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Evaluation of enzymatic cell treatments for application of CARD-FISH to methanogens

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Abstract

Several enzymatic permeabilization protocols (utilizing lysozyme, proteinase K, achromopeptidase, or recombinant pseudomurein endopeptidase [PeiW]) were evaluated for application of in situ hybridization with horseradish peroxidase-labeled oligonucleotide probes and catalyzed reporter deposition (CARD-FISH) to methanogens. In this study, twelve methanogens were selected that have typical cell surface structures: pseudomurein, surface layer, methanochondroitin and sheath. Among the treatments tested, PeiW treatment was observed to be the most effective one, although methanogens having a sheath were stained heterogeneously and methanogens having methanochondroitin were not permeabilized. On the other hand, lysozyme, proteinase K, and achromopeptidase treatments were ineffective or caused cell-lysis, resulting in weak or no signals. Applicability of PeiW treatment was further evaluated using an anaerobic granular sludge sample. The detection rate of *Archaea* by CARD-FISH increased remarkably after the treatment. Based on the results obtained in this study, we propose PeiW treatment as a novel permeabilization method for CARD-FISH application to methanogens.

Keywords: Catalyzed reporter deposition; Fluorescence in situ hybridization; Methanochondroitin; Methanogens; Pseudomurein; Pseudomurein endopeptidase PeiW; Sheath; Surface layer

1. Introduction

Methane is one of the most important greenhouse gases and it has been estimated that approximately 74% of global methane discharge is from biological processes such as bioactivity of methanogens (Cicerone and Oremland, 1988). Culture independent molecular analyses based on rRNA gene and methanogenesis specific genes (e.g., methyl coenzyme M reductase) revealed that methanogens inhabit various anaerobic environments such as anaerobic fermenting reactors, rice paddy soils, bogs, landfills, etc. (Chen et al., 2003; Hales et al., 1996; Lueders et al., 2001; Sekiguchi et al., 1998, 1999). Significance of understanding ecology of methanogens is increasing because they not only produce greenhouse effect gas, but also play important roles in carbon cycle under anaerobic environments. To investigate their ecology in situ, whole-cell fluorescence in situ hybridization (FISH) is a useful technique due to its superior characteristics for identification, enumeration, and spatial distribution analysis (Amann et al., 1995). Nevertheless, it is usually difficult to detect methanogens in oligotrophic environments by FISH with fluorescently labeled rRNA-targeted probes (Chen et al., 2003) unlike those in eutrophic environments (Sekiguchi et al., 1999). One of the reasons for this may be low rRNA content of the cells (Amann et al., 1995).

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FISH combined with catalyzed reporter deposition (CARD-FISH) was introduced to microbial community analysis a decade ago and is known to increase signal intensity dramatically (Schönhuber et al., 1997). The principle of signal amplification is based on the deposition of a large number of fluorescently labeled tyramide by enzymatic catalysis of horseradish peroxidase (HRP). The detection rates of probe-hybridized cells were significantly increased by the application of CARD-FISH (Ishii et al., 2004; Pernthaler et al., 2002; Sekar et al., 2003). Furthermore, CARD-FISH also allows in situ detection of mRNA in addition to rRNA (Kubota et al., 2006; Pernthaler and Amann, 2004). However, the penetration of HRP-labeled probes into fixed cells is usually problematic due to the huge molecule of HRP compared to fluorescence dyes (Amann et al., 1992; Ishii et al., 2004; Pernthaler et al., 2002; Schönhuber et al., 1997; Schönhuber et al., 1999; Sekar et al., 2003). Therefore, appropriate permeabilization protocols for diffusion of HRP-labeled probes into the cells are essential for CARD-FISH application. To date, enzymatic treatments such as lysozyme, proteinase K and achromopeptidase treatments have been reported for some prokaryotes (Ishii et al., 2004; Pernthaler et al., 2002; Schönhuber et al., 1999; Sekar et al., 2003; Teira et al., 2004). However, appropriate CARD-FISH protocols for methanogens have not been developed so far.

Approximately, a hundred species of methanogens have been isolated till date. Cell surface structures of methanogens are diverse and entirely different from the bacterial ones. The surface structures are roughly divided into four groups, as pseudomurein, surface layer (s-layer), methanochondroitin, and sheath (König, 1988 and reference therein). Members of the family *Methanobacteriales* and the genus *Methanopyrus* have pseudomurein whereas members of the genus *Methanosarcina* have methanochondroitin matrix outside the s-layer. Likewise, the genus *Methanospirillum* and the genus *Methanosaeta* have a proteinaceous sheath in addition to individual cell envelopes and most of other methanogens have s-layer of protein or glycoprotein. In this study, we investigated several different permeabilization protocols (utilizing lysozyme, proteinase K, achromopeptidase, or recombinant pseudomurein endopeptidase [PeiW]) and proposed the most appropriate one for the application of CARD-FISH to methanogens. Furthermore, we also investigated applicability of this treatment to environmental samples using anaerobic granular sludge.

2. Materials and methods

2.1. Sample preparations

For this study, *Methanococcus vannielii* (DSM1224), *Methanoculleus bourgensis* (DSM3045), *Methanolobus vulcani* (DSM3029), *Methanogenium organophilum* (DSM3596), *Methanospirillum hungatei* (DSM864), *Methanosaeta concilii* (DSM3671), *Methanosaeta thermophila* (DSM6194), *Methanobacterium bryantii* (DSM863), *Methanobrevibacter arboriphilus* (JCM9316), *Methanothermobacter thermautotrophicus* (DSM1053), *Methanosarcina barkeri* (DSM800), and *Methanosarcina mazei* (JCM9314) were purchased from Deutsche Sammlung von Mikro-organismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and Japan Collection of Microorganisms (JCM, Wako, Japan). The cells were cultivated in medium as described elsewhere (Sekiguchi et al., 2000). Different principal substrates were provided to the microorganisms as acetate for the genus *Methanosaeta*, methanol for the genus *Methanosarcina*, and hydrogen for the others. *M. mazei* was also cultivated in HS-MA medium to grow as single cells (Sowers et al., 1993). Granular sludge was collected from a mesophilic laboratory-scale upflow anaerobic sludge blanket (UASB) reactor treating artificial wastewater. The samples were fixed with 3% paraformaldehyde in phosphate-buffered saline (PBS; 130 mM NaCl, 10.8 mM Na_2HPO_4, 4.2 mM NaH_2PO_4 [pH7.2]) for 3–4 h for pure cultures and 12 h for granular sludge sample at 4 °C and stored in 50% ethanol with PBS at −20 °C.

2.2. Embedding and cell permeabilization

The fixed samples were embedded in low melting point agarose as described elsewhere (Ishii et al., 2004; Pernthaler et al., 2002). For the granular sludge sample, five granules were taken into a 1.5 ml tube, crushed, and subjected to ultrasonication prior to embedding. The samples were then dehydrated in 50, 80, and 96% ethanol for 5, 1, and 1 min, respectively. For permeabilization, the following treatments were performed: (i) lysozyme treatment (10 mg/ml in 100 mM Tris–HCl [pH 7.5], 50 mM EDTA [pH 8.0]) at 37 °C for 40 min (Pernthaler et al., 2002); (ii) low concentration (20 μg/ml in 10 mM Tris–HCl [pH 7.5]) or high concentration (1 mg/ml) of proteinase K treatment for 10 min at room temperature (Schönhuber et al., 1999; Teira et al., 2004); (iii) achromopeptidase treatment (60 U/ml in 10 mM NaCl, 10 mM Tris–HCl [pH 8.0]) for 30 min at 37 °C (Sekar et al., 2003); (iv) the lysozyme treatment followed by the achromopeptidase treatment (Ishii et al., 2004; Sekar et al., 2003); and (v) recombinant pseudomurein endopeptidase (PeiW; 16 U/ml in 50 mM HEPES [pH 7.0], 5 mM dithiothreitol, 21 mM Na_2S) for 15 min at 60 °C (Nakamura et al., 2006). The recombinant PeiW was prepared and the enzyme activity was measured as described by Nakamura et al. (2006). After the treatments, slides were immersed in PBSX (0.05% Triton X-100 in PBS) for 5 min, in ultra-pure water for 1 min, and in 96% ethanol for 1 min and eventually air-dried.

2.3. In situ hybridization with Cy3-labeled probes

FISH with the ARC915 probe (5′-GTG CTC CCC CGC CAA TTC CCT -3′; Stahl and Amann, 1991), the MX825 probe (5′-TCG CAC CGT GGC CGA CAC CTA GC -3′; Raskin et al., 1994) and the MB1174 probe (5′-TAC CGT CGT CCA CTC CTT CCT C-3′; Raskin et al., 1994) was performed as described previously (Sekiguchi et al., 1999). The probes were labeled with Cy3 at the 5′ end.

2.4. In situ hybridization with HRP-labeled probes and tyramide signal amplification

In situ hybridization with the HRP-labeled ARC915 probe (ThermoHybaid, Ulm, Germany) was performed with minor