

Cell proliferation at 122°C and isotopically heavy CH₄ production by a hyperthermophilic methanogen under high-pressure cultivation

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We have developed a technique for cultivation of chemolithoautotrophs under high hydrostatic pressures that is successfully applicable to various types of deep-sea chemolithoautotrophs, including methanogens. It is based on a glass-syringe-sealing liquid medium and gas mixture used in conjunction with a butyl rubber piston and a metallic needle stuck into butyl rubber. By using this technique, growth, survival, and methane production of a newly isolated, hyperthermophilic methanogen *Methanopyrus kandleri* strain 116 are characterized under high temperatures and hydrostatic pressures. Elevated hydrostatic pressures extend the temperature maximum for possible cell proliferation from 116°C at 0.4 MPa to 122°C at 20 MPa, providing the potential for growth even at 122°C under an *in situ* high pressure. In addition, piezophilic growth significantly affected stable carbon isotope fractionation of methanogenesis from CO₂. Under conventional growth conditions, the isotope fractionation of methanogenesis by *M. kandleri* strain 116 was similar to values (−34‰ to −27‰) previously reported for other hydrogenotrophic methanogens. However, under high hydrostatic pressures, the isotope fractionation effect became much smaller (< −12‰), and the kinetic isotope effect at 122°C and 40 MPa was −9.4‰, which is one of the smallest effects ever reported. This observation will shed light on the sources and production mechanisms of deep-sea methane.

carbon isotope fractionation | deep-sea hydrothermal vent | hyperthermophile | methanogenesis | piezophilic

Microbial methanogenesis in the deep sea is a key process in the carbon cycle of Earth. It contributes to the CH₄ pool (free gas and methane hydrate), a potential energy source and alternative to petroleum (1, 2) as well as a strong greenhouse gas with a potential for rapid release (3), in deep-sea and subseafloor sediments. Methanogens are known to have several methanogenic types using different substrates of H₂, acetate, methanol, CO, and so on. Hyperthermophilic hydrogenotrophic methanogens play a major role in primary production of ecosystems in deep-sea hydrothermal areas in the present Earth (4, 5) and may represent the most ancient type of microorganisms flourishing in the Archean Earth (6–10).

Despite the significance of methanogens in the deep-sea and subseafloor ecosystems, the ecophysiological and biogeochemical characteristics of their *in situ* habitats have been little understood. It has been quite difficult to incorporate high hydrostatic pressures into experiments involving gaseous substrates such as H₂ and CO₂. If this difficulty can be overcome by any specific apparatus (11, 12), the subsequent handling of microbiological experiments under high hydrostatic pressures remains a great technical barrier. Thus, growth characterization of only thermophilic methanogens *Methanocaldococcus jannaschii* and *Methanothermococcus thermolithotrophicus* under high pressures has been successfully achieved, and only their piezophilic responses of growth and methane production have

been investigated (13, 14). Other than these studies, investigation of methanogens and other chemolithoautotrophs under high hydrostatic pressures has been not conducted.

Here, we develop a microbiological cultivation and incubation technique in gas-rich fluid under a high hydrostatic pressure corresponding to deep-seafloor and subseafloor habitats. The capability of this technique is tested for several indigenous deep-sea chemolithoautotrophs, including a member of the genus *Methanopyrus*, a hyperthermophilic methanogen that had been the most hyperthermophilic microorganism on Earth, growing in temperatures up to 110°C (15, 16), until *Pyrolobus fumarii* (17) and the hyperthermophilic archaeon strain 121 (18) renewed the record of upper temperature limit for life. As previously reported for *M. jannaschii* (13) and other hyperthermophilic heterotrophs (19, 20), the piezophilic response to the elevated hydrostatic pressure can strongly affect the growth physiology, such as temperature ranges of growth and cellular metabolic and biochemical functions. In this study, the growth, survival, and methane production of *Methanopyrus kandleri* strain 116, a newly isolated *Methanopyrus* strain from a deep-sea hydrothermal habitat in the Kairei hydrothermal field in the Central Indian Ridge (CIR Kairei field), are characterized under high hydrostatic pressures equivalent to its potential *in situ* habitats.

Results

Development of a Cultivation Technique Under High Hydrostatic Pressures. An easy handling cultivation technique was established [supporting information (SI) Figs. S1 and S2]. After 3- to 4-day incubations, all of the tested chemolithoautotrophs were successfully grown in the piezophilic cultivation syringes with final cell yields of a level similar to that of those grown under the optimum condition without hydrostatic pressure. Thus, our technique would be applicable to deep-sea and deep-subseafloor chemolithoautotrophs with any type of metabolism.

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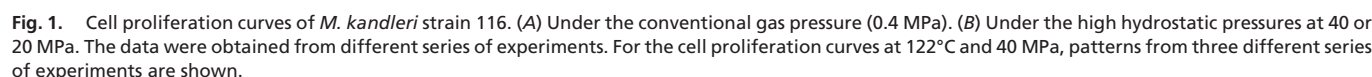
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Data deposition: *Methanopyrus kandleri* strain 116 was deposited in the Japan Collection of Microorganisms (deposition no. 15049). The 16S rRNA gene sequence of *Methanopyrus kandleri* strain 116 was deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases (accession no. AB301476).

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Growth Characteristics of *M. kandleri* Strain 116 Under a High Hydrostatic Pressure. The effect of hydrostatic pressure on the growth of *M. kandleri* strain 116 was characterized by using the piezo-

Survival Capability of *M. kandleri* Strain116 Above the Growth Temperature Range. The piezophilic ability of *M. kandleri* strain 116 to survive under high temperatures was examined by using the piezophilic cultivation syringe (Fig. 2). Under a gas pressure of 0.4 MPa, a 10^8 -cell population of *M. kandleri* strain 116 completely lost viability after 8 h at 121°C and ≈ 2 h at 130°C (Fig. 2). However, under the elevated hydrostatic pressure of 30 MPa, the same population remained viable for >24 h at 121°C and 3 h at 130°C (Fig. 2). The thermal death of strain 116 at 121°C and 30 MPa appeared to contradict the potential growth at 122°C and

carbon fixation, respectively (Fig. 3A). However, under high hydrostatic pressures, the isotope fractionation effect of methanogenesis became much smaller ($<-12\text{‰}$) than the values under the conventional growth conditions (Fig. 3A). In contrast, the isotope fractionation of carbon fixation under the same condition was estimated to be -14‰ to -16‰ (Fig. 3A). The elevated hydrostatic pressure did not significantly affect the isotope fractionation of carbon fixation but did have a strong impact on the isotope fractionation of methanogenesis.

The effect of hydrostatic pressure on isotope fractionation of methanogenesis was further characterized (Fig. 3B). With an increasing hydrostatic pressure, the isotope fractionation became smaller (Fig. 3B). The average of isotope fractionation values at each hydrostatic pressure was reciprocally correlated with the hydrostatic pressure (Fig. 3B). The isotopic fractionation of methanogenesis by *M. kandleri* strain 116 was correlated with the H_2 concentration dissolved in the medium fluid (Fig. S7). Finally, the isotope fractionation of methanogenesis was also highly correlated with the Gibbs free-energy change (ΔG) estimated from the culture conditions (Fig. 3C and Table S3).

Discussion

The development of high-pressure cultivation methods can be traced back to the pioneering work on deep-sea microorganisms by Zobell and Morita (21). For 50 years, the only deep-sea chemolithoautotrophs cultivated at high temperature and hyperbaric-pressure were the thermophilic hydrogenotrophic methanogens *M. jannaschii* (13) and *M. thermolithotrophicus* (14). Under the high pressures equivalent to their deep-sea habitats, both the optimum and maximum temperatures for growth and CH_4 production were significantly elevated (13). Because of their easier manipulation, all of the other deep-sea microorganisms investigated under high pressures so far have been heterotrophs, and similar phenomena have been observed for other hyperthermophilic heterotrophs (19, 20). However, the complex design of the high-temperature, high-pressure bioreactors is a major obstacle for various types of ecophysiological experiments, and the construction of a less laborious cultivation and incubation system would greatly facilitate such studies.

In this study, a *M. kandleri* strain 116 was isolated from a deep-sea hydrothermal field in the Central Indian Ridge (5). The deep-sea hydrothermal field is located at water depths of 2,420–2,450 m, and the potential indigenous habitats of the strain are expected in the ambient depths of seafloor or the deeper subseafloor. The growth experiments using our technique clearly demonstrated that the isolate was piezophilic. The elevated hydrostatic pressure (40 or 20 MPa) shifted the potential growth temperature range of *M. kandleri* strain 116 $\approx 5^\circ\text{C}$ higher than that in the nonpiezophilic condition; the increased temperature range enabled several rounds of cell division and concomitant methane production of *M. kandleri* strain 116 at up to 122°C (Figs. 1 and 2, Fig. S6, and Table S2). Because the upper growth temperature limit could strongly depend on the culture conditions as observed in this study, the comparison of the highest temperature record among the microorganisms grown under different conditions may not be substantial. However, it may also be important to document that the growth of *M. kandleri* strain 116 at 122°C under high pressures is the highest ever recorded, which would establish a basis of understanding the limit of life and biosphere on this planet.

The elevated hydrostatic pressure affected not only the growth temperature range but also the survival capability during exposure to higher temperatures than the growth range of *M. kandleri* strain 116. Before the isolation of a hyperthermophile strain 121 (18), *P. fumarii* had the highest survival capability at high temperatures; it could revive after incubation at 121°C for 1 h (17). This result was especially striking because most mesophiles are killed by autoclaving at 121°C for 20 min. Not only does strain

121 grow at 121°C , it survives exposure to 130°C for 2 h (18). At low pressures, *M. kandleri* strain 116 also survived exposure to 130°C for 2 h (Fig. 2). However, at 30 MPa, strain 116 survived after 3 h (Fig. 2). Thus, under high hydrostatic pressures, *M. kandleri* strain 116 was also extremely thermotolerant.

The stable carbon isotope fractionation of CH_4 produced by methanogens is an important indicator of microbial activity on the modern earth and a chemical fossil of microbial activity on ancient Earth. The compositional and isotopic classification (22) is used for identifying the sources of gaseous hydrocarbons found in oil and gas wells and subsurface methane hydrate deposits, even though the responsible microorganisms have yet to be identified in many cases (23). Isotopically “light” CH_4 has been proposed to be an indicator of ancient microbial production in deep-sea hydrothermal systems in the early Earth, ≈ 3.5 Ga (7). Recently, McCollom and Seewald (24) demonstrated that the isotopically light CH_4 is produced by an abiotic Fisher–Tropsch-type reaction under the high temperatures and pressures common in hydrothermal systems. This result suggests that isotopically light CH_4 may be not a reliable indicator of microbial methanogenesis in deep-sea hydrothermal environments (25). However, the isotopic fractionation of microbiologically produced CH_4 under high hydrostatic pressures has been never investigated, so it is also possible that the isotopically light CH_4 may not be produced by methanogens in the deep-sea hydrothermal environments.

Using our technique, we examined the stable carbon isotopic fractionation of methanogenesis and carbon fixation under high hydrostatic pressures. The isotope fractionation of methanogenesis and carbon fixation by *M. kandleri* strain 116 under conventional growth conditions were $\approx -27\text{‰}$ to -34‰ for the methanogenesis and -19 to -23‰ for the carbon fixation, respectively (Fig. 3A). These values were very similar to those previously reported for *M. kandleri* strain AV19 and many other hydrogenotrophic methanogens (26, 27). However, under high hydrostatic pressures, the isotope fractionation effect of methanogenesis became much smaller ($<-12\text{‰}$) than the values under the conventional growth conditions (Fig. 3A). The kinetic isotope effect for methanogenesis of *M. kandleri* strain 116 at 122°C and 40 MPa was -9.4‰ , which is one of the smallest values ever reported. Thus, the small isotope fractionation may have generated isotopically “heavy” CH_4 in the deep-sea hydrothermal environments that could have been misclassified as a magmatic source (25).

The reduced isotope fractionation of methanogenesis under high hydrostatic pressures might be associated with the increased concentration of dissolved H_2 resulting from the elevated hydrostatic pressure. The isotope fractionation of methanogenesis was correlated with the concentration of dissolved H_2 in the medium (Fig. S7). A similar relationship between H_2 concentration and the carbon isotope fractionation has been demonstrated in *Methanothermobacter marburgensis* (28, 29). Valentine *et al.* (28) suggested that “differential reversibility” of the hydrogenotrophic methanogenesis pathway would have an impact on the kinetic isotope effect of the whole pathway and that the differential reversibility might be strongly associated with H_2 -concentration-dependent ΔG . Penning *et al.* (29) indeed showed the strong correlation between the carbon isotope fractionation and the ΔG of hydrogenotrophic methanogenesis based on pure and coculture experiments of several methanogens. In this study, the isotope fractionation of methanogenesis by *M. kandleri* strain 116 was also highly correlated with the ΔG estimated from the culture conditions (Fig. 3C and Table S3). Because the soluble H_2 concentration in the liquid phase was the most significant parameter to control the ΔG of hydrogenotrophic methanogenesis under different growth conditions, the correlation may be explained not by the thermodynamic constraints but by the kinetic effect of the elevated H_2 concentration.

However, the correlation between the isotopic fractionation and the ΔG of methanogenesis could provide an important insight: the environmental energy status yielding the large ΔG in natural deep-sea microbial habitats might be associated with isotopically heavy biogenic CH_4 .

M. kandleri strain 116 represents a member of the dominant hyperthermophilic methanogen population in the subseafloor microbial ecosystem in the CIR Kairei field (5). Based on the physical properties and the compositional and isotopic data of H_2 , CO_2 , and CH_4 in the two types of hydrothermal fluids, the potential isotope fractionation of CH_4 production by the subseafloor methanogen population was estimated to be $\approx -16\text{‰}$ (5). Because not only *Methanopyrales* but also *Methanococcales* members could be the predominant methanogens in the subseafloor ecosystem of the CIR Kairei field (5), the patterns in isotopic fractionation of methanogenesis between the different groups of methanogens should be clarified to estimate the *in situ* isotope fractionation of CH_4 production in natural microbial communities. Future investigation of other deep-sea methanogens will provide a great insight into the sources and production mechanisms of CH_4 in the CIR Kairei field and even in the global deep seafloor and subseafloor environments.

Materials and Methods

Design and Establishment of Piezophilic Cultivation Technique. A batch fluid cultivation system under a high hydrostatic pressure was designed by using a combination of syringe and piston (Fig. S1). An apparatus made of a glass syringe (Hamilton), a hand-made butyl rubber piston, a stainless steel needle (27 G), and a butyl rubber stopper was prepared (Fig. S1). All of the butyl rubber parts were repeatedly autoclaved (121°C and 15 min) with distilled water, 1 M HCl, and 1 M NaOH solutions in advance to minimize contamination of nonbiological products such as CH_4 by the butyl rubber during the experiments. The cultivation syringe was pressurized in the distilled- and deionized-water filling in a titanium hydrostatic pressure vessel (Teramex), and then the vessel was incubated at a given temperature up to 110°C in a temperature-controlled dry oven (Fig. S1). If the incubation temperature was over 110°C, a stainless steel, high-temperature, high-pressure reactor (up to 500°C and 60 MPa) (Nitto Ko-atsu) was used instead of a small titanium pressure vessel.

To test for gas leaking from the cultivation syringe and preservation of reductive fluid, 1 ml of synthetic seawater (MP basal medium as described in *SI Methods* in *SI Text*) containing 0.05% (wt/vol) sodium sulfide and 0.5 mg liter⁻¹ of resazurin saturated with 100% Ar and 3 ml of a gas mixture of H_2 (80%) plus CO_2 (20%), H_2 (70%) plus CO_2 (20%) plus CH_4 (10%), or H_2 (40%) plus CO_2 (20%) plus CH_4 (40%) were placed in the cultivation syringe and were incubated at 50 MPa for 12, 24, and 48 h at temperatures from 4°C to 122°C. After the incubation, the synthetic seawater was not colored by resazurin, the gas components were extracted, and the concentration was measured by methods described below. In most cases, >80% of the initial concentrations of the gas species were preserved in the fluid of the cultivation syringe even after 48 h at 122°C, but H_2 and CH_4 were relatively escapable from the syringe with increasing time of incubation at higher temperatures.

Cultivation of Various Chemolithoautotrophs Under High Hydrostatic Pressures. Representative deep-sea chemolithoautotrophs were cultivated under high hydrostatic pressures by using the piezophilic cultivation syringe system. Previously described deep-sea chemolithoautotrophs of *Sulfurovum lithotrophicus* (30), *Hydrogenimonas thermophila* (31), and *Methanoterris formicicum* (32) were chosen. In addition, mesophilic and extremely thermophilic chemolithoautotrophic sulfate-reducing bacteria (*Desulfovibrio* sp. and *Desulfatator* sp.) that had been isolated by our research group from deep-sea hydrothermal vent environments in the Mid-Atlantic Ridge (water depth of $\approx 2,400$ m) and the Central Indian Ridge (water depth of $\approx 2,400$ m) were tested (see *SI Methods* in *SI Text*).

Isolation and Characterization of a Hyperthermophilic Methanogen Strain 116. Methods for isolation and characterization of a hyperthermophilic hydrogenotrophic methanogen strain 116 were described in *SI Methods* in *SI Text*.

Growth Characteristics Under Nonpiezophilic and Piezophilic Conditions. All of the growth experiments of strain 116 under nonpiezophilic and piezophilic conditions were performed with a 120-ml serum bottle and a 5-ml cultivation

syringe, respectively. The experiments for growth characteristics other than the temperature–pressure effects on growth, survival, and methane production were conducted as described in *SI Methods* in *SI Text*.

The effect of temperature on the growth of strain 116 was examined with MPFe medium under both nonpiezophilic (0.4 MPa) and piezophilic (40 MPa) conditions. In the piezophilic growth experiments, 1 ml of liquid medium with the cell inoculum and 3 ml of 100% H_2 were included in the cultivation syringe. Growth was monitored by direct cell counting by using DAPI and methane production in the medium. Under the conventional gas-pressure condition, multiples of cultures (10–14 serum bottles) were simultaneously incubated, and duplicate cultures were periodically sampled during the incubation. The cell numbers of duplicate cultures in the serum bottles were determined by using 1 ml of subsampled aliquots, and the rest of the culture fluids in the bottles were applied to the gas extraction (*SI Methods* in *SI Text*). In the piezophilic experiments, multiples of cultures in the piezophilic cultivation syringes (four separate cultivation syringes in a pressure vessel and a total of 16–24 separate cultivation syringes in 4–6 pressure vessels) were prepared and incubated. Four cultivation syringes were taken from a decompressed pressure vessel at a certain period of incubation, and 0.1 ml of the culture medium in the culture syringe was extracted by using a disposable syringe and needle (27 G) through the butyl rubber piston. The extracted culture fluids were used for direct cell-counting using DAPI. In some series of experiments, the rest of culture fluid in the cultivation syringe was applied to the gas extraction (*SI Methods* in *SI Text*). The production of nonbiological CH_4 during the incubation was checked by using the parallel medium bottles and syringes without the cell inoculum. The detectable CH_4 production was observed only when accompanied by the cellular proliferation. The reproducibility of growth was checked with at least two different series of experiments, and the temperature of the medium or the pressure vessels was monitored by self-recording a platinum thermoresistance probe or a platinum thermocouple probe during the incubation. In the case of the experiment at 122°C, two hydrostatic pressures (20 and 40 MPa) were tested.

The effect of hydrostatic pressure on growth of strain 116 was examined with MPFe medium at 105°C under hydrostatic pressures of 2, 10, 20, 30, 40, and 50 MPa by using the cultivation syringe system. In addition, the effect of H_2 concentration on the growth of strain 116 was also characterized with MPFe medium at 100°C under the gas mixtures of H_2 (80%) plus CO_2 (20%) (3 atm), H_2 (24%) plus N_2 (56%) plus CO_2 (20%) (3 atm), H_2 (7.2%) plus N_2 (72.8%) plus CO_2 (20%) (3 atm), and H_2 (2.2%) plus N_2 (77.8%) plus CO_2 (20%) (3 atm). Based on the solubility of H_2 in water (33), the H_2 concentration dissolved in the liquid medium was assumed to be 2.27, 0.68, 0.2, and 0.06 mM, respectively. In a similar manner, the H_2 concentration dissolved in MPFe medium (1 ml of liquid medium and 3 ml of 100% H_2) under hydrostatic pressures was calculated to be 14.2 mM at 2 MPa, 71 mM at 10 MPa, and 123 mM at >20 MPa.

Survival Capability of Strain 116 Above the Potential Growth Temperature Range. The survival capability of strain 116 above its growth temperature range was examined under different hydrostatic pressures (0.4 MPa and 30 MPa). The culture at the late exponential phase grown with MPFe medium at 100°C under the gas pressure of 3 atm was used for these experiments. The culture bottles were repressurized by a gas mixture of H_2 (80%) plus CO_2 (20%) to 3 atm. Then the cultures were incubated in the bottles at 121°C and 130°C in dry ovens. For the incubation under a high hydrostatic pressure, 3 ml of the cultures was taken into 5 ml of the piezophilic cultivation syringe. The cultivation syringe without gas volume was incubated with a stainless steel, high-temperature, high-pressure reactor at 121°C and 130°C at 30 MPa. After the incubation, the culture was inoculated into fresh MPFe medium prepared in test tubes, and serial dilution cultures were conducted at 95°C. Viable cell counts were estimated from the maximal dilution series of tubes, giving the growth of strain 116.

Extraction of Gas Components and Stable Carbon Isotope Composition Analyses. Gas components (H_2 , ΣCO_2 , and CH_4) in the medium under nonpiezophilic and piezophilic conditions were extracted as described in *SI Methods* in *SI Text*.

The concentrations of the extracted gaseous carbons (CH_4 and ΣCO_2) were measured by a gas chromatography Micro GC CP2002 (GL Sciences) with a thermal conductivity detector. The concentration and the stable isotope composition of the filtered cellular carbon were analyzed by Taiyo–Nissan using a SerCon ANCA-GSL elemental analyzer-isotope ratio mass spectrometer (SerCon). The stable isotope composition of CH_4 and ΣCO_2 in the extracted gas was also analyzed by Taiyo–Nissan using a SerCon ANCA-ORCHID gas chromatograph isotope ratio mass spectrometer (GC-IRMS) (SerCon) in the cases of relatively abundant recovered CH_4 . The measurement errors were <1‰ for $\delta^{13}\text{C}$ of cellular carbon isotopic analysis and <0.3‰ and <0.5‰ for $\delta^{13}\text{C}(\Sigma\text{CO}_2)$ and $\delta^{13}\text{C}(\text{CH}_4)$ in the GC-IRMS, respectively. The more sensitive

