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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.

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 *r*ecognition of *in*dividual 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-

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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 1.5–4.0 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 to 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 *Methanobacterium 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

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]
mcr A 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	4.0
			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	4.0
			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	4.0
			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.