucleotide probes has been successfully carried out for mRNAs (3, 5, 6, 16) and high copy plasmids (19, 37), but not for chromosomal encoded single copy genes, probably due to its low sensitivity.

TSA-FISH is one promising method for the detection of low abundant molecules using single oligonucleotide probes and has been used in environmental microbiology (3, 16, 24, 29). Signals in TSA-FISH are amplified by the deposition of a large number of fluorescently labeled tyramide molecules using enzymatic catalysis with horseradish peroxidase
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Recently, Kubota et al. (16) developed two-pass TSA-FISH for prokaryotes, which has higher sensitivity than TSA-FISH, by iteration of the TSA reaction with an immunochemical reaction, and successfully detected mRNA in a methanogen with reliably strong signals using an epifluorescent microscope. Van de Corput et al. (35) described that 50–60 copies of target molecules were enough to obtain strong signals by two-pass TSA-FISH with single oligonucleotide probes targeting the human cytomegalovirus immediate early gene. More recently, Hoshino et al. (13) reported that the practical detection limit of planktonic cells was as low as 14±2 target molecules per cell by TSA-FISH with single oligonucleotide probes using a confocal laser scanning microscope (CLSM). These reports encouraged us to detect single copy genes by two-pass TSA-FISH using epifluorescent microscopy although CLSMs and epifluorescent microscopes differ greatly in terms of excitation power, signal detector systems and so on.

This paper describes the detection of a single copy gene encoded on a prokaryotic chromosome by FISH with a single oligonucleotide probe. Two-pass TSA-FISH and locked-nucleic-acid (LNA)-incorporated oligodeoxynucleotide probes (LNA/DNA probes) were employed to achieve high sensitivity and high nucleic acid hybridization affinity, respectively (Fig. 1). The approach was evaluated by detecting the methyl coenzyme M reductase (mcr) gene in a methanogen.

Materials and Methods

Microorganisms and fixation

The strains used in this study were Methanococcus maripaludis strain S2 (JCM 13030) and Methanococcus vannielii (JCM 13029). The genome of strain S2 has been sequenced (11). The cells were cultivated in media recommended by the culture collection (Japan collection of microorganisms, Wako, Japan), and harvested in a logarithmic growth phase, fixed in a 4% (w/v) paraformaldehyde solution for 12 h at 4°C, and stored in an ethanol/phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, 1.47 mM KH₂PO₄ [pH 7.2]) solution at −20°C. Cloning of the mcrA gene and preparation of Escherichia coli

Escherichia coli cells carrying plasmids with a partial mcrA gene were prepared as described elsewhere (16) with some modifications. DNA of M. maripaludis and M. vannielii was extracted using the Mag Extractor Genome kit (Toyobo, Tokyo, Japan). The mcrA gene was amplified with the two specific primer sets for each organism (Table 1) in a mixture of PCR solution (0.025 U µL⁻¹ Taq polymerase, 0.5 pmol µL⁻¹ of each primer, 1×PCR buffer, 200 µM dNTPs [Applied Biosystems, Foster City, CA, USA]) and the appropriate amount of extracted genomic DNA. The PCR profile was 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. Subsequently, the PCR products were purified with the Min Elute PCR purification kit (Qiagen, Tokyo, Japan) and ligated into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA, USA). Ligation reactions and the subsequent transformation of TOP10 competent cells (Invitrogen) were carried out according to the manufacturer’s recommendations. Clones with the appropriate insert were cultivated in Luria Bertani (LB) broth with ampicillin (50 µg mL⁻¹) overnight at 37°C. Subsequently, 50 µL of the culture was added to 5 mL of fresh LB broth. Chloramphenicol was then added to the cultures when the OD₆₀₀ had reached 0.4–0.5. Finally, the E. coli cells were harvested 4 h after the addition of chloramphenicol, fixed with paraformaldehyde, and stored in the ethanol/PBS solution at −20°C until further use. For control experiments, E. coli carrying self-ligated plasmids was also cultivated.

TSA-FISH

TSA-FISH was performed in accordance with previous reports with some modifications (16, 24). The fixed cells were embedded in low melting point agarose on each well of a 10-well glass slide (Matsunami, Osaka, Japan) and air-dried at 60°C as described elsewhere (18). Samples were dehydrated through an ethanol series (50, 80 and 96% v/v ethanol for 3, 1 and 1 min, respectively). To

Fig. 1. Flow diagram of single copy gene detection by two-pass TSA-FISH with a single oligonucleotide probe. Step 1: hybridization of a probe to a target gene. Step 2: immunochemical reaction with HRP-labeled anti-DIG antibody. Step 3: first TSA reaction with tyramide-DNP. Step 4: second immunochemical reaction with HRP-labeled anti-DNP antibody. Step 5: second TSA reaction with tyramide-Cy3.
digest RNA in cells, RNase treatment was carried out (0.5 mg mL\(^{-1}\) in 10 mM Tris-HCl [pH 7.5], and 15 mM NaCl at 37°C for 30 min; Nippon Gene, Tokyo, Japan). After the treatment, the slides were immersed in TNT buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.3% Tween 20) for 10 min and in ultra pure water for 1 min, and dehydrated in 96% ethanol for 1 min. For the permeabilization of \(E.\) coli cells, the cells were treated with lysozyme (0.1 mg mL\(^{-1}\) in 100 mM Tris-HCl [pH 7.5], and 50 mM EDTA [pH 8.0]) at room temperature for 1 min, and washed as presented above. No permeabilization was carried out for the methanogens as described previously (18).

Twenty micro-liters of hybridization buffer (1×SSC [15 mM sodium citrate plus 150 mM sodium chloride; pH 7.5], formamide [varying from 0 to 80%; v/v]), 10% [w/v] dextran sulfate, 1% [w/v] blocking reagent [Roche Diagnostics, Mannheim, Germany], 0.01% [w/v] sodium dodecyl sulfate [SDS], 1×Denhardt’s solution [Sigma-Aldrich, Steinheim, Germany], and 0.2 mg mL\(^{-1}\) sheared salmon sperm DNA [Ambion, Austin, TX, USA] was applied to each well on the glass slides. Then, slides were placed into a chamber humidified with 1×SSC and formamide (the same concentration as the hybridization buffer), and prehybridization was carried out for 30 min at 46°C. After incubation, hybridization buffer was replaced with 20 µL of hybridization buffer with 0.1 µM of digoxigenin (DIG)-labeled probes. In order to increase signal intensity, DIG was labeled at both the 5’ and 3’ ends. The denaturation of probes and chromosomes and subsequent hybridization were carried out by incubating at 95°C for 20 min followed by at 46°C overnight in the humidified chamber. To remove excess probe, the slide was immersed in washing buffer 1 (1×SSC [pH 7.5] and formamide [the same concentration as the hybridization buffer]) for 1 h (twice, 30 min each) at 48°C and then in washing buffer 2 (0.1×SSC [pH 7.5] and 0.01% [w/v] SDS) for 30 min (twice, 15 min each) at 48°C.

For the immunochemical reaction, the slide was immersed in TNT buffer for 10 min at room temperature. After excess buffer was removed, BB buffer (1% [w/v] blocking reagent [Roche Diagnostics] and 1% [w/v] BSA [Sigma Aldrich] in PBS) was applied to each well and incubated for 1 h at room temperature. Subsequently, the BB buffer was replaced with a mixture of 1 part HRP-labeled anti-DIG antibody (Roche Diagnostics) and 2,500 parts BB buffer (final concentration of the antibody, 0.02 U mL\(^{-1}\)). After incubation for 90 min at room temperature, the slide was immersed in TNT buffer for 30 min (twice, 15 min each) at room temperature with mild agitation.

Tyramide signal amplification was carried out by incubating freshly prepared tyramide-Cy3 working solution (a mixture of 1 part of tyramide and 50 parts of amplification buffer containing 10% [w/v] dextran sulfate, 0.1% [w/v] blocking reagent [Roche Diagnostics] in amplification diluent [Perkin Elmer, Waltham, MA, USA]) for 15 min at 37°C. Afterwards, the slide was immersed in TNT buffer for 30 min (twice, 15 min each) at 48°C, in ultra pure water for 1 min at room temperature, in 96% ethanol (v/v) for 1 min and finally air-dried.

**Two-pass TSA-FISH**

Two-pass TSA-FISH was carried out based on the protocol described by Kubota et al. (16). The experimental procedure prior to the TSA reaction was the same as that for TSA-FISH. The first round of tyramide signal amplification was performed with tyramide-dinitrophenyl (DNP) (Perkin Elmer). Subsequent to washing in TNT buffer for 30 min (twice, 15 min each) at 48°C, a second immunochemical reaction was carried out. An HRP-labeled anti-DNP antibody (Perkin Elmer) was used instead of the anti-DIG antibody, the dilution ratio was decreased to 1 to 100, and the incubation time was shortened to 30 min. After washing in TNT buffer for 30 min (twice, 15 min each) at 48°C, mild agitation, a second TSA reaction with tyramide-Cy3 and subsequent washing were carried out as described above.

**Epifluorescence microscope and digital camera**

For microscopic observation and digital photographs, 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, Rockingham, VT, USA) was used.

**Results and Discussion**

**Probes**

The type of probe is crucial in gene detection. In this study, we employed LNA/DNA probes with higher affinity and specificity than DNA probes and greater design flexibility and lower costs than peptide nucleic acid (PNA) probes.
Silahtaroglu et al. (30) investigated the effects of LNA content and position on in situ hybridization, and found that probes with a substitution of every other LNA are the best in terms of hybridization efficiency and signal intensity. According to their strategy, there are two patterns of substitution. In this study, probes were designed to avoid self-hybridization, especially LNA-LNA hybrids, based on the predictions from in silico analyses of probe sequences using LNA tools (33). Eventually, four LNA/DNA probes targeting the *M. maripaludis* mcrA gene and one LNA/DNA probe targeting the *E. coli* rrn gene were designed according to previously reported sequences (Table 1). These probes target the antisense of each gene to avoid hybridization to RNA, though RNase treatment is involved. The probes were labeled with DIG at both the 5' and 3' ends to increase the number of reporter molecules for antibodies. Increasing the DIG labeling might cause lower specificity (36). Furthermore, antibodies may not be accessible to DIG due to the availability of space even if more labeling is carried out.

**Validation of probes suitability for in situ hybridization**

To examine whether the LNA/DNA probes were suitable for in situ hybridization, we prepared *E. coli* harboring plasmids with the partial mcrA gene (200–500 copies per cell). The *E. coli* cells were successfully detected by TSA-FISH using all the probes except the L-MR1 probe. Different fluorescent intensities indicate different hybridization efficiencies. The L-ME2 probe showed the highest signal intensity and most of the cells were stained (Fig. 2A). On the other hand, weak signals and low detection rates were obtained with the L-MR1 probe (Fig. 2D). All probes showed strong signals after two-pass TSA-FISH (Fig. 2B and E). There were no signals from *E. coli* carrying self-ligated plasmids (Fig. 2C and F) or after DNase treatment (data not shown), ensuring that the probes hybridized to the mcrA gene in plasmids, not to other molecules.

(4, 17, 25, 30).
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obtained only when two-pass TSA-FISH was applied (Fig. 3A). Interestingly, the gene was detected using any probe indicating that the detection was independent of the hybridization efficiency of the probe as determined using E. coli with plasmids (see above), probably due to the superior sensitivity of two-pass TSA-FISH. The number of signals from cells was decreased to almost zero under increased stringent hybridization conditions (Fig. 4A) or after DNase treatment, suggesting that the signals were probably from the probes hybridized to the target genes. However, nonspecific signals from glass slides and few cells were also obtained (Fig. 3A).

To ensure the signals were derived from probes, first, we employed a poly-HRP-labeled anti-DIG antibody (Roche Diagnostics) instead of the single HRP-labeled anti-DIG antibody to increase the signal-to-noise ratio. However, signal intensity was only slightly increased; indeed, the background noise significantly increased (data not shown). The poly-HRP-labeled anti-DIG antibody must be larger than the single HRP-labeled antibody, suggesting less permeability. Hence, we concluded that use of the poly-HRP-labeled anti-DIG antibody is not beneficial for improving the signal-to-noise ratio.

Nonspecific signals were also obtained after two-pass TSA-FISH without probes, mainly caused by the nonspecific binding of HRP-labeled anti-DIG antibodies (data not shown). Therefore, the optimization of immunochemical reactions and subsequent washing procedures were carried out. Different antibody concentrations (0.5, 0.1 and 0.02 U mL$^{-1}$) and reaction times (30, 60 and 90 min) at room temperature or 4°C were tested and the best result was obtained when 0.02 U mL$^{-1}$ of antibody was incubated for 90 min at room temperature (data not shown). Washing procedures after immunochemical reactions were optimized by adding different kinds of detergents such as Tween 20, Triton X-100 and SDS in TN buffer (100 mM Tris-HCl [pH 7.5] and 150 mM NaCl), at various concentrations (0.05%, 0.1%, 0.3% and 1%). These detergents attenuated the nonspecific signals; however, a high concentration of SDS (1%) caused cell lysis. A better result was obtained when 0.3% Tween 20 in TN buffer was used. Note that use of an antibody stored long-term (at 4°C for approximately six months) after being dissolved in ultra pure water lowered the signal intensity from cells and increased the number of nonspecific signals. After these optimization procedures, nonspecific signals from glass slides were almost eliminated; nevertheless, some nonspecific signals from cells still remained (less than 1% of total cells were detected by two-pass TSA-FISH without probes). The detection rates with each probe were approximately 10%; hence, we concluded that detection of the mcr gene in M. maripaludis was carried out (Fig. 3B).

Detection rates

During development, detection rates were approximately 10% using each probe and even lower under low stringent conditions (low formamide concentrations in hybridization buffers). Elevating the denaturing temperature from 80°C to 95°C resulted in detection rates of up to approximately 25% (Fig. 4A). Hybridization with the four probes simultaneously did not increase detection rates. These results explain the importance of chromosomal denaturation for higher detection rates. We also found that not only a high denaturing temperature, but also a high concentration of formamide in the hybridization buffer is necessary, as shown by the low detection rates at low formamide concentrations (Fig. 4A). In our protocol, chromosomes and probes are simultaneously denatured; therefore, a hybridization buffer should be prepared with a high concentration of formamide. LNA/DNA probes were an appropriate choice for our approach since they are able to hybridize under stringent conditions due to high affinity whereas DNA probes are not (4, 17, 30). In fact, detection using DNA probes was not successful in this study (data not shown). Further improved denaturation procedures, for example, the use of a PNA opener (32), might help to increase detection rates.

In this study, the rRNA gene, existing 7 copies per chromosome of E. coli (28), was also detected by two-pass TSA-FISH with the L-NonEUB LNA/DNA probe and a detection rate of more than 50% was achieved (Fig. 3D). These results suggest a certain number of target molecules, which is unknown as yet, to be required to achieve a high detection rate. Thus, the application of in situ target nucleic acid amplification methods is one way to improve detection rates. The
efficiency of immunochemical reactions and the labeling efficiency of DIG to probes are also considered factors.

Mismatch discrimination

Mismatch discrimination ability is also important, and therefore was investigated using *M. vannielii*, which has one or three mismatches to the probes used in this study, as a non-target microorganism (Table 1). Profiles of the detection rates with the L-ME2 and L-MR1 probes are shown in Fig. 4. When the L-MR1 or L-ME3 probe, having 3 mismatches to *M. vannielii*, was used, *M. maripaludis* was distinguishable from *M. vannielii*. It was also discriminated when a single mismatch probe, L-ME2 or L-ME1, was used. Nevertheless, detection rates for *M. vannielii* could not achieve 0%, but were less than 1% (Fig. 4). These detection rates were almost the same as those for two-pass TSA-FISH without probes (see above); hence, the signals were derived from non-removable nonspecific binding of enzyme-labeled antibodies rather than nonspecifically hybridized probes, suggesting that a single mismatch can be discriminated by this technique. The incorporation of LNA bases is known to improve mismatch discrimination ability (14, 38), which is usually described as ΔTm (Tm, melting temperature; ΔTm=Tmₐ as perfectly matched duplexes–Tmₐ as mismatch duplexes). Although the specificity of FISH can be controlled by both Tm and Td (dissociation temperature), it is obvious that the greater the ΔTm, the higher the specificity. PNA probes, showing great mismatch discrimination in low salt environments (25), can also be used as an alternative.

Concluding remarks

Hoshino et al. (13) reported that TSA-FISH could practically detect as few as 14±2 copies per cell using a CLSM system. The primary advantage of a CLSM over an epifluorescence microscope is the reduced fluorescence emitted from outside of the plane of focus, achieving a higher signal-to-noise ratio. Consistent with their study, gene detection by TSA-FISH resulted in no visible signals using an epifluorescence microscope, giving a lower signal-to-noise ratio than a CLSM system, and only two-pass TSA-FISH allowed visualization. However, detection rates were only around 15% under specific conditions and nonspecific signals derived from nonspecific antibody binding could not be eliminated completely. Competitor probes were used to improve both specificity and detection rate, but they did not behave as they do in rRNA FISH. Less than 1% of a target population is often more difficult to detect by FISH, and more than 10% of targets are necessary to apply this technique. Hence, the application of this technique to environmental samples seems restricted at this moment. As shown in the experiment targeting the *E. coli* rRNA gene, one of the keys to elevating the detection rate is to increase the number of target molecules. In situ nucleic acid amplification techniques are reasonable to overcome this problem. However, these techniques require two or more oligonucleotides for priming and probing (12, 15, 22, 23). We have tried in situ whole genome amplification using random primers, followed by two-pass TSA-FISH with single oligonucleotide probes. In this way, only single probes are necessary, but this approach was not successful due to insufficient genome amplification in paraformaldehyde- or ethanol-fixed cells (data not shown).

Another approach is to use polynucleotide probes with or without subsequent signal amplification (24, 26, 34, 40). Polynucleotide probes can be multiply labeled with reporter molecules; hence, increased sensitivity (higher signal-to-noise ratio) is achieved. One disadvantage of polynucleotide probes is lower mismatch discrimination ability compared with oligonucleotide probes (39). Some researchers reported a threshold of 70–76% (7) or 78–85% (20) sequence identity. This is not suitable for rRNA FISH, especially when a phylogenetically close group is targeted. However, in the case of functional genes, it might be suitable since low nucleic acid sequence identity often results in high similarity when the sequence is converted to amino acids (8, 21, 27). RING-FISH has been developed for gene detection using polynucleotide probes, but it is difficult to control its sensitivity and specificity (26, 40). To our knowledge, a combination of TSA-FISH or two-pass TSA-FISH with polynucleotide probes has not been developed for gene detection yet though it could potentially overcome the problems associated with RING-FISH and also those found in this study.

In summary, though some limitations still remain, two-pass TSA-FISH with single oligonucleotide probes has the potential to detect single copy genes on chromosomes in whole-fixed cells with high specificity without pre-amplification of target nucleic acids.

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References

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