Presence of a Novel Methanogenic Archaeal Lineage in Anaerobic Digesters Inferred from *mcrA* and 16S rRNA Gene Phylogenetic Analyses

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ABSTRACT

To find taxonomically novel methanogenic archaea, a clone analysis targeting *mcrA* gene (a functional molecular marker of methanogenic archaea) was conducted for four anaerobic granular sludges. Several *mcrA* gene phylotypes were clearly different from those of other groups of known methanogens. These were found to belong to an unidentified group called as MCR-2b group. Comparative phylogenetic analysis of deduced amino acid of McrA and 16S rRNA gene sequences revealed that the MCR-2b McrA group is possibly derived from a novel methanogen group; the 16S rRNA gene sequences have been classified into an uncultured archaeal group WCHA1-57. The result suggests that WCHA1-57 archaeal group may represent a putative new order of the methanogenic archaeal group that contributes to methane production in anaerobic digesters.

Keywords: anaerobic granular sludge, methanogen, *mcrA* gene

INTRODUCTION

Methanogenic archaea (methanogens) play an important role in the terminal step of the degradation of organic substances in anaerobic environments. In addition, because of their ability to produce methane, methanogens are regarded as a potential cause of elevated levels of atmospheric greenhouse gases, alternatively, a usable microorganism to produce energy from organic wastes (Liu and Whitman, 2008). Therefore, methanogens have been extensively studied in the fields of microbiology and biogeochemistry.

To date, over 100 methanogen species have been isolated and characterized (Liu and Whitman, 2008). All known methanogens belong to the phylum *Euryarchaeota* and are phylogenetically classified into the following seven orders: *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanocellales* and *Methanomassiliicoccales*. Although many methanogen isolates have been reported so far, many yet-uncultivated species of methanogens are widely distributed in various anaerobic environments, as found by culture-independent molecular studies based on the methanogen-specific marker gene *mcrA*; some of these species may represent novel phylogenetic orders of methanogens (Steinberg and Regan, 2009; Fry *et al.*, 2009). Therefore, to comprehensively understand methanogens,

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investigation of uncultured methanogens represented as unidentified *mcrA* genes is a significant challenge.

The *mcrA* gene encodes the alpha subunit of methyl-coenzyme M reductase, which catalyzes the last step of the methane production pathway and is present in all methanogens characterized so far. Therefore, the *mcrA* gene has been widely used as a molecular marker for the detection, distribution, phylogenetic diversity, and population dynamics of methanogens. Comparative phylogenetic studies have clearly shown that the phylogenetic trees of *mcrA* gene (or deduced McrA amino acid sequence) and 16S rRNA gene have a similar topology (Luton *et al.*, 2002). Thus, it would be possible to collate the *mcrA* sequences obtained from environmental samples with the corresponding phylogenetic groups of the 16S rRNA gene, even if the *mcrA* gene sequences are currently affiliated with uncultured groups.

In this study, we aimed to find taxonomically new order of methanogens by obtaining unidentified *mcrA* genes and comparing these sequences with the corresponding 16S rRNA gene sequences and phylogeny. For this purpose, we performed *mcrA* and 16S rRNA gene-based clone analyses on four anaerobic granular sludges. Our comparative phylogenetic analysis suggests the existence of a novel methanogen group among the anaerobic digesters, which may represent the eighth order of methanogen.

MATERIALS AND METHODS

Anaerobic granular sludge samples

Four anaerobic granular sludge samples were collected and analyzed in this study (Table 1). The detailed operational conditions for a pilot-scale upflow anaerobic sludge blanket used for treating municipal sewage (sample MS) have been described previously (Takahashi *et al.*, 2011). Samples AQ and LAQ were obtained from upflow sludge blanket (USB) reactors treating seawater from the fish tanks of aquariums. Sample AN was obtained from a denitrification microbial community that was cultivated as seed sludge for commercial USB reactors used for treating aquarium seawater. For the denitrification microbial community, acetate was added as carbon and energy source. Although denitrification was the primary microbial reaction in samples AQ, LAQ and AN, several previous studies have reported that methanogenesis and denitrification occur simultaneously, and that phylogenetically diverse methanogens are present in denitrification processes (Mosquera-Corral *et al.*, 2001; Sun *et al.*, 2008). Therefore, we used the denitrification sludges for this study. All the sludge samples were carefully washed once with phosphate-buffered saline (PBS: 130 mM NaCl, 10 mM Na₂HPO₄, 1.7 mM KH₂PO₄, 2.7 mM KCl [pH 7.4]) and then subjected to further experiments.

DNA extraction, PCR and clone library construction

Extraction of DNA from the granular sludge samples was performed using an ISOIL for beads beating kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. PCR amplification was performed using the Premix Ex Taq Hot Start Version (TaKaRa Bio Inc., Japan), and the reaction mixture for PCR was prepared according to the manufacturer's instruction. For PCR amplification, the primer pairs Mlf (5'-GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC-3'; Luton *et al.*,

2002)/MLr (5'-TTC ATT GCR TAG TTW GGR TAG TT-3'; Luton *et al.*, 2002) and Arc9F (5'-CYG GTY GAT CCY GCC RG-3'; Kato *et al.*, 2011)/ 1492R (5'-GGH TAC CTT GTT ACG ACT T-3'; a slightly modified version of a primer reported by Weisburg *et al.*, 1991) were used for construction of *mcrA* gene and archaeal 16S rRNA gene clone libraries, respectively. The PCR conditions were as follows: initial denaturation at 95°C for 60 sec, followed by 25 to 35 cycles of denaturation at 98°C for 10 sec, annealing for 30 sec (at 50°C for *mcrA* gene, 55°C for 16S rRNA gene) and extension at 72°C (60 sec for *mcrA* gene, 90 sec for 16S rRNA gene). To reduce possible bias caused by PCR amplification, the PCR products obtained at minimized PCR cycle numbers ranging from 25 to 35 cycles at five-cycle intervals were used. The PCR cycle numbers for amplification of the *mcrA* genes and 16S rRNA gene of sample MS were 35 and 30, respectively. The PCR products were purified using a MinElute PCR purification kit (Qiagen, Venlo, Netherlands) followed by cloning using a TOPO TA cloning kit (Invitrogen, Waltham, MA, USA). The *mcrA* and 16S rRNA gene sequences were determined using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems, Waltham, MA, USA) and an automated sequence analyzer (3730xl Genetic Analyzer, Applied Biosystems).

Phylogenetic analysis

Recovered *mcrA* gene and 16S rRNA gene clone sequences were classified into each phylotype using a threshold of 97% sequence similarity. The representative sequences of the phylotypes were subjected to similarity analysis with the NCBI's BLASTN program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The phylogenetic analyses were performed with the ARB program (Ludwig *et al.*, 2004). A 16S rRNA gene-based phylogenetic tree was constructed by applying the neighbor-joining method with Jukes-Cantor distance correction. A phylogenetic tree based on the deduced McrA amino acid sequence was constructed by the neighbor-joining method with 167 amino acid positions and the percentage of acceptance of mutations distance correction. In the phylogenetic analyses, the gene sequences from *Methanopyrus kandleri* were used as the outgroup. Bootstrap analyses of 1,000 trial replicates were performed with the MEGA version 6.06 package (Tamura *et al.*, 2013) to estimate the confidence of the tree topologies. The sequences reported in this study have been deposited in the GenBank/EMBL/DDBJ database under the accession numbers LC002077 to LC002191.

Table 1 - Anaerobic granular sludge samples used in this study.

Sample name	Treating wastewater	Reactor operation temperature [°C]	Reactor volume [L]	Carbon source	Loading rates for organic substances [g-COD/L/day] ^d	Influent NO ₃ ⁻ conc. [mg-N/L]	C/N ^a
MS	Pilot-scale municipal sewage	Ambient	1,178	Sewage 272 mg-COD/L ^b	0.82	B.D.	N.D.
AQ	Pilot-scale aquarium tank seawater	25	8.8	0.9 mM Acetate ^c	0.56	14	1.3
LAQ	Lab-scale aquarium tank seawater	25	2.0	1.3 mM Acetate ^c	0.81	23	1.3
AN	Lab-scale acetate and nitrate-amended synthetic seawater	Ambient	1,000	2.5 mM Acetate	1.5	30	2.0

B.D.: below detection limit < 0.05 mg-NO₃/L.

N.D.: not determined.

^aC/N: carbon to nitrogen ratio of wastewater.

^bCOD: chemical oxygen demand.

^cAcetate was major carbon source, but fish food residues and fish feces were potential carbon sources.

^dThe values were calculated based on the reactor volume.

Fluorescence *in situ* hybridization (FISH)

Fixation of the granular sludge samples was performed immediately after sampling. After washing the sludge samples with PBS buffer, the samples were fixed with 2% paraformaldehyde in PBS for 12 h at 4°C and stored in 50% ethanol with PBS at –20°C. Prior to FISH, the fixed granular sludge samples were dispersed by a manually operated homogenizer and an ultrasonic homogenizer (Model UH-50, SMT Co. Ltd, Tokyo, Japan, at 15 and 0 W for 15 sec alternately, six cycles on ice). The 16S rRNA-targeted oligonucleotide probes used in this study are listed in Table 2. Standard FISH was performed according to a previous report (Sekiguchi *et al.*, 1998), with modification by the addition of 1% (w/v) blocking reagent (Roche Diagnostics, Switzerland) to the hybridization buffer. In this study, we used 5' singly or 5', 3' doubly labeled oligonucleotide probes for the standard FISH. Both ends of the oligonucleotide probes were labeled with Alexa Fluor 488. Catalyzed reporter deposition (CARD)-FISH with horseradish peroxidase (HRP)-labeled probes was performed based on a method described previously (Kubota *et al.*, 2006). For the CARD-FISH experiments, the fixed samples were embedded in 0.1% low melting point agar (MetaPhor agarose, Cambrex, USA), and the following pretreatments for the incremental penetration of the HRP-labeled probe into fixed cells were performed: (i) proteinase K treatment (at 2, 10, 30, 50, 80, 100, 125, 160, and 200 µg/mL and 1 mg/mL in 100 mM Tris-HCl [pH 7.5] and 50 mM EDTA [pH 8.0] for 10 min at room temperature); (ii) HCl treatment (at 1, 10, and 100 mM and 1 M for 1 min at room temperature); and (iii) no pretreatment. To inactivate endogenous peroxidase, the cell samples were incubated with H₂O₂ solution (final concentration, 0.3% (v/v) in methanol) for 30 min at room temperature. As a negative control, the sludge samples were hybridized with an HRP-labeled nonsense probe, NON338 (Wallner *et al.*, 1993), which did not produce a CARD-FISH signal in the samples. The hybridization condition of probe Arc864 was determined by Clone-FISH (Schramm *et al.*, 2002), where the Clone-FISH sample was prepared as described in Kubota *et al.* (2006). Following all of the *in situ* hybridization steps, the FISH samples were finally counterstained with 4', 6-diamidino-2-phenylindole (DAPI; 1 µg/mL for 10 min at room temperature) to distinguish the microbial cells. An Olympus microscope (BX51F, Olympus, Japan) with a color CCD camera system (DP72, Olympus) was used for the microscopic observation.

Table 2 - 16S rRNA-targeted oligonucleotide probes used in this study.

Probe name	Target group	Sequence (5' to 3')	%FA	Reference
EUB338 ^a	<i>Bacteria</i>	GCT GCC TCC CGT AGG AGT	20	Amann <i>et al.</i> (1990)
EUB338-I ^a		GCA GCC TCC CGT AGG AGT		Daims <i>et al.</i> (1999)
EUB338-II ^a		GCA GCC ACC CGT AGG TGT		Daims <i>et al.</i> (1999)
EUB338-III ^a		GCT GCC ACC CGT AGG TGT		Daims <i>et al.</i> (1999)
NON338	Negative control for CARD-FISH	ACT CCT ACG GGA GGC AGC		Wallner <i>et al.</i> (1993)
ARC915 ^b	<i>Archaea</i>	GTG CTC CCC CGC CAA TTC CT	50 [50] ^c	Stahl and Amann (1991)
Arc864 ^b	WCHA1-57 ^d	CCC TAC AGC ACA GGG CCA	20° [60] ^{c,e}	Chouari <i>et al.</i> (2005)

^aThese probes were used in a mixture at an equal amount (mol).

^bThis probe is 5', 3' doubly labeled oligonucleotide probe was used in standard FISH analysis.

^cNumber in parenthesis is formamide (FA) concentration of the hybridization buffer in CARD-FISH analysis.

^dThe name of uncultured group according to reported by Dojka *et al.* (1998).

^eThese FA concentrations were determined by Clone-FISH experiment.

RESULTS AND DISCUSSION

Exploration of taxonomically novel methanogen group

To find taxonomically novel methanogen (unidentified *mcrA* gene) groups at least on the order level, *mcrA* gene-based clone analysis was conducted on four anaerobic granular sludge samples. About 90 clones from each library were randomly selected, sequenced and phylogenetically analyzed (Table 3 and Fig. 1a). Diverse *mcrA* gene phylotypes were detected, and almost all of the phylotypes belonged to previously identified methanogen groups namely, *Methanomicorbiales*, *Methanosarcinales*, *Methanobacteriales* and *Methanomassiliicoccales*. However, we also found that some *mcrA* phylotypes in all the anaerobic granular samples, except for the sample LAQ (Table 3 and Fig. 1a), were distantly related with any described methanogenic orders, and they were clustered into an unidentified *mcrA* group called MCR-2b group (Steinberg and Regan, 2009). The clonal abundance of the MCR-2b phylotypes was found to be 1.2 to 4.3%.

Since the sample MS had the highest relative clonal abundance of MCR-2b phylotypes, an archaeal 16S rRNA gene-based clone analysis was conducted on this sample to collate the MCR-2b group of *mcrA* gene phylotypes with 16S rRNA gene-based phylogenetic classification. Seventy-seven clones analyzed were classified into five phylotypes (Table 3). These phylotypes were affiliated with the orders *Methanosarcinales*, *Methanomicrobiales* and an uncultured archaeal group called WCHA1-57 (Fig. 1b). There was an inconsistency found for phylotype composition between the *mcrA* gene and 16S rRNA gene-based libraries of the sample MS: phylotypes related to *Methanobactreiales* were detected in the *mcrA* gene-based library, whereas *Methanobacteriales*-related phylotypes were not retrieved in the 16S rRNA gene-based library. This inconsistency may be caused by biases associated with PCR amplification and cloning. The phylotypes within the orders *Methanosarcinales* and *Methanomicrobiales* were very closely related to the sequences of previously isolated methanogens (members of the genera *Methanosaeta*, *Methanomethylovorans* and *Methanospirillum*) with high sequence similarities (> 98%). The clonal frequency of the phylotypes closely related to previously isolated methanogens accounted for 92.2%. The uncultured archaeal group WCHA1-57 (also called WSA2 or ARC I) belongs to the phylum *Euryarchaeota*, and the group has been considered as an uncultured archaeal lineage at the subphylum, order, or class levels (Sekiguchi, 2006). The WCHA1-57 phylotype detected in this study (i.e., MS sludge-16S1_2 in Fig. 1b) was the most closely related to the clone KuA13 obtained from oil-contaminated groundwater (sequence identity 99.6%; GenBank accession number AB077223; Watanabe *et al.*, 2002). The relative abundance of the WCHA1-57 phylotype in the clone library was 7.8%. Since the WCHA1-57 phylotype is the only uncultured archaeal group among the detected 16S rRNA gene phylotypes, the MCR-2b *mcrA* gene sequences would be derived from the host methanogen, the 16S rRNA genes of which represent the WCHA1-57 phylotype.

Table 3 - Phylogenetic affiliations of *mcrA* and 16S rRNA genes clones obtained from anaerobic granule sludge samples.

Phylogenetic affiliation	Sample name							
	MS		LAQ		AQ		AN	
	Number of clones	Number of phylotypes ^a	Number of clones	Number of phylotypes	Number of clones	Number of phylotypes	Number of clones	Number of phylotypes
<i>mcrA</i>								
<i>Methanomicrobiales</i>	34	19	- ^b	-	3	3	22	5
<i>Methanosarcinales</i>								
<i>Methanosaetaceae</i>	18	4	-	-	4	2	11	8
<i>Methanosarcinaceae</i>	2	2	-	-	3	1	-	-
<i>Methanomassiliicoccales</i>	1	1	-	-	2	2	5	4
<i>Methanobacteriales</i>	20	7	54	7	36	8	27	4
<i>Methanobacteriales mrtA</i>	12	3	37	7	37	11	27	8
MCR-2b ^c	4	1	-	-	1	1	2	2
Total	91	37	91	14	86	28	94	31
16S rRNA								
<i>Methanomicrobiales</i>	2	1						
<i>Methanosarcinales</i>								
<i>Methanosaetaceae</i>	68	2						
<i>Methanosarcinaceae</i>	1	1						
WCHA1-57 ^d	6	1						
Total	77	5						

^aA phylotype was defined as > 97.0 % sequence identity.^b-, Not detected.^cThe name of uncultured group according to reported by Steinberg and Regan (2009).^dThe name of uncultured group according to reported by Dojka *et al.* (1998).

Fig. 1 - Phylogenetic trees of (a) deduced McrA amino acid sequences and (b) archaeal 16S rRNA gene sequences, featuring the position of the MCR2-b *mcrA* and WCHA1-57 16S rRNA gene phylotypes obtained in this study. Phylotypes in boldface were obtained in this study. The head of each phylotype name indicates the sample name. The numbers in parentheses indicate the number of identical clones obtained per number of clones analyzed for each phylotype. The accession numbers are shown after each phylotype name. The scale bars indicate 10% sequence divergence. The symbols at the nodes show the bootstrap values (> 70% indicated only) obtained after 1,000 replicates.

To verify this implication, a comparative phylogenetic analysis of the deduced McrA amino acid and the 16S rRNA gene sequences was performed. The result showed that the MCR-2b McrA clade likely corresponded to the WCHA1-57 archaeal clade (Fig. 1). This implies that the host archaea of WCHA1-57 16S rRNA gene phylotypes are methanogens. Previous observations also support the possibility of WCHA1-57 archaea being a methanogen group. Chouari *et al.* (2005) reported that the enrichment of WCHA1-57 (ARC I) phylotypes in cultivations using formate or H₂/CO₂ as substrates, which are typical substrates for methanogenic archaea, although the methane production of the enrichments was not checked in the literature. In addition, many WCHA1-57-related 16S rRNA gene sequences have been retrieved from anaerobic digesters (Chouari *et al.*, 2005; Steinberg and Regan, 2008; Rivière *et al.*, 2009; Schauer-Gimenez *et al.*, 2010). In some anaerobic digesters treating municipal sewage sludge, the WCHA1-57 phylotype population represented one of the predominant archaeal components, and the relative clonal abundance was > 70% in the archaeal clone libraries (Chouari *et al.*, 2005; Rivière *et al.*, 2009). These observations indicate that WCHA1-57 archaea make up one of the important microbial populations in anaerobic digestion systems. Many WCHA1-57 phylotypes have been found not only in anaerobic digesters but also in a variety of anoxic environments such as oil-contaminated sites (Watanabe *et al.*, 2002; Ramos-Padrón *et al.*, 2011), lake sediments (Chan *et al.*, 2005) and landfills (Huang *et al.*, 2003; Clementino *et al.*, 2006). Similarly, MCR-2b *mcrA* phylotypes have been identified in various anaerobic environments, especially in the anaerobic digesters (Nettmann *et al.*, 2008; Steinberg and Regan, 2009).

Although the comparative phylogenetic analysis suggests an interrelationship between the MCR-2b *mcrA* group and the WCHA1-57 16S rRNA gene group, one cannot exclude the possibility that WCHA1-57 archaea do not function as methanogens. The *mcrA* gene in WCHA1-57 archaea may have been acquired through horizontal gene transfer of *mcrA* alone from a methanogenic lineage, or it may be an evolutionary relict that is no longer effective for the methane production in WCHA1-57 archaea (inactivation and/or deletion of genes essential for methanogenesis other than *mcrA*).

Attempt to identify WCHA1-57 archaea harboring the *mcrA* gene using FISH techniques

In order to verify whether WCHA1-57 archaea actually harbor the *mcrA* gene affiliated with the MCR-2b clade, a molecular-based analysis with the following techniques was attempted: (i) WCHA1-57 archaeal cells are visually detected by FISH with a specific 16S rRNA targeted probe; (ii) FISH-positive cells are sorted by fluorescence-activated cell sorting (FACS); and (iii) the *mcrA* gene sequences retrieved from sorted cells are analyzed. This approach was based on a combination of FISH and FACS analysis that has been used for the linking of phylogeny and metabolic activity (Mota *et al.*, 2012).

First, we tried to detect WCHA1-57 archaeal cells by standard FISH, using a specific probe (Arc864), in three anaerobic sludges that contained MCR-2b *mcrA* phylotypes (i.e., samples MS, AQ and AN). Unfortunately, we did not obtain any signal with the Arc864 probe in triplicate experiments, although we detected archaeal cells having several morphologies by the archaeal universal probe ARC915 (data not shown). Moreover, we employed CARD-FISH, a highly sensitive FISH technique, with and

without cell wall treatment for sample MS. However, we did not obtained a positive signal. These observations suggest that the WCHA1-57 archaeal cells express a very low content of rRNA in the anaerobic digesters and/or they may have a unique cell wall that is less permeable to the HRP-labeled oligonucleotide probes (Kubota, 2013). Chouari *et al.* (2005) reported the successful FISH detection of WCHA1-57 archaeal cells in enrichment cultures. Assuming that WCHA1-57 archaeal cells would actually grow in their enrichment cultures, a sufficient number of rRNAs could be present in the cells and the archaeal cells would thus be successfully detected by the standard FISH technique. Thus, a low rRNA content of WCHA1-57 archaeal cells in the anaerobic digesters might be a critical reason for the unsuccessful detection of WCHA1-57 archaeal cells by any of the FISH techniques used in this study. Because of this failure to detect WCHA1-57 archaeal cells, it was difficult to conduct further experiments mentioned above. In the future, we will try to employ a more sensitive FISH technique that can detect single-copy genes encoded on a chromosome (Moraru *et al.*, 2010; Kawakami *et al.*, 2012), to clarify whether WCHA1-57 archaea actually harbor the *mcrA* gene.

CONCLUSION

In this study, *mcrA* gene-based clone analysis was performed to find taxonomically novel methanogens from anaerobic granular sludges. Several novel *mcrA* gene sequences distantly related with the *mcrA* gene sequences of known methanogen groups were retrieved from the anaerobic granular sludge samples. All the unique *mcrA* gene sequences were affiliated with an unidentified *mcrA* group called MCR-2b. Comparative phylogenetic analysis of the deduced MCR-2b McrA amino acid and the 16S rRNA gene sequences suggests that the MCR-2b McrA group may be derived from a new taxon of methanogens, represented by the phylogenetic group of 16S rRNA gene sequences as WCHA1-57. Despite the attempts of molecular characterization by FISH techniques, it remains unclear whether or not WCHA1-57 archaea actually harbor the *mcrA* gene of MCR-2b.

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