# Cell proliferation at 122°C and isotopically heavy $CH_4$ production by a hyperthermophilic methanogen under high-pressure cultivation

Ken Takai*[†], Kentaro Nakamura[‡], Tomohiro Toki[§], Urumu Tsunogai[¶], Masayuki Miyazaki*, Junichi Miyazaki*, Hisako Hirayama*, Satoshi Nakagawa*, Takuro Nunoura*, and Koki Horikoshi*

*Subground Animalcule Retrieval Program and [‡]Institute for Research of Earth Evolution, Japan Agency for Marine–Earth Science and Technology, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan; [§]Department of Science, Ryukyu University, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan; and [¶]Department of Earth and Planetary System Science, Faculty of Science, Hokkaido University, N10 W8, Kita-ku, Sapporo 060-0810, Japan

Edited by James M. Tiedje, Michigan State University, East Lansing, MI, and approved May 12, 2008 (received for review January 6, 2008)

**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 $CO_2$. 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 $CH_4$ 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 $H_2$, 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 $H_2$ and $CO_2$. 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.

Author contributions: K.T. designed research; K.T., K.N., T.T., U.T., M.M., J.M., H.H., S.N., and T.N. performed research; K.T., K.N., T.T., and U.T. contributed new reagents/analytic tools; K.T., K.N., T.T., U.T., and M.M. analyzed data; and K.T., K.N., T.T., U.T., and K.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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).

[†]To whom correspondence should be addressed. E-mail: kent@jamstec.go.jp.

This article contains supporting information online at www.pnas.org/cgi/content/full/0712334105/DCSupplemental.

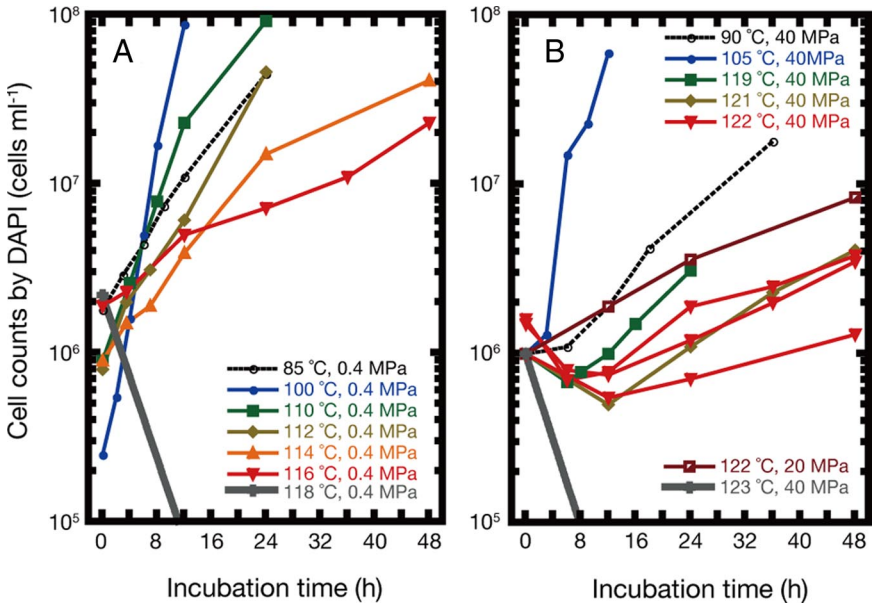


**Fig. 1.** Cell proliferation curves of *M. kandleri* strain 116. (*A*) Under the conventional gas pressure (0.4 MPa). (*B*) Under the high hydrostatic pressures at 40 or 20 MPa. The data were obtained from different series of experiments. For the cell proliferation curves at 122°C and 40 MPa, patterns from three different series of experiments are shown.

**Isolation and Taxonomic Characterization of Strain 116.** A hyperthermophilic hydrogenotrophic methanogen, strain 116, was isolated from an *in situ* colonization system deployed in black smoker fluid of the Kairei hydrothermal field (5). Cells of strain116 were long rods with a width of 0.5–0.7 $\mu$m and a length of 6–8 $\mu$m, occurring singly in the exponential growth phase but as filamentous forms of up to 20 cells in the stationary growth phase (Fig. S3). Strain 116 was nonmotile, and no flagellum was observed under any growth conditions (Fig. S3). Strain 116 was a hyperthermophilic, strictly hydrogenotrophic methanogen that was not able to use any methanogenic substrates other than $H_2$ and $CO_2$.

Under a gas pressure of 0.4 MPa, strain 116 grew at 85°C–116°C with an optimum temperature for growth of 100°C (Figs. 1 and 2). The optimum pH for growth was 6.3–6.6. Strain 116 required the existence of NaCl for growth in the range 0.5–4.5% (wt/vol), and the optimum NaCl concentration was 3.0% (wt/vol).

Strain 116 used ammonium or nitrate as the sole nitrogen source. Selenium, tungsten, and vitamins were not required as the growth cofactors, whereas metallic iron was required for growth. The metallic iron was substituted by other hydrogenogenic, iron-containing minerals and rocks such as pyrite, pyrrotite, and peridotite. Thus, metallic iron, pyrite, pyrrotite, or peridotite could be a growth cofactor of strain 116, whereas none of them could serve as an electron donor of strain 116 (see *SI Results* in *SI Text*).

The phylogenetic analysis of the 16S rRNA gene sequence indicated that strain 116 was the most closely related with *M. kandelri* type strain AV19 (Fig. S4). The DNA-DNA relatedness between strains 116 and AV19 was determined to be 79.5%. Thus, strain 116 should be taxonomically classified into *M. kandleri*. When compared with strain AV19, however, the physiological properties of strain 116 significantly differed. Strain 116 grows at much higher temperatures (up to 116°C) under nonpiezophilic conditions than does strain AV19, and strain 116 requires the presence of metallic iron or any of the hydrogenic, iron-containing minerals for growth.

**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 piezophilic cultivation system (Fig. S5). *M. kandleri* strain 116 was piezophilic, optimally growing at 105°C at hydrostatic pressures between 20 and 30 MPa (Fig. S5). The hydrostatic-pressure range could be equivalent to the *in situ* hydrostatic pressures of their potential deep-sea and subseafloor habitats.

The piezophilic response of *M. kandleri* strain 116 to the growth temperature range was examined. Under a hydrostatic pressure of 40 MPa, strain 116 grew between 90°C and 122°C, and the optimum temperature for growth was 105°C (Figs. 1 and 2 and Fig. S6). At 122°C and 40 MPa, strain 116 showed a potential for several rounds of cell division even after 48 h (Fig. 1). This pattern of cell proliferation at 122°C and 40 MPa was verified by many different series of experiments (Fig. 1), and the concomitant methane production according to the cell-number increase was identified (Fig. S6 and Tables S1 and S2). The cell proliferation and even the methane production were not detected in the growth experiment at 123°C (Fig. 1). In addition, when the growth experiment was conducted at 122°C and 20 MPa (optimum hydrostatic pressure for growth), more convincing signatures for the growth were obtained (Figs. 1 and 2). Thus, it is concluded that strain 116 is able to grow at up to 122°C under high hydrostatic pressures.

The significantly enhanced growth and methane production rates under a high hydrostatic pressure could be explained by the increased concentration of dissolved $H_2$ in the medium fluid (estimated to be from 2.27 mM at 0.4 MPa to 123 mM at 40 MPa) rather than as an effect of the elevated hydrostatic pressure. However, the upper shift of the growth temperature range was addressed as a response to the elevated hydrostatic pressure.

**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

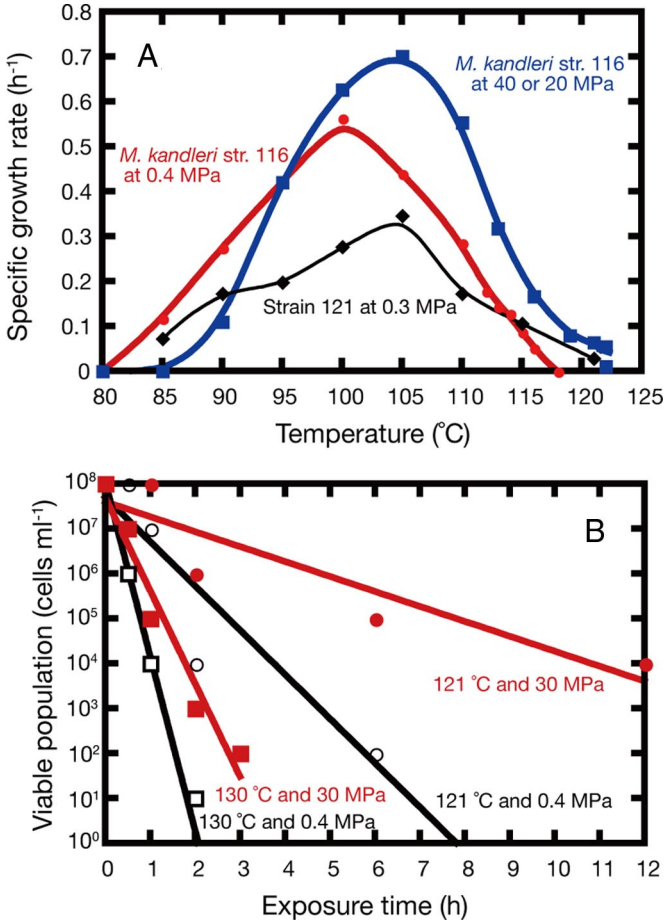


**Fig. 2.** Growth temperature range and thermotolerance of *M. kandleri* strain 116. Shown are the effect of temperature on the growth rate estimated from the cell proliferation curves (Fig. 1) (*A*) and the effect of hydrostatic pressure on viable cell density recovered after exposure to higher temperatures than growth (*B*). (*A*) Red and blue lines indicate the results at 0.4 MPa and at 40 MPa or 20 MPa, respectively. Black lines indicate the growth rate of a hyperthermophilic archeaon strain 121 (18). The specific growth rate under the piezophilic cultivation conditions was estimated only from 3–4 data points. (*B*) Red and black lines indicate the survival curves at 30 and 0.4 MPa, respectively.

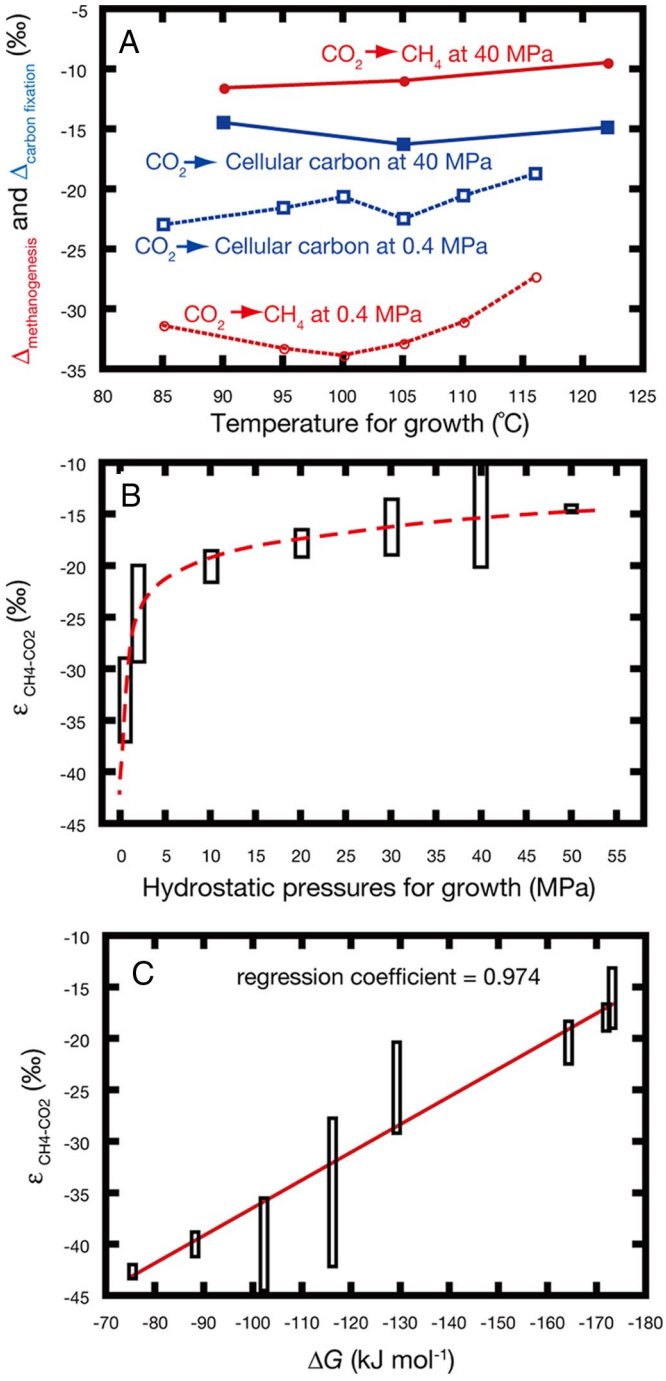


**Fig. 3.** Stable carbon isotope fractionation of *M. kandleri* strain 116 under different growth conditions. (*A*) Kinetic isotope effects of methanogenesis (red lines) and carbon fixation (blue lines) at different growth temperatures at 0.4 MPa (dotted lines) and 40 MPa (solid lines). (*B*) $\varepsilon_{CH4\text{-}CO2}$ under different hydrostatic pressures at 105°C. Red line shows a regression determined by double reciprocal plot. (*C*) Correlation between $\varepsilon_{CH4\text{-}CO2}$ and $\Delta G$ at different conditions. Red line shows a linear regression. (*B* and *C*) Bars indicate the range of $\varepsilon_{CH4\text{-}CO2}$ values at a given condition.

20 or 40 MPa (Fig. 2). However, the survival experiments under the high hydrostatic pressure were conducted by using the nearly full grown culture and medium fluid in the absence of additional growth substrates such as $H_2$ and $CO_2$. Without the growth substrates, strain 116 would not increase the viable cells at 121°C even under high hydrostatic pressure. Thus, the cell proliferation observed at the approximate potential maximum growth temperature under the high pressures might be evidence that the cell division proceeds faster to some extent than the cell death at that temperature. This conclusion may also explain why the cell number of strain 116 decreased during the lag time under the piezophilic growth at around the potential maximum growth temperature (Fig. 1).

**Isotopic Characteristics of Methanogenesis and Carbon Fixation of Strain 116.** Stable carbon isotopic fractionation of $CH_4$ and cellular carbons from the inorganic carbon source ($\Sigma CO_2$) was characterized under various growth conditions. Shift in cell number, $\Sigma CO_2$, $CH_4$ concentration, and the stable carbon isotope composition of $\Sigma CO_2$, $CH_4$, and cellular carbon during the growth of *M. kandleri* strain 116 under different cultivation conditions is indicated in Tables S1 and S2. The kinetic isotope effects of production of $CH_4$ (methanogenesis) and cellular carbon from the $\Sigma CO_2$ (carbon fixation) were calculated according to the Rayleigh model (Fig. 3*A*). The isotope fractionation effects of methanogenesis and carbon fixation by *M. kandleri* strain 116 under conventional growth conditions were $\approx -27‰$ to $-34‰$ for the methanogenesis and $-19‰$ to $-23‰$ for the

MICROBIOLOGY

carbon fixation, respectively (Fig. 3*A*). However, under high hydrostatic pressures, the isotope fractionation effect of methanogenesis became much smaller ($<-12‰$) than the values under the conventional growth conditions (Fig. 3*A*). In contrast, the isotope fractionation of carbon fixation under the same condition was estimated to be $-14‰$ to $-16‰$ (Fig. 3*A*). 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. 3*B*). With an increasing hydrostatic pressure, the isotope fractionation became smaller (Fig. 3*B*). The average of isotope fractionation values at each hydrostatic pressure was reciprocally correlated with the hydrostatic pressure (Fig. 3*B*). 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 ($\Delta G$) estimated from the culture conditions (Fig. 3*C* 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. thermlithotrophicus* (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 ≈5°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 $\approx -27‰$ to $-34‰$ for the methanogenesis and $-19$ to $-23‰$ for the carbon fixation, respectively (Fig. 3*A*). 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 ($<-12‰$) than the values under the conventional growth conditions (Fig. 3*A*). 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 $\Delta G$. Penning *et al.* (29) indeed showed the strong correlation between the carbon isotope fractionation and the $\Delta G$ 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 $\Delta G$ estimated from the culture conditions (Fig. 3*C* and Table S3). Because the soluble $H_2$ concentration in the liquid phase was the most significant parameter to control the $\Delta G$ 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 $\Delta G$ of methanogenesis could provide an important insight: the environmental energy status yielding the large $\Delta G$ 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 $\approx -16‰$ (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 *Methanotorris 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 ≈2,400 m) and the Central Indian Ridge (water depth of ≈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 reproductiveness 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 ($H_2$, $\Sigma CO_2$, 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 $\Sigma CO_2$) 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 ration mass spectrometer (SerCon). The stable isotope composition of $CH_4$ and $\Sigma CO_2$ in the extracted gas was also analyzed by Taiyo–Nissan using a SerCon ANCA-ORCHID gas chromatograph isotope ration mass spectrometer (GC-IRMS) (SerCon) in the cases of relatively abundant recovered $CH_4$. The measurement errors were <1‰ for $\delta^{13}C$ of cellular carbon isotopic analysis and <0.3‰ and <0.5‰ for $\delta^{13}C(\Sigma CO_2)$ and $\delta^{13}C(CH_4)$ in the GC-IRMS, respectively. The more sensitive

GC-IRMS analysis of $CH_4$ extracted from the piezophilic cultivation syringe was carried out by using a Mat-Finnigan 252 mass spectrometer coupled to a gas chromatograph (GC-IRMS system) as previously described (34). The analytical errors in these analyses were $<0.1‰$ and $<0.4‰$ for $\delta^{13}C(\Sigma CO_2)$ and $\delta^{13}C(CH_4)$ in the GC-IRMS, respectively.

Because a small but detectable gas leak from the piezophilic cultivation syringe was observed during the incubation at high temperatures, nonbiological isotope fractionation of $CH_4$ and $\Sigma CO_2$ under the piezophilic cultivation condition was repeatedly checked by using the piezophilic cultivation syringes including 1 ml of MPFe medium consisting of 12 mM soluble $CH_4$ [$\delta^{13}C(CH_4) = -41.9 \pm 0.1‰$ at initial] and 100 mM of $\Sigma CO_2$ [$\delta^{13}C(\Sigma CO_2) = -24.7 \pm 0.1‰$ at initial], respectively, without the cell inoculum at 122°C and 40 MPa for 48 h. The isotopic compositions of $CH_4$ and $\Sigma CO_2$ after 12, 24, and 48 h were $-41.9 \pm 0.3‰$, $-41.6 \pm 0.1‰$, $-41.0 \pm 0.1‰$, and $-24.0 \pm 0.1‰$, $-24.7 \pm 0.1‰$, $24.3 \pm 0.2‰$, respectively. These results strongly suggested that the nonbiological isotope fractionation effect would be negligible for estimation of isotopic fractionation by strain 116, in particular at the relatively early stage of growth (until 24 h).

**Calculation of Stable Isotope Fractionation.** Based on the concentrations and the carbon isotopic compositions of the cellular carbons, $\Sigma CO_2$ and $CH_4$, kinetic isotope effects ($\Delta$) of methanogenesis and carbon fixation by strain 116 under different growth conditions were calculated by the following equations:

$$\Delta_{\text{methanogenesis}} = -1{,}000(1 - 1/\alpha_{\text{methanenogensis}}) \quad [1]$$

$$\Delta_{\text{carbon fixation}} = -1{,}000(1 - 1/\alpha_{\text{carbon fixation}}), \quad [2]$$

where $\alpha_{\text{methanenogensis}}$ and $\alpha_{\text{carbon fixation}}$ were the fractionation factors for methanogenesis and carbon fixation. Both $\alpha_{\text{methanenogensis}}$ and $\alpha_{\text{carbon fixation}}$ were calculated by the equations described in *SI Methods* in *SI Text*. In this study, however, not all of the experimental conditions provided enough reliable data of the carbon isotope compositions and the concentrations of $CH_4$, $CO_2$, and cellular carbon to the calculate the fractionation factors. In such cases, the $\varepsilon$ value ($\varepsilon_{CH4\text{-}CO2}$) of methanogenesis from $\Sigma CO_2$ was calculated by the following equation described by House *et al.* (26):

$$\varepsilon_{CH4\text{-}CO2} = 1{,}000\ln\{(\delta^{13}C(\Sigma CO_2)_t + 1{,}000)/(\delta^{13}C(CH_4)_t + 1{,}000)\}. \quad [3]$$

**Thermodynamic Calculation of $\Delta G$.** Calculations of $\Delta G$ were carried out as described in *SI Methods* in *SI Text*.

**ACKNOWLEDGMENTS.** We thank the captain and crew of the R/V Yokosuka and the operation team of the DSV Shinkai 6500 for their technical expertise in sampling in the CIR Kairei field, Mr. Katsuyuki Uematsu for electron microscopic observation, and Prof. William B. Whitman for his editing and comments on this manuscript. This work was supported by Grant-in-Aid for Science Research 18684027 (to K.T.) and by Grant-in Aid for the 21st Century Centers of Excellence Program ''Neo-Science of Natural History'' (to U.T.) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

1. Kvenbolden KA (1995) A review of geochemistry of methane in natural gas hydrate. *Org Geochem* 23:997–1008.
2. Milkov AV (2004) Global estimates of hydrate-bound gas in marine sediments: How much is really out there? *Earth-Science Rev* 66:183–197.
3. Overpeck JT, Cole JE (2006) Abrupt change in Earth's climate system. *Annu Rev Environ Resour* 31:1–31.
4. Nealson KH, Inagaki F, Takai K (2005) Hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs): Do they exist and why should we care? *Trends Microbiol* 13:405–410.
5. Takai K, *et al.* (2004) Geochemical and microbiological evidence for a hydrogen-based, hyperthermophilic subsurface lithoautotrophic microbial ecosystem (HyperSLiME) beneath an active deep-sea hydrothermal field. *Extremophiles* 8:269–282.
6. Takai K, *et al.* (2006) Ultramafics-Hydrothermalism-Hydrogenesis-HyperSLiME (UltraH$^3$) linkage: A key insight into early microbial ecosystem in the Archean deep-sea hydrothermal systems. *Paleontol Res* 10:269–282.
7. Ueno Y, Yamada K, Yoshida N, Maruyama S, Isozaki Y (2006) Evidence from fluid inclusions for microbial methanogenesis in the early Archaean era. *Nature* 440:516–519.
8. Shock EL (1997) High-temperature life without photosynthesis as a model for Mars. *J Geophys Res* 102:23687–23694.
9. Sleep NH, Bird DK (2007) Niches of the pre-photosynthetic biosphere and geologic preservation of Earth's earliest ecology. *Geobiology* 5:101–117.
10. Sherwood Lollar B, *et al.* (2007) Hydrogeologic controls on episodic $H_2$ release from Precambrian fractured rocks—Energy for deep subsurface life on Earth and Mars. *Astrobiology* 7:971–986.
11. Bernhardt G, Jaenicke R, Lüdemann H-D (1987) High-pressure equipment for growing methanogenic microorganisms on gaseous substrates at high temperature. *Appl Environ Microbiol* 53:1876–1879.
12. Miller JF, *et al.* (1988a) High-pressure-temperature bioreactor for studying pressure-temperature relationship in bacterial growth and productivity. *Biotechnol Bioeng* 31:407–413.
13. Miller JF, Shah NN, Nelson CM, Ludlow JM, Clark DS (1988b) Pressure and temperature effects on growth and methane production of the extreme thermophile *Methanococcus jannaschii*. *Appl Environ Microbiol* 54:3039–3042.
14. Bernhardt G, *et al.* (1988) Effect of carbon dioxide and hydrostatic pressure on the pH of culture media and the growth of methanogens at elevated temperature. *Appl Microbiol Biotechnol* 28:176–181.
15. Huber R, Kurr M, Jannasch HW, Stetter KO (1989) A novel group of abyssal methanogenic archaebacteria (*Methanopyrus*) growing at 110°C. *Nature* 342:833–834.
16. Kurr M, *et al.* (1991) Methanopyrus kandleri, gen. and sp. nov. represents a novel group of hyperthermophilic methanogens, growing at 110°C. *Arch Microbiol* 156:239–247.
17. Blöchl E, *et al.* (1997) Pyrolobus fumarii, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. *Extremophiles* 1:14–21.
18. Kashefi K, Lovley DR (2003) Extending the upper temperature limit for life. *Science* 301:934.
19. Canganella F, Gonzalez JM, Yanagibayashi M, Kato C, Horikoshi K (1997) Pressure and temperature effects on growth and viability of the hyperthermophilic archaeon *Thermococcus peptonophilus*. *Arch Microbiol* 168:1–7.
20. Nelson CM, Schuppenhauer MR, Clark DS (1991) Effects of hyperbaric pressure on a deep-sea archaebacterium in stainless steel and glass-lined vessels. *Appl Environ Microbiol* 57:3576–3580.
21. Zobell CE, Morita RY (1957) Barophilic bacteria in some deep sea sediments. *J Bacteriol* 73:563–568.
22. Claypool GE, Kvenvolden KA (1983) Methane and other hydrocarbon gases in marine sediment. *Annu Rev Earth Planet Sci* 11:299–327.
23. Inagaki F, *et al.* (2006) Biogeographical distribution and diversity of microbes in methane hydrate-bearing deep marine sediments on the Pacific Ocean Margin. *Proc Natl Acad Sci USA* 103:14146–14169.
24. McCollom TM, Seewald JS (2006) Carbon isotope composition of organic compounds produced by abiotic synthesis under hydrothermal conditions. *Earth Planet Sci Lett* 243:74–84.
25. McCollom TM, Seewald JS (2007) Abiotic synthesis of organic compounds in deep-sea hydrothermal environments. *Chem Rev* 107:382–401.
26. House CH, Schop JW, Stetter KO (2003) Carbon isotopic fractionation by Archaeans and other thermophilic prokaryotes. *Org Geochem* 34:345–356.
27. Botz R, Pokojski HD, Schmitt M, Thomm M (1996) Carbon isotope fractionation during bacterial methanogenesis by $CO_2$ reduction. *Org Geochem* 25:255–262.
28. Valentine DL, Chidthaisong A, Rice A, Reeburgh WS, Tyler SC (2004) Carbon and hydrogen isotope fractionation by moderately thermophilic methanogens. *Geochim Cosmochim Acta* 68:1571–1590.
29. Penning H, Plugge CM, Galand PE, Conrad R (2005) Variation of carbon isotope fractionation in hydrogenotrophic methanogenic microbial cultures and environmental samples at different energy status. *Global Change Biol* 11:2103–2113.
30. Inagaki F, Takai K, Nealson KH, Horikoshi K (2004) Sulfurovum lithotrophicum gen. nov., sp. nov., a novel sulfur-oxidizing chemolithoautotroph within the epsilon-Proteobacteria isolated from Okinawa Trough hydrothermal sediments. *Int J Syst Evol Microbiol* 54:1477–1482.
31. Takai K, Nealson KH, Horikoshi K (2004) Hydrogenimonas thermophila gen. nov., sp. nov., a novel thermophilic, hydrogen-oxidizing chemolithoautotroph within the epsilon-Proteobacteria, isolated from a black smoker in a Central Indian Ridge hydrothermal field. *Int J Syst Evol Microbiol* 54:25–32.
32. Takai K, Nealson KH, Horikoshi K (2004) Methanotorris formicicus sp. nov., a novel extremely thermophilic, methane-producing archaeon isolated from a black smoker chimney in the Central Indian Ridge. *Int J Syst Evol Microbiol* 54:1095–1100.
33. Jauregui-Haza UJ, Pardillo-Fontdeevila EJ, Wilhelm AM, Delmas H (2004) Solubility of hydrogen and carbon monooxide in water and some organic solvents. *Latin Am Appl Res* 34:71–74.
34. Tsunogai U, Yoshida N, Ishibashi J, Gamo T (2000) Carbon isotopic distribution of methane in deep-sea hydrothermal plume, Myojin Knoll Caldera, Izu-Bonin arc: Implications for microbial methane oxidation in the oceans and applications to heat flux estimation. *Geochim Cosmochim Acta* 64:2439–2452.