Detection of *Archaea* in an upflow anaerobic sludge blanket reactor by in situ hybridization chain reaction–fluorescence in situ hybridization

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Abstract
Detection of functional genes and mRNAs using highly sensitive fluorescence in situ hybridization (FISH) is necessary to further our understanding of the in situ physiological potential of prokaryotes. Although highly sensitive FISH requires cell-wall treatment for the penetration of enzyme-labeled probes or polynucleotide probes into target cells, a universal cell-wall treatment method has not yet been reported. We developed an in situ hybridization chain reaction (HCR)–FISH for improving probe permeability for sensitive FISH. The signal intensity of in situ HCR was stronger than that of standard FISH. The probe permeability of in situ HCR–FISH was higher than that of catalysed reporter deposition (CARD)–FISH in *Methanosaeta concilii*, which requires cell-wall treatment for CARD–FISH. To test the applicability of in situ HCR–FISH for detecting environmental samples, it was applied to anaerobic sludge in an upflow anaerobic sludge blanket reactor that was treating sewage. The detection rate with in situ HCR–FISH (18% ± 3%) was almost the same as that with standard FISH (22% ± 4%) and higher than that with CARD–FISH (9% ± 3%). These results indicated that in situ HCR–FISH is superior to CARD–FISH for detecting prokaryotes with low cell permeability.

Keywords
Methanogen, Upflow anaerobic sludge blanket, Sensitive fluorescence in situ hybridization, In situ hybridization chain reaction–fluorescence in situ hybridization

INTRODUCTION

Fluorescence in situ hybridization (FISH) is routinely used for identification, enumeration, and spatial distribution of prokaryotes in bioreactors such as an upflow anaerobic sludge blanket (UASB) (Sekiguchi *et al*., 1999). To further understand the in situ physiological potential of prokaryotes, detection of functional genes or mRNAs is necessary. However, it is usually difficult to detect functional genes and mRNAs by standard FISH using fluorescently labeled oligonucleotide probes. Recently, catalysed reporter deposition (CARD)–FISH, two-pass tyramide signal amplification (TSA)–FISH, and recognition of individual genes (RING)–FISH have been reported for detecting functional genes or mRNAs (Kubota *et al*., 2006; Kubota *et al*., 2008; Zwirglmaier *et al*., 2003). However, highly sensitive FISH requires high-molecular-weight enzyme-

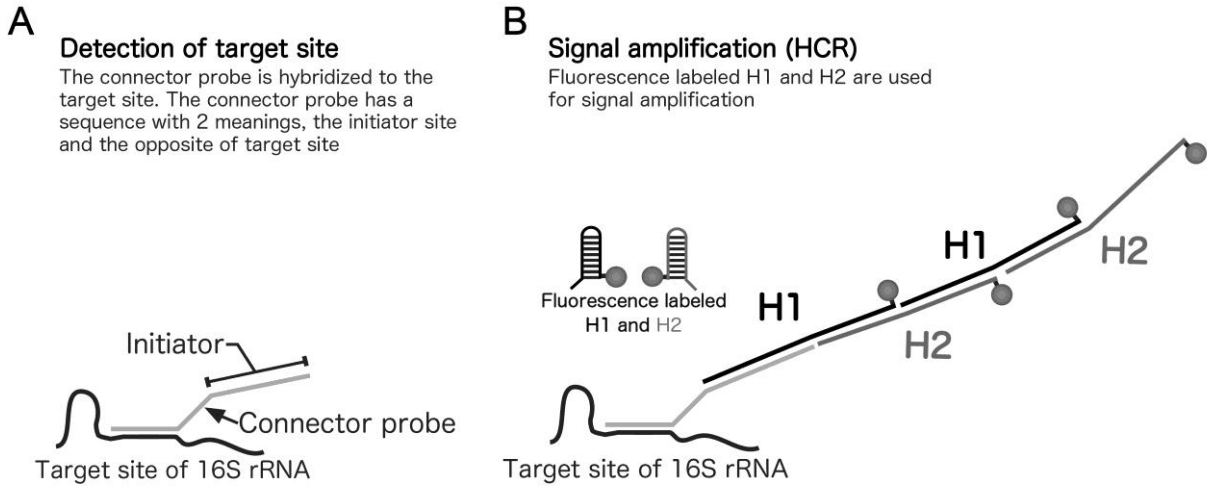

Fig. 1 Princple of in situ HCR-FISH. (A) The connector probe was hybridized to target site for detection. (B) The amplifier probes (H1 and H2) were hybridized to the connector probe and extension from the connector probe. The specificity of in situ HCR-FISH was determined at hybridization conditon of the connector probe. The HCR conditon was was constant not by target cells.

labeled probes or polynucleotide probes for signal enhancement, and it is difficult for such probes to penetrate the target cells. Moreover, a universal cell-wall treatment method for all cell wall types has not yet been reported, and it is difficult to apply highly sensitive FISH to prokaryotes such as methanogens in UASBs (Kubota *et al*., 2008). To overcome this problem, we developed a novel highly sensitive FISH that combines a hybridization chain reaction (HCR) with standard FISH (in situ HCR–FISH) without enzymes or polynucleotide probes. HCR was first reported for the detection of single-stranded DNA (Dirks *et al*., 2004). HCR does not use an enzymatic reaction for detecting its target; rather, it uses only two hairpin probes (approximately 50mers). Thus, we considered that the hairpin probes may penetrate cells with low permeability. While we were developing our novel sensitive FISH, in situ HCR was reported for detecting eukaryotic mRNA (Choi *et al.*, 2010). Therefore, we combined our FISH technique with this newly reported protocol (Fig. 1). In this study, as the first step in detecting functional genes and mRNAs, we optimized the protocol of in situ HCR–FISH for detecting prokaryotic rRNA, and we detected *Arachea* in anaerobic sludge to test the applicability of in situ HCR to environmental samples.

MATERIALS AND METHODS

Sample collection and preparation

The strains used in this study were *Methanococcus vannielii* (JCM 13029) and *Methanosaeta concilii* (JCM10134). An anaerobic sludge sample was collected from a UASB reactor treating sewage. These samples were harvested in logarithmic growth phase, fixed in 4% paraformaldehyde solution for 12 h at 4°C, and stored in an ethanol/phosphate-buffered saline [137 mM NaCl, 8.1 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4 (pH 7.2)] solution at −20°C.

FISH, CARD–FISH, and in situ HCR–FISH

The 16S rRNA-targeted oligonucleotide probes and optimized formamide (FA) concentration used this study are listed in Table 1. FISH and CARD–FISH were performed as described

Table 1. Probes used in this study

Methods	Probes	Probe sequence (5'-3')※1	Length	%FA※2	Reference
FISH	ARC915	GTGCTCCCCCGCCAATTCCT	20	40	Sekiguchi *et al.*, 1999
CARD-FISH	ARC915	GTGCTCCCCCGCCAATTCCT	20	40	Kubota *et al.,* 2008
In situ HCR-FISH	ARC915-connector	CCGAATACAAAGCATCAACGACTAGAAAAAAGTGCTCCCCCGCCAATTCCT	51	40	This study
	H1	TCTAGTCGTTgatgctttgtattcggCGACAGATAAccgaatacaaagcatc	52	0	Modified choi *et al*., 2010
	H2	ccgaatacaaagcatcAACGACTAGAgatgctttgtattcggTTATCTGTCG	52	0	Modified choi *et al*., 2010

※1 Small letters are stem structure of HCR probes. Under bar is first hybridization site.
※2 %FA shows formamide concentration (v/v).

previously (Sekiguchi *et al*., 1999; Kubota *et al*., 2006). In situ HCR–FISH was optimized for prokaryotes as described below. The fixed cells were embedded in low-melting agarose in each well of a 10-well glass slide and air dried at 60°C in a dehydrator. Samples were dehydrated in an ethanol series [50%, 80%, and 96% (v/v) for 3, 1, and 1 min, respectively]. Hybridization buffer 1 (15 µl; 20 mM Tris-HCl, 0.9 M NaCl, 40 % (v/v) FA, 0.01% SDS) containing 0.5 µM connector probe was applied to each well. The slides were placed into a chamber and incubated overnight at 46°C. After incubation, the slides were washed in hybridization buffer 1 for 30 min at 48°C and then in hybridization buffer 2 (50 mM Na_2HPO_4, 0.9 M NaCl, 0.01% SDS) for 5 min at room temperature. Fluorescence-labeled H1 and H2 were prepared as described previously, and 5 µM fluorescence-labeled H1 and H2 were incubated for 1 min 30 s at 95°C and for 30 min at 25°C. Hybridization buffer 2 containing 5 µM fluorescence-labeled H1 and H2 were applied to each well and incubated for 2 h at 46°C. The slides were then washed in hybridization buffer 2 for 30 min at 10°C. Finally, the slides were immersed in ultra-pure water for 30 s and 96% ethanol (v/v) for 30 s at 10°C and dried at 4°C.

Microscopic evaluation

Following FISH, CARD–FISH, and in situ HCR–FISH, samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and an epifluorescence microscope (BX50F; Olympus, Tokyo, Japan) with a colour CCD camera (DP70; Olympus) was used to record the data.

Signal intensity and detection rates

More than 700 DAPI-stained cells were counted to calculate the detection rates, which were defined as the ratios of FISH-, CARD–FISH-, and in situ HCR–FISH-positive cells to DAPI-stained cells. All experiments were performed in triplicate.

RESULTS AND DISCUSSION

Application of in situ HCR–FISH

The first in situ HCR–FISH protocol was evaluated by targeting ARC915 site of the rRNA in fixed cells from log-phase cultures of *M. vannielii*. Positive signals of in situ HCR–FISH were obtained, and the signal intensity was stronger than that of standard FISH (Fig. 2). To test for nonspecific hybridization of the hairpin probes, in situ HCR–FISH was performed without the connector probe; no nonspecific signal was detected (data not shown). Furthermore, under high stringency hybridization conditions with the connector probe, no nonspecific signals were detected (data not shown). These initial results indicated that the in situ HCR–FISH protocol was suitable for application in prokaryotic cells.

Probe permeability of in situ HCR–FISH

To examine the probe permeability of in situ HCR–FISH, *M. concilii*, which requires cell-wall treatment for CARD–FISH, was detected using in situ HCR–FISH and CARD–FISH without any cell-wall treatment. Rod-shaped *M. concilii* cells were detected without any cell-wall treatment by in situ HCR–FISH (Fig. 3). However, the CARD–FISH signals were from particles and were spotty within the cells. These results indicated that the fluorescent dye-labeled probes used in situ HCR–FISH penetrated the cells

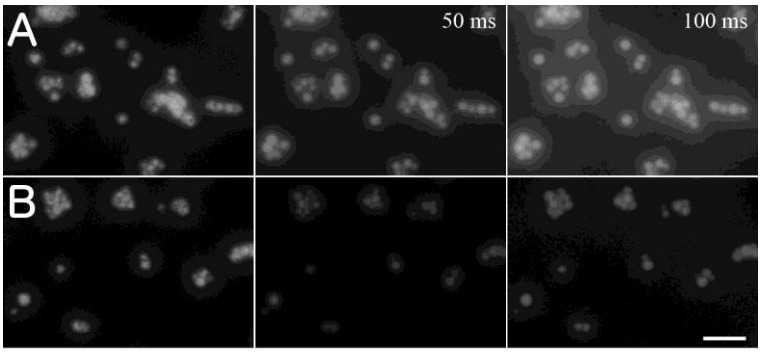

Fig. 2 Detection of *M. vanneilii* targeted ARC915 site by in situ HCR-FISH (A) and FISH (B). Photomicrographs of DAPI stained cells (left panels) and epiflurescence (right panels) show identical fields. Exposure times are indicated at the upper right of epifluorescent micrographs. Bar represents 10 µm.

more readily than the horseradish peroxidase-labeled probe used for CARD–FISH.

Detection of *Archaea* in a UASB reactor

Finally, the anaerobic granular sludge in a UASB reactor was used to evaluate the applicability of in situ HCR–FISH to environmental samples. The detection rates of ARC915 site-stained cells by FISH, CARD–FISH, and in situ HCR–FISH are shown in Table 2. The detection rate with CARD–FISH was lower than that with standard FISH. In contrast, the detection rate with in situ HCR–FISH was almost the same as that with standard FISH ($p > 0.05$), and the signal intensity with in situ HCR–FISH was higher than that with FISH. These results showed that in situ HCR–FISH could be used to detect cells that were undetectable by CARD–FISH due to low cell permeability and that in situ HCR–FISH could be successfully performed with environmental samples from anaerobic granular sludge.

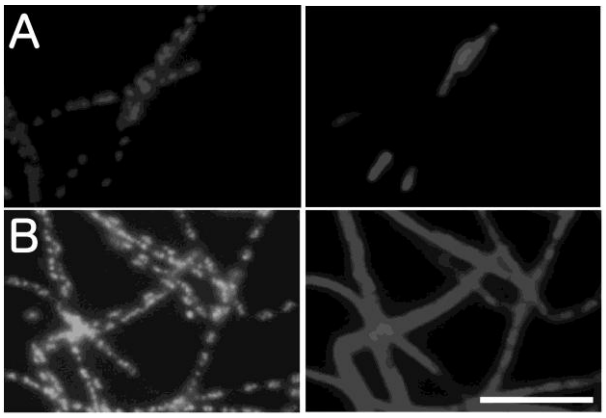

Fig. 3 Detection of *M. concilii* by CARD-FISH (A) and in situ HCR (B), without cell wall treatment. Photomicrographs of DAPI stained cells (left panels) and epiflurescence (right panels) show identical fields. Exposure times were adjusted to each method. Bar represents 10 μm.

Table 2. Detection rates (%) of *Archaea* in UASB reactor by each method without cell wall treatment

Methods	FISH	CARD–FISH	In situ HCR–FISH
Detection rates	22 ± 4	9 ± 3	18 ± 3

SUMMARY

In situ HCR–FISH can be used in the same manner as FISH for anaerobic granular sludge samples without any cell-wall treatment and can be utilized for highly sensitive FISH. We consider that with further improvement of the signal-amplification step, in situ HCR–FISH has the potential to detect mRNAs or functional genes. The course of decomposition and microbial metabolic pathways in anaerobic sludge may be revealed by combining this technique with single-cell analyses, such as microautoradiography (MAR)–FISH, nano-secondary ion mass spectrometry (SIMS), and single-cell genomics.

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