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Physiological and isotopic characteristics of nitrogen fixation by hyperthermophilic methanogens: Key insights into nitrogen anabolism of the microbial communities in Archean hydrothermal systems

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Abstract

Hyperthermophilic hydrogenotrophic methanogens represent one of the most important primary producers in hydrogen (H_2)-abundant hydrothermal environments in the present-day ocean and throughout the history of the Earth. However, the nitrogen sources supporting the development of microbial communities in hydrothermal environments remain poorly understood. We have investigated, for the first time, methanogenic archaea commonly found in deep-sea hydrothermal environments to understand their physiological properties (growth kinetics, energetics, and metal requirements) and isotopic characteristics during the fixation of dinitrogen (N_2), which is an abundant but less-bioavailable compound in hydrothermal fluids. Culture experiments showed that *Methanocaldococcus* strain (Mc 1-85N) (Topt = 85 °C) and *Methanothermococcus* strain (Mt 5-55N) (Topt = 55 °C) assimilated N_2 and ammonium, but not nitrate. Previous phylogenetic studies have predicted that the *Methanocaldococcus* and *Methanothermococcus* lineages have nitrogenases, key enzymes for N_2 fixation, with biochemically uncharacterised active site metal cofactors. We showed that Mt 5-55N required molybdenum for the nitrogenase to function, implying a molybdenum-bearing cofactor in the strain. Molybdenum also stimulated diazotrophic (i.e., N_2-fixing) growth of Mc 1-85N, though further experiments are required to test whether the strain contains a molybdenum-dependent nitrogenase. Importantly, Mc 1-85N exhibited an apparently lower requirement of and higher tolerance to molybdenum and iron than Mt 5-55N. Furthermore, both strains produced more ^{15}N-depleted biomass (-4‰ relative to N_2) than that previously reported for diazotrophic photosynthetic prokaryotes. These results demonstrate that diazotrophic hyperthermophilic methanogens can be broadly distributed in seafloor and subseafloor hydrothermal environments, where the availability of transition metals is variable and where organic carbon, organic nitrogen, and ammonium are generally scarce. The emergence and function of diazotrophy, coupled with methanogenesis, in the early Earth is also consistent with the nitrogen isotopic records of 3.5 billion-year-old hydrothermal deposits.

Keywords: nitrogen fixation, ammonium assimilation, nitrogen fixation rate, metal requirements, isotopic systematics, methanogen, nitrogen cycle, early Earth

1. Introduction

Deep-sea hydrothermal systems provide a variety of microbial habitats, and the focused and diffusing hydrothermal fluids contain microorganisms from reduced hot subseafloor environments (Deming and Baross, 1993; Summit and Baross, 1998; Takai and Nakamura, 2010; 2011). Microbiological and chemical components entrained in the discharging hydrothermal fluids and included in the subseafloor fluids are key signals for understanding the composition and function of indigenous microbial communities living in the subseafloor (Karl et al., 1989; Cowen et al., 2003; Butterfield et al., 2004; Takai et al., 2004; Orcutt et al., 2011). Many hyperthermophilic (optimal growth temperature: 70-120 °C) and thermophilic (optimal growth temperature: 50-70 °C) microorganisms have been isolated from seafloor hydrothermal environments, and these microorganisms utilise a variety of energy, carbon and nitrogen sources (e.g., Jones et al., 1983; Neuner et al., 1990; Huber et al., 1992; Nakagawa et al., 2003). Thermodynamic calculations and microbial community compositions in the hydrothermal mixing zones suggest that the chemolithotrophic energy potentials obtained from the hydrothermal fluids and the ambient seawater would control the development of chemolithotrophic microbial communities associated with hydrothermal activities (McCollom and Shock, 1997; Shock and Holland, 2004; Tivey et al., 2004; Takai and Nakamura, 2010; 2011). In addition to the chemolithotrophic energy state, the abundance and availability of biologically essential elements, such as nitrogen, phosphorus and transition metals, would significantly affect the composition and function of the microbial communities (Takai and Nakamura, 2010). However, the nitrogen sources supporting the development of chemolithotrophic microbial communities in the seafloor and subseafloor hydrothermal environments remain poorly understood.

The nitrate (NO_3^-) concentration in diffusing hydrothermal fluids (< 120 °C) is generally lower than that expected from a simple mixing of a magnesium-zero end-member hydrothermal fluid (0 μM) and the ambient deep-sea water (40 μM) (Johnson et al., 1988; Karl et al., 1989; Bourbonnais et al., 2012a). For instance, the nitrate concentration is less than 20 μM in the low-temperature (20 °C) diffusing fluids of the Galapagos spreading centre (Johnson et al., 1988). The non-conservative nitrate depletion most likely originates from biological consumption because many microorganisms can utilise nitrate via assimilatory and/or dissimilatory reduction (Nakagawa et al., 2003; Nakagawa

et al., 2005; Bourbonnais et al., 2012b). Nitrogen isotopic ratios of the nitrate in the diffusing fluids have been reported only from the Juan de Fuca Ridge, and they seem to increase from 6‰ (the value of nitrate in deep-sea water) to 10‰ as the degree of non-conservative nitrate depletion increases (Bourbonnais et al., 2012a). The ammonium (NH_4^+) concentration in high temperature (> 150 °C) hydrothermal fluids in unsedimented systems is typically similar to that of the ambient deep-sea water (1 μM or less), but it is occasionally as high as 15 μM in certain fields (German and Von Damm, 2003; Bourbonnais et al., 2012a). The exception is hydrothermal fluids (> 300 °C) venting from the Endeavour Segment on the Juan de Fuca Ridge, where decomposition of organic matter in sediments buried at an early stage of the ridge formation has been proposed to be the candidate source of ammonium (1000 μM, Lilley et al., 1993; Bourbonnais et al., 2012a). Nitrogen isotopic ratios of ammonium in hydrothermal fluids have been reported only from the Juan de Fuca Ridge, and they are 6.7 ± 1.0‰ (n = 16) at the Axial Volcano and 3.7 ± 0.6‰ (n = 37) at the Endeavour Segment (Bourbonnais et al., 2012a). Dissolved dinitrogen (N_2) is more abundant in hydrothermal fluids than nitrate and ammonium (400-3400 μM in magnesium-zero end-member hydrothermal fluids and 590 μM in deep-sea water) (Charlou et al., 1996; 2000; 2002). Isotopic ratios of N_2 in hydrothermal fluids have been reported only from the Tonga-Kermadec Arc, and they are slightly depleted in ^{15}N relative to deep-sea water (0‰) by 2‰ (de Ronde et al., 2011).

Previous studies have shown that a limited number of hyperthermophilic and thermophilic microorganisms can assimilate N_2 via a nitrogenase enzyme complex that catalyses N_2 reduction to ammonia (diazotrophy) (Belay et al., 1984; Mehta and Baross, 2006; Steunou et al., 2006; Hamilton et al., 2011). Furthermore, the phylogenetic diversity of nitrogenase genes (*nifH*) in deep-sea hydrothermal fluids has pointed to the presence of methanogenic archaea and anaerobic bacteria (clostridia, sulphate-reducing proteobacteria) as potential nitrogen fixers in the subseafloor microbial communities (Mehta et al., 2003).

The discovery of diazotrophic hyperthermophilic methanogens (Mehta and Baross, 2006) has highlighted the potential ubiquity and important role of these organisms in H_2-abundant marine hydrothermal environments throughout Earth history. Hyperthermophilic methanogens represent one of the most predominant primary producers in the deep-sea hydrothermal environments with hydrothermal fluid chemistries that are characterised by highly enriched H_2 (more than approximately 1 mM) (Takai et al., 2004; Flores et al., 2011). Furthermore, hyperthermophilic

methanogenesis has been theoretically and empirically predicted as one of the most ancient chemolithotrophic energy metabolisms supporting the earliest ecosystem associated with the ocean hydrothermal systems on the Hadean Earth (Russell and Martin, 2004; Ferry and House, 2006; Takai et al., 2006; Sleep and Bird, 2007; Martin et al., 2008; Russell et al., 2010). In fact, geological evidence of ancient methanogenesis in seafloor and subseafloor hydrothermal environments has been furnished by hydrothermal deposit records that date to 3.5 billion years ago (giga-annum, Ga) (Ueno et al., 2006).

Although many studies have reported on the ecophysiology and biochemistry of methanogenesis metabolisms and functions (Garcia et al., 2000; Thauer et al., 2008), the physiology of nitrogen fixation in hyperthermophilic methanogens remains to be elucidated, including the rate and energetics of nitrogen fixation and the biological requirement of transition metals used in the nitrogenase cofactors (e.g., molybdenum (Mo) and iron (Fe)). In addition, the isotopic systematics of nitrogen fixation in hyperthermophilic methanogens should be investigated to explain the role of the global biogeochemical nitrogen cycle throughout Earth's history.

In the present-day ocean, more than 70% of biological nitrogen compounds are provided by microbial nitrogen fixation (1-3 × 10^{14} gN/y) (Brandes and Devol, 2002). By contrast, on the early Earth, the potential nitrogen sources for living forms should have been produced by abiotic processes, such as atmospheric production of nitric oxide by lightning (Navarro-González et al., 2001), photochemical production of hydrogen cyanide (Zahnle et al., 1986), multistep conversion of nitric oxide and hydrogen cyanide to ammonium in the ocean (Zahnle et al., 1986; Summers and Chang et al., 1993; Brandes et al., 1998; Summers 2005; Brandes et al., 2008; Singireddy et al., 2012), shock synthesis of amines and amino acids (Furukawa et al., 2008), and hydrothermal synthesis of ammonia from N_2 reduction (Brandes et al., 1998, Schoonen and Xu, 2001; Smirnov et al., 2008). These prebiotic sources of biologically available nitrogen may have been sufficient immediately after the origin of life, but such abiotically produced nitrogen pools were likely drained by the early expansion of microbial populations and habitats. This process may have triggered the onset of biological nitrogen fixation. Based on the phylogenetic analyses of nitrogenase sequences, two possible hypotheses for the origin of nitrogen fixation have been proposed (Leigh, 2000; Raymond et al., 2004; Boyd et al., 2011b). One hypothesis proposes that the Mo-Fe-type nitrogenase was present in the last universal common ancestor (LUCA origin model) (Leigh, 2000; Raymond et al., 2004),

whereas the other claims that the Mo-Fe-type nitrogenase was derived from the ancestral methanogens (methanogen origin model) (Boyd et al., 2011b). To trace the time and place of the possible onset of biological nitrogen fixation, researchers have used not only an approach based on molecular evolution but also an approach involving the exploration of chemical fossils (isotopic signatures) in the geological record (Beaumont and Robert, 1999; Nishizawa et al., 2007). However, because the isotopic characteristics of nitrogen fixation in methanogens have, until now, been completely unknown, the interpretation of the geological record has been equivocal.

We report, for the first time, the physiological properties and isotopic characteristics of nitrogen anabolisms, including nitrogen fixation, in hyperthermophilic and thermophilic methanogenic genera found in global hydrothermal environments (*Methanocaldococcus* and *Methanothermococcus* spp.) (Takai et al., 2004; Flores et al., 2011; Ver Eecke et al., 2012). These methanogens, together with anaerobic archaeal methanotrophs, are known to encode for nitrogenase homologs that do not cluster phylogenetically with previously characterised nitrogenases with iron-molybdenum (FeMo), iron-vanadium (FeV) or iron-iron (FeFe) cofactors (Dekas et al., 2009; Boyd et al., 2011a; Dos Santos et al., 2012). Cultivation experiments were conducted under various conditions (e.g., under varying concentrations of Mo, Fe, N_2 and H_2 in the culture media) to potentially reproduce present and past oceanic and hydrothermal environments. The results include the novel finding that diazotrophic methanogens produce biomass that is more depleted in ^{15}N than diazotrophic photosynthetic prokaryotes (Minagawa and Wada, 1986; Macko et al., 1987; Carpenter et al., 1997; Beaumont et al., 2000; Zerkle et al., 2008; Bauersachs et al., 2009). The relatively large isotopic fractionation effect of the methanogens and its evolutionary implications are also discussed.

2. Methods

2-1. Isolation and phylogenetic characterisation of methanogenic strains

We used two strains of hyperthermophilic and thermophilic methanogens isolated from the Kairei field on the Central Indian Ridge. A hyperthermophilic methanogen was isolated from an *in situ* cultivation system (ISCS) deployed in 362 °C black smoker fluid from the Kali chimney at the Kairei Field. A slurry sample of the ISCS substratum was inoculated into a nitrogen-fixing medium (see section 2-2 for the chemical composition) prepared in test tubes with a gas phase of N_2 (0.1

MPa), CO_2 (0.1 MPa) and H_2 (0.2 MPa). A positive enrichment culture was obtained from the test tube incubated at 85 °C, and coccoid cells with F420-dependent autofluorescence were observed. Similarly, a thermophilic methanogen was isolated from an outer portion of the Kali chimney structure. The chimney sample was inoculated into a nitrogen-fixing medium prepared in test tubes with a gas phase of N_2 (0.1 MPa), CO_2 (0.1 MPa) and H_2 (0.2 MPa). A positive enrichment culture was obtained from the test tube incubated at 55 °C, and coccoid cells with F420-dependent autofluorescence were observed. The dilution-to-extinction method (Takai et al., 2008) was used to purify these strains using the same medium at 85 and 55 °C, respectively. A phylogenetic analysis of the 16S rRNA gene sequences revealed that the strain grown at 85 °C belonged to the genus *Methanocaldococcus* and was closely (99% similarity) related to a strain of *Methanocaldococcus* FS406-22. The analysis also showed that the strain grown at 55 °C belonged to the genus *Methanothermococcus* and was closely (99% similarity) related to a strain of *Methanothermococcus* okinawensis. We assigned the name *Methanocaldococcus* sp. kairei 1-85N (grown at 85 °C) (described as Mc 1-85N hereafter) to the former strain and the name *Methanothermococcus* sp. kairei 5-55N (grown at 55 °C) (described as Mt 5-55N hereafter) to the latter strain. Nitrogen fixation by Mc 1-85N and Mt 5-55N was verified by uptake of ^{15}N-labelled N_2 into cellular nitrogen under cultivation with ^{15}N-labelled N_2 as the sole nitrogen source.

2-2. Medium preparation

We conducted the diazotrophic cultivation of the methanogens in a nitrogen-fixing medium. The medium contained (g per litre): NaCl, 30; KH_2PO_4, 0.09; K_2HPO_4, 0.09; $MgCl_2/6H_2O$, 3.0; $MgSO_4/7H_2O$, 4.0; $CaCl_2$, 0.8; KCl, 0.33; $NiCl_2$, 0.002; Na_2SeO_3, 0.002. A solution of trace minerals (10 mL) was added to a litre of the medium. The trace mineral solution contained (g per litre): $MgSO_4/7H_2O$, 3; $MnSO_4/H_2O$, 0.5; $CoSO_4/7H_2O$, 0.18; $CaCl_2/2H_2O$, 0.1; $ZnSO_4/7H_2O$, 0.18; $CuSO_4/5H_2O$, 0.01; $KAl(SO_4)_2/12H_2O$, 0.02; H_3BO_3, 0.01; $NiCl_2/6H_2O$, 0.025; $Na_2SeO_3/5H_2O$, 0.0003. Subsequently, 20 mL of the medium was dispensed into a 160-mL glass serum bottle and autoclaved at 121 °C for 20 min. Na_2MoO_4 and $FeCl_3$ solutions (filter-sterilised) were then added to the medium on a clean bench. In the basic experiment, the gas phase consisted of N_2 (0.1 MPa), CO_2 (0.1 MPa) and H_2 (0.2 MPa). The medium was buffered with 12 mM $NaHCO_3$ solution (filter-sterilised) to a final pH of 6.0 (at room temperature) and was reduced by the addition of

192 $Na_2S/9H_2O$ solution to a final concentration of 2.1 mM. The glass bottle was sealed with a sterile
193 butyl rubber stopper and crimped with an aluminium seal. The concentration of ammonium
194 incorporated as an impurity of the medium and a carryover in the inoculum was less than 5 μM, and
195 the concentration of ammonium after diazotrophic cultivation was generally less than 6 μM. For
196 comparison purposes, a negative control experiment was conducted under a gas phase of Ar (0.1
197 MPa), CO_2 (0.1 MPa) and H_2 (0.2 MPa). Additionally, a series of ammonium-supplemented
198 cultivation experiments was conducted by adding NH_4Cl (100 μM–10 mM) to the medium. The gas
199 phase consisted of Ar (0.1 MPa), CO_2 (0.1 MPa) and H_2 (0.2 MPa) or N_2 (0.1 MPa), CO_2 (0.1 MPa)
200 and H_2 (0.2 MPa) when the initial concentration of the ammonium in the medium was 10 mM. By
201 contrast, the gas phase consisted of CO_2 (0.1 MPa) and H_2 (0.2 MPa) when the initial concentration
202 of the ammonium was below 1 mM (i.e., 100, 200, 1000 μM).
203 In a growth experiment under high-pressure, 5 mL or 10 mL of the medium was dispensed into
204 a 30 mL Sulfinert-coated stainless steel tube (Swagelok, Ohio) with an instrument plug valve capable
205 of operating up to 413 bar and 121 °C. The headspace was replaced by N_2 (2 MPa), CO_2 (0.1 MPa)
206 and H_2 (2 MPa). The final concentrations of $NaHCO_3$ and $Na_2S/9H_2O$ were adjusted to 12 mM and
207 2.1 mM, respectively.
208

209 **2-3. Cultivation experiments**

210 We pre-cultured the methanogen strains under the same conditions as those of the designed
211 cultivation experiment and inoculated the strains into fresh media. The cultivation temperatures were
212 85 °C for Mc 1-85N and 55 °C for Mt 5-55N. The duration of the cultivations ranged from 9.5 to 43
213 hours. We estimated the initial concentrations of the gaseous components dissolved in the media are
214 1.3 mM (H_2) and 0.4 mM (N_2) in the basic experiments and 13 mM (H_2) and 8 mM (N_2) in the
215 high-pressure experiments (Wiesenburg and Guinasso 1979). The measured pH values of the media
216 during cultivation always ranged from 5.7 to 6.1. The Mo speciation in sulphidic water was
217 experimentally investigated at 25 °C, and most of the Mo (VI) (99.9% or more) was found to exist as
218 oxythiomolybdate ($MoO_{4-x}S_x^{2-}$) at a neutral pH with 0.5 mM H_2S (Erickson and Helz, 2000).
219 Although there is no study on Mo speciation in sulphidic water at high temperatures, most of the Mo
220 in our media would likely exist as oxythiomolybdate under the experimental conditions. The growth

221 was tracked by direct counting of DAPI-stained cells. The uncertainty associated with the cell counts
222 was estimated to be 20-30% (one standard deviation) through replicate measurements.
223

224 **2-4. Chemical and isotopic analyses**

225 To investigate the isotopic characteristics of nitrogen fixation, the concentrations and isotopic
226 compositions of particulate nitrogen (PN) and total nitrogen (TN: sum of PN and dissolved nitrogen
227 compounds, except for N_2) were analysed for Mc 1-85N and Mt 5-55N. After cultivation, the
228 particulate matter (primarily cells) in the medium was collected by filtration through a GF/F filter
229 (pre-combusted at 450 °C for 4 h). Subsequently, the amount and the isotopic ratio of PN were
230 measured by combustion of the GF/F filter at 1,000 °C in a FLASH EA 1112 elemental analyser on
231 line with a Finnigan DELTAplus Advantage mass spectrometer at JAMSTEC. The precision
232 achieved with repeated analyses of in-house standards (alanine, glycine, and histidine) was typically
233 greater than 10% for the PN concentration and greater than 0.4‰ for $\delta^{15}N$. The PN concentration
234 should be considered a minimum estimate because it is possible that some fractions of the particulate
235 matter passed through the GF/F filter (Hewson et al., 2004).
236 To measure the concentration and isotopic ratio of TN, the TN in the medium was converted to
237 N_2O via two reaction steps. First, the TN was converted to nitrate by the persulphate oxidation
238 method, and the resulting nitrate was then converted to N_2O by the denitrifier method (Koba et al.,
239 2010). After purification by gas chromatography (Agilent HP6890) with a Poraplot column (25 m ×
240 0.32 mm), the concentration and isotopic ratio of the resultant N_2O were analysed with a Finnigan
241 DELTAplus XP mass spectrometer at the Tokyo University of Agriculture and Technology
242 (Nishizawa et al., 2013). The overall precision achieved with repeated analyses of the same sample
243 was typically 10% for the TN concentration and greater than 0.7‰ for $\delta^{15}N$. The nitrogen isotopic
244 ratios of PN and TN were expressed as $\delta^{15}N$ relative to the substrate used in the cultivation (N_2 in the
245 diazotrophic condition, NH_4Cl in the non-diazotrophic condition). The $\delta^{15}N$ value of the N_2 substrate
246 relative to air was -14.15 ± 0.05‰ (n = 2). This value was determined before the cultivation
247 experiment by a Finnigan MAT253 mass spectrometer in dual-inlet mode. The $\delta^{15}N$ value of the
248 NH_4Cl substrate relative to air was -5.8 ± 0.4‰ (8.4‰ relative to the N_2 substrate; n = 7), as
249 determined by the online combustion method described above.

The ammonium concentration in the medium was determined by the indophenol blue method (reproducibility: ± 5%) (Solorzano 1969). The methane (CH_4) concentration in the headspace was measured by gas chromatography coupled with a thermal conductivity detector (GC-TCD, reproducibility: ± 2%) (GL Science GC-3200). The total Fe and Mo concentrations in the medium were measured with ICP-MS (internal precision: better than 5%) (Agilent 7500ce).

2-5. Calculations

The cell-specific growth rate was calculated from the slope of the growth curve in exponential phase and was reported as μ (h^{-1}). The cell-specific nitrogen uptake rate in the exponential phase, ρN (mol N × $cell^{-1}$ × min^{-1}), was calculated from the relation $\rho N = \mu \times Q_N$ (eq. 1), where Q_N denotes cellular nitrogen content (mol N × $cell^{-1}$) (Tuit et al., 2004). The ρN was determined from replicate experiments (n = 3–7).

The amount of chemical energy potentially available to the methanogen was calculated from the change in the Gibbs free energy associated with methanogenesis ($4H_2(aq) + CO_2(aq) \rightarrow CH_4(aq) + 2H_2O$). We used the following equation: Potential energy yield = RT * ln(K/Q) (eq. 2). R is the universal gas constant, T is the temperature in Kelvin, and K represents the equilibrium constant, which is calculated from the standard Gibbs free energy of methanogenesis ($\Delta G^o r$) at the cultivation temperature and pressure, using the relation $\Delta G^o r = -RT * \ln K$. The activity product Q was calculated from the relation $Q = a_{CH4} /(a_{CO2} * a_{H2}^4)$. The symbols a_{CH4}, a_{CO2} and a_{H2}^4, respectively, denote the activities of CH_4, CO_2 and H_2 dissolved in the medium and were calculated from molar concentrations (m) and activity coefficients (γ) ($a = m * \gamma$). The amounts of H_2 and ΣCO_2 (sum of gaseous CO_2, aqueous CO_2, HCO_3^- and CO_3^{2-}) consumed during our experiment were calculated from the measured amount of CH_4 produced. We neglected the contribution of cellular carbon to the amount of ΣCO_2 consumed due to the low amount of cellular carbon produced from ΣCO_2 (less than 1.4%). Solubility of H_2 and CH_4 in the medium was calculated from Wiesenburg and Guinasso (1979). Values of $\Delta G^o r$, γ, and CO_2 speciation in the medium during experiment (ionic strength = 0.65 M) were calculated using the Geochemist's Workbench computer code (Bethke, 2008).

The isotopic ratio of TN produced during the growth of methanogens ($\delta^{15}N$ (TN pro.)) was calculated using the following equation: $\delta^{15}N$ (TN pro.) = ([TN] × $\delta^{15}N$ (TN) – [TN]' × $\delta^{15}N$ (TN)') /

([TN] − [TN]') (eq. 3). Symbols [TN] and [TN]' denote concentrations of TN at T and T' hours after cultivation starts (T > T').

The isotope enrichment factors ($\varepsilon_{P/S}$) of nitrogen fixation and ammonium uptake were calculated using the following equation: $\delta^{15}N_{P,ac} = \delta^{15}N_{S,0} - f \times \ln(f) \times (1 - f)^{-1} \times \varepsilon_{P/S}$ (eq. 4) (Mariotti et al., 1981). The $\delta^{15}N_{P,ac}$ and $\delta^{15}N_{S,0}$ symbols denote the nitrogen isotopic ratios of accumulated product (TN for the nitrogen fixation experiment and PN for the ammonium uptake experiment) and substrate (N_2 for the nitrogen fixation experiment and NH_4Cl for the ammonium uptake experiment), respectively. The symbol "f" denotes the remaining fraction of substrate. In the ammonium uptake experiment, the f value was calculated from the ammonium concentrations in the medium before and after cultivation. By contrast, equation 4 can be approximated by $\delta^{15}N_{P,ac} = \delta^{15}N_{S,0} + \varepsilon_{P/S}$ in the nitrogen fixation experiment if the f value is greater than 0.98 (eq. 5).

3. Results

3-1. Rates and metal requirements of nitrogen fixation

Both Mc 1-85N and Mt 5-55N utilised N_2 and ammonium as the sole nitrogen source, but not nitrate (Figures 1a, b; Table 1). The diazotrophic growth of Mc 1-85N was observed in the presence of broad ranges of Mo and Fe concentrations (Mo = 5 nM–1 mM; Fe = 100 nM–10 mM) (Figure 1a and Table 1). In the media with higher Mo concentrations (10-1,000 μM), growth followed a simple exponential curve until the H_2 was largely consumed. In the media with lower Mo concentrations (5 nM to 1 μM), growth initially followed a simple exponential curve and then reached the stationary phase before H_2 was depleted. Thus, it is likely that diazotrophic growth is directly linked to the availability of Mo in the medium.

Under the diazotrophic growth condition with a Fe concentration of 1 mM or less in the medium, the cell-specific growth rate in the exponential growth phase was almost constant (0.28 ± 0.03 h^{-1}, n = 12), irrespective of the Mo concentrations added, but it was five times lower than that under the non-diazotrophic growth condition in the presence of 10 mM of ammonium (1.5 h^{-1}).

By contrast, Mt 5-55N exhibited diazotrophic growth under narrower ranges of Mo and Fe concentrations in the media (Mo = 1 μM–10 μM; Fe = 10 μM–100 μM) (Figure 1b and Table 1). Interestingly, Mt 5-55N grew well at a low Mo concentration (Mo = 5 nM; Fe = 100 μM) (Table 1)

in the presence of 1 mM ammonium, which is an Mo concentration where the diazotrophic growth of Mt 5-55N was prevented. This result indicated that Mt 5-55N requires relatively higher amounts of Mo to activate its nitrogenase function. The specific growth rate in the exponential growth phase was $0.27 \pm 0.02\ h^{-1}$ in the diazotrophic growth condition, which was similar to that of Mc 1-85N.

The cell-specific nitrogen uptake rates in the exponential growth phase were estimated to be 2-11 x 10^{-17} mol N × $cell^{-1}$ × min^{-1} for Mc 1-85N (n = 7) and 8 x 10^{-17} mol N × $cell^{-1}$ × min^{-1} for Mt 5-55N (n = 2), (cells = 1 μm in diameter for both strains) under the diazotrophic growth condition. The cell-specific nitrogen uptake rates of Mc 1-85N and Mt 5-55N are one order of magnitude higher than that of a marine diazotrophic cyanobacterium, *Crocosphaera watsonii* strain WH8501 (0.2-1.0 × 10^{-17} mol N × $cell^{-1}$ × min^{-1}, n = 4) (cell = 2.5-6 μm in diameter), but they are only approximately half the rate of a marine diazotrophic cyanobacterium, *Trichodesmium erythraeum* (19 × 10^{-17} mol N × $cell^{-1}$ × min^{-1}, n = 1) (cell = 6-22 μm wide × approx. 10 μm long) (Tuit et al., 2004) (Table 2). Although the exact cell volumes of these microorganisms in the diazotrophic experiment were not directly measured, the results suggest that the nitrogen uptake rates per unit cell volume of these methanogens would be much higher than those of marine cyanobacteria (Table 2). The C/N molar ratios of Mc 1-85N and Mt 5-55N, respectively, ranged from 4.1 to 8.4 (generally 4.1 to 6.4) and 4.0 to 7.3 (generally 4.1 to 5.5) under the various growth conditions.

3-2. Energetics of nitrogen fixation

The amount of CH_4 produced was linearly correlated with the amount of PN during the exponential growth of Mc 1-85N (Figure 2). The slope of the relationship between the amounts of CH_4 and PN represents the growth yield. The growth yield of Mc 1-85N under the diazotrophic condition was 36 ± 3 mg N × (mol CH_4)$^{-1}$ (n = 11) and was approximately one-fifth of that under the ammonium-replete condition (10 mM of NH_4^+) (169 ± 11 mg N × (mol CH_4)$^{-1}$, n = 5). Interestingly, the growth yield of Mc 1-85N was 84 ± 15 mg N × (mol CH_4)$^{-1}$ when the ammonium concentration in the medium was below 200 μM (n = 6; data not shown). The growth yield of Mt 5-55N under the ammonium-replete condition was 170 ± 40 mg N × (mol CH_4)$^{-1}$ (n = 3).

During the exponential growth of Mc 1-85N, the potential energy yield from methanogenesis decreased from 132 to 92 kJ × (mol CH_4)$^{-1}$ as H_2 concentration in the medium decreased from 1360

337 to 140 μM (n = 8; Table EA-1). In contrast, the constant growth yields of Mc 1-85N are consistent
338 with the observation that hydrogenotrophic methanogens generally produce the same number of ATP
339 per molecule of CH_4 generated, independent of the Gibbs energy change of methanogenesis (e.g.,
340 Thauer et al., 2008). The growth yield of Mc 1-85N under the diazotrophic condition is
341 higher-than-expected because, in theory, the number of ATP molecules needed for nitrogen fixation
342 is about ten times higher than that needed for ammonia assimilation (Cabello et al., 2009).
343

344 **3-3. Isotopic characteristics of nitrogen fixation and extracellular ammonium assimilation**
345 **3-3-1. Mc 1-85N**

346 Under the diazotrophic condition, the concentrations of PN and TN increased during growth
347 (Table 3). The concentrations of PN in the exponential phase (10, 12 and 16.5 h) were nearly equal to
348 those of TN within the analytical uncertainties. After 10 h, the $\delta^{15}N$ value of TN was constant at -4‰
349 (relative to the N_2 substrate) and was close to that of PN. The $\delta^{15}N$ (TN) value in the early growth
350 phase (2‰ at 6 h) was higher than that in the later growth phases. The $\delta^{15}N$ value of TN produced
351 during the growth of the methanogen ($\delta^{15}N$ (TN pro.)) was estimated from equation 3 to be -6.5‰
352 (relative to the N_2 substrate) for the period from 6 to 10 h. The $\delta^{15}N$ (TN pro.) value was -3.7‰ for
353 the period from 10 to 12 h and -4.5‰ for the period from 12 to 16.5 h (Table 3). The $\delta^{15}N$ values of
354 PN produced in the experiments were -3.9 ± 0.5‰ (relative to the N_2 substrate) (1SD; n = 48) under
355 the various metal conditions (Figure 3a).

356 The positive $\delta^{15}N$ (TN) value at 6 h might be due to binary mixing of a ^{15}N-enriched
357 ammonium contaminant, initially present in the medium, with ^{15}N-depleted cellular nitrogen. The
358 mass contribution of the ammonium contaminant (70 ng N/mL) to TN was less than 12% at 6 h.
359 Hence, the $\delta^{15}N$ value of the ammonium contaminant should have been more than 42‰ (relative to
360 the N_2 substrate) if the $\delta^{15}N$ value of the cellular nitrogen was -4‰ (the same $\delta^{15}N$ value as the PN
361 after 10 h). Such a high $\delta^{15}N$ (NH_4^+) value seems to be unlikely because the $\delta^{15}N$ (NH_4^+) values of
362 the inoculum and the medium were likely less than NH_4Cl (+8.4‰; Section 2-4). Alternatively, the
363 $\delta^{15}N$ (TN) value at 6 h may be explained by the combination of a very small amount of isotopic
364 fractionation during nitrogen fixation in the early exponential phase and the presence of the
365 ammonium contaminant, which was slightly enriched in ^{15}N.

In the high-pressure experiments under the diazotrophic conditions, Mc 1-85N exhibited evident growth, and the TN concentration increased from 0.2 to 46 μg N/mL at the maximum (9 days after inoculation). The $\delta^{15}N$ (TN pro.) value was -3.9 ± 0.9‰ (1SD; n = 5) in the three independent cultivations. No significant difference was observed for the $\delta^{15}N$ (TN pro.) values between the high-pressure condition (H_2 = 2 MPa, N_2 = 2 MPa) and the normal pressure condition (H_2 = 0.2 MPa, N_2 = 0.1 MPa) (Figure 3b).

By contrast, the magnitude of nitrogen isotope fractionation of ammonium uptake generally decreased as the concentration of ammonium in the medium decreased. The $\varepsilon_{cell/NH4+}$ value was -16 ± 1‰ in a concentration range from 10 mM to 8 mM NH_4^+ (f = 0.82–0.98; n = 4), -14 ± 1‰ in 1,070 to 670 μM NH_4^+ (f = 0.78–0.87; n = 5) and -7 ± 1‰ in 200 to 70 μM NH_4^+ (f = 0.58–0.76; n = 3) (Figure 4).

3-3-2. Mt 5-55N

Under the diazotrophic condition, the concentrations of PN and TN increased during growth (Table 3). The concentration of PN in the exponential growth phase (16 h) was nearly equal to that of TN, within the analytical uncertainty. The isotopic ratios of PN and TN were relatively constant throughout growth. The $\delta^{15}N$ (TN pro.) value was -3.4‰ for the period from 13 to 16 h and -4.2‰ for the period from 16 to 26.5 h. The $\delta^{15}N$ values of PN produced in the experiments were -3.7 ± 0.5‰ (1SD; n = 17) under the various metal conditions (Figure 3a). The $\varepsilon_{cell/NH4+}$ value was -17 ± 1‰ in a concentration range from 10 mM to 9 mM (f = 0.92–0.99; n = 6) (Figure 4).

4. Discussion

4-1. Factors influencing isotopic fractionation during nitrogen fixation and intracellular ammonia assimilation

In the nitrogen fixation experiments, the concentrations of cellular nitrogen (i.e., PN) produced were 1-10 μg N/mL, while those of ammonium initially present in the media were ≤ 70 ng N/mL. The cellular nitrogen produced from the assimilation of the ammonium contamination could thus alter the overall $\delta^{15}N$ (PN) values by +1‰ at the most, assuming that the $\delta^{15}N$ values of the ammonium contamination are close to the NH_4Cl reagent (8.4‰, Section 2-4). Furthermore, the

395 amounts of N_2 consumed in diazotrophic cultivations were small (< 2% of the initial amounts of N_2).
396 The overall isotopic fractionations between PN and N_2 were thus estimated to be -3.9 ± 0.5‰ (n =
397 48) and -3.7 ± 0.5‰ (n = 17) for Mc 1-85N and Mt 5-55N, respectively, using equation 5.
398 The overall isotopic fractionations by the methanogens were larger than those of photosynthetic
399 prokaryotes (-1.4 ± 0.9‰, n = 51; Minagawa and Wada, 1986; Macko et al., 1987; Carpenter et al.,
400 1997; Beaumont et al., 2000; Zerkle et al., 2008; Bauersachs et al., 2009) (Kruskal-Wallis, p-value <
401 0.001) (Figure 5). It is interesting to consider what factors may cause the differing amounts of overall
402 isotopic fractionation by methanogens and photosynthetic prokaryotes during nitrogen fixation and
403 cellular nitrogen assimilation. Nitrogen fixation consists primarily of a two-step process: the
404 diffusion of N_2 into the cell (step 1) and the reduction of N_2 to ammonia by nitrogenase in the
405 cytoplasm (step 2). Cellular nitrogen assimilation requires an additional step (step 3): the
406 assimilation of ammonia into cellular nitrogen compounds via glutamate and/or glutamine (Figure
407 A1-a). Several studies have shown that a certain fraction of the ammonia produced by nitrogenase is
408 not assimilated, but rather, it is excreted from diazotrophic cyanobacteria, leading to the formation of
409 a dissolved nitrogen pool (the sum of the dissolved organic nitrogen and dissolved inorganic nitrogen
410 other than N_2) in the surrounding environment (Glibert and Bronk 1994). Thus, the isotopic ratio of
411 PN reflects not only the isotopic fractionation of nitrogen fixation (ε_{step_1}, ε_{step_2}) but also that of
412 intracellular ammonia assimilation ($\varepsilon_{step_3,}$) if the ammonia produced in step 2 is not entirely
413 converted to cellular nitrogen compounds. By contrast, the isotopic ratio of TN produced during
414 diazotrophy reflects the net isotopic fractionation of nitrogen fixation, not that of intracellular
415 ammonia assimilation.
416 In the exponential growth phases of Mc 1-85N and Mt 5-55N, TN was composed almost
417 completely (> 90%) of PN, and the $\delta^{15}N$ values of PN and TN represented rather similar values
418 (Table 3). Thus, the isotopic fractionation of intracellular ammonia assimilation (ε_{step_3}) is negligible,
419 and the difference between the isotopic ratios of PN and substrate N_2 should exactly represent the
420 isotopic fractionation of nitrogen fixation. Furthermore, the isotopic ratio of TN produced by Mc
421 1-85N was not affected by different concentrations of N_2 in the medium (380-7,600 μM; Figure 3b),
422 suggesting that the isotopic fractionation effect during step 1 had little influence on the overall
423 isotopic fractionation. These results collectively indicate that the ^{15}N-depleted cellular nitrogen of

methanogens primarily reflects an isotopic fractionation occurring during N_2 reduction by nitrogenase (ε_{step_2}).

The ε_{step_2} value did not vary with the different growth temperatures of the methanogens (55 and 85 °C) or the different concentrations of Mo (5 nM–1 mM), Fe (100 nM–10 mM) and H_2 (up to 13 mM). Isotopic fractionation during nitrogen fixation by the methanogens is thus predicted to be constant (-4‰) in natural hydrothermal environments that have broad gradients of temperature and metal concentrations due to the mixing of hot hydrothermal fluids and cold seawater.

The isotopically lighter cellular nitrogen of the methanogens likely results from a more negative ε_{step_2} value compared with the photosynthetic prokaryotes. Based on the phylogenetic distribution of nitrogenase gene sequences, the *Methanocaldococcus* and *Methanothermococcus* lineages are predicted to have nitrogenases with biochemically uncharacterised active site metal cofactors, whereas the photosynthetic prokaryotes have nitrogenases with FeMo-cofactors (Boyd et al., 2011a; Dos Santos et al., 2012). Our experiments suggest that the uncharacterised metal cofactors are not vanadium dependent because the culture media for the methanogens lacked vanadium. In contrast, our experiments suggest that Mt 5-55N requires molybdenum for the nitrogenase to function because the diazotrophic growth of Mt 5-55N was inhibited in a low Mo condition (Mo = 15 nM; Fe = 100 μM; H_2 = 0.2 MPa; N_2 = 0.1 MPa; NH_4^+ < 10 μM), whereas it grew well with ammonium under a lower Mo condition (Mo = 5 nM; Fe = 100 μM; H_2 = 0.2 MPa; N_2 not added; NH_4^+ = 1 mM) (Table 1). Mt 5-55N should thus contain Mo-bearing nitrogenase, consistent with the theoretical prediction that the uncharacterised active site metal cofactors are analogous to the FeMo-cofactor (McGlynn et al., 2013). The structural observation of the nitrogenase of Mt 5-55N is, however, required to demonstrate this inference. N_2 reduction to ammonia by the FeMo-cofactor is a multistep reaction via N_xH_y intermediates (Chatt et al., 1978; Seefeldt et al., 2009; Figure A1-b). The first step of N_2 reduction possibly limits the overall rate of ammonia production because the triple bond of N_2 is highly stable (e.g., 948 kJ/mol in free state). The high stability of N_2 likely induces, to some extent, desorption of N_2 from the FeMo-cofactor, creating the following reaction flows: free N_2 (N≡N) ⇌ N_2 adsorbed on the FeMo-cofactor (N≡N-Fe) → N_xH_y intermediates. Thus, the potential major factors influencing the ε_{step_2} value are the isotopic fractionations associated with forward and backward reactions of N≡N-Fe (i.e., reduction to NH=N-Fe and desorption, respectively) and a ratio of the forward and backward reaction flows (Rees 1973). Assuming the reduction steps of N_2 of the

uncharacterised active site metal cofactor are identical to those of the FeMo-cofactor, we expect the methanogens to have more negative ε_{step_2} values than photosynthetic prokaryotes in the following three cases:

i) when the magnitude(s) of isotopic fractionation(s) associated with forward and/or backward reaction(s) of N_2 on the uncharacterised cofactor is (are) larger than that on the FeMo-cofactor;

ii) when the ratio of the forward and backward reaction flows of N_2 on the uncharacterised cofactor is different from the ratio on the FeMo-cofactor;

iii) or a combined effect of i) and ii).

For further discussion, detailed information is required about local coordination of N_2 on the uncharacterised cofactor, and the kinetics of N_2 reduction steps and adsorption/desorption on the uncharacterised cofactor.

4-2. Factors influencing isotopic fractionation during extracellular ammonium assimilation

The apparent isotopic fractionations during extracellular ammonium assimilation of Mc 1-85N and Mt 5-55N under the ammonium-replete condition (10 mM of NH_4^+) were -16‰ and -17‰, respectively (Figure 4). These $\varepsilon_{cell/NH4+}$ values of the methanogens are similar to those previously reported in bacteria grown under ammonium-replete conditions ($\varepsilon_{cell/NH4+}$ = -15‰ at 4–70 mM NH_4^+ for *Anabaena vinelandii, Anabaena sp*. and *Vibrio harveyi*) (Delwiche and Steyn, 1970; Macko et al., 1987; Hoch et al., 1992). With a level of ammonium greater than several millimolar (> 3 mM) supplemented in a medium, *V. harveyi* used glutamate dehydrogenase to assimilate intracellular ammonium, primarily infiltrated as ammonia by membrane diffusion (Hoch et al., 1992). Consequently, the $\varepsilon_{cell/NH4+}$ value of *V. harveyi* appears to represent the combined effects of the isotopic equilibrium between ammonium and ammonia and the kinetic isotope fractionation of glutamate dehydrogenase activity. By contrast, the methanogens likely use the GS-GOGAT pathway to assimilate intracellular ammonium, based on the genetic information of *Methanocaldococcus* and *Methanothermococcus* lineages (Table EA-2). Hence, the $\varepsilon_{cell/NH4+}$ values of the methanogens under ammonium-replete conditions likely represent the combined effects of the isotopic equilibrium between ammonium and ammonia and the kinetic isotope fractionation of GS-GOGAT activity.

The $\varepsilon_{cell/NH4+}$ value of Mc 1-85N increased from -14‰ to -7‰ with decreasing ammonium concentrations in the medium from 1 mM to 100 μM (Figure 4). Similarly, an $\varepsilon_{cell/NH4+}$ value of -4‰ has been reported in *V. harveyi* grown in a medium containing only 20 μM of NH_4^+ (Hoch et al., 1992). The increase in the $\varepsilon_{cell/NH4+}$ value of *V. harveyi* with decreasing ammonium concentrations is mainly due to switching the extracellular ammonium uptake mechanism from passive membrane diffusion to an active, inter-membrane transport (Hoch et al., 1992). Genes for a putative ammonium transporter are identified in the genomes of *Methanocaldococcus* and *Methanothermococcus* lineages (Table EA-2). The increased $\varepsilon_{cell/NH4+}$ value of Mc 1-85N under the ammonium-depleted condition would thus originate from a small magnitude of isotopic fractionation by active ammonium transport, as predicted in the case of *V. harveyi*.

4-3. Implications for the ecological roles and evolutionary physiology of diazotrophic methanogens

The differing growth responses of Mc 1-85N and Mt 5-55N to the transition metal concentrations under the diazotrophic conditions may provide interesting insights into the ecological significance and evolutionary physiology of these diazotrophic methanogens. For diazotrophic growth, Mc 1-85N showed an apparently lower requirement of and higher tolerance to transition metals (Mo = 5 nM–1 mM; Fe = 100 nM–10 mM) than did Mt 5-55N (Section 4-1). It remains uncertain whether the different responses to the transition metal concentrations for diazotrophic growth are due primarily to the differences in cellular physiology between the hyperthermophilic and thermophilic *Methanococcales* lineages or due solely to the corresponding differences between the Mc 1-85N and Mt 5-55N strains. Nevertheless, this result implies that diazotrophic primary production of hyperthermophilic methanogens within *Methanococcales* may be more feasible in a broad spectrum of seafloor and subseafloor environments associated with H_2-rich crustal hydrothermal activities.

The apparently lower Mo requirement of Mc 1-85N for diazotrophic growth is most likely associated with the possible existence and function of a high Mo affinity of inter-membrane transporter or channel proteins adapted to environments having very low concentrations of Mo. In contrast, a high Mo tolerance has been shown in *Anabaena vinelandii*, and it was found that cellular

Mo homeostasis was achieved by both the regulation of the extracellular Mo uptake rate and the storage of excess amounts of Mo by Mo-storage proteins (up to 100 μM) (Pienkos and Brill, 1981; Bellenger et al., 2011). Thus, the high tolerance to transition metals, such as Mo and Fe, shown by hyperthermophilic methanogens may be dependent on mechanisms similar to those found in *A. vinelandii*.

The concentration of soluble iron (Fe^{2+}) in seawater may have decreased from 1-100 μM before 2.5 Ga to 1 nM today due to the progressive oxidation of the ocean (Holland 1973, 1984; Beukas and Klein, 1990; Canfield 2005). In contrast, the iron concentrations of high-temperature (> 250 °C) hydrothermal fluids in the Precambrian ocean are still in question. The current consensus appears to be that the high-temperature hydrothermal fluids in the Precambrian ocean had about the same range of iron concentrations as those observed in modern hydrothermal systems (generally 1-20 mM) (e.g., Seyfried et al., 1991; Douville et al., 2002). In contrast, geological observations and hydrothermal experiments suggest that reactions between basalt and CO_2-rich seawater in high-temperature zones (> 250 °C) could have generated alkaline, iron-poor (1 μM) hydrothermal fluids at mid-ocean ridges before 3 Ga (Shibuya et al., 2010; 2013a, b). In the latter model, the iron source of Archean banded iron formations is considered to be acidic to neutral, iron-rich hydrothermal fluids generated by the rock-dominant water/rock reactions expected in oceanic plateau/island arc settings (Shibuya et al., 2013a). In either case, the potential range of iron concentrations in the hydrothermal mixing zones (50-120 °C) at the Precambrian mid-ocean ridges would fall within the iron concentration range that allows diazotrophic growth of Mc 1-85N. Hyperthermophilic methanogens may thus have lived in the hydrothermal mixing zones throughout Earth's history, without suffering from iron limitation or iron toxicity.

Mo-depleted (less than 10 nM) habitats may be typical of the seafloor and subseafloor environments associated with crustal hydrothermal circulation for the present-day Earth and even for the hydrothermal and non-hydrothermal oceanic environments in the Precambrian Eon. In the present-day oxic seawater, Mo dissolves as molybdate (MoO_4^{2-}), and its concentration is 110 nM (Morris, 1975; Collier, 1985). By contrast, Mo concentrations in high-temperature (> 300 °C), H_2S-abundant fluids from two hydrothermal fields are reported to be 2-5 nM, likely due to the precipitation of Mo sulphides (Kishida et al., 2004). The primary source of Mo in the present-day ocean is riverine molybdate, generated by the oxidative dissolution of Mo sulphides in the

continental crust (Bertine and Turekian, 1973; Taylor and McLennan, 1995), suggesting that the Mo concentrations in the Precambrian ocean under the limited to low O_2 levels of the atmosphere should have been low. Based on the abundance of Mo in Precambrian shale, the Mo concentration in the Precambrian ocean is roughly estimated to be less than 10 nM before 800 Ma (Scott et al., 2008). Due to experimental limitations, this study did not examine diazotrophy of Mc 1-85N in the presence of Mo below 5 nM. It should be noted, however, that the concentration of molybdate in our sulphidic medium during the experiment was likely lower than that of total Mo (the sum of the dissolved and insoluble forms of Mo in the medium), due to the precipitation of particle-reactive oxythiomolybdate (Section 2-3). This finding implies diazotrophy of Mc 1-85N in the presence of molybdate below 5 nM, and it highlights the possible abundance and function of diazotrophy by hyperthermophilic methanogens in the hydrothermal mixing zones of the Precambrian ocean.

4-4. Implications for nitrogen sources of microbial communities in early Archean hydrothermal environments

The isotopic records in geological samples may imply the possible emergence and function of nitrogen fixation by hyperthermophilic methanogens in early Archean hydrothermal environments (Figure 6a). The isotopic ratio of the atmospheric N_2 that was dissolved in seawater and preserved in fluid inclusions has been determined from various geological samples deposited during different ages, and it has been found to be virtually constant during the past 3.5 Gyr (-2–0‰; Sano and Pillinger, 1991; Nishizawa et al., 2007). In contrast, ^{15}N-depleted organic matter (as low as -6‰) is found in early Archean cherts generally produced by deep-sea hydrothermal activities (Beaumont and Robert, 1999; Ueno et al., 2004). The slight ^{15}N-depletion of the organic matter compared to N_2 is consistent with nitrogen fixation in the ancient hydrothermal environments.

We should be aware that abiotic processes might have created some of the organic nitrogen compounds in the early Archean. Experimental studies have shown that abiotic reactions create organic nitrogen compounds (amides, amino acids and nitriles) under some hydrothermal conditions (e.g., Hennet, et al., 1992; Yanagawa and Kobayashi 1992; Marshall 1994; Rushdi and Simoneit 2004; Huber and Wächtershäuser, 2006). However, it is still debatable whether the abiotic reactions had quantitatively created the organic nitrogen compounds in natural hydrothermal systems in the

early Earth because the previous experimental studies had assumed geologically unusually high concentrations of reactants (e.g., carbon monoxide, potassium cyanide, formic acid, oxalic acid, and ammonium-salts) (Bada et al., 2007; Aubrey et al., 2009). Furthermore, the nitrogen isotopic fractionations during these reactions (e.g., Fischer-Tropsch-type reactions and Strecker synthesis) have not been investigated. Although we cannot eliminate the possibility of abiotic synthesis of organic nitrogen compounds in natural hydrothermal environments, it is premature to assess the abiotic origins of organic nitrogen compounds in Archean hydrothermal deposits.

In contrast, multiple lines of evidence have suggested that potential hyperthermophilic methanogens were alive in such seafloor and subseafloor environments associated with hydrothermal activities in the early Earth. In the Pilbara Craton, numerous silica vein intrusions are found in the surrounding basaltic greenstones in the Dresser Formation, and they are interpreted as the remnants of seafloor hydrothermal conduits at 3.5 Ga (Isozaki et al., 1997; Nijman et al., 1999; Van Kraendonk et al., 2001). The silica veins contain a substantial amount of organic matter and putative microfossils with a $\delta^{13}C$ of -36‰ and ^{13}C-depleted CH_4 (as low as -56‰) within primary fluid inclusions (Ueno et al., 2001; 2004; 2006). The ^{13}C-depleted organic matter and the CH_4 indicate potential activity of hyperthermophilic methanogens at 3.5 Ga, but they would not have been produced from abiotic Fischer-Tropsch-type reactions because of the absence of effective catalysts (native metals and magnetite) for the reactions during the silica vein formation (Ueno et al., 2004; 2006b; see an alternative view by Sherwood Lollar and McCollom, 2006). Thus, this evidence is consistent with the inference that the ^{15}N-depleted organic matter (as low as -4‰; Ueno et al., 2004) in the silica veins is also derived from possible hyperthermophilic methanogen populations.

The study of the primary fluid inclusions in the silica veins from the Dresser Formation has shown that the isotopic ratio of N_2 was -3 to +1‰ (Nishizawa et al., 2007), which is a ratio comparable to that of N_2 typically dissolved in deep-sea hydrothermal fluids on the present-day Earth (de Ronde et al., 2011). Hence, the $\delta^{15}N$ values of the organic matter potentially produced from the diazotrophy of hyperthermophilic methanogen populations are estimated from -7 to -3‰, using the $\delta^{15}N$ values of N_2 for the Archean hydrothermal fluids and the isotopic fractionation effect between N_2 and cellular nitrogen compounds determined in this study (Figure 6b). Note that we cannot directly compare the $\delta^{15}N$ values of the potential methanogen populations to those of the organic matter in the silica veins.

The nitrogen compounds in sedimentary environments consist primarily of organic nitrogen compounds and fixed ammonium. The fixed ammonium is a fraction of the ammonium released from organic matter during diagenesis and subsequently fixed in clay minerals (Hall 1999). In addition, during the metamorphic processes of the sedimentary rocks, the quantities and isotopic ratios of the initial nitrogen compounds can change via thermal volatilisation (Hall 1999). Thus, to correctly interpret the relationship between the nitrogen isotopic ratios of the initial organic compounds in the past and the organic matter in the present, we must consider the possible isotopic fractionations during diagenesis and metamorphism at different spatial and temporal scales.

The isotopic fractionation of metamorphism on sedimentary nitrogen compounds was evaluated by stepwise combustion experiments of the silica veins from the Dresser Formation (Pinti et al., 2001, 2009). Based on an inverse relationship between the concentration and the isotopic ratio of nitrogen released from the silica veins at temperatures above 500 °C, Pinti et al. (2009) estimated that the $\delta^{15}N$ value of the pre-metamorphic sedimentary nitrogen in the silica veins had been between -7 and -4‰.

In addition, the long-term (most likely, microbial and thermal) maturation of organic matter during burial diagenesis would release ammonium and could alter the isotopic ratio. However, previous studies have shown that the $\delta^{15}N$ values of ammonium (both the free and fixed forms) are typically close to those of the bulk sedimentary nitrogen and organic matter (± 1‰; Williams et al., 1995; Freudenthal et al., 2001; Prokopenko et al., 2006). These observations imply that burial diagenesis would provide minimal isotope fractionation and that the ammonium released from the source organic matter may have an isotopic ratio similar to that of the source organic matter, as much as ± 1‰.

In the early phase of deposition, organic matter is more or less influenced by the decompositional functions of microbial communities (early diagenesis). Two types of microbial decompositional processes are known to alter the $\delta^{15}N$ values of the initial organic matter. One process is the microbial decomposition of organic matter under aerobic conditions, which typically results in the ^{15}N enrichment of sedimentary nitrogen by +3 to +4‰ (Altabet and Francois, 1994; Nakatsuka et al., 1997; Möbiuset al., 2010). The other process is the decomposition of organic matter by anaerobic microbial populations, which typically leads to limited or slight ^{15}N depletion of sedimentary nitrogen (-2 to 0‰) (Lehmann et al., 2002; Higgins et al., 2010; Möbiuset al., 2010). In

the case of the anoxic Archean ocean and hydrothermal environments, it is hypothesised that anaerobic microbial decomposition caused the nitrogen isotopic alteration of the organic matter in the silica veins during early diagenesis.

Based on our discussion of the possible isotopic alteration processes, such as metamorphism, burial diagenesis and early diagenesis, we predict that the nitrogen isotopic ratios of the initial organic matter at the time of vein formation in the Dresser Formation should range from -8 to -1‰ (i.e., -7-1+0 = -8‰, -4+1+2 = -1‰). This range is very similar to the estimated $\delta^{15}N$ range of the organic compounds produced by the diazotrophic hyperthermophilic methanogens (-7 to -3‰). Furthermore, N_2 is expected to be the predominant nitrogen compound in the hydrothermal fluids of the Dresser Formation because the silica veins are thought to have been deposited in an Archean mid-ocean ridge (Kitajima et al., 2001), implying ammonium-poor hydrothermal fluids with little influence from sedimentary organic matter on the seafloor. Even if methanogens had assimilated tiny amounts of ammonium, the $\delta^{15}N$ value of the resultant organic matter may have been lower than the estimated $\delta^{15}N$ value of the initial organic matter (-8 to -1‰). This is because the isotopic ratio of ammonium in the fluid inclusions is -10‰ (Nishizawa et al., 2007) and the assimilation of the ammonium causes further ^{15}N depletion in the resultant organic matter (< -10‰; Figure 4). We should note, however, that we could not entirely exclude the possibility of ammonium assimilation by subpopulations of the potential microbial community in the Dresser hydrothermal environments. Such a process may have caused a spread in $\delta^{15}N$ values of the initial organic matter toward -10‰. However, nitrogen isotopic ratios of the organic matter in the Dresser silica veins require both the presence of an initial organic matter whose $\delta^{15}N$ value is close to N_2 (-3 to +1‰) and the involvement of nitrogen fixation in organic synthesis. We thus indicate the possible emergence and function of diazotrophy by hyperthermophilic methanogens at 3.5 Ga.

Nitrogen fixation by ancient hyperthermophilic methanogens may have been conserved over the long history of organic evolution. This conserved ability may be relevant to the ecological significance of such methanogens in present-day oceanic hydrothermal systems. The ammonium concentrations in the high temperature (> 150 °C) fluids of typical basalt-hosted systems are low, with values at 15 μM or less (German and Von Damm, 2003; Bourbonnais et al., 2012a). This observation is consistent with the experimental results that suggest ammonium yields from abiotic N_2 reduction were low (≤ 2.5%) under hydrothermal conditions (at 120-500 °C and 27-1000 bars in the

presence of magnetite, iron sulphides, iron metal, or nickel metal) (Brandes et al., 1998; Schoonen and Xu, 2001; Smirnov et al., 2008). Thus, this substantial hydrothermal fluid chemistry has most likely been unchanging over the history of the Earth. The low ammonia concentrations in the fluids may have stimulated nitrogen fixation, as observed in methane seeps where ammonium concentrations are 30-200 μM (Miyazaki et al., 2009; Dekas et al., 2013). Additionally, it is uncertain whether substantial amounts of the nitrogen compounds originating from atmospheric chemistry were transported to the ancient deep-sea hydrothermal systems, where they could persistently support primary production by chemosynthetic microbial communities at a global scale. Nitric oxide, hydrogen cyanide, and their decomposition products (nitrate, nitrite, and ammonium) are the potential nitrogen products of the early atmosphere (Zahnle et al., 1986; Summers and Chang et al., 1993; Navarro-González et al., 2001; Summers and Khare, 2007). Based on the kinetics of the theoretical production/decomposition of ammonium and nitrite on the early Earth, Summers (1999) suggested that ammonium concentrations in the early ocean were approximately 2 μM under various temperature, pH and ferrous iron concentration conditions. In addition, the diazotrophic methanogens of Mc 1-85N and Mt 5-55N lack the metabolic ability to utilise nitrate, a product of nitric oxide, as the sole nitrogen source. In terms of the amount of elemental utilisation essential for the production of substantial biomass, nitrogen fixation should have evolved, at least for these types of diazotrophic methanogens and for any other microbial components of the ancient microbial ecosystems in the ocean, at 3.5 Ga or even earlier.

Acknowledgments

We thank R. Senda and K. Suzuki for experimental assistance. We also thank T. Shibuya, K. Kawagucci, K. Nakamura, J. Glass, and A. Kraepiel for fruitful discussion. Comments by T. McCollom and two anonymous reviewers greatly improved the manuscript. This research was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (22540499 to M. N.).

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Figure legends

Figure 1: Growth curves of a) *Methanocaldococcus* sp. kairei 1-85N (Mc 1-85N) at 85 °C and b) *Methanothermococcus* sp. kairei 5-55N (Mt 5-55N) at 55 °C. Solid arrows in Figure 1a show the points of depletion of H_2 in the culture bottle. Except for a negative control experiment for Mc 1-85N at Fe = 1 mM, Mo = 100 nM, Ar = 0.1 MPa, CO_2 = 0.1 MPa and H_2 = 0.2 MPa, the initial partial pressures of N_2, CO_2 and H_2 in the headspace were set at 0.1 MPa, 0.1 MPa and 0.2 MPa, respectively.

Figure 2: a) Relationship between the amounts of CH_4 and particulate nitrogen during cultivation of Mc 1-85N at 85 °C. Error bars for the amounts of CH_4 and particulate nitrogen are smaller than the symbol sizes, except for the three points shown. b) Relationship between amounts of CH_4 and particulate carbon during cultivation of Mc 1-85N at 85 °C. Error bars for the amounts of CH_4 and particulate nitrogen are smaller than the symbol size, except for the three points shown. The symbols are the same as in Figure 1.

Figure 3: Stable nitrogen isotopic ratios of a) particulate nitrogen during the diazotrophic growth of Mc 1-85N and Mt 5-55N at a varying concentration of Mo in the medium under the atmospheric pressure condition, and b) total nitrogen produced during the diazotrophic growth of Mc 1-85N at various partial pressures of H_2 and N_2 in the media. In each graph, the second Y-axis (right side) is shown on a scale corresponding to the isotope enrichment factor of nitrogen fixation.

Figure 4: Nitrogen isotopic fractionation during extracellular ammonium uptake by Mc 1-85N and Mt 5-55N grown at different ammonium concentrations. Except for the results at the high ammonium concentration (10 mM), each symbol in the figure represents the result from a cultivation experiment. The isotope enrichment factor is plotted against the range of ammonium concentrations during each experiment.

Figure 5: Nitrogen isotopic ratios of cellular nitrogen compounds of diazotrophs relative to the substrate N_2. Data were obtained for non-heterocystous cyanobacteria from Carpenter et al. (1997) and Bauersachs et al. (2009) (square: *Lyngbya* sp.; star: *Crocosphaera* sp.; diamond: *Cyanothece* sp.; inverted triangle: *Gloeothece* sp.; X: *Myxosarcina* sp.; filled circle: *Trichodesmium thiebautii*; open circle: *Trichodesmium* IMS101), for heterocystous cyanobacteria from Minagawa and Wada (1986), Macko et al. (1987), Beaumont et al. (2000), Zerkle et al. (2008) and Bauersachs et al. (2009) (square: *Anabaena variabilis*; star: *Anabaea cylindrica*; diamond: *Anabaena* sp. strain IF; inverted triangle: *Calothrix sp.*; X: *Nodularia* sp.; filled circle: *Nostoc* sp.) and for purple non-sulphur bacteria from Beaumont et al. (2000) (*Rhodobacter capsulatus*). Data for thermophilic and hyperthermophilic methanogens (average value ± standard deviation) are from this study.

Figure 6: Nitrogen isotopic ratios of a) various geological samples from different ages, and b) organic matter and fluid inclusions in the 3.5 Ga hydrothermal silica veins from the North Pole area in the Pilbara Craton, with the estimated range of nitrogen isotopic ratios of initial organic compounds at the time of deposition, and of cellular organic compounds produced by possible diazotrophic hyperthermophilic methanogens from hydrothermal fluid N_2 at that time. The isotopic range of the atmospheric N_2 during 3.5 Gyr is from Sano and Pillinger (1991) and Nishizawa et al. (2007). The nitrogen isotopic data of sedimentary rocks are from Calvert et al. (1996), Beaumont and Robert (1999), Jenkyns et al. (2001), Pinti et al. (2001), Levman and von Bitter (2002), Jia and Kerrich (2004), Kuypers et al. (2004), Ueno et al. (2004), Meyers and Bernasconi (2005), Nishizawa et al. (2005), Papineau et al. (2005), Ohkouchi et al.(2006), Junium and Arthur (2007), Garvin et al. (2009), Godfrey and Falkowski (2009), Papineau et al. (2009), Pinti et al. (2009) and Thomazo et al. (2011). In b), the nitrogen isotopic ratio data for organic matter and N_2 preserved in the 3.5 Ga hydrothermal silica veins are from Ueno et al., (2004) and Nishizawa et al., (2007).

Figure A1: a) A schematic illustration of nitrogen fixation and intracellular ammonia assimilation. b) A schematic illustration of postulated N_2 binding and reduction to NH_3 at an Fe site in the FeMo-cofactor of nitrogenase by limiting alternating (top) and distal (bottom) mechanisms proposed by Seefeldt et al. (2009) and Chatt et al. (1978), respectively.

Table(s)

Table 1: Growth characteristics of hyperthermophilic and thermophilic methanogens.

Fe (nM)	Mo (nM)	NH_4^+ (μM)	NO_3^- (μM)	N source(s)	μ (h^{-1})[a]	n[b]	Maximum cell yield (cell/ml)[c]
***Methanocaldococcus* sp. kairei 1-85N; T = 85 °C (This study)**							
$H_2/CO_2/N_2$ (50/25/25 mol%; 400 kPa)							
25	100	<10	<5	N_2	–	1	NG
100	5	<10	<5	N_2	0.31±0.07	3	+
100	100	<10	<5	N_2	0.27±0.01	4	+
100,000	1,000	<10	<5	N_2	0.32±0.03	4	++
100,000	100,000	<10	<5	N_2	0.23±0.01	7	+++
1,000,000	15	<10	<5	N_2	0.29±0.05	3	+
1,000,000	100	<10	<5	N_2	0.26±0.01	4	+
1,000,000	1,000	<10	<5	N_2	0.29±0.01	3	++
1,000,000	10,000	<10	<5	N_2	0.28±0.02	7	++
1,000,000	1,000,000	<10	<5	N_2	0.32±0.09	3	++
10,000,000	10,000	<10	<5	N_2	0.17±0.02	4	++
10,000,000	100,000	<10	<5	N_2	0.31±0.02	3	++
10,000,000	1,000,000	<10	<5	N_2	0.12±0.01	5	++
$H_2/CO_2/Ar$ (50/25/25 mol%; 400 kPa)							
1,000,000	100	<10	<5	None	–	1	NG
100,000	1,000	10,000	<5	NH_4^+	1.5±0.05	3	+++
100,000	1,000	<10	1000	NO_3^-	–	2	NG
H_2/CO_2 (66/33 mol%; 300 kPa)							
100,000	5	1,000	<5	NH_4^+	0.81±0.12	6	++
100,000	1,000	10	100	NH_4^+, NO_3^-	–	2	<1 x 10^6
100,000	1,000	<10	100	NO_3^-	–	1	NG
***Methanothermococcus* sp. kairei 5-55N; T = 55 °C (This study)**							
$H_2/CO_2/N_2$ (50/25/25 mol%; 400 kPa)							
100	100	<10	<5	N_2	–	2	NG
1,000	1,000	<10	<5	N_2	–	2	NG
10,000	1,000	<10	<5	N_2	0.25±0.01	4	++
10,000	10,000	<10	<5	N_2	0.26±0.01	3	++
100,000	15	<10	<5	N_2	–	2	NG
100,000	100	<10	<5	N_2	–	2	NG
100,000	1,000	<10	<5	N_2	0.29±0.05	4	++
100,000	10,000	<10	<5	N_2	0.29±0.02	4	++
100,000	100,000	<10	<5	N_2	–	2	NG
1,000,000	100	<10	<5	N_2	–	2	NG
1,000,000	10,000	<10	<5	N_2	–	2	NG
100,000	5	10,000	<5	NH_4^+, N_2	–	2	++
100,000	1,000	10,000	<5	NH_4^+, N_2	0.51±0.02	4	++
1,000,000	100	10,000	<5	NH_4^+, N_2	–	2	NG
$H_2/CO_2/Ar$ (50/25/25 mol%; 400 kPa)							
100,000	1,000	<0.01	1000	NO_3^-	–	1	NG
H_2/CO_2 (66/33 mol%; 300 kPa)							
100,000	1,000	100	100	NH_4^+, NO_3^-	–	2	+
100,000	1,000	10	100	NH_4^+, NO_3^-	–	2	NG
100,000	5	1,000	<5	NH_4^+	–	2	++
***Methanocaldococcus* FS406-22; T = 90 °C (Mehta and Baross, 2006)**							
5,500,000	4,800	–	–	N_2	0.22	3	
5,500,000	4,800	13,000	14,000	NH_4^+, NO_3^-	0.37	3	
***Methanocaldococcus jannaschii*; T = 90°C (Jones et al., 1983)**							
10,000	410	5,000	not added	NH_4^+	1.5	3	

a: Cell-based growth rate in exponential phase (mean ± standard deviation). -: Not measured.

b: Number of replicate determinations of the cell-based growth rate.

c: +++: (1-5) x 10^8 cell/ml, ++: (1-10) x 10^7 cell/ml, +: (1-10) x 10^6 cell/ml, NG: No growth.

Table 2: Nitrogen uptake rates of diazotrophic microorganisms in exponential phase.

	Methanogen		**Cyanobacteria**		**Soil bacteria**
Strain	1	2	3	4	5
Nitrogen source	N_2	N_2	N_2	N_2	N_2
T [^oC]	85	55	28	28	22
approximate cell volume [μm^3] [a]	0.5-4	0.5-4	8-110	280-3800	0.5-4
μ [h^{-1}] (cell-based) [b]	0.27 ± 0.04	0.25 ± 0.02	0.01-0.02	0.02	0.25[h]
Q_N [fmolN/cell] [c]	6-24	18-22	7-30	529	17[h]
ρN [10^{-17}molN/cell/min] [d]	2-11	8	0.2-1[g]	19[g]	7[h]
$\rho N'$ [$10^{-17}\mu m^3$ cell volume/min] [e]	0.5-21	2-15	0.002-0.1	0.005-0.07	2-13
N [f]	7	2	4	1	1

Strain. 1: *Methanocaldococcus* sp. kairei 1-85N (This study); 2: *Methanothermococcus* sp. kairei 5-55N (This study); 3: *Crocosphaera watosonii* strain WH8501 (Tuit et al., 2004); 4: *Trichodesmium erythraeum* (Tuit et al., 2004); 5: *Azotobacter vinelandii* (Bellenger et al., 2011).

a. cell size of strain 1: 1-2 µm in diameter, 2: 1-2 µm in diameter, 3: 2.5-6 µm in diameter, 4: 6-22 µm wide × 10 µm long, 5: 1-2 µm in diameter; **b**. Cell-based growth rate in exponential phase (mean ± standard deviation); **c**. Cellular nitrogen content; **d**. Cell-based nitrogen uptake rate in exponential phase; **e**. Cell-based nitrogen uptake rate per unit cell volume calculated from ρN and approximate cell volume; **f**. Number of replicate determinations of μ; **g**. The rate is estimated from daily-averaged cell-specific N assimilation rate; **h**. Maximum rate.

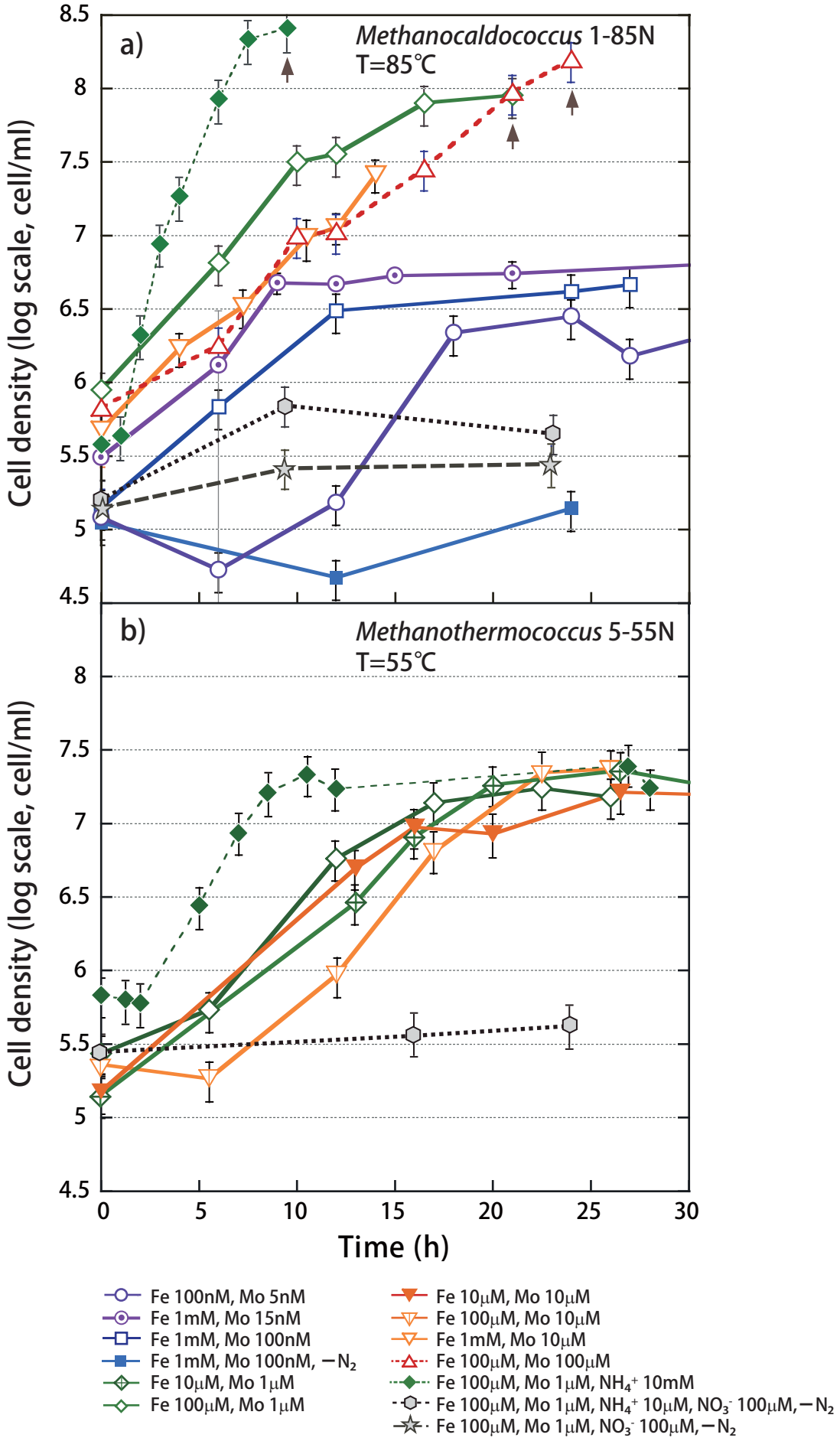
Table 3: Time course of concentrations and isotope ratios of particulate nitrogen compounds (PN) and total nitrogen (TN) during diazotrophic growth of methanogens.

Time (h)	Cell density (cell/ml)	TN [a] (μg N/ml)	PN (μg N/ml)	$\delta^{15}N$ (TN) [a] (‰)	$\delta^{15}N$ (TN pro.) [b] (‰)	$\delta^{15}N$ (PN) (‰)
***Methanocaldococcus* sp. kairei 1-85N**						
T = 85 °C, Fe = 100,000 nM, Mo = 1000 nM, $H_2/CO_2/N_2$ (50/25/25; 400 kPa)						
0	8.9×10^5	–	–	–	–	–
6	6.6×10^6	0.6	–	1.9	–	–
10	3.2×10^7	2.3 ± 0.3	2.4	–4.1 ± 0.4	–6.5 ± 0.7 (6 ~ 10 h)	–4.4
12	3.6×10^7	3.8 ± 0.5	3.7	–4.0 ± 0.7	–3.7 ± 0.2 (10 ~ 12 h)	–4.2
16.5	8.0×10^7	11.3 ± 1.1	9.5	–4.3 ± 0.5	–4.5 ± 0.1 (12 ~ 16.5 h)	–4.3
21	9.1×10^7	–	8.1	–	–	–4.3
***Methanothermococcus* sp. kairei 5-55N**						
T = 55 °C, Fe = 10,000 nM, Mo = 1000 nM, $H_2/CO_2/N_2$ (50/25/25; 400 kPa)						
0	1.4×10^5	–	–	–	–	–
13	2.9×10^6	0.8	–	–4.5	–	–
16	8.2×10^6	2.1	2.5	–3.8	–3.4 ± 0.1 (13 ~ 16 h)	–3.7
26.5	2.3×10^7	4.7	4.3	–4.0	–4.2 ± 0.1 (16 ~ 26.5 h)	–3.4
30.5	1.8×10^7	–	4.1	–	–	–3.4
48	3.2×10^7	–	4.7	–	–	–3.1

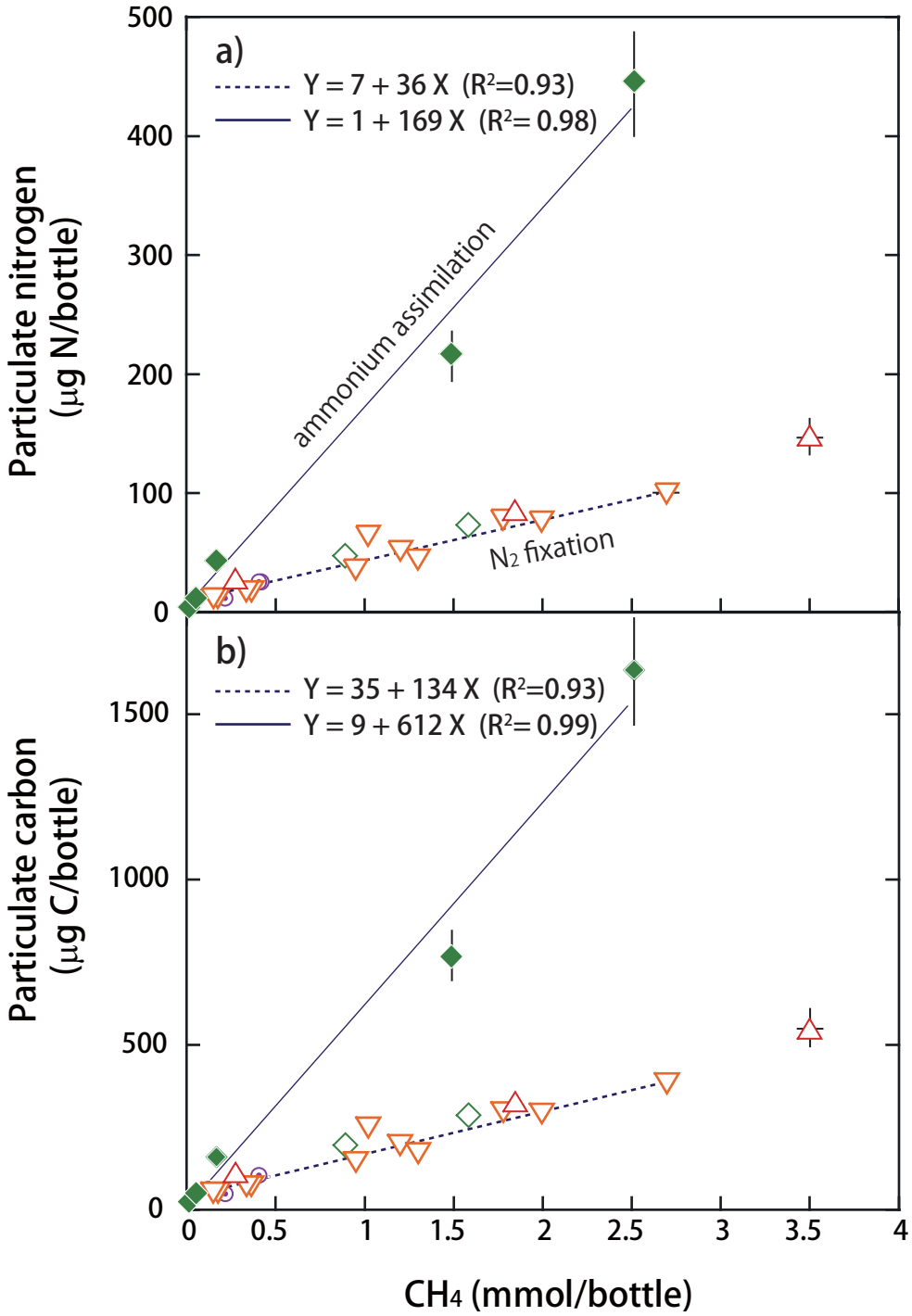
$\delta^{15}N$ (TN), $\delta^{15}N$ (TN pro.), and $\delta^{15}N$ (PN) values are expressed relative to N_2 substrate in cultivation bottle, respectively.

a. Uncertainty shows standard deviation of duplicate or triplicate measurements; **b**. $\delta^{15}N$ (TN pro.) denotes isotopic ratio of TN produced during growth, and is estimated by following equation: $\delta^{15}N$ (TN pro.) = ([TN] * $\delta^{15}N$ (TN) – [TN]'* $\delta^{15}N$ (TN)')/ ([TN] – [TN]'). Symbols [TN] and [TN]' denote concentrations of TN at T and T' hours after cultivation starts (T > T').

Figure(s)



Nishizawa et al., Fig. 1



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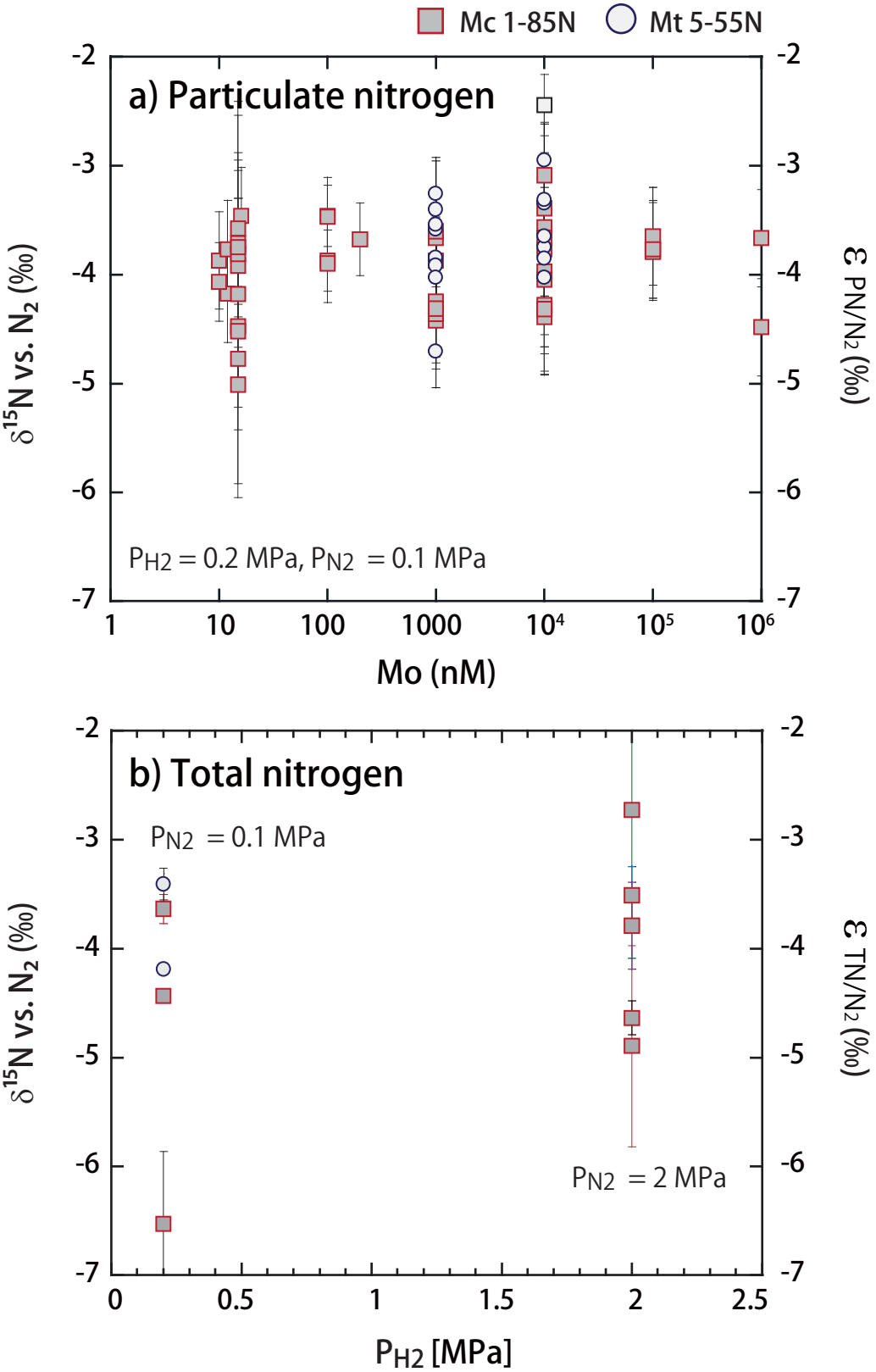

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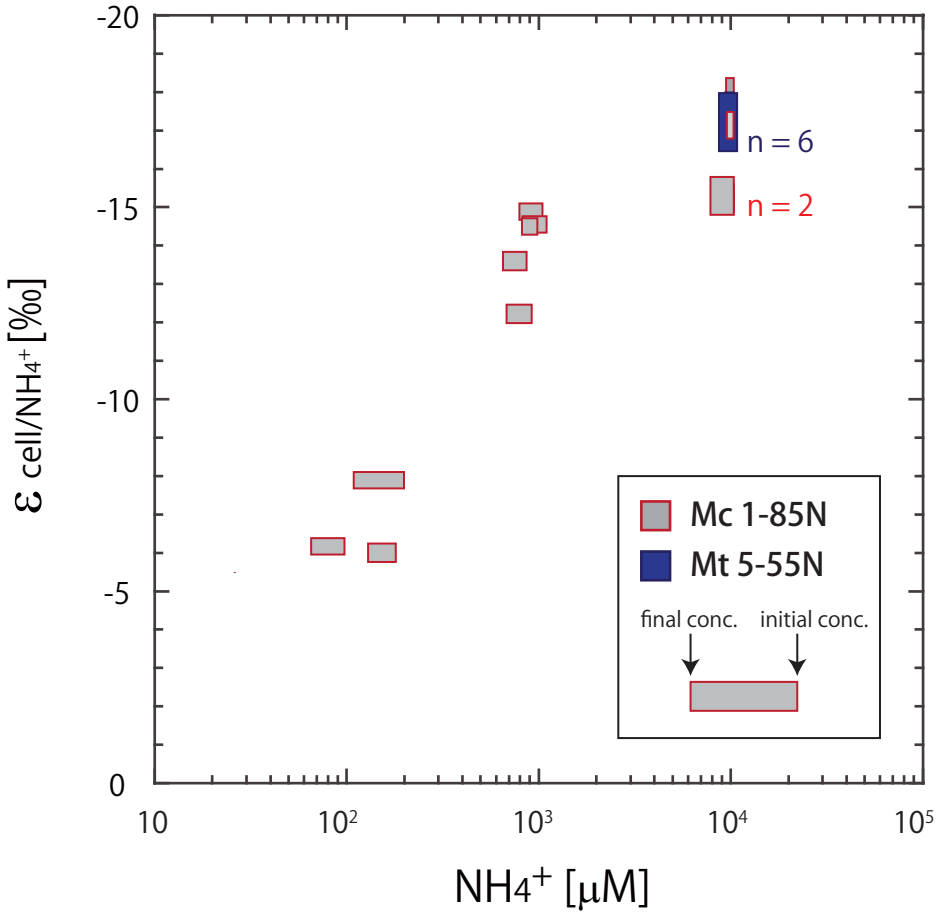

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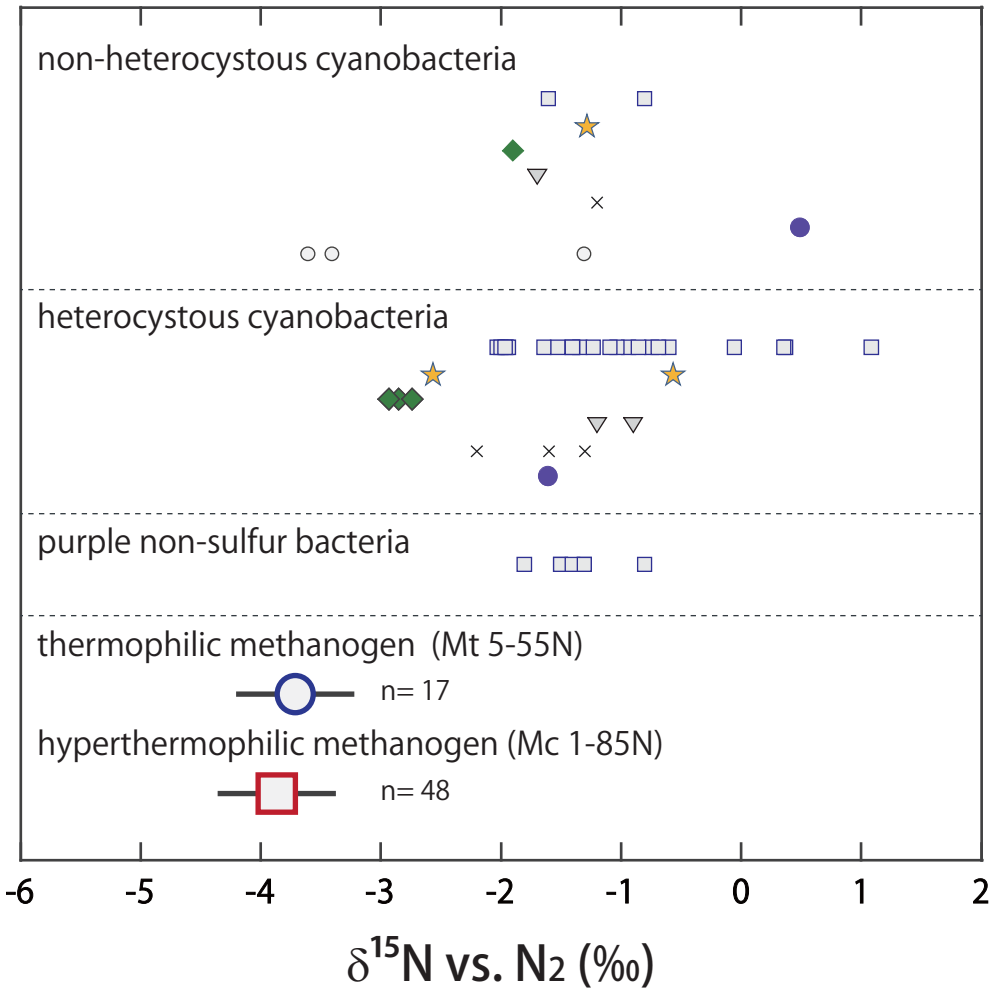

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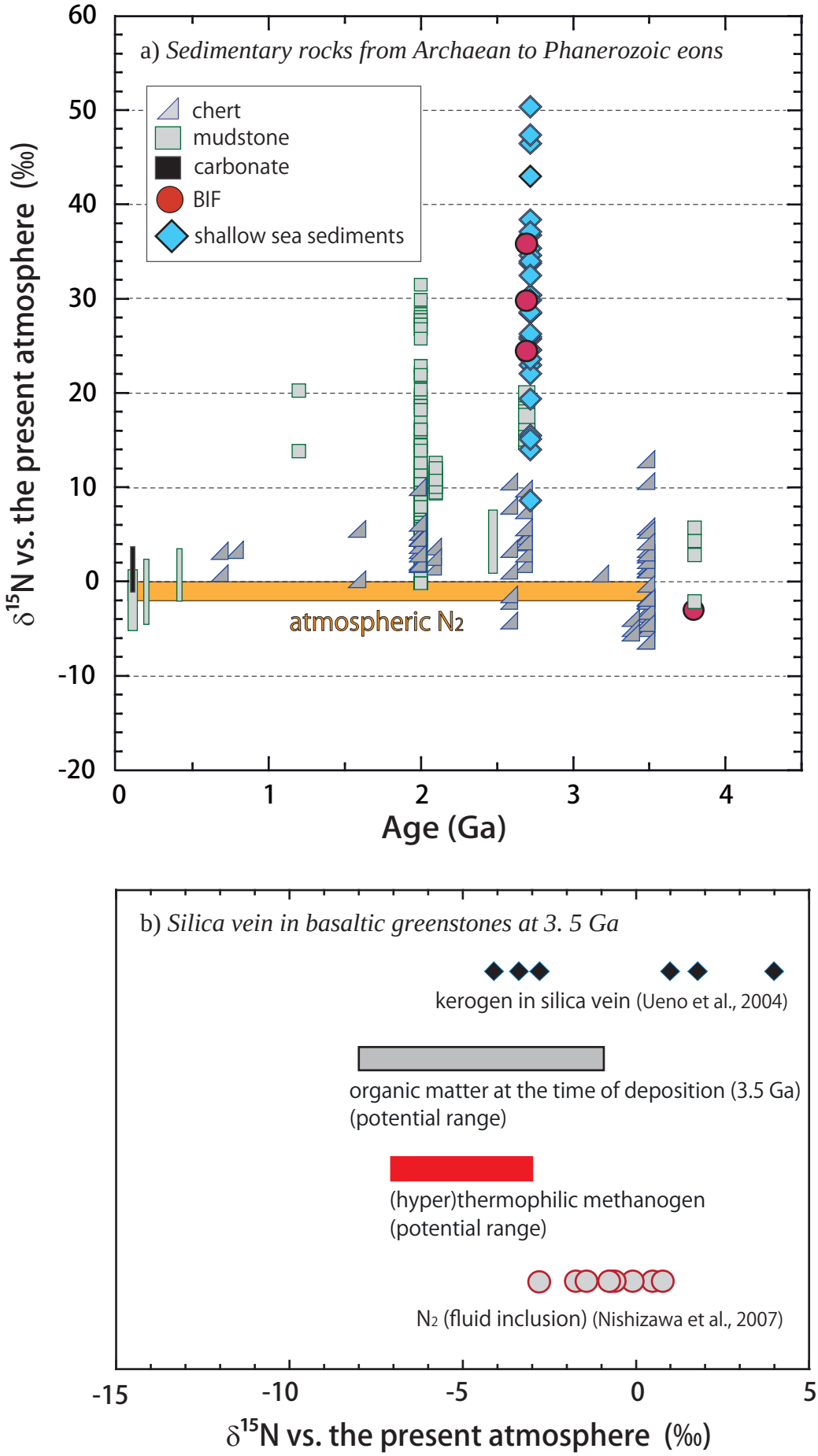
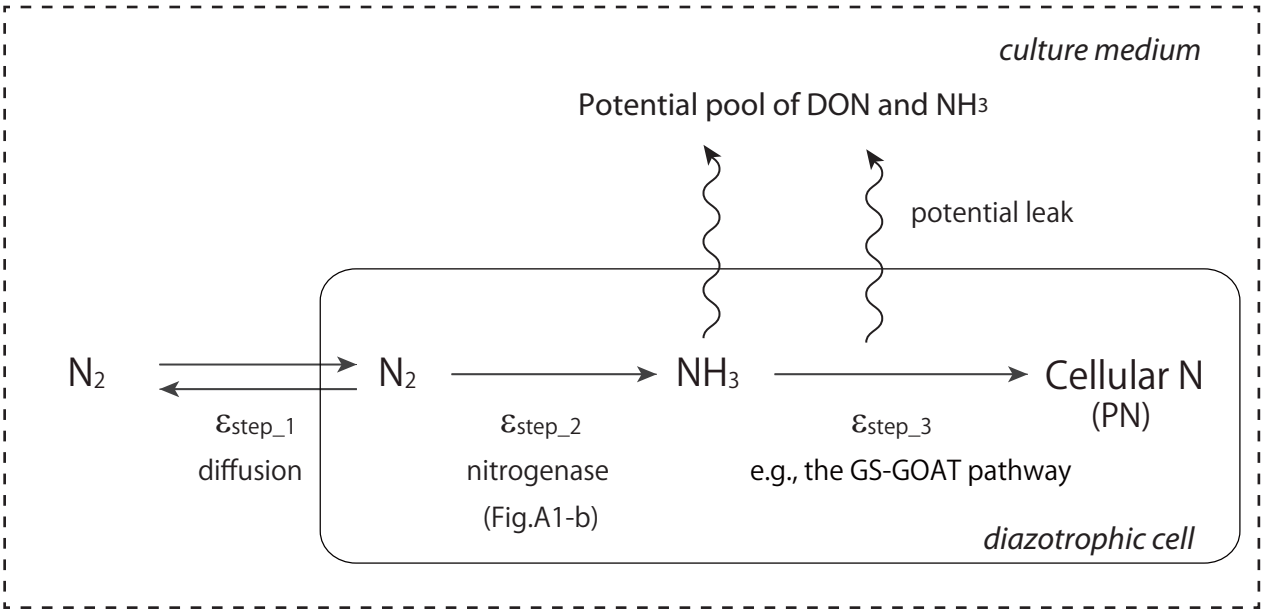


Nishizawa et al., Fig. 6

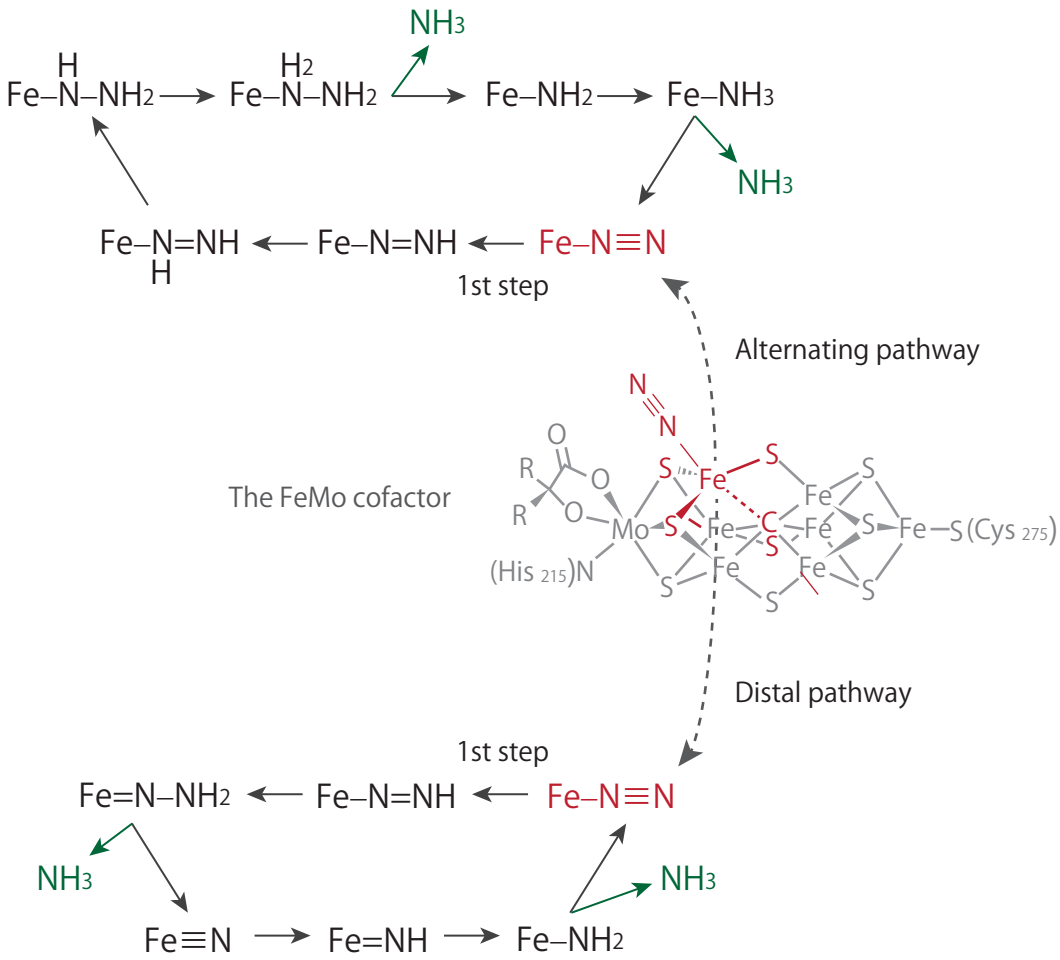
a) N_2 fixation and intracellular ammonia assimilation



Isotopic measurements

Particlulate Nitrogen (PN) = Cellular N, Total Nitrogen (TN) = cellular N+DON+NH_3

b) N_2 binding and reduction to NH_3 at an Fe site in the FeMo cofactor of nitrogenase



Nishizawa et al., Fig. A1