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Methanogen community in Zoige wetland of Tibetan plateau and phenotypic characterization of a dominant uncultured methanogen cluster ZC-I

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Summary

Zoige wetland of Tibetan plateau is characterized by being located at a low latitude (33°56′N, 102°52′E) region and under the annual temperature around 1°C. Previous studies indicated that Zoige wetland was one of the CH_4 emission centres in Qinghai-Tibetan plateau; in this study, the methanogen community in this low-latitude wetland was analysed based on the homology of 16S rRNA and *mcrA* genes retrieved from the soil. The results indicated that members of *Methanosarcinales* and *Methanomicrobiales* constituted the majority of methanogens, and a novel uncultured methanogen cluster, Zoige cluster I (ZC-I) affiliated to *Methanosarcinales*, could be dominant. Using quantitative polymerase chain reaction (qPCR) assay, ZC-I methanogens were estimated to be 10^7 cells per gram of soil, accounting for about 30% of the total *Archeae*. By combining culturable enrichment with qPCR assay, the quantity of ZC-I methanogens in the methanogenic enrichment with acetate, H2/CO_2, methanol or trimethylamine was determined to increase to 10^8 cells ml^{-1}, but not with formate, which indicated that ZC-I methanogens could use the four methanogenic substrates. The growth rates at 30°C and 15°C were not pronounced different, implying ZC-I to be the cold-adaptive methanogens. The broad substrate spectrum identified the ZC-I methanogens to be a member of *Methanosarcinaceae*, and could represent a novel sub-branch specifically inhabited in cold ecosystems. Fluorescence *in situ* hybridization (FISH) images also visualized ZC-I methanogens the sarcina-like aggregate of the spherical cells. The prevalence and flexibility in substrate utilization and growth temperature suggested ZC-I methanogens to be an important player in the methanogenesis of Zoige wetland.

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Introduction

Nature wetlands are the important methane source as where inhabit abundant methanogenic *Archaea* (Matthews and Fung, 1987). Wetlands in cold area, like permafrost and tundra, contribute a large proportion of global methane emission as about 75% of Earth's biosphere is under permanently low temperature (Cavicchioli, 2006). Numerous studies about the methanogenic pathway and methanogen structure in paddy field and on boreal permafrost and peatlands indicated that acetoclastic methanogenesis was prevalent at low temperature and *Methanosarcinales* constituted the majority of the methanogens, and *Methanomicrobiaceae* constituted the main hydrogenotrophic methanogens (Schulz *et al.*, 1997; Falz *et al.*, 1999; Fey and Conrad, 2000; Fey *et al.*, 2004; Kotsyurbenko *et al.*, 2004; Cadillo-Quiroz *et al.*, 2006). More precisely, Hoej and colleagues (2005) reported that *Methanosarcina* was infrequently detected in the high arctic wetlands Spitsbergen, Norway, while *Methanosaeta* was the dominant acetoclastic methanogen. Study on different types of boreal wetlands with acidic pH in Finland did reveal the distinct methanogen communities (Galand *et al.*, 2005), most likely correlated to the different vegetations. Therefore cold wetlands located in different regions of the earth could harbour a unique predominant methanogen community to mediate a distinct methanogenic pathway.

Zoige wetland of Tibetan plateau is a type of cold wetland distinct from the boreal ones by located at a low-latitude (33°56′N, 102°52′E) region; however, it is under permanent low temperature at an annual average temperature around 1°C due to the high altitude of 3400–3600 m. Previous studies indicated that the annual methane emission in Qinghai-Tibetan plateau was estimated to be 0.56–1 Tg (Jin *et al.*, 1999; Ding *et al.*, 2004; Ding and Cai, 2007), and Zoige wetland and the head-stream site of Yangtze River and Yellow River could be the

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methane emission centres of the plateau (Jin *et al*., 1999). Chen and colleagues (2008) recently reported that the mean methane emission rate of 14.45 mg CH_4 m^{-2} h^{-1} (0.17–86.78 mg CH_4 m^{-2} h^{-1}) was measured in Zoige wetland covered by two dominant vegetations (*Carex muliensis* and *Eleocharis valleculosa*) during growing season. These implied that psychroactive methanogens could inhabit in the wetland soil; however, the methanogen community structure in this low-altitude cold wetland is poorly understood.

In the present study, the methanogen community structure in Zoige wetland soil was analysed based on the homology of 16S rRNA and *mcrA* genes retrieved from the soil. Furthermore, by combining culturable enrichment with uncultured approaches, a dominant uncultured methanogen cluster affiliated to *Methanosarcinales*, which could be the cold ecosystem-specific methanogen cluster, was phenotypically characterized.

Results

Analysis of methanogen community structure in Zoige wetland

To get insight of the methanogen community structure in Zoige wetland soil, total DNA was extracted from the soil covered by *E. valleculosa*, a dominant grass in the wetland, and then phylogenetic diversities of archaeal 16S rRNA gene and methyl-coenzyme M reductase subunit A (*mcrA*) gene, a gene exclusively existing in methanogens, were analysed. A pair of *Archaea*-specific 16S rRNA primers 915F/1492R and a pair of *mcrA* gene primers MCRf/MCRr (Table 1) were applied to amplify the respective gene. Totally 55 16S rRNA gene fragments were retrieved and the sequence homology analysis (Fig. 1A) indicated that the majority were from the members of two orders of methanogens, of 25 sequences related to *Methanosarcinales* and 15 sequences related to *Methanomicrobiales*. The remaining sequences were retrieved from unidentified *Euryarchaeota* (four sequences) and uncultured *Crenarchaeota* (seven sequences). The 16S rRNA sequences of an uncultured methanogen cluster within *Methanosarcinales*, designated as Zoige Cluster I (ZC-I), accounted for 18 out of 55 clones and were remotely related to the cultured members of *Methanosarcinales* at the highest similarity of 16S rRNA with *Methanococcoides burtonii* (92%), a psychrophilic methanogen isolated from Antarctic lake. Therefore ZC-I could represent a dominant uncultured methanogen group in Zoige wetland. Similar result of methanogen diversity was obtained from the deduced McrA protein homology analysis (Fig. 1B). Among the 24 *mcrA* sequences, an uncultured methanogen group comprised of nine *mcrA* sequences, possibly the equivalent of ZC-I cluster in 16S rRNA phylogenetic tree, was also clustered with *Methanosarcinales*. The equivalence of ZC-I cluster in both phylogenetic trees could be supported by that higher ratio (19 out of 25) of *mcrA* sequences (GenBank Accession No. EU430345–EU430369) retrieved from a ZC-I methanogens highly enriched acetate culture (inoculated with 10^{-7} dilution soil in Table 2) fell in the 16S rRNA ZC-I cluster equivalent. The second dominant *mcrA* group consisted of eight sequences could be an uncultured methanogen group affiliated to *Methanomicrobiales* and more *mcrA* sequences (four) than 16S rRNA sequences (two) were clustered to RC-I.

Quantification of the uncultured methanogen cluster ZC-I in the in situ *soil*

As higher ratio of ZC-I methanogens sequences was determined both in 16S rRNA and in *mcrA* gene libraries, quantitative real-time polymerase chain reaction (PCR) assays were developed to determine the 16S rRNA gene copies of ZC-I methanogens and total *Archaea* in Zoige wetland soil by using the ZC-I cluster-specific 16S rRNA

Table 1. Oligonucleotide primers and probes used in this study.

Primer or probe	Specificity	Sequence[b] (5′ to 3′)	Target genes	Base position[a]	Application	Reference
915F	*Archaea*	AAAGGAATTGGCGGGGGAGCAC	16S RNA	913–934	PCR	Amann *et al.* (1995)
1492R	Universal	TACGGYTACCTTGTTACGACTT	16S RNA	1492–1513	PCR	Amann *et al.* (1995)
MCRf	Methanogens	TAYGAYCARATHTGGYT	*mcrA*	ND	PCR	Springer *et al.* (1995)
MCRr	Methanogens	ACRTTCATNGCRTARTT	*mcrA*	ND	PCR	Springer *et al.* (1995)
Arc787	*Archaea*	ATTAGATACCCSBGTAGTCC	16S RNA	787–806	qPCR	Yu *et al.* (2005)
Arc1059	*Archaea*	GCCATGCACCWCCTCT	16S RNA	1044–1059	qPCR	Yu *et al.* (2005)
MethanoF	ZC-I cluster	TCAGGCAACGAGCAAGACC	16S RNA	1094–1112	qPCR	This study
MethanoR	ZC-I cluster	GATATTGATACGCGATTACTAC	16S RNA	1351–1372	qPCR	This study
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	16S RNA	915–934	FISH	Raskin *et al.* (1994)
Probe ZC-I	ZC-I cluster	GCCAAAAAGCGTGCCCTCATTC	16S RNA	1281–1302	FISH	This study

a. Position in the 16S of *Escherichia coli* (Brosius *et al*., 1978).
b. Y = C/T; R = A/G; N = A/C/G/T; S = C/G; B = C/G/T; W = A/T.
ND, not determined.

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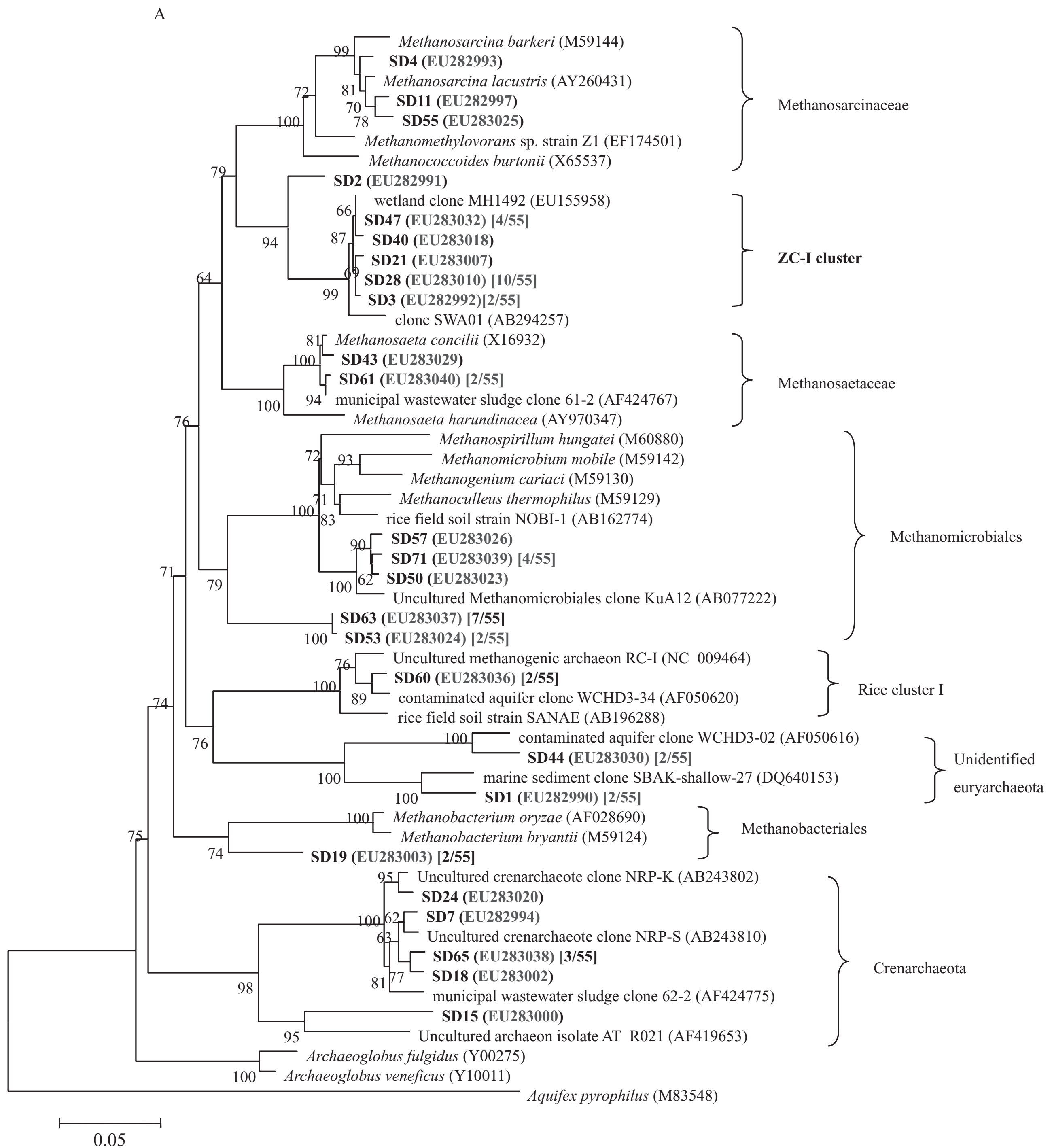

Fig. 1. A. Phylogenetic tree of archaeal 16S rRNA gene sequences (bold) retrieved from Zoige wetland soil based on the homology of partial sequences in length of 599 bp. The tree was rooted with *Aquifex pyrohilus* and constructed using neighbour-joining method and the clustering nodes were also recovered in maximum likelihood method. Numbers at nodes represent levels of bootstrap support (%) based on neighbour-joining analysis of 1000 replicated data sets. GenBank accession numbers are given in parentheses and numbers in square brackets indicate the clone number out of the total clones. Bar represents 5% sequence divergence.
B. Phylogenetic tree of methanogen *mcrA* sequences (bold) retrieved from Zoige wetland soil based on the homology of deduced amino acid sequences in length of 158 amino acids. The tree was rooted with *Methanopyrus kandleri* and constructed using neighbour-joining method. Numbers at nodes represent levels of bootstrap support (%) based on neighbour-joining analysis of 1000 replicated data sets. GenBank accession numbers are given in parentheses and numbers in square brackets indicate the clone number out of the total clones. Bar represented 5% sequence divergence.

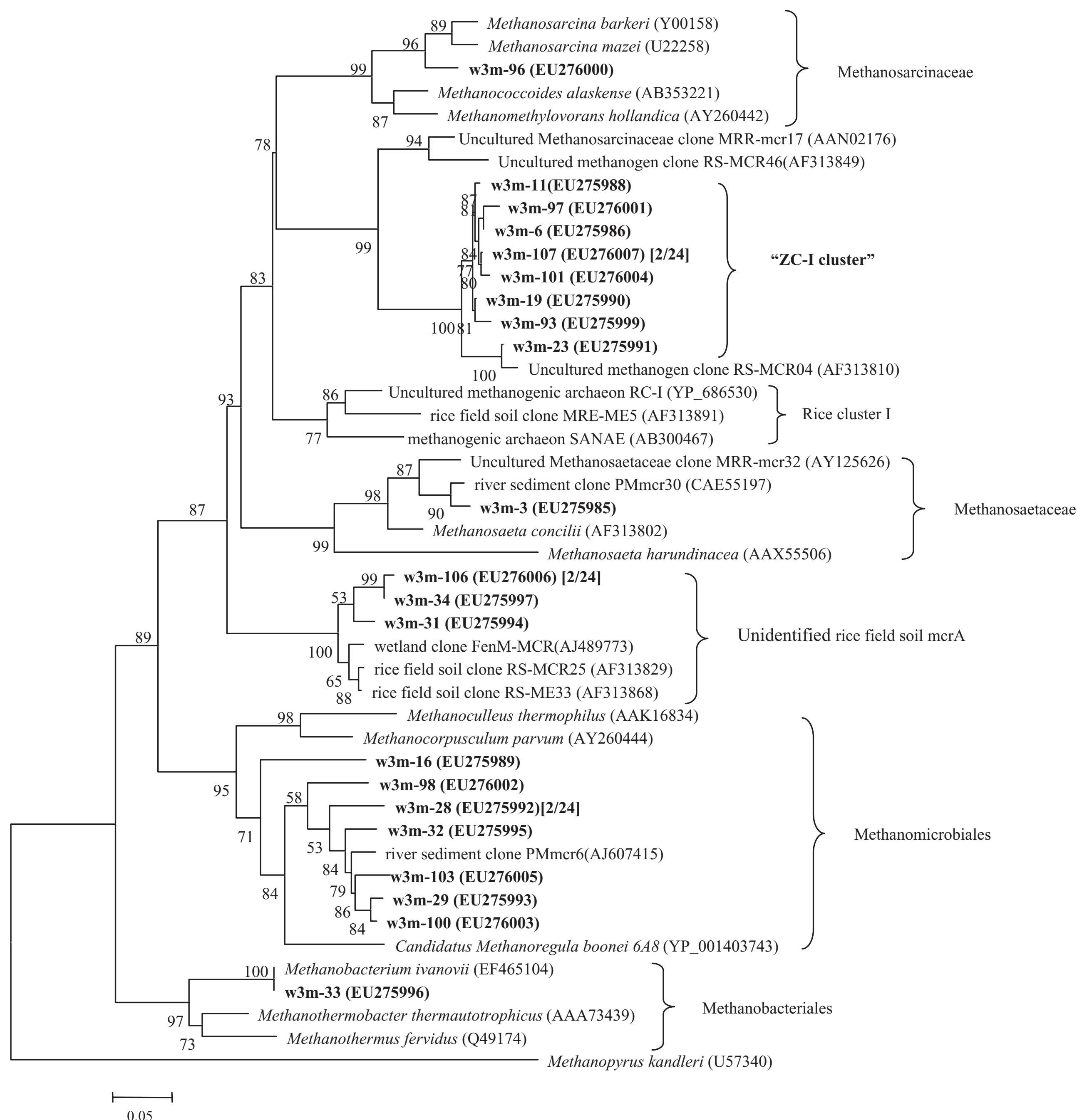

Fig. 1. *Continued*

primer set MethanoF/MethanoR and a universal *Archaea* 16S rRNA primer set Arc787/Arc1059 listed in Table 1.

The wetland soil sampled from *E. valleculosa* rhizosphere was 10-fold serially diluted till 10^{-8} magnitude, and then ZC-I methanogens and total *Archaea* in all the dilutions were quantified by quantitative polymerase chain reaction (qPCR) assays. The result showed that the 16S rRNA gene copies of ZC-I methanogens and total *Archaea* were $(7.76 \pm 1.13) \times 10^7$ per gram of soil and $(2.66 \pm 0.34) \times 10^8$ per gram of soil (Fig. 2), respectively, which indicated that the cell number of ZC-I methanogens in the soil could be 10^7 magnitude g^{-1}. Hence the percentage of ZC-I methanogens in total *Archaea* in the soil was

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Table 2. 16S rRNA copy numbers of ZC-I methanogens and total *Archaea* in various methanogenic enrichments inoculated with 10-fold serial diluted soil and incubated at 15°C for 60 days.

Substrate (20 mmol l^{-1} or 10^5 Pa)	10^{-1} dilution			10^{-3} dilution			10^{-5} dilution			10^{-7} dilution		
	ZC-I ($\times 10^6$)	*Archaea* ($\times 10^6$)	Z/A (%)	ZC-I ($\times 10^4$)	*Archaea* ($\times 10^4$)	Z/A (%)	ZC-I ($\times 10^2$)	*Archaea* ($\times 10^2$)	Z/A (%)	ZC-I ($\times 10^1$)	*Archaea* ($\times 10^1$)	Z/A (%)
Acetate	219.8 ± 6.1	452.3 ± 8.8	48.6 ± 2.3	183.3 ± 5.6	323.3 ± 8.9	56.7 ± 3.4	192.1 ± 4.5	302.5 ± 6.3	63.5 ± 2.9	211.9 ± 3.7	271.1 ± 5.9	78.2 ± 2.7
Methanol	205.7 ± 5.3	493.3 ± 9.1	41.7 ± 1.9	196.9 ± 2.2	374.3 ± 7.5	52.6 ± 1.7	187.6 ± 2.9	317.4 ± 4.1	59.1 ± 1.8	231.7 ± 4.1	343.8 ± 4.8	67.4 ± 2.2
Trimethylamine	236.6 ± 7.1	458.5 ± 8.2	51.6 ± 2.6	242.4 ± 3.8	412.9 ± 6.3	58.7 ± 1.9	214.2 ± 3.0	328.5 ± 4.2	65.2 ± 2.1	219.5 ± 3.4	297.1 ± 3.7	73.9 ± 1.8
H_2/CO_2	151.5 ± 4.8	342.8 ± 6.2	44.2 ± 2.3	132.8 ± 3.2	263.5 ± 4.2	50.4 ± 2.1	167.1 ± 2.7	270.8 ± 3.1	61.7 ± 1.8	163.7 ± 3.8	228.3 ± 4.9	71.7 ± 3.2
Formate	7.1 ± 1.3	98 ± 4.6	7.2 ± 1.8	8.4 ± 0.9	144.3 ± 7.1	5.8 ± 0.8	7.9 ± 1.5	203.5 ± 2.3	3.9 ± 0.7	ND	ND	ND

Z/A, ZC-I/*Archaea*; ND, not detected.

calculated to be 29.37 ± 5.88% in the dilution magnitude of 10^{-1}–10^{-4}, while the percentage was elevated to about 40% in 10^{-7} dilution due to the total *Archaea* being reduced to $(1.77 \pm 0.17) \times 10^8$ copies in the dilution magnitude. This further confirmed the high quantity of ZC-I methanogens in the soil and implied that it was active in methanogenesis of the wetland.

Identification of substrate spectrum of the uncultured methanogen cluster ZC-I

As cluster ZC-I could be active in methanogenesis of Zoige wetland, its substrate spectrum was identified in this experiment. The soil sampled from *E. valleculosa* rhizosphere was 10-fold serially diluted to 10^{-7} magnitude to the broths containing each of the five methanogenic substrates (20 mmol l^{-1}) and cultured at 15°C. After 60 days of incubation, methane accumulation of 18.1 ± 1.1 mmol l^{-1} in methanol, 41.3 ± 1.7 mmol l^{-1} in trimethylamine, 18.6 ± 0.8 mmol l^{-1} in acetate and 11.8 ± 2.3 mmol l^{-1} in H2/CO_2 was measured in the enrichments inoculated with 10^{-1} dilution of the soil; however, methane accumulation was only 4.3 ± 0.7 mmol l^{-1} in formate enrichment, only 2.5 mmol l^{-1} more than that in blank (1.8 ± 0.3 mmol l^{-1}). Then ZC-I methanogens and total *Archaea* were quantified in the enrichments inoculated with various dilutions of soil slurry. The results (Table 2) showed that by comparing with those in the *in situ* soil (Fig. 2), the quantity of ZC-I methanogens was increased in all the enrichments except for formate enrichment. And 16S rRNA copies of ZC-I methanogens were detected as more than 2×10^8 per millilitre of cultures of methanol, trimethylamine and acetate and 1.5×10^8 per millilitre of culture of H_2/CO_2 enrichment inoculating with 10^{-1} dilution of soil. This indicated that ZC-I methanogens could use methanol, trimethylamine, acetate and H_2/CO_2 to produce methane, but not formate. Although the absolute quantity of ZC-I methanogens decreased in the four enrichment cultures with the increasing dilution magnitude of the inoculated soil, the percentage of ZC-I in total *Archaea* all increased in proportional to the dilution magnitude and to account for 67–78% in the 10^{-7} soil dilution enrichments (Table 2), further verifying the predominance of ZC-I methanogens in the soil. The uncultured methanogen ZC-I shared the common metabolic characteristics of members in the genus of *Methanosarcina* by having a broad substrate spectrum, and the phenotypic characterization was accordance with its phylogenetic affiliation to *Methanosarcinales* as well.

Growth temperature of the uncultured methanogen cluster ZC-I

To test the growth temperature of ZC-I methanogens, the wetland soil was inoculated into 60 mmol of acetate and cultivated either at 15°C or at 30°C. CH_4 production was

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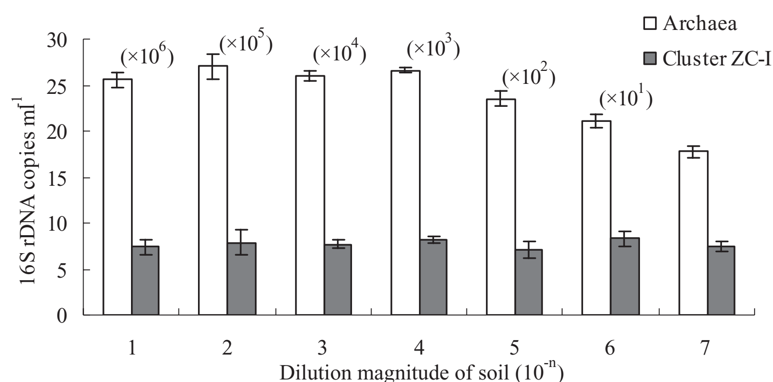

Fig. 2. 16S rDNA copy numbers of cluster ZC-I methanogens and total *Archaea* in 10-fold serial dilutions of the *in situ* rizhosphere soil of *Eleochalis valleculosa*. Numbers on the top of each bar refer to the logarithm values of 16S rDNA copy numbers (mean ± SE of three DNA extractions).

followed until 60 days of incubation (Fig. 3A), and the average methane production rates were measured to be 0.9 mmol l^{-1} CH_4 day^{-1} at 15°C and 1.2 mmol l^{-1} CH_4 day^{-1} at 30°C respectively. Then the increase of ZC-I methanogens in the enrichments during incubation period was estimated by qPCR assay. The assay showed that the quantity of ZC-I methanogens was increased at a rate of $(5.8 \pm 0.4) \times 10^6$ copies per day at 15°C and $(8.1 \pm 0.7) \times 10^6$ copies per day at 30°C (Fig. 3B), respectively, suggesting that ZC-I methanogens grew mesophilically while adapted to lower temperature, such as 15°C, the *in situ* soil temperature in this study.

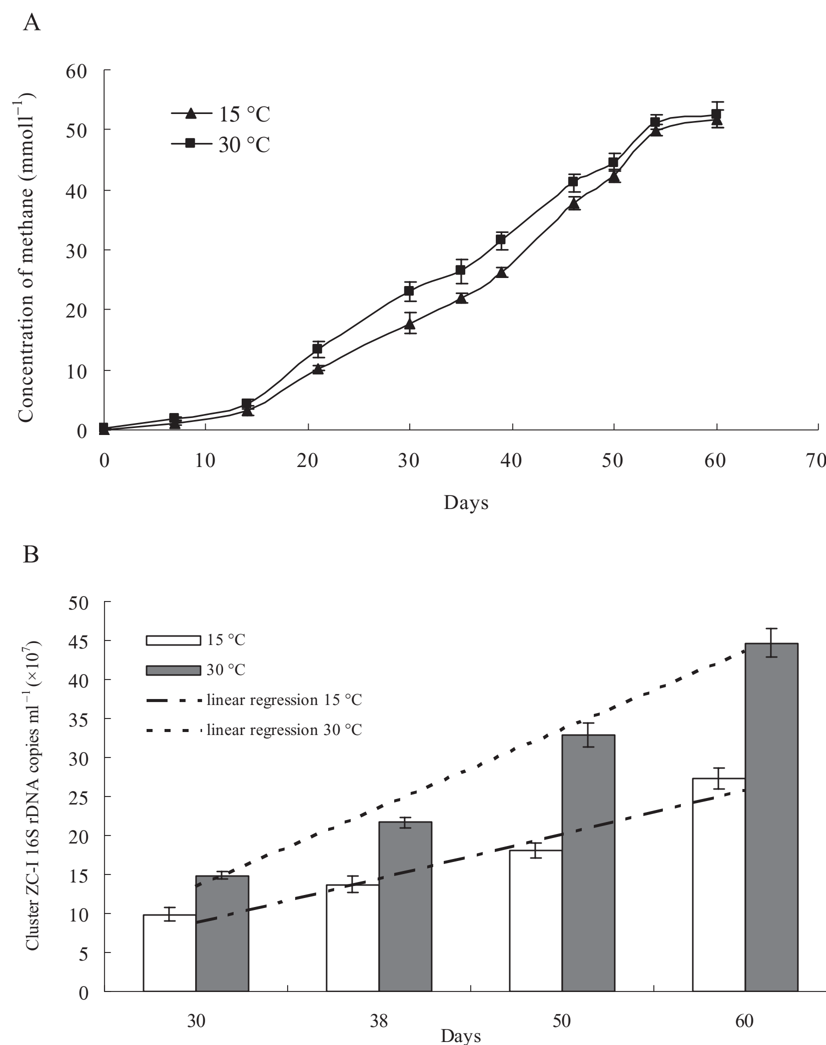

Fig. 3. Methane production and the 16S rDNA copy numbers of ZC-I cluster in the enrichment with acetate (60 mmol l^{-1}) at 15°C or 30°C.
A. Methane production plots against incubation time (days) (mean ± SE of three parallel tests).
B. 16S rDNA copy numbers of ZC-I cluster per millilitre of culture. Dashed linear regressions were calculated to show the increase tendency of 16S rDNA copies (mean ± SE of three DNA extractions).

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Visualization of the uncultured methanogen cluster ZC-I by fluorescence in situ *hybridization (FISH)*

Although purified culture of ZC-I methanogens was not obtained so far, both increases of the 16S rRNA copies and percentage in total *Archaea* in the enrichments indicated that ZC-I methanogens were highly enriched. Therefore fluorescence *in situ* hybridization (FISH) was performed to visualize the morphology of ZC-I methanogens by using ZC-I-specific probe ZC-I, and of *Archaea* by an *Archaea* universal probe ARC915 (Table 1) respectively. Fluorescence *in situ* hybridization image of the acetate enrichment showed that sarcina-like aggregates formed by spherical cells emitted strong red fluorescence (probed to ZC-I) amid the cells with green fluorescence (probe ARC915) (Fig. 4A and B). The FISH image further verified ZC-I methanogens as a kind of methanosarcina. Both *Archaea* and ZC-I methanogens accounted for high ratio in DAPI-stained enrichment (Fig. 4C), indicating that the majority of *Archaea* in the enrichment was comprised of methanogens, as they were highly enriched by methanogenic substrates.

Discussion

Zoige wetland of Tibetan plateau covers an area of 2.8×10^4 km^2, and this peatland contains high organic matter attribute to the high coverage of vegetation and under permanently low temperature. As a typical low-latitude cold wetland, Zoige wetland could harbour a unique methanogen community. Both 16S rRNA and *mcrA* gene homology analyses showed that members in the orders of *Methanosarcinales* and *Methanomicrobiales* constituted the majority of methanogens, while *Methanobacteriales* were at low ratio. Methanogen community of Zoige wetland studied in this study was similar to those of a petroleum reservoir in Canada and Siberian Arctic (Grabowski *et al.*, 2005; Ganzert *et al.*, 2007), so that *Methanomicrobiales* could be the cold selective hydrogenotrophic methanogens. Similar observations were also reported in profundal lake sediments (Nusslein *et al.*, 2001), natural boreal wetlands (Kotsyurbenko *et al.*, 2004) and the boreal peatlands of Finland (Galand *et al.*, 2005). Furthermore, 16S rRNA sequences related to the uncultured methanogen cluster ZC-I retrieved from Zoige wetland were also detected in other ecosystems,

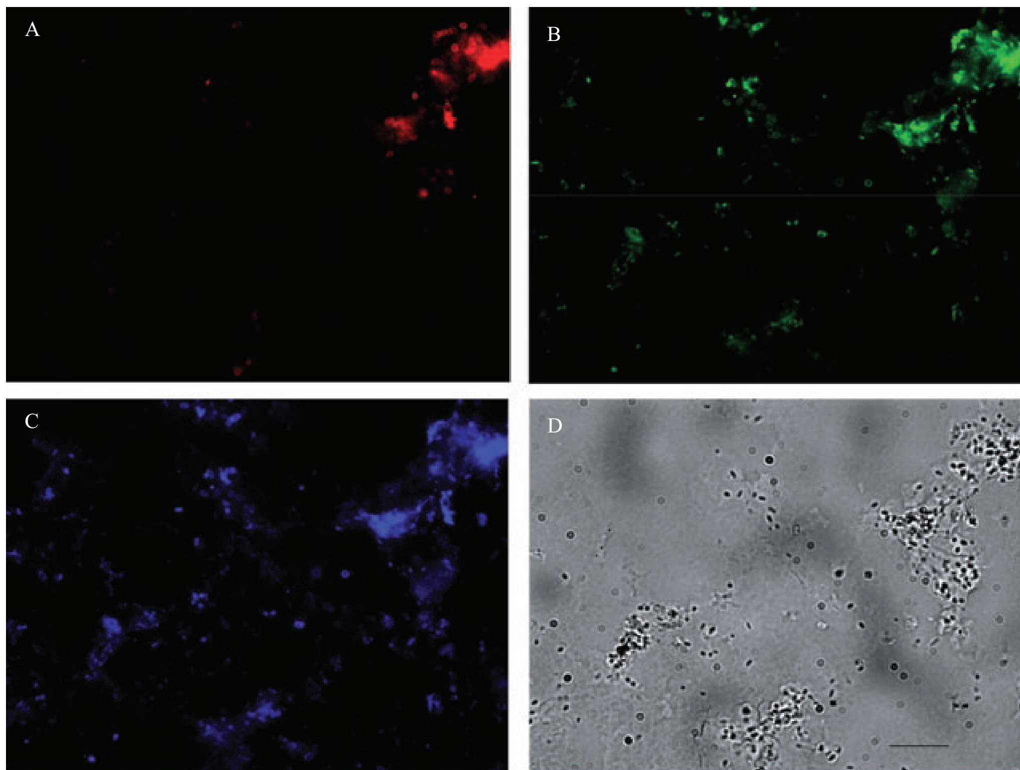

Fig. 4. Epifluorescence micrographs of whole-cell hybridization of the enrichment culture growing in 60 mmol l^{-1} of acetate inoculated with 10^{-7} dilution of the soil. The same microscopic field is shown (A) after hybridization with a 5′-Cy3-labelled oligonucleotide probe specific for ZC-I methanogens; (B) after hybridization with a 5′-FAM-labelled oligonucleotide probe of total *Archaea*; (C) after staining of universal DNA with DAPI; (D) as phase-contrast image. The bar indicates a size of 10 μm.

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like clone SWA01 (AB294257) obtained from a deep coal seam groundwater of northern Japan (Shimizu *et al.*, 2007) and clone MH1492 (EU155958) from a minerotrophic fen (H. Cadillo-Quiroz, E. Yashiro, J.B. Yavitt and S.H. Zinder, unpubl. data). This suggested that methanogens in cluster ZC-1 could distribute widely, but frequently occurred in cold ecosystems.

Although they were all peatlands, Zoige wetland could harbour a methanogen community slightly different from those of boreal cold wetlands, like the acidic peatland of Siberia (Kotsyurbenko *et al.*, 2004), where RC-II was a dominant methanogen cluster. The reason that RC-II was not detected in Zoige wetland probably was due to either the real absence of RC-II or different primers applied in this study. The methanogen structure in Zoige wetland was also distinct from those in the peatlands of Finland, where *Methanosaeta*, RC-I and Fen cluster were prevalent in fens and bog (Galand *et al.*, 2005) respectively. The distinct methanogen groups in different wetlands could be correlated to the field vegetation types, those would either excrete unique compound mixture or accumulate grass-specific biomass, the latter would provide methanogenic substrates via multiple species involved biodegradation relay. Rooney-Varga and colleagues (2007) also indicated a linkage between vegetation type and archaeal community composition in Alaskan peatlands.

The uncultured methanogen cluster ZC-I was quantified to be 10^7 per gram of soil according to three or four 16S rRNA gene copies in *Methanosarcinales* (Maeder *et al.*, 2006) and estimated to account for about 30% of total *Archaea* inhabited in the wetland. Therefore they could represent the majority of *Methanosarcinales* members in Zoige wetland. ZC-I methanogens were phenotypic characterized as the psychroactive *Methanosarcina* by utilizing diverse methanogenic substrates except for formate. Fluorescence *in situ* hybridization also visualized a sarcina-like cell aggregation, but never observed the *Methanosaeta*-like long filament cell shape, confirming ZC-I methanogens the membership of *Methanosarcina* and possibly a novel group of *Methanosarcinales.*

Growth temperature experiment indicated ZC-I methanogens to be mesophilic while with better cold adaptability as growth rate at 30°C (1.2 mmol CH_4 day^{-1}) was only 1.3 times faster than at 15°C (0.9 mmol CH_4 day^{-1}). In addition, although ZC-I methanogens grew faster at higher temperature, the acetoclastic methanogenesis rates of the wetland soil were similar at both temperatures (Fig. 3A), suggesting that other psychroactive acetoclastic methanogen(s) could inhabit in the soil. It is believed that the psychroactive (psychrotolerant) microorganisms, including methanogens dominant in cold terrestrial ecosystems, are equipped by the potential to develop within a wide range of temperature and can play a significant role in organic matter turnover. It is likely that the diverse metabolizing capability and the flexibility in adapting the ambient temperature give ZC-I methanogens the robustness and richness in the cold wetland, so as to have a significant contribution in methane emission of Zoige wetland.

Experimental procedures

Soil sampling

The sampling site, Zoige wetland, is located at Tibetan plateau at 33°56′N, 102°52′E and at an altitude of 3430–3460 m. This peatland is covered dominantly by *C. muliensis* and *E. valleculosa*, and the soil temperatures are determined between 6°C and 15°C from June to September in the depth of 10–30 cm (Yang and Dong, 1993) and pH was 7.0–7.5. The soil covered by *E. valleculosa* in the depth of 20–30 cm was sampled at the end of August 2005 and filled into sterile serum bottles sealed with butyl rubber stopper, and then kept in a cool box during transportation.

Media and growth experiments

The pre-reduced basal medium was prepared as described previously (Zehnder and Wuhrmann, 1977), but omitting rumen fluid and titanium solution. Nine millilitres of the medium was dispensed into screw-capped tubes sealed with butyl rubber stoppers and the gas phase was N_2 for routine cultivation unless indicated. Various methanogenic enrichments were obtained by addition of the following substrates into the basal medium, 20 mmol l^{-1} each of acetate, formate, methanol and trimethylamine, and 1.01×10^5 Pa H_2/CO_2 (80:20) respectively. A mixture of ampicillin (final concentration, 2 mg ml^{-1}) and kanamycin (final concentration, 1 mg ml^{-1}) was added to inhibit bacteria growth (Kotelnikova *et al.*, 1998). One millilitre of soil slurry was inoculated into 9 ml of various methanogenic media inside of an anaerobic box (Forma Anaerobic System 1029) and incubated either at 15°C or at 30°C. CH_4 production was measured for all the enrichments during the incubation period.

Analytical technique

Methane gas was measured by gas chromatograph GC-14B (Shimadzu) as described previously (Zhang *et al.*, 2004). The temperature parameters were set as follows: 50°C for column, 80°C for injector and 100°C for detector.

DNA extraction, PCR amplification and phylogenetic analysis

DNAs were extracted from soil samples or methanogenic enrichment cultures using a modified cetyltrimethyl ammonium bromide method as described previously (Zhou *et al.*, 1996). Briefly, samples were suspended in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA] with 7.5% chelex (Sigma), 50 mM EDTA (pH 7.0), 2% SDS and 200 μg of proteinase K to 750 μl extract mixture. After the chelex layer removing upon centrifugation, the mixture was added by 100 μl of 5 M

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NaCl and 80 μl of cetyltrimethyl ammonium bromide (10% w/v in 0.7 M NaCl) and incubated at 65°C for 30 min. Then crude DNA was purified with DNA gel purification kit (Qiagen, America) and dissolved in TE buffer as 25–50 μg ml^{-1}.

16S rRNA genes were PCR amplified using *Archaea* universal forward primer 915F and Prokaryote universal reverse primer 1492R (Table 1), and using the DNA preparation as template. Polymerase chain reaction mixture (50 μl) contained 5 μl of Taq buffer, 1 μl of dNTP (5 mM, final concentration), 1 μl of each primer (0.2 μM, final concentration), 1.5 μl of template DNA, 1 μl of *Taq* DNA polymerase (5 U, TaKaRa, Japan) and 39.5 μl of ddH_2O. Polymerase chain reaction amplification was performed at Bio-rad MyCycler, and the thermo-cycling parameters were as follows: initial denaturation at 94°C for 7 min followed by 30 cycles of denaturation at 94°C for 60 s, annealing at 50°C for 60 s and extension at 72°C for 90 s. Methanogen-specific *mcrA* genes were amplified using a pair of primer MCRf/MCRr (Table 1). The thermo-cycling parameters were the same as for 16S rRNA gene except annealing at 45°C for 45 s with 35 cycles.

Polymerase chain reaction products were purified as described previously (Kemnitz *et al*., 2004). The purified 16S rRNA fragments of about 599 bp and *mcrA* gene fragments of about 485 bp were cloned into pUCm-T vector, respectively, and sequenced by Sangon Biological Engineering Technology and Services (Shanghai, China).

Chimera sequences of 16S rRNA genes were identified by Chimera Check of Ribosomal Database Project II (release 8.1) (Cole *et al*., 2005). The 16S rRNA and *mcrA* gene sequences were submitted to GenBank to search for the similar sequences using BLAST algorithm. The best matching sequences were retrieved from the database and aligned and similarity analysis was performed by CLUSTAL X (Thompson *et al*., 1994). The phylogenetic tree was constructed using neighbour-joining method implemented in MEGA 4.0 (Tamura *et al*., 2007). The topologies of the resultant tree were evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 re-sampling.

Quantitative PCR experiments

The principle of qPCR was described elsewhere (e.g. Raeymaekers, 2000; Suzuki *et al*., 2000). In the following qPCR experiments, polymerase chain reactions were carried out on ABI Prism 7000 sequence detection (Applied Biosystems), and all the qPCR reactions were performed in eight strip PCR tubes (Axygen, America). Reaction signals were generated by binding of SYBR green to double-stranded DNA.

Primers used for quantitative real-time PCR analysis were designed using softwares of Premier express 2.0, Primer Premier 5.0 and Oligo 6.0. A pair of primer MethanoF/MethanoR was two specific fragments of the 16S rRNA gene of the uncultured methanogen cluster ZC-I (Table 1). Specificity of primer set MethanoF/MethanoR was evaluated by obtaining amplification exclusively from cluster ZC-I clones, but neither clones from other clusters in the 16S rRNA library nor pure methanogen species phylogenetically related to the sequences retrieved from the wetland, including *Methanomethylovorans hollandica* DSM 15978^T, *Methanosarcina mazei* DSM 2053^T and *Methanosaeta harundinacea* JCM 13211^T. The primer specificity was further confirmed by sequencing the amplicons (279 bp) to be a fragment of 16S rRNA gene from ZC-I cluster (data not shown).

Clone SD47 inserted with 16S rRNA fragment (EU283032) of cluster ZC-I and clone with the complete 16S rRNA (EF174501) of *M. hollandica* Z1 were used as the quantitative standards of cluster ZC-I and total *Archaea* respectively. The plasmids with both 16S rRNA fragments were extracted by Tianprep Mini Plasmid Kit (Tiangen Biotech) and purified using 3S Spin Agarose Gel DNA Purification Kit (Shanghai Biocolor Bioscience and Technology Company). The DNA preparations were quantified by NanoDrop® ND-1000 UV-Vis Spectrophotometer (America). 16S rRNA copy numbers were calculated according to the following equation (Stubner, 2002; Kolb *et al*., 2003; Yu *et al*., 2006) by assuming an average molecular weight of 660 Da for a base pair in double-stranded DNA (He *et al*., 2003).

$$\text{16S rDNA (copy numbers ml}^{-1}) = \frac{\text{16S rDNA concentration (g ml}^{-1}) \times 6 \times 10^{23}\ \text{(copy mol}^{-1})}{\text{16S rDNA amplicon size (bp)} \times 660\ \text{(g 16S rDNA mol}^{-1}\ \text{bp}^{-1})}$$

The plasmids were then 10-fold serially diluted to $10–10^{10}$ 16S rRNA molecules per microlitre and used to generate standard curves of 16S rRNA copies for ZC-I methanogens and total *Archaea*.

Quantitative polymerase chain reaction experiment was performed by using primer sets of Arc787/Arc1059 and MethanoF/MethanoR to determine the 16S rRNA copy numbers of total *Archaea* and ZC-I methanogens respectively. Each qPCR mixture contained 12.5 μl of SYBR green master 2× mix (Applied Biosystems), 1 μl of DNA template prepared above and 100 nM of each primer and ddH_2O to a final volume of 25 μl. The PCR initiated at 50°C for 2 min to optimize AmpErase uracil-N-glycosylase activity, followed by denaturation at 95°C for 10 min and 40 cycles of amplification as following, DNA denaturation at 95°C for 30 s, primer annealing at 57°C for 40 s and elongation at 72°C for 40 s. Fluorescence data were collected during elongation step. The reactions were all performed in three replicates.

FISH experiments

Probe ZC-I was a 22 bp ZC-I cluster-specific 16S rRNA fragment and designed using the 'Probe design' tool of ARB program package and probe function program of RDP (Cole *et al*., 2003). The specificity of probe ZC-I was verified by exclusively hybridizing to the clones in ZC-I cluster, but no hybridization signals either with the clones of non-ZC-I cluster in the 16S rRNA gene library or with the cultured methanogens including *M. hollandica* DSM 15978^T, *M. mazei* DSM 2053^T and *M. harundinacea* JCM 13211^T.

The enrichment cultures used for FISH experiments were treated as described by Chouari and colleagues (2003). A stringent wash step was performed for 20 min at 48°C. Slides were visualized using a Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Nucleotide sequence accession numbers

The 16S rRNA and *mcrA* gene nucleotide sequences have been deposited in GenBank nucleotide sequences database

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