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Quantification of the methane concentration using anaerobic oxidation of methane coupled to extracellular electron transfer

Yaohuan Gao^a, Hodon Ryu^b, Bruce E. Rittmann^c, Abid Hussain^a, Hyung-Sool Lee^a

^aDepartment of Civil and Environmental Engineering, University of Waterloo, Waterloo, Ontario N2L 3G1, Canada

^bNational Risk Management Research Laboratory, U.S. Environmental Protection Agency, 26 W. Martin Luther King Drive, Cincinnati, OH 45268, USA

^cBiodesign Swette Center for Environmental Biotechnology, Arizona State University, Tempe, AZ 85287-5701, USA

Abstract

A biofilm anode acclimated with growth media containing acetate, then acetate + methane, and finally methane alone produced electrical current in a microbial electrochemical cell (MxC) fed with methane as the sole electron donor. *Geobacter* was the dominant genus for the bacterial domain (93%) in the biofilm anode, while methanogens (*Methanocorpusculum labreanum* and *Methanosaeta concilii*) accounted for 82% of the total archaeal clones in the biofilm. Fluorescence *in situ* hybridization (FISH) imaging clearly showed a biofilm of mixed bacteria and archaea, suggesting a syntrophic interaction between them for performing anaerobic oxidation of methane (AOM) in the biofilm anode. Measured cumulative coulombs were linearly correlated to the methane-gas concentration in the range of 10–99.97% ($R^2 = 0.99$) when the measurement was sustained for at least 50 min. Thus, cumulative coulombs over 50 min could be used to quantify the methane concentration in gas samples.

Keywords

Extracellular electron transfer; Anaerobic oxidation of methane; Reverse methanogenesis; Methane concentration; Sensors

1. Introduction

Anaerobic oxidation of methane (AOM) plays an important role in global methane flux, potentially consuming 5–20% of global methane production (Valentine and Reeburgh, 2000). Anaerobic methanotrophic archaea (ANME) can build consortia with sulfate-reducing bacteria, denitrifying bacteria, Anammox bacteria, or metal-reducing bacteria to complete AOM (Amos et al., 2012, Beal et al., 2009, Haroon et al., 2013, House et al., 2011, Milucka et al., 2012, Moran et al., 2008, Norði et al., 2013, Rasigraf et al., 2012, Segarra et al., 2013, Wu et al., 2011), while some bacteria (*e.g.*, *Candidatus Methylothermobacter oxyfera*) are able to carry out AOM without partners (Haroon et al., 2013, Wu et al., 2011). At least two pathways have been proposed for AOM. The first pathway is reverse methanogenesis by ANME archaea that are coupled with syntrophic bacteria (*e.g.*, sulfate reducers) (Hallam et

al., 2004, Thauer and Shima, 2008, Wegener et al., 2015). In addition to ANME, that methanogens may be able to carry out reverse methanogenesis has been known for some time (Moran et al., 2005, Zehnder and Brock, 1979), and more recent literature directly and indirectly suggest reverse methanogenesis by methanogens (Grossman et al., 2002, Sivan et al., 2016, Taewoo Yi et al., 2012). The second pathway is intracellular oxygenic methane oxidation, which occurs in the case of *Candidatus Methyloirabilis oxyfera* (Ettwig et al., 2010).

While AOM coupled to sulfate reduction and denitrification has been intensively studied (Ettwig et al., 2010, Haroon et al., 2013, Jagersma et al., 2012, Segarra et al., 2013, Shi et al., 2013, Thauer, 2011), AOM coupled with the reduction of metal oxides has received less attention, although several works reported that AOM can be coupled with metal oxides reduction (Beal et al., 2009, Egger et al., 2015). AOM linked to metal-oxide reduction has an enormous potential for methane consumption in subsurface environments (Beal et al., 2009), and this supports that extracellular electron transfer (EET) can be linked with AOM. AOM via EET opens up the possibility that AOM may occur at an anode of a microbial electrochemical cell (MxC). If this is true, the rate of AOM might be monitored as the electrical current in an MxC enriched with an AOM consortium. Since the rate of AOM has been shown to be proportional to the concentration of methane (Nauhaus et al., 2005, Timmers et al., 2015), the electrical current, which reflects the rate of methane oxidation during anodic respiration, also could be a measure of the methane concentration.

The goals of this study were threefold. The first objective was to confirm that the coupling of AOM and EET to electrodes can generate current in an MxC; the second objective was to identify candidate players performing AOM + EET in the biofilm anode; and the final objective was to assess an AOM-EET MxC potentially as a methane sensor.

2. Material and methods

2.1 Microbial electrochemical cell (MxC)

A dual-chamber MxC was used for the experiment: an anode chamber of 140 ml and a cathode chamber of 122 ml. Carbon fibers (24 K Carbon Tow, Fibre Glast, USA) were employed as the anode, and a stainless steel mesh was used as the cathode. Details of the MxC configuration can be found in Fig. S1.

2.2. Inoculation and reactor operation

The MxC was inoculated with return activated sludge from the Waterloo wastewater treatment plant (Ontario, Canada) and run in continuous mode for over four months with an acetate medium (Torres et al., 2008); the medium was composed of 50 mM phosphate buffer (pH 7.4, Na_2HPO_4 and KH_2PO_4), 0.7 mM NH_4Cl , 25 mM CH_3COONa , and a mineral solution having the final concentration (per L): 5 mg EDTA, 11.6 mg MgCl_2 , 5.9 mg $\text{Mn}_2\text{Cl}_2 \cdot 4\text{H}_2\text{O}$, 0.8 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 1.14 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 mg ZnCl_2 , 0.1 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1 mg $\text{AlK}(\text{SO}_4)_2$, 0.1 mg H_3BO_3 , 0.2 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.01 mg Na_2SeO_3 , 0.1 mg $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, and $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$. After sterilization, the medium was purged with nitrogen (Praxair, 99.999%) for 30 min and $\text{FeCl}_2 \cdot 2\text{H}_2\text{O}$ (20 μM) and

$\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ (77 μM) were then added via a sterile syringe filter (Advantec 0.2 μm , PTFE, Cole-Parmer). The anode potential was poised at -0.19 V vs. the standard hydrogen electrode (SHE) using an Ag/AgCl reference electrode (rE-5B, BASi, USA) and a potentiostat (Biologic VSP, SnowHouse Solutions Inc., Canada). The same medium lacking acetate was used as the catholyte. After the steady-state current reached $\sim 8\text{ A/m}^2$ with the acetate medium, nitrogen gas was introduced from the top sampling port of the anode chamber, while the anolyte was expelled from the bottom side opening. The deoxygenated medium lacking acetate was then injected into the anode chamber by using a 60-ml syringe. The fresh mineral medium was under continuous nitrogen sparging during this process.

Since the initial attempts to acclimate an anodic biofilm with pure methane as the sole carbon and electron source failed, a transition stage was designed. In this stage, methane gas (99.97%, Praxair, Canada) was continuously bubbled into the anode chamber for methane saturation, but with intermittent injections of acetate to reach 0.16–0.42 mM in the anolyte. Acetate was injected only after the current was below 0.05 A/m^2 . Once AOM activity was evident, methane was supplied as the sole electron donor, and AOM operation was carried out continuously for over three years; the current density generated from AOM in day 580 to day 660 was presented in Fig. S2. Methane gas (99.97%) was alternated with nitrogen gas (Praxair, 99.999%) to confirm that current generation was from AOM, and the results (presented later in Fig. 1) confirm that current was generated only when AOM occurred. For these experiments, nitrogen gas (Praxair, 99.999%) was provided to the anode at flow rates of 6.7–8.3 ml/min. until the current became close to zero.

2.3. Correlating methane concentration with electrical signals in the MxC

After the current generation stabilized at $1.6\text{--}2.7\text{ mA/m}^2$ in the MxC run with a methane-saturated medium, the MxC was tested as a methane sensor. The relationship of cumulative coulombs generated from the MxC with different levels of methane (10%, 52.7%, 62.8%, and 99.97%) balanced with either helium or nitrogen gas (Praxair, 99.999%) was evaluated. Two of these methane gas mixtures (52.7% and 62.8%) were prepared by mixing pure methane (99.97%) and nitrogen or helium gas (99.999%) in a 1-L Pyrex® storage bottle using rotameters (PTFE-Glass Flowmeter on tripod bases, 150 ml/min, Cole-Parmer) to fix the volumes of each gas. The other two methane gases were purchased from a supplier with certified compositions (Praxair). For these gases in cylinders, the gas was supplied directly to the anode at a controlled flow rate of 12.4 ml/min. (MGC MilliGas Counter); for these homemade gas mixtures, the methane gas was supplied to the anode through a loop system comprised of a 1-L Pyrex® storage bottle and a peristaltic pump (MasterFlex 7553–80, Cole-Parmer). The methane gas was circulated inside this loop from the bottle to the anode chamber at a flow rate of $11.7 \pm 1.6\text{ mL/min}$. (see Fig. S3 in Supplementary Information). The loop system minimized mass-transport limitation for methane between liquid and gas phases. Between tests of two different methane gas mixtures, the anode chamber was sparged with nitrogen gas (Praxair, 99.999%) until the current density was only from endogenous decay, $0.62 \pm 0.17\text{ }\mu\text{A}$, after two to three days of nitrogen sparging. All tests were started after the current declined to this decay current.

The current was recorded every 2 min for 2 h (with methane sparging, predetermined time period) in order to build a calibration curve between the methane concentrations and the cumulative coulombs. The current generated from endogenous decay alone (no methane) was subtracted from the observed current with methane. After the shortest reaction time having the coefficient of determination (R^2) over 0.99 was selected, the calibration curve was then validated with three gas samples randomly prepared by mixing pure methane and pure nitrogen gases. The three gas samples were directly supplied to the anode chamber to mimic actual monitoring conditions in the field. Methane percentage of the gas samples was quantified with a GC equipped with thermal conductivity detector (TCD) in triplicate. Operating conditions for the GC were the same as reported by Gao et al. (2014).

2.4. Cloning and Sanger sequencing

To identify dominant microorganisms mainly responsible for AOM coupled to EET, the biofilm from anode fibers was collected using a sterilized spatula inside an anaerobic chamber (Coy Type B Vinyl, Mandel Scientific, Canada) at the end of the experiments. Frequent biofilm sampling can significantly perturb small current density in the MxC, and hence the biofilm was not sampled with time. The biofilm was stored at $-80\text{ }^{\circ}\text{C}$ prior to DNA extractions. DNA extraction was performed using Mo Bio PowerLyzer™ PowerSoil® kit (MO BIO Laboratories) according to the manufacturer's protocol. The DNA concentration was measured using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies). DNA extracts were stored at $-20\text{ }^{\circ}\text{C}$ until further processing.

The universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 787R (5'-GGACTACCAGGTATCTAAT-3') were used to amplify 16S rRNA genes of bacteria as described before (Ley et al., 2008, Ryu et al., 2012). The universal archaeal primers 25F (5'-CYGGTTGATCCTGCCRG-3') and 958R (5'-YCCGGCGTTGAMTCCAATT-3') were used to amplify 16S rRNA genes of archaea (Buchholz-Cleven et al., 1997, Elshahed et al., 2004). PCR amplifications were performed (25- μL reaction volume) using the polymerase TaKaRa Ex Taq™ (Takara Bio Inc.) in a Bio-Rad Tetrad Peltier Thermal Cycler (Bio-Rad, Hercules) using the following cycling conditions: one initial denaturation step at $95\text{ }^{\circ}\text{C}$ for 5 min, and 30 cycles of 1 min at $95\text{ }^{\circ}\text{C}$, 1 min at $56\text{ }^{\circ}\text{C}$, and 1 min at $72\text{ }^{\circ}\text{C}$, with a final extension step of 12 min at $72\text{ }^{\circ}\text{C}$. PCR products were visualized on 1.5% agarose gels using GelStar Nucleic Acid gel stain (Lonza, Rockland, ME, USA). Selected PCR products were cloned into pCR®4 TOPO® vector and transformed to TOPO10 chemically competent *E. coli* cells as described by the manufacturer (Invitrogen). Individual *E. coli* clones were then sub-cultured into 300 μL of Luria Broth containing 50 $\mu\text{g}/\text{mL}$ ampicillin and screened for inserts using M13 PCR.

The PCR products were submitted to the Children's Hospital DNA Core Facility (Cincinnati, USA) for sequencing using an Applied Biosystems Prism 3730XL DNA analyzer. Raw sequences were processed using Sequencher 4.10.1 software (Gene Codes). Chimeric sequences detected using Bellerophon (Huber et al., 2004) were not included in further analyses. The clone libraries were compared using Naive Bayesian rRNA Classifier version 2.5 of Ribosomal Database Project (RDP) with 95% confidence threshold.

Sequences of different representative taxa obtained in this study were deposited in GenBank under the following accession numbers: [KF254802-KF254839](#).

2.5. Fluorescence in situ hybridization (FISH)

Domain-specific and 16S rRNA-targeted oligonucleotide probes for bacteria and archaea (Raskin et al., 1994, Richter et al., 2007) were used for FISH. For the *in situ* hybridization, the probe for general archaea was labeled with fluorescein (green fluorescence), and the probe for general bacteria was labeled with TAMARA (red fluorescence), as summarized in Table S1. The anode fibers were fixed for 1 h with 2% paraformaldehyde, washed with phosphate-buffered saline, and then fixed on gelatin-coated glass slides. Serial ethanol washes (50%, 80%, and 96%) were used to dehydrate the samples. Hybridizations were performed at 46 °C for 5 h with hybridization buffer (0.9 mol/L NaCl, 20 mmol/L Tris-HCl [pH 7.2], 0.01% sodium dodecyl sulfate) containing 5 ng of each labeled probe per mL. The hybridization stringency was adjusted by adding formamide to the hybridization buffer, *i.e.* 5% for EUB338 and 35% for ARC915 (Sekiguchi et al., 1999). The washing step was conducted at 48 °C for 30 min with washing buffer containing the same components as the hybridization buffer except for the probes. The image (Fig. S4 in Supplementary Information) was obtained with a Zeiss epifluorescence microscope (Axio Scope. A1) and a camera (AxioCam CM1).

3. Results and discussion

3.1. Current generation from the AOM-MxC

Fig. 1 shows that current density ranged from 1.6 to 2.3 mA/m² when methane gas (99.97%) was continuously fed to the anode chamber of the MxC (after ~3 years acclimation and operation). When the methane gas was switched to nitrogen gas (99.999%) on day 13, the current density gradually decreased with time and reached zero on day 18; the gradual decline of current density was probably caused by endogenous decay (An and Lee, 2013). The current density recovered as methane gas was supplied to the anode chamber again, reaching 1.9–2.0 mA/m² on day 24. These results support that microorganisms in the biofilm anode anaerobically oxidized methane and used the anode as the terminal electron sink.

3.2. Microbial community and FISH image of the biofilm anode

Table 1 lists the strains identified by cloning and Sanger sequencing of the biofilm on the anode. Strains from the *Geobacter* genus (*G. sulfurreducens* and *G. pickeringii*) were dominant within the domain of bacteria, comprising 93% of bacterial clones. The *Geobacter* genus dominated the bacteria in the biofilm anode because they are well-known anode-respiring bacteria able to perform EET to anodes (Lovley, 2012). The available genomes of *Geobacter* (e.g., *G. sulfurreducens* and *G. pickeringii*) do not indicate genes related to AOM (Badalamenti and Bond, 2015, Methé et al., 2003); thus, *Geobacter* in the biofilm would not have directly utilized methane as an electron donor.

Methanogens (*Methanocorpusculum labreanum* of *Methanomicrobiales* and *Methanosaeta concilii* of *Methanosarcinales*) accounted for 82% of the total archaeal clones. None of the identified methanogens, including *Methanocorpusculum* and *Methanosaeta*, is from the

known ANME consortia. However, some studies have identified that ANME lineages 1 and 2 are distantly related to the orders of *Methanomicrobiales* and *Methanosarcinales* (Knittel et al., 2005, Michaelis et al., 2002). Besides, it is documented that certain methanogens could have the ability to conduct reverse methanogenesis (Blazewicz et al., 2012, Moran et al., 2005, Zehnder and Brock, 1979), which might be coupled to the reductions of oxidants (e.g., sulfate, nitrite, or nitrate), allowing reverse methanogenesis to proceed exergonically (Haroon et al., 2013, Thauer, 2011).

The abundances of *Methanocorpusculum*, *Methanosaeta*, and the *Geobacter* genera in the biofilm anode suggest that the methanogens may have been carrying out reverse methanogenesis coupled to EET. The standard redox potential at pH 7 for methane is -0.24 V, and anode potential was fixed at -0.19 V during the experiments. Thus, a potential gradient of 0.05 V was available for an AOM-EET consortium, and its Gibbs free energy at pH 7 ($G^{\circ'}$) was -38.6 kJ/mol CH_4 if energy loss in EET was negligible (Lee et al., 2016, Malvankar et al., 2012, Reguera et al., 2006). This energy yield is higher than a threshold Gibbs free energy of -10.6 to -35 kJ/mol CH_4 for a consortium of fermenters and methanogens (Conrad and Wetter, 1990, Thauer et al., 2008), and it is almost twofold higher than $G^{\circ'}$ for AOM coupled to sulfate reduction ($\text{SO}_4^{2-}/\text{H}_2\text{S}$, -21 kJ/mol CH_4) (Thauer, 2011).

The FISH image (Fig. S4) clearly shows archaea (green) and bacteria (red) in close proximity to each other in the anode biofilm. This FISH image implies that methanogens (*Methanocorpusculum* and *Methanosaeta*) and ARB (*Geobacter*) may have developed a syntrophic interaction. Close proximity favored direct- or intermediate-dependent (e.g., via H_2) electron transfer from methanogens to ARB and to the anode (Beal et al., 2009, Liu et al., 2012). This study cannot distinguish the main mechanism by which AOM-microorganisms transfer electrons to ARB, but it implies a syntrophy between methanogens and ARB.

3.3. Determining the reaction time and a linear correlation of cumulative coulombs with methanem concentration

Fig. 2 shows that the current density began to increase immediately upon test-gas sparging, but it did not reach steady state in 2 h. This non-steady state current density in response to AOM could occur due to slow AOM kinetics (Scheller et al., 2010) in the biofilm anode, mass transport limitation of methane molecules to the biofilm, or both. The limiting factor for the non-steady current density could not be assessed, but it seems that current (or current density) is not a good indicator to estimate methane gas concentration for the non-steady current density in the MxC used in this study. Perhaps current density could be used for estimating the methane concentration in an MxC that is able to produce steady current density from methane; further study is required to address limiting factors for steady current density in methane-fed MxCs. The cumulative coulombs instead of the non-steady current density were found to be able to well correlate methane concentration in the MxC.

Fig. 3 compares the coefficient of determination (R^2) for linear curves correlating methane gas percentage with cumulative coulombs under reaction times from 30 min to 60 min. As expected, the increase in cumulative coulombs was proportional to the CH_4 percentage. For

reaction times of 30 and 40 min, linearity was poor and the coefficients were relatively low at 0.80–0.83. Linearity clearly improved when the reaction time was extended to 50 and 60 min, and the coefficient increased to ~0.99. The trend of linearity and R² (~0.99) continued for the reaction up to 100 min (data not shown). As longer time could not substantially improve the correlation, A time period of 50 min was chosen to estimate the methane concentration of an unknown gas: $y = 782.04x + 0.0356$, where y is methane percentage in a gas mixture and x is cumulative coulombs.

3.4. Quantification of methane gas concentration using the MxC

Fig. 4 compares the methane concentrations measured with the GC-TCD with the coulomb-based method. The values agreed to within 5.5–9.3%, with two coulomb-based values lower than the GC-TCD value and one higher. This result demonstrates that an MxC enriched with AOM-EET microorganisms (methanogens and *Geobacter*) has the potential to be applied an *in situ* methane sensor, at least for high methane concentration ranges. Biofilm anodes performing AOM-EET actually oxidize aqueous methane, which indicates that this new sensor can also be adapted to assess methanogenesis in natural and engineered systems.

This work focused on relatively high methane concentration as a proof-of-concept study. Methane concentration in natural environments is often very dilute; hence, the sensitivity and accuracy of this new MxC sensor should be further examined with methane gases at low levels (e.g., a few hundred ppm). A reaction time of 50-min. was selected to quantify methane gas concentration with cumulative coulombs generated from AOM-EET in the MxC, but shorter reaction time (e.g., 5–10 min) will be preferred as an in-situ sensor. Miniature-MxCs (mL scale), with higher electrode surface area to electrolyte volume ratio, may be able to reduce the reaction time for obtaining quantifiable cumulative coulombs from AOM. Although several parameters should be further studied, this work is the first to demonstrate that MxCs could measure high percentages of methane gas concentration via AOM coupled to EET in biofilm anodes.

4. Conclusions

A biofilm anode enriched with *Geobacter* and methanogens generated electric current from methane as the sole electron donor, probably via AOM coupled to EET pathway. FISH imaging supports a potential syntrophy between metal reducers and methanogens in the biofilm anode. Cumulative coulombs from AOM-EET reactions were linearly correlated to the methane-gas concentration in the range of 10–99.97% ($R^2 = 0.99$) after at least 50 min of reaction time in the MxC. This study is the first to demonstrate that MxCs are able to measure high concentrations of methane gas with electrical signals generated from AOM-EET.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Methane-dependent generation of electric current in the MxC operated with alternating supplies of methane and nitrogen gases. The fluctuations of the electric current were caused by variations of temperature (24 ± 6 °C) in the laboratory.

Fig. 2. Current density in response to different methane concentrations introduced after the 1-h mark. Methane gases were provided for the anode chamber in the MxC for over two hours; data beyond 2-h mark are not included here.

Fig. 3.

Linear plots and coefficients of determination correlating methane concentration with cumulative coulombs in the MxC: (A) 30 min, (B) 40 min, (C) 50 min, and (D) 60 min.

Fig. 4. Quantification of the gas-phase CH₄ percentages in three gases by the GC-TCD (green bars) and the coulomb-based method (blue circles). Error bars denote one standard deviation from the mean (triplicate measurements) for the GC-TCD results. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1.

Distributions of bacterial and archaeal 16S rRNA genes by cloning and sequencing for the anaerobic methane-oxidizing MxC.

Domain	Order	Species (No. of clones)	GAN ^c (identity)
Bacteria	<i>Fibrobacter/Acidobacteria</i> group	<i>Candidatus Cloacimonas</i> sp. (n = 3) ^a	KP258984 (94%) or AF338764 (99%)
		<i>Geobacter sulfurreducens</i> (n = 53)	(98%)
	<i>Desulfuromonadales</i>	<i>Geobacter pickeringii</i> (n = 28)	CP009788 (95%)
		<i>Geobacter</i> spp. (n = 5) ^a	AE017180 (92%)
		<i>Bacteroidales</i> (n = 1)	CP006772 (94%) or GU083566 (99%)
	<i>Spirochaetales</i>	<i>Paludibacter propionigenes</i> (n = 1)	CP002345 (97%) or GU455014 (99%)
		<i>Spirochaetaceae</i> (n = 1) ^b	HQ689204 (88%)
		<i>Methanocorpusculum labreanum</i> (n = 60)	NR074173 (99%)
Archaea	<i>Methanomicrobiales</i>	<i>Methanocorpusculum sinense</i> (n = 1)	NR104804 (98%)
		<i>Methanocorpusculum</i> spp. (n = 3) ^a	NR074173 (92%)
		<i>Methanosarcinales</i>	CP002565 (99%)
	<i>Methanosarcinales</i>	<i>Methanosaeta concilii</i> (n = 12)	CP002565 (99%)
		<i>Methanosaeta</i> spp. (n = 2) ^a	CP002565 (92%)
		<i>Candidatus Methanoplasma termitum</i> (n = 6)	CP010070 (97%)
	<i>Methanomassiliicoccales</i>	<i>Methanomassiliicoccus luminyensis</i> (n = 3)	NR118098 (95%)

^a Genus.

^b Family.

^c GenBank accession number.