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Estimation of methanogenesis by quantification of coenzyme F430 in marine sediments

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Coenzyme F430 is the hydrocorphinoid nickel complex which acts as active site in methyl-coenzyme M reductase (MCR). The MCR-F430 complex catalyzes the last step of methanogenesis: reduction of methyl-coenzyme M to methane. Since F430 is a common enzyme for mathanogens, it can be a function-specific biomarker to estimate biomass and activities of methanogens in environmental samples. A recently developed high sensitive method for coenzyme F430 analysis and a further purification step revealed F430 concentrations at 69 and 88 meters below seafloor (mbsf) off Shimokita Peninsula (529 and 31.3 fmol g-wet^{-1}) and at 60 and 275 mbsf at Nankai Trough (31.4 and 26 fmol g-wet^{-1}). Estimated methanogenic biomass and activities based on reported F430 content and activities in methanogens and prokaryotic cell weight, were ranging from 3.9×10^6 to 2.7×10^6 cells g-wet^{-1} and from 1.7 to 11 μmol d^{-1} g-wet^{-1}.

Keywords: coenzyme F430, methane hydrate, methanogens, function-specific compound analysis, marine sediment

Introduction

During the last four decades, methanogenesis in marine sediments has been intensively studied because of its importance to understand carbon cycle on the Earth surface. So far, the studies revealed that methane is mainly of biogenic rather than abiogenic origin among surface to several hundred meters below seafloor (Kvenvolden, 2002; Milkov, 2004). The biogenic methane is produced by methanogenic archaea (methanogens) which utilize C_1 and C_2 compounds such as carbon dioxide, acetate, or methanol for carbon substrates.

The carbon substrates for methanogenesis are produced during the degradation of sedimentary organic matter by a sequence of microbial processes (Whiticar., 1999). Since the methanogenesis is the last step of the degradation of organic matter in anaerobic environments, investigation of their quantitative distribution and activities of methanogens are important to understand global carbon cycles (Thauer *et al*., 2008).

The microbially derived methane can be a major constituent of methane hydrate at continental margin. The globally deposited methane hydrate is estimated to be ~100,000 Gt (Kvenvolden, 2002; Milkov, 2004), which is a potent energy resource. Since the major source of methane in the hydrate is of methanogen origin, investigation of distribution and activities of methanogens leads to deeper understanding of the starting point of methane hydrate formation as well as migration and accumulation of methane.

In subsurface marine sediments, available techniques to detect methanogen's activity are limited. Molecular biologic techniques have been used to investigate microbial community structure and function-specific gene analysis. However, in many cases methanogen have not been detected in marine sediments although methanogenesis is a major metabolic process in anoxic subseafloor sediments, suggesting either a low population of methanogens or unknown methanogens which cannot be detected with available primers (Biddle *et al*., 2006; Fry *et al*., 2008; Valentine, 2011).

Analyses of archaeal membrane lipids and their isotopic composition are powerful tools to estimate biomass and source organisms. However, any membrane lipid species are not representative for methanogens. Known constituents of membrane lipids of methanogens are archaeol, cardoarchaeol, *sn*-2- and *sn*-3-hydroxyarchaeols, and macrocyclic archaeol (Koga *et al*.,

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1993 and references therein). Hydroxyarchaeols have been found in one or a few species of genus *methanosphaera*, *methanococcus*, *methanosarcina*, *methanolobus*, *methanohalophilus*, and *methanothrix*, while macrocyclic archaeol have been found only in *methanococcus jannaschii* although theses core lipids are known as specific biomarker for methanogens. On the other hand, archaeol and cardoarchaeol are common in not only methanogens and diffused in archaeal domain. These facts imply that methanogen-like isotopic composition cannot be a robust evidence for the presence of methanogens.

Recently we developed analysis of coenzyme F430 by coenzyme F430 is the hydrocorphinoid nickel complex which acts as active site in methyl-coenzyme M reductase (MCR). The MCR-F430 complex catalyzes the last step of methanogenesis: reduction of methyl-coenzyme M to methane. Since F430 is a common function-specific compound in methanogenic pathways including hydrogenotrophic, aceticlastic and methylotrophic methangenesis (Thauer, 1998), all methanogens including uncultured methanogens should utilize F430 for methanogenesis. Recent studies suggested that anaerobic methane oxidizing archaea (ANME) also utilize F430 and its homologue for the reversed methanogenesis (Krüger *et al*., 2003; Mayr *et al*., 2008; Shima *et al*., 2012). Thus, F430 is a function-specific compound for both methanogenesis and anaerobic methane oxidation, which has a potential to be a practical biomarker compound for estimation of biomass and activity of methanogens and ANME in subsurface environments.

Recently, we have developed a highly sensitive method for the analysis of F430 in the environmental samples with an on-line liquid chromatography-mass spectrometry. The triple quadrupole mass spectrometer enables F430 detection as low as 0.1 fmol. It corresponds to 6×10^2 to 1×10^4 cells of methanogens if applying reported F430 contents in methanogen cultures and estimated prokaryotic cell weight in natural environments (Diekert *et al*., 1981; Lipp *et al*., 2008; Whitman *et al*., 1998). This highly sensitive method enables detection of F430 in various environmental samples including microbial mats, ground water, paddy soils, and marine sediments, providing the basic information for the understanding of methanogenesis (Kaneko *et al*., 2014).

In this study, the developed coenzyme F430 analysis will be applied to quantify coenzyme F430 in methanogenic marine sediments. Especially for a sediment sample, organic matrices interfered precise detection of coenzyme F430. New technique to remove the organic matrices will also be reported. In addition, methanogenic biomass and activity were estimated from available reference data including coenzyme F430 content in a cell and specific activity.

Materials and Methods

Sample description

A granule sample consisting of high-density methanogenic archaea including genus *Methanobacterium*, *Methanosaeta* and *Methanomicrobiales* (code MBK, Kaneko *et al*., 2014; Takano *et al*., 2013) was supplied from Sumitomo Heavy Industry Ltd., Yokosuka, Japan.

Two sediment samples were collected from 69 and 88 m below the seafloor (mbsf, Site 9001; core section 8-4 and 10-4) off the Shimokita Peninsula, Japan (41°10.6380′ N, 142°12.08′ E) during the shakedown cruise CK06-06 of the D/V Chikyu (Aoike, 2007).

Another two sediment samples were collected from 60 and 275 mbsf (Site 0004; core section 7H7 and 29R2) at Nankai Trough (33°13.2278′ N, 136°43.3312′ E) during expedition 316 of Integrated Ocean Drilling Program (IODP, Kinoshita *et al*., 2009).

Extraction of F430

Samples (wet) were extracted with 1% formic acid (pH 2; Wako Pure Chemical Industries Ltd.) by ultrasonication for 30 min on ice, followed by centrifugation (×10,000 *g*; 30 min at 4°C) to recover the supernatant. This step was repeated three times. The combined supernatant was introduced to an anion exchange column (Q Sepharose column; GE Healthcare) that had been equilibrated with 50 mM of Tris/HCl (pH 7.5) and washed with deionized water prior to use. The recovered eluent was introduced to a C_{18} SPE column (Sep-Pack; Waters Corp.) that had been equilibrated with methanol (overnight) and conditioned with 5 mL of 1% formic acid. An absorbed yellowish band (F430 fraction) on the column was eluted with 100% methanol. The recovered F430 fraction was dried and stored at –20°C prior to further treatment. The dried F430 fraction was reacted with BF_3/methanol in a closed vial at 40°C for 3.5 h to convert F430 to its pentamethyl ester (F430M). Water was added to the vial and the aqueous phase was extracted three times with dichloromethane (DCM). The organic phase was recovered after centrifugation and dried under N_2 stream. The recovery of F430M was >95%.

Quantification by liquid chromatography-mass spectrometry (LC-MS/MS)

LC-MS/MS was performed using an Agilent HPLC 1260 Infinity coupled to a 6460 Triple Quadrupole (QQQ) LC/MS system (Kaneko *et al*., 2014). Briefly, F430M was analyzed in positive ion mode by electrospray ionization (ESI) with an Agilent JetStream. Source and sheath gas temperatures were set at 300°C and 250°C, respectively. Source and sheath gas flow rates were set to 5 and 11 L min^{-1}, respectively. Capillary and nozzle voltages were

set at 3500 and 500 V, respectively. For multiple reaction monitoring (MRM) analysis, the fragmentor voltage was 180 V and the collision energy was 0 V (zero-collision energy MRM, Kaneko *et al*., 2014), which is substantially SIM (selective ion monitoring)-SIM analysis. Both precursor and product ions of F430 were set to *m/z* 975.4 for F430M, 1021.4 for methylthio-F430. Compound separation by HPLC was conducted using a ZORBAX Eclipse XDB-C_{18} (4.6 × 250 mm; 5 μm p.s., Agilent Technologies). Mobile phases were 10 mM ammonium acetate (A) and acetonitrile (B). The flow rate was 0.5 mL min^{-1}. The gradient condition was started at 0% B followed by 30% B after 3 min and then 90% B after 90 min.

Concentration of coenzyme F430 was calculated by external standard method using concentration known coenzyme F430 standard. Coenzyme F430 for the standard was extracted from methanogenic granule and purified with a preparative HPLC. Purity and concentration of the standard solution was determined by nuclear magnetic resonance spectroscopy (NMR) and isotope ratio mass spectrometry coupled with elemental analysis (EA-IRMS) in earlier study (Kaneko *et al*., 2014). The concentration of the coenzyme F430 standard is 0.79 ± 0.03 μmol mL^{-1}.

Silica gel column chromatography

Silica gel column chromatography was performed for the F430M fraction to remove organic matrices in the sample. Silica gel (Sigma-Aldorich, 200–400 mesh) was activated by combustion at 450°C for 5 h, then deactivated with 1 wt% H_2O prior to use. Silica gel (1.4 mL) stored in hexane solution was loaded to a grass tube (6 mm i.d.) and conditioned with 3 bed volume of methanol. The F430M fraction dissolved in methanol was gently loaded on the silica gel column. The column was washed with 3 mL (2 bed volume) of methanol (F1) and acetonitrile (F2), followed by elution of F430M (F3) with 3 ml (2 bed volume) of 100 mM $NaClO_4$ in acetonitrile/H_2O (80/20 v/v). F3 fraction was dried under N_2 stream. Recovery of F430M and removal of $NaClO_4$ was simultaneously performed by liquid-liquid extraction with H_2O and dichloromethane.

Results and Discussion

Application in marine sediments

Presence of methanogens in sediments off Shimokita Peninsula has been suggested in previous studies. Methanogens belonging to the genus *Methanobacterium*, *Methanosarcina*, *Methanococcoides*, and *Methanobrevibacter* have been isolated from sediment at a depth of 107 m using a down-flow hanging sponge (DHS) reactor (Imachi *et al*., 2011). In our earlier study, F430 was also detected at 97 and 107 mbsf (Kaneko *et al*., 2014; Takano *et al*., 2013).

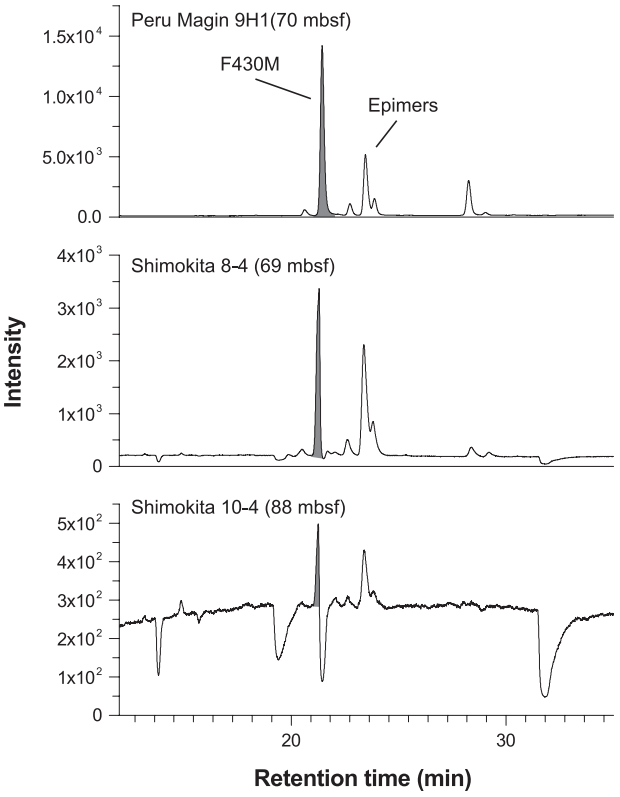

Fig. 1. MRM chromatograms of F430M fraction from 69 and 88 mbsf in sediments off Shimokita Peninsula.

In this study, we investigated distribution of F430 at four sedimentary layers (69 and 88 mbsf off Shimokita Peninsula and 60 and 275 mbsf at Nankai Trough). In all sediments, F430 was successfully detected (Fig. 1). In particular, F430 concentration at 69 mbsf is 530 fmol g-wet^{-1} sediment, which is 10 times higher than those reported in our previous study (60 fmol g-wet^{-1} at 97 mbsf and 40 fmol g-wet^{-1} at 107 mbsf, Table 1).

Since the concentration of sedimentary F430 can reflect methanogens biomass *in situ* based on observation in nature (Kaneko *et al*., 2014), high concentration of F430 at 69 mbsf suggests a more abundant population of methanogens relative to deeper horizon.

Although F430 was detected at 88 mbsf, relatively high background and its negative shift nearby F430 prevents from precise quantification of F430. These features would be derived from complex organic matrices in the sample. Especially, the negative shift of the baseline would be caused by ion suppression due to decreasing ionization efficiency of F430 by organic interferences. Co-eluting organic matrix can also cause reduction of coenzyme F430 ionization efficiency. Hence, the affect of organic matrices should be removed to get better chro-

Table 1. Coenzyme F430 concentrations in environmental samples

Sample name	Sample type	Concentration		References
MBK	Granules	3.30E+07	fmol/g-wet	Kaneko *et al.* (2014)
Anjo E2	Paddy soil	2.02E+03	fmol/g-wet	
Kawatabi	Paddy soil	8.70E+02	fmol/g-wet	
Chikugo CF	Paddy soil	3.08E+02	fmol/g-wet	
Chikugo RSC	Paddy soil	1.06E+03	fmol/g-wet	
Ita-wari	Groundwater	8.11E+02	fmol/L	
Peru 9H1	Marine sediments	1.92E+03	fmol/g-wet	
Shimokita 11-4	Marine sediments	6.33E+01	fmol/g-wet	
Shimokita 8-4	Marine sediments	5.29E+02	fmol/g-wet	This study
Shimokita 10-4	Marine sediments	3.13E+01	fmol/g-wet	
Nankai 7H7	Marine sediments	3.14E+01	fmol/g-wet	
Nankai 29R2	Marine sediments	2.60E+01	fmol/g-wet	

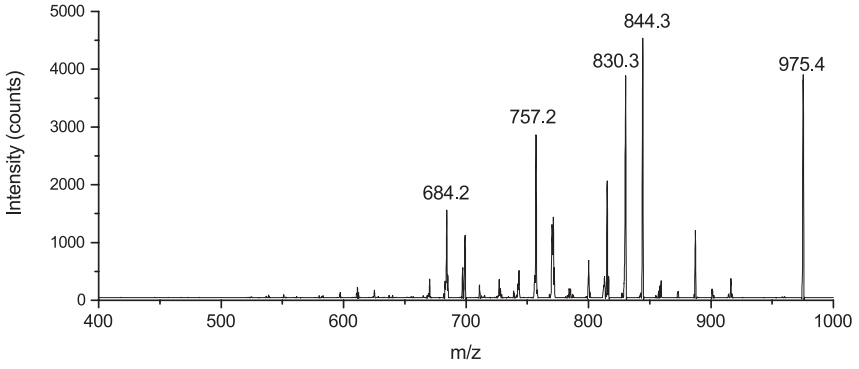

Fig. 2. Mass spectram of F430M when 66 V of collision energy was applied.

matogram.

One reason for high background on the chromatogram may be zero-collision energy MRM applied in this study. Applying normal MRM method may be helpful for reduction of interference from organic matrix on the chromatogram. However, it is hard to occur product ions even if high collision energy is applied. In our earlier study, a fragment ion with $m/z = 844.3$ was a most significant product when 66 V of collision energy was applied, but parent ion is still abundant (Fig. 2, Kaneko *et al.*, 2014). Applying normal MRM method (975.4 → 844.3) sacrifices the sensitivity and does not mean substantial resolution of reduction of ionization efficiency and ion suppression. More fundamental resolution to get a better chromatogram would be a removal of organic matrices before ionization.

Silica gel column chromatography to remove organic matrices

Silica gel column chromatography has been used to classify organic molecules and remove organic matrices in samples. This technique can be widely applicable from nonpolar to polar lipids and porphyrins (tetrapyrrole compounds). Coenzyme F430M is well absorbed on silica gel column and is not eluted by organic solvents with high elution force including dichloromethane, methanol and acetonitrile but elute with 0.1 M $NaClO_4$ acetonitrile/H_2O (80/20 v/v). Recovery during silica gel chromatography was 71% ± 0.9 ($n = 4$).

Silica gel column chromatography was tested in the F430 fraction extracted from sediment at 88 mbsf off Shimokita Peninsula to remove organic matrices. In this study, an aliquot of the sample was loaded on the silica gel column and separated into three fractions. On the MRM chromatogram of F1 (methanol fraction), F430 was not detected while increased background and negative peaks of ion suppression were appeared (Fig. 3). On the chromatogram of F2 (acetonitrile), F430 as well as increased background and ion suppression were not observed. On the chromatogram of the final fraction F3 (0.1 M $NaClO_4$ in acetonitrile/H_2O), F430 and its homologues were detected with better peak shape compared with original samples, and high background and negative peaks were no longer detected. Thus, the silica gel chromatography is useful to remove organic matrices.

The result of quantification of F430 in F3 is 97.9 fmol

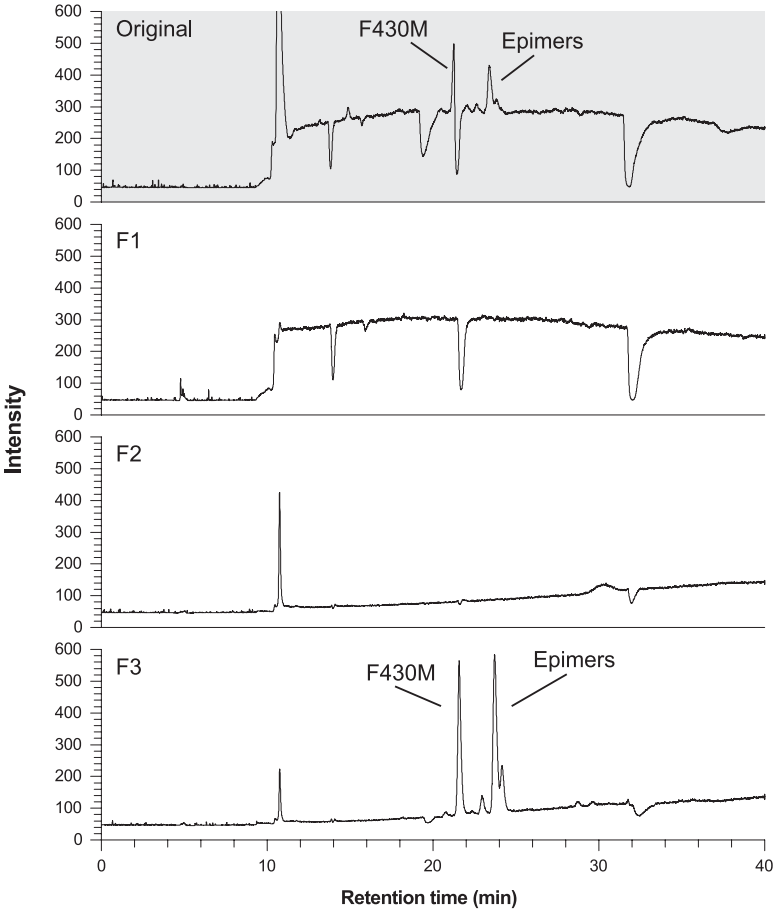

Fig. 3. MRM chromatogram of F430M fraction at 88 mbsf in sediments off Shimokita Peninsula (original), and methanol fraction (F1), acetonitrile fraction (F2) and Acetonitrile/H_2O (100 mM NaCl) fraction (F3) on silica gel chromatography.

g^{-1}, suggesting that organic interferences causing ion suppression lead to underestimation of F430 concentration in samples.

Estimating in situ methanogenic biomass and activity

Coenzyme F430 is thermally unstable and finally epimerized to thermodynamically stable form of 12,13-diepi F430 via 13-epi F430. At the thermodynamic equilibrium (100°C), 88% of coenzyme F430 epimerizes to 12,13-diepi F430 and the process occurs in day scale even at room temperature (Diekert *et al*., 1981; Mayr *et al*., 2008). Such a rapid deformation coenzyme F430 implies that it does not accumulate in the native form. The native form of coenzyme F430 can be presents as both free form and MCR-bound form. The free form of coenzyme F430, however, is a precursor of MCR-bound F430 and binding of F430 to apo-protein is an irreversible process (Ankel-Fuchs *et al*., 1984). Residence time of such an intermediate would be short in cells. Even if it is long due to low metabolic activity in marine sediments, coenzyme F430 would be epimerized. Furthermore, protein matrix of MCR moderate nonplanar deformation and epimerization of coenzyme F430 and incorporation of 12,13-diepi F430 and tunes the chemistry of the active site of Ni ions (Mbofana and Zimmer, 2006). Thus, coenzyme F430 detected in marine sediments should represent MCR-binding F430 in living cells when we assume that the rate of epimerization at the conditions of marine sediments is enough fast.

Coenzyme F430 contents in methanogens have been investigated previously in various cultures of methanogens including *Methanobacterium thermoautotrophicum*, *Methanobrevibacter smithii*, *Methanococcus vannielii*, *Methanospirillum hungatii*, and *Methanosarcina barkeri*. The F430 content ranges from 800 nmol $g^{-1}_{\text{dry cell}}$ (*M. thermoautotrophicum* and *M. barkeri*) to 227 nmol $g^{-1}_{\text{dry cell}}$ (*M. vannielii*) with average of 580 ± 240 nmol $g^{-1}_{\text{dry cell}}$ (Diekert *et al*., 1981). The prokaryotic cell weight is estimated to range from 2 $\times 10^{-13}$ g in soil to 36 $\times 10^{-15}$ g in marine sediment (Lipp *et al*., 2008; Whitman *et al*., 1998). Hence, abundance of the methanogen cell per g-sediment can be estimated from

Table 2. The activities of F430 expressed by culture-based studies

μmol/min/μmol F430	Methanogen specimen	Targets	References
17500	*M. thermoautotrophicum* strain Marburg	Cell suspension	Schönheit *et al*. (1980)
7500	*M. thermoautotrophicum* strain Marburg	Cell suspension	Perski *et al*. (1981)
8000	*M. thermoautotrophicum* strain Marburg	Cell suspension	Perski *et al*. (1982)
19	*M. thermoautotrophicum* strain Marburg	F430M	Jaun and Pfaltz (1988)
150–300	*M. thermoautotrophicum* strain Marburg	MCR	Ellermann *et al*. (1988)
375	*M. thermoautotrophicum* strain Marburg	MCR	Ellermann *et al*. (1989)
15000	*M. thermoautotrophicum* strain Marburg	MCR	Goubeaud *et al*. (1997)

The activities of F430 were expressed in a unit of μmol/min/μmol F430.
For conversion of the activity from literature data, following parameters were used: MCR = 300 kDa; protein in Methanogen = 50% of dry cell; MCR = 12% of cell protein; 1 mg MCR = 0.0067 μmol F430.

F430 concentration by using the following equation:

$$n = \frac{C_{\text{F430 in sample}}}{C_{\text{F430 in culture}} \times m_{\text{cell}}} \left(\text{cells g}^{-1}\right)$$

where $C_{\text{F430 in sample}}$ and $C_{\text{F430 in culture}}$ denote F430 concentrations in sample and culture, respectively and m_{cell} denotes a mean weight of the cell. In fact, estimated cell abundance from F430 concentration seems to well correlate with that estimated by microbiological techniques including cell counting and gene concentration in various environmental samples (paddy field, microbial mat, groundwater (Kaneko *et al*., 2014).

Cell densities calculated from F430 concentration are 2.7×10^7 and 4.7×10^6 cells g-wet^{-1} 69 and 88 mbsf of offshore Shimokita Peninsula while 4.7×10^6 and 3.9×10^6 cells g-wet^{-1} at 60 and 275 mbsf of Nankai Trough. A comparison with total prokaryotic cell at Shimokita Peninsula based on a developed cell enumeration technique using hydrofluoric acid treatment and automated fluorescent image analysis (Morono *et al*., 2009) suggests that methanogen biomass account for about 30 and 10% of total prokaryotic biomass at 69 and 88 mbsf.

The amount of F430 should precisely reflect the amount of MCR because 2 molecules of F430 are individually bounded to two active sites of single MCR. The rate of methanogenesis can also be correlated with concentration of MCR as well as F430 as described by Michaelis-Menten model. The specific activity of MCR (or F430) has been investigated using cell suspension, extracted MCR and F430M to be about 15,000 μmol methane min^{-1} μmol-F430^{-1} (Table 2, Goubeaud *et al*., 1997; Ellermann *et al*., 1988, 1989; Jaun and Pfaltz, 1988; Perski *et al*., 1981, 1982; Schönheit *et al*., 1980). Applying the specific activity of F430 to the concentration of F430 from sediment samples, the potential methanogenic activity is calculated to be 11 and 2.1 μmol d^{-1} g-wet^{-1} at 69 and 88 mbsf offshore Shimokita Peninsula, and 0.7 and 0.6 μmol d^{-1} g-wet^{-1} at Nankai Trough. These estimated methanogenic rate are substantially higher than those based on the tracer experiments using ^{14}C-labeled carbon substrates which is basically pico mol level (~1×10^{-5} nmol d^{-1} cm^{-3} to 1240 nmol d^{-1} cm^{-3}, Fry *et al*., 2008; Parkes *et al*., 2000, 2005; Yoshioka *et al*., 2010). This discrepancy of estimated methanogenic potential can be responsible to both overestimation based on coenzyme F430 concentration and underestimation based on the culture experiment with ^{14}C tracer. The validity of our calculated results cannot show in this stage. Further experiments are required for better estimation of methanogenic potential *in situ* as described below.

Future works

The protocols to estimate methanogen's biomass and activity described in this study have some limitations. Great care must be taken when the methods are applied to subseafloor sediments because of accumulation of debris from past-living organisms. Preservation of biomarkers to detect living prokaryotic signals has been often argued in previous study (e.g., Lipp and Hinrichs, 2009; Schouten *et al*., 2010; Xie *et al*., 2014). Intact coenzyme F430 may not accumulate in natural environment as a fossil compound due to its unstable nature (Thauer and Bonacker, 1994; Diekert *et al*., 1981). Since F430 is readily degraded by both biological and abiological processes after cell death, the degradation rate in the terrestrial environments can be faster than DNA and polar intact lipids. However, the degradation rates of F430 in marine sediments where temperature, oxygen and microbial activities are low have not been investigated. The utility of F430 as a biomarker to investigate modern methanogenesis has to be assessed by degradation experiment of F430 at the conditions of marine sediments in the future study.

In this study, conversion of methanogen's biomass and activities from F430 was conducted using reference data of culture-based studies. However F430 contents in a cell and its activity in marine sediments will not necessarily be the same as in a culture where substrate is abundant.

Strictly controlled culture-based studies and *in situ* cultivation at easy-to-access (e.g., paddy field) are required for precise estimation of methanogenic biomass and activities in subseafloor sediments.

Conclusions

In this study, we investigated coenzyme F430 concentration and estimated methanogenic biomass and activity in marine sediments offshore Shimokita Peninsula and Nankai Trough. A recently developed high sensitive analytical method enabled detection of F430 in marine sediments where the concentration of F430 is extremely low. However, organic matrices seem to affect ionization during LC-MS analysis and prevent from precise quantification of F430 at near lower detection limit. Silica gel column chromatography is useful to remove organic matrices for better quantification. Theoretically, methanogenic biomass and activities *in situ* can be calculated from F430 concentration however estimated biomass and activities in marine sediments were quite higher than those estimated by other microbiological and ^{14}C tracer methods. Further studies to clarify relationship between F430 concentration and biomass/activities and degradation rate of F430 in anoxic marine sediments are required for better estimation of methanogenesis rate *in situ*.

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