Growth Kinetics, Carbon Isotope Fractionation, and Gene Expression in the Hyperthermophile Methanocaldococcus jannaschii during Hydrogen-Limited Growth and Interspecies Hydrogen Transfer

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Running Head: H2-limited growth of M. jannaschii

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ABSTRACT

Hyperthermophilic methanogens are often H₂ limited in hot subseafloor environments and their survival may be due in part to physiological adaptations to low H₂ conditions and interspecies H₂ transfer. The hyperthermophilic methanogen *Methanocaldococcus jannaschii* was grown in monoculture at high (80-83 μM) and low (15-27 μM) aqueous H₂ concentrations and in co-culture with the hyperthermophilic H₂ producer *Thermococcus paralvinellae*. The purpose was to measure changes in growth and CH₄ production kinetics, CH₄ fractionation, and gene expression in *M. jannaschii* with changes in H₂ flux. Growth and cell-specific CH₄ production rates of *M. jannaschii* decreased with decreasing H₂ availability and decreased further in co-culture. However, cell yield (cells produced per mole of CH₄ produced) increased six-fold when *M. jannaschii* was grown in co-culture relative to monoculture. Relative to high H₂ concentrations, isotopic fractionation of CO₂ to CH₄ (εCO₂-CH₄) was 16‰ larger for cultures grown at low H₂ concentrations and 45‰ and 56‰ larger for *M. jannaschii* growth in co-culture on maltose and formate, respectively. Gene expression analyses showed H₂-dependent methylene-tetrahydromethanopterin (H₄MPT) dehydrogenase expression decreased and coenzyme F₄₂₀-dependent methylene-H₄MPT dehydrogenase expression increased with decreasing H₂ availability and in co-culture growth. In co-culture, gene expression decreased for membrane-bound ATP synthase and hydrogenase. The results suggest that H₂ availability significantly affects the CH₄ and biomass production and CH₄ fractionation by hyperthermophilic methanogens in their native habitats.
**IMPORTANCE** Hyperthermophilic methanogens and H$_2$-producing heterotrophs are collocated in high-temperature subseafloor environments such as petroleum reservoirs, mid-ocean ridge flanks, and hydrothermal vents. Abiotic flux of H$_2$ can be very low in these environments and there is a gap in our knowledge about the origin of CH$_4$ in these habitats. In the hyperthermophile *Methanocaldococcus jannaschii*, growth yields increased as H$_2$ flux, growth rates, and CH$_4$ production rates decreased. The same trend was observed increasingly with interspecies H$_2$ transfer between *M. jannaschii* and the hypothermophilic H$_2$ producer *Thermococcus paralvinellae*. With decreasing H$_2$ availability, isotopic fractionation of carbon during methanogenesis increased, resulting in isotopically more negative CH$_4$ with a concomitant decrease in H$_2$-dependent methylene-tetrahydromethanopterin dehydrogenase gene expression and increase in F$_{420}$-dependent methylene-tetrahydromethanopterin dehydrogenase gene expression. The significance of our research is in understanding the nature of hyperthermophilic interspecies H$_2$ transfer and identifying biogeochemical and molecular markers for assessing the physiological state of methanogens and possible source of CH$_4$ in natural environments.

**INTRODUCTION**

Each year, approximately 1 Pg of CH$_4$ is produced globally through methanogenesis, largely by methanogens growing syntrophically with fermentative microbes that hydrolyze biopolymers (1), but little is known about the magnitude or mechanism of methanogenesis through thermophilic H$_2$ syntrophy or interspecies H$_2$ transfer. Deep-sea hydrothermal vents are known habitats for thermophilic
methanogens (2). It was also estimated that 35% of all marine sediments are above 60°C (3) suggesting that these environments likewise provide a large global biotope for thermophiles. Microcosms containing low-temperature hydrothermal fluid as well as an archaeal co-culture derived from a high-temperature oil pipeline each produced CH₄ through interspecies H₂ transfer at 80°C when supplemented with organic compounds, both without added H₂ (4, 5). Both showed that CH₄ was produced from a mixed microbial community consisting of the hyperthermophilic H₂-producing heterotroph *Thermococcus* and the (hyper)thermophilic, hydrogenotrophic methanogens *Methanocaldococcus*, *Methanothermococcus*, and *Methanothermobacter*. Molecular and culture-dependent analyses show that *Thermococcus* and thermophilic methanogens are collocated in hydrothermal vents (5-11), produced waters from high-temperature petroleum reservoirs (12-19), and mid-ocean ridge flanks (20). In high-temperature, organic-rich environments such as petroleum reservoirs, collocated H₂-producing heterotrophs are the primary source of H₂ (21) but very little is known about this process at high temperatures or how thermophilic syntrophy affects environmental signals.

In this study, growth and CH₄ production kinetic, carbon isotope fractionation, and gene expression data were examined together for a hyperthermophilic methanogen under conditions ranging from monoculture growth at high and low H₂ concentrations to co-culture growth with a H₂-producing partner. The hyperthermophile *M. jannaschii* was grown in monoculture in a chemostat under H₂-replete and H₂-limited conditions based on previous kinetic experiments (9). It was also grown with the H₂-producing hyperthermophilic heterotroph *Thermococcus paralvinellae* using maltose and formate.
separately as the growth substrates (22). The purpose was to determine if *M. jannaschii*

cell yield (amount of biomass produced per mole of CH$_4$ produced, $Y_{CH4}$) increases

when cultures are shifted from H$_2$-replete to H$_2$-limited growth conditions and if $Y_{CH4}$

remains high or increases further during interspecies H$_2$ transfer. This study also

examined if interspecies H$_2$ transfer stimulates the growth rate or cell yield of *T.

paralvinellae* or ameliorates its H$_2$ inhibition relative to its growth in monoculture.

Furthermore, isotopic carbon fractionation was examined to determine if the CH$_4$ is

isotopically lighter when H$_2$ flux is reduced as previously observed in moderately

thermophilic methanogens (23-25). Finally, differential gene expression analysis using

RNA-Seq was used to determine if changes occur in *M. jannaschii* for the expression of

genes for carbon assimilation, CH$_4$ production, or energy generation when H$_2$

decreases in availability. This study demonstrates the utility of measuring growth kinetic

parameters, carbon isotope fractionation, and differential gene expression patterns for

two species grown in co-culture. The data elucidate how hyperthermophilic

methanogens behave in a high H$_2$ flux environment such as those found at some

hydrothermal vents versus a low H$_2$-flux environment such as petroleum reservoirs.

RESULTS

**Growth parameters for mono- and co-cultures.** A summary of the growth conditions

is provided in Table 1. In monoculture, the specific growth rate of *M. jannaschii* in the

chemostat decreased from $1.04 \pm 0.12$ h$^{-1}$ (± standard error) when grown on 80-83 μM

H$_2$ to $0.50 \pm 0.09$ h$^{-1}$ when grown on 15-27 μM H$_2$ (Fig. 1A). Cell concentrations in the

medium and H$_2$ and CH$_4$ concentrations in the headspace remained constant.
throughout growth in the chemostat (Fig. S1). Attempts to grow *M. jannaschii* in co-culture with *T. paralvinellae* in the chemostat when either maltose or formate was the energy source, with and without stirring and gas sparging of the medium with CO₂ and N₂, were unsuccessful. This was likely due to the open reactor that permits gas to flow out of the reactor without any gas pressure increase. Co-culture growth was readily established in sealed bottles that contained 1 atm of gas pressure at room temperature. At 82°C, the gas pressure in the bottle was 1.2 atm, which slowed H₂ efflux from the growth medium to the headspace. Therefore, the co-cultures were grown in sealed bottles with the same volume of medium and headspace as the chemostat. The growth rates of *M. jannaschii* decreased further when it was grown in co-culture with *T. paralvinellae* to 0.12 ± 0.01 h⁻¹ and 0.22 ± 0.03 h⁻¹ when *T. paralvinellae* was grown on maltose and formate, respectively (Fig. 1A). Relative to H₂ concentrations when *T. paralvinellae* was grown in the bottles in monoculture, nearly all the H₂ was removed from the co-culture bottles and CH₄ was produced (Fig. S2).

The cell-specific CH₄ production rate decreased 3.6-fold when *M. jannaschii* was grown on 15-27 μM H₂ (139 ± 8 fmol cell⁻¹ h⁻¹, ± standard error) relative to growth on 80-83 μM H₂ (496 ± 21 fmol cell⁻¹ h⁻¹) (Fig. 1B). The rates decreased further when grown in co-culture on maltose (21.3 ± 2.7 fmol cell⁻¹ h⁻¹) and on formate (24.8 ± 4.3 fmol cell⁻¹ h⁻¹) (Fig. 1B). However, the growth yields (YCH₄) for *M. jannaschii* grown in co-culture were significantly higher when grown on maltose (9.1 ± 1.9 cells [×10¹²] per mol CH₄, ± standard error) and formate (13.5 ± 2.0 cells [×10¹²] per mol CH₄) than growth yields in monoculture on 15-27 μM H₂ (2.1 ± 0.2 cells [×10¹²] per mol CH₄) and 80-83 μM H₂ (1.5 ± 0.1 cells [×10¹²] per mol CH₄) (Fig. 1C). Summaries of the growth and CH₄ production
kinetics data for *M. jannaschii* are available in the Supplemental Material (Fig. S1 and S2, Tables S1 and S2).

There was no change in the specific growth rate or maximum cell concentration of *T. paralvinellae* when it was grown with or without *M. jannaschii* or with change in carbon source (Fig. 2A, Fig. S2). The specific growth rates of *T. paralvinellae* grown on maltose in monoculture and in co-culture were 0.16 ± 0.01 h\(^{-1}\) (± standard error) and 0.22 ± 0.02 h\(^{-1}\), respectively, while growth rates on formate in monoculture and in co-culture were 0.18 ± 0.05 h\(^{-1}\) (± standard error) and 0.16 ± 0.02 h\(^{-1}\), respectively (Fig. 2A). Furthermore, when grown on maltose, there was no change in the growth yield (Table S3) or cell-specific acetate production rate of *T. paralvinellae* when grown in monoculture (0.94 ± 0.16 pmol cell\(^{-1}\) h\(^{-1}\), ± standard error) relative to growth in co-culture (1.05 ± 0.15 pmol cell\(^{-1}\) h\(^{-1}\)) (Fig. 2B). However, when grown on maltose, *T. paralvinellae* produced formate (in addition to H\(_2\) and acetate) when grown in monoculture (0.60 ± 0.18 pmol cell\(^{-1}\) h\(^{-1}\)) but not when grown in coculture (Fig. 2C). The cell-specific H\(_2\) production rate was higher when *T. paralvinellae* was grown in monoculture on formate (130.9 ± 11.1 fmol cell\(^{-1}\) h\(^{-1}\), ± standard error) relative to monoculture growth on maltose (0.9 ± 0.1 fmol cell\(^{-1}\) h\(^{-1}\)) (Table S3). A summary of the growth and metabolite production kinetics data for *T. paralvinellae* is available in the Supplemental Material (Fig. S2, Tables S3). There was no growth of *M. jannaschii* when it was incubated in monoculture in medium supplemented with only 0.01% yeast extract or 0.1% sodium formate and 0.01% yeast extract with N\(_2\):CO\(_2\) in the headspace. These additions also did not stimulate the growth of *M. jannaschii* in monoculture when a H\(_2\):CO\(_2\) headspace was provided.
Carbon isotope fractionation. The final $\delta^{13}C_{CO_2}$ values were -24.4 to -21.6‰ in the co-culture bottles and -33.3 to -28.2‰ in the chemostat (Table 1). The final $\delta^{13}C_{CO_2}$ of the *M. jannaschii* monocultures in bottles were +19.2 to +22.6‰, demonstrating a substantial draw down of the reactant. $\delta^{13}C_{CH_4}$ values became increasingly negative with increasing H$_2$ limitation during cell growth. The $\delta^{13}C_{CH_4}$ in the chemostat were -55.9 to -55.8‰ when *M. jannaschii* was grown on 80-83 μM H$_2$ and decreased to -75.7 to -72.5‰ when grown on 15-27 μM H$_2$. The corresponding $\varepsilon_{CO_2-CH_4}$ values increased from 28.5 to 29.3‰ during high H$_2$ growth to 43.7 to 45.9‰ during low H$_2$ growth (Table 1).

Similarly, $\delta^{13}C_{CH_4}$ values became more negative with increasing H$_2$ limitation during co-culture cell growth. In monoculture with 1.92 atm of initial H$_2$ in the headspace at 82°C, the $\delta^{13}C_{CH_4}$ from *M. jannaschii* was -34.2 to -32.9‰ (Table 1). $\delta^{13}C_{CH_4}$ decreased to -91.2 to -89.0‰ when *M. jannaschii* was grown in co-culture with *T. paralvinellae* on maltose and to -99.4‰ when grown in co-culture on formate. The corresponding $\varepsilon_{CO_2-CH_4}$ values increased from 22.1 to 23.0 ‰ during monoculture growth in a serum bottle to 73.5 to 85.1‰ during growth in co-culture with *T. paralvinellae* (Table 1).

Transcriptomic analyses. RNA-Seq mapped 1,866 transcripts to the *M. jannaschii* genome. The thirteen samples that span four growth conditions were analyzed based on principal component analysis (PCA) (Fig. S3A) and t-Distributed Stochastic Neighbor Embedding (t-SNE) (Fig. S3B) results. Pairwise comparisons of *M. jannaschii* grown in monoculture on high and low H$_2$ showed up to 12 genes to be differentially expressed (adjusted $p < 0.01$ and $|\log_2 FC| > 1$) with 1 gene down-regulated and 11 genes up-regulated during growth on low H$_2$ relative to growth on high H$_2$ (Table S4). Under low H$_2$ conditions, F$_{420}$-dependent methylene-tetrahydromethanopterin
(H₄MPT) dehydrogenase (mtd, MJ_RS0555, NCBI RefSeq database) gene expression increased 3.5-fold (Fig. 3A). There was no significant change in gene expression for H₂-dependent methylene-H₄MPT dehydrogenases (hmd, MJ_RS04180 and hmdX, MJ_RS03820) (Fig. 3B, Fig. S4) or for any of the methyl-CoM reductase A I or II genes (mcrA, MJ_RS00415 and MJ_RS04540) (Fig. S5) for M. jannaschii grown in monoculture on high and low H₂ in the chemostat. The genes that encode for a GTP binding protein (MJ_RS01180), bacteriohemerythrin (MJ_RS03980), radical SAM protein (MJ_RS04390), a signal recognition particle (MJ_RS05550), a transcriptional regulator (MJ_RS06225), and four hypothetical proteins were up-regulated on low H₂ while a gene that encodes for a histone (MJ_RS04990) was up-regulated on high H₂ (Table S4).

For co-cultures grown on maltose, 97% of the reads mapped unambiguously to the T. paralvinellae genome and 1.5% mapped to the M. jannaschii genome. For co-cultures grown on formate, 67% of the reads mapped unambiguously to the T. paralvinellae genome and 29% mapped to the M. jannaschii genome. These proportions generally matched the proportions of T. paralvinellae and M. jannaschii cells in each co-culture type based on cell concentration estimates (Fig. S2). Merged pairwise comparisons of M. jannaschii gene expression for cultures grown in monoculture and M. jannaschii grown in co-culture with T. paralvinellae showed up to 338 genes to be differentially expressed (adjusted $p < 0.01$ and $|\log_{2}FC| > 1$) with 146 up-regulated genes and 192 down-regulated genes when grown in co-culture relative to growth in monoculture on high and low H₂ (Table S5). However, we cannot rule out the
possibility that some of these gene expression changes are caused by the switch from the chemostat to bottles.

F$_{420}$-dependent methylene-H$_{4}$MPT dehydrogenase (mtd, MJ_RS05555) gene expression was up-regulated 4.3-fold in co-culture relative to *M. jannaschii* grown in monoculture conditions (Fig. 3A). In contrast, gene expression of H$_{2}$-dependent methylene-H$_{4}$MPT dehydrogenases (*hmd*, MJ_RS04180 and *hmdX*, MJ_RS03820) were both down-regulated 2.1-fold in *M. jannaschii* grown in co-culture relative to *M. jannaschii* grown in monoculture (Fig. 3B, Fig. S4). There was no change in gene expression for the *mcrI* and *mcrII* genes (Fig. S5). Gene expression for a hypothetical protein with a predicted RNA-binding domain (MJ_RS03480) showed a 22.5-fold increase in co-cultures relative to monocultures (Fig. S6). Expression of 6 of the 9 *M. jannaschii* genes that encode for a V-type ATP synthase (MJ_RS01130-MJ_RS01165, MJ_RS03255) were down-regulated when cultures were grown in co-culture relative to expression in *M. jannaschii* grown in monoculture (Fig. 4). Similarly, expression of 14 genes in a putative operon for membrane-bound, ferredoxin-dependent hydrogenase were also down-regulated in *M. jannaschii* cultures grown in co-cultures relative to cultures grown in monoculture (Fig. 4). These genes include Eha subunits A and B (MJ_RS02795-02800), an oxidoreductase (MJ_RS02755), a dehydrogenase (MJ_RS02765), and a catalytic subunit (MJ_RS02730).

**DISCUSSION**

Microorganisms in nature live in complex communities and biogeochemically impact their environment through interspecies metabolic interactions. Most of what is
known about the kinetics and physiology of methanogenesis at varying H$_2$ concentrations and in co-culture comes from studies of the thermophile *Methanothermobacter thermoautotrophicus* and the mesophile *Methanococcus maripaludis*. Growth rates of both organisms decreased when they were H$_2$ limited relative to H$_2$-replete growth. However, growth yields ($Y_{\text{CH}_4}$) increased when the cultures were H$_2$ limited (26-28). Prior to this study, growth yields had not been measured for any methanogen during interspecies H$_2$ transfer or for any hyperthermophilic methanogens under varying H$_2$ concentrations.

To determine *M. jannaschii* metabolism and kinetics under H$_2$-replete and H$_2$-limited growth conditions, as defined in a previous study (9), continuous growth in chemostats was established. The decrease in specific growth rate and cell-specific CH$_4$ production rate of *M. jannaschii* when grown in monoculture under H$_2$-limited conditions show that growth and methanogenesis rates are limited by H$_2$ concentration. This trend continued when *M. jannaschii* was grown in co-culture with *T. paralvinellae* suggesting that interspecies H$_2$ transfer led to further H$_2$ limitation of methanogenesis. However, the cell yield for *M. jannaschii* increased when the cells were grown in co-culture relative to growth in monoculture. This is consistent with previous studies that show higher cell yields for *M. thermoautotrophicus* and *M. maripaludis* upon H$_2$ limitation, but there is no consensus on a physiological explanation (26-28). During methanogenesis, methyl-H$_4$MPT is either converted to methyl-CoM for production of CH$_4$ and energy generation on the cytoplasmic membrane or to acetyl-CoA for biosynthetic reactions (Fig. 5). Depending on the H$_2$ concentration, hydrogenotrophic methanogens decide between maximum growth rate and maximum growth yield. This pattern can be explained by the
rate-yield tradeoff which creates two divergent ecological strategies, namely 1) slow growth but efficient metabolism and high yields when resources are scarce and 2) fast growth but inefficient metabolism and low yields upon rich resources. The rate-yield tradeoff is suggested to be integral to evolution and the coexistence of species (29). It was proposed previously but not demonstrated that syntrophic growth of methanogens with a fermentative partner is optimized for cell yield rather than growth rate (27). In this study, *M. jannaschii* grew and produced CH$_4$ solely on the H$_2$ produced by *T. paralvinellae* and the cell yield of *M. jannaschii* increased in co-culture compared to growth in monoculture.

*Thermococcus* species use maltose for biosynthesis and energy generation that yields acetate and CO$_2$ as well as H$_2$ and a proton/sodium motive force via a membrane-bound hydrogenase (30, 31). However, they are auxotrophic for certain amino acids that it must be supplied from the environment (32, 33). *T. paralvinellae* increased gene expression of a membrane-bound formate hydrogenlyase operon and produced formate when inhibited by exogenous H$_2$ suggesting that it converts H$_2$ to formate when H$_2$ inhibited (22). *T. paralvinellae* also separately used formate as an energy source in the absence of maltose, produced H$_2$, and generated a proton/sodium motive force but required 0.01% yeast extract in the growth medium (22). Consequently, the cell-specific H$_2$ production rate was ~100-fold higher when cultures were grown on formate.

Morris *et al.* (34) defined microbial syntrophy as ‘obligately mutualistic metabolism’ and included co-culture growth between the hyperthermophilic H$_2$-producer *Pyrococcus furiosus* and various hyperthermophilic methanogens, including *M.*
T. paralvinellae, as an example based on increased cell concentrations of both organisms in co-culture relative to each in monoculture (35). Unlike T. paralvinellae, *P. furiosus* lacks formate hydrogenlyase as a mechanism to overcome H₂ inhibition (36) and may be more dependent upon syntrophy to ameliorate H₂ inhibition. In this study, when *T. paralvinellae* was grown with *M. jannaschii*, growth in co-culture did not stimulate the growth rate, growth yield, or maximum cell concentration of *T. paralvinellae*. This suggests the relationship between *T. paralvinellae* and *M. jannaschii* is not obligately mutualistic and therefore more accurately represents interspecies H₂ transfer rather than syntrophy. However, there was no formate production when *T. paralvinellae* was grown in co-culture on maltose with *M. jannaschii*, and *M. jannaschii* cannot grow on formate (37, this study), so *M. jannaschii* does appear to ameliorate H₂ inhibition in *T. paralvinellae* when grown in co-culture.

It was shown previously that the fractionation of carbon isotopes between CO₂ and CH₄ increased with decreasing concentrations of H₂ availability, or more accurately with decreasing Gibbs energy for the methanogenesis reaction (23). The ε_{CO₂-CH₄} fractionation factor for the thermophile *Methanothermobacter marburgensis* increased from 22 to 39‰ at high H₂ concentrations to 58 to 64‰ at limiting H₂ concentrations (23, 24). It was proposed that variations in the carbon isotopic fractionation factor are controlled by the extent of reversibility of the methanogenesis pathway, which was proposed to increase with decreasing Gibbs energy availability (23). In this study, the CH₄ produced was isotopically more negative and the ε_{CO₂-CH₄} fractionation factor increased when *M. jannaschii* was grown in the chemostat with low H₂ relative to high H₂ conditions. Similarly, in bottles, the CH₄ was isotopically more negative and ε_{CO₂-CH₄}
was much larger when *M. jannaschii* was grown in co-culture with *T. paralvinellae* relative to when it was grown in monoculture with an initial estimated aqueous H\(_2\) concentration of 1.2 mM. The most negative CH\(_4\) in this study was produced when *M. jannaschii* was grown in co-culture and H\(_2\) fluxes are presumably at their lowest rates. Previous studies showed that during CO\(_2\) fixation and methanogenesis (Fig. 5) in *M. thermoautotrophicus* and *M. maripaludis*, gene expression for H\(_2\)-dependent methylene-H\(_4\)MPT dehydrogenase (*hmd*) decreased while expression of cofactor F\(_{420}\)-dependent methylene-H\(_4\)MPT (*mtd*) increased when growth was H\(_2\)-limited relative to H\(_2\)-replete growth (27, 28, 38). It was suggested that the Mtd reaction is the more reversible of the two methylene-H\(_4\)MPT dehydrogenase reactions, which facilitates enhanced carbon isotope fractionation by methanogenesis pathway reversal in these methanogens under H\(_2\)-limited conditions (23). The proteome of *M. jannaschii* contained a lower abundance of Hmd and higher abundances of Mtd and four flagellar proteins in early logarithmic growth phase when grown in batch phase under H\(_2\)-limited conditions relative to H\(_2\)-replete conditions, but both Hmd and Mtd were found at high relative abundances in late logarithmic growth phase when grown on H\(_2\)-replete conditions (39). During H\(_2\) syntrophy, the *M. thermoautotrophicus* proteome had more Mtd and less Hmd relative to monoculture growth on H\(_2\)-replete conditions (40). There were no significant changes in gene expression or protein abundance for Hmd and Mtd in *M. maripaludis* during H\(_2\) syntrophy relative to a H\(_2\)-limited monoculture (41).

In this study, RNA-Seq was used to determine changes in gene expression profiles in *M. jannaschii* for carbon assimilation, CH\(_4\) production, and energy generation pathways when there were changes in H\(_2\) availability. When *M. jannaschii* was grown
under H$_2$-limited conditions and in co-culture, mtd expression was significantly upregulated and hmd expression was significantly downregulated in co-culture cells compared to monoculture cells. This suggests a preference for F$_{420}$ as an electron carrier in the methanogenesis pathway under H$_2$-limited conditions. The increase in cell yield in co-culture was not supported by a change in the expression of genes in the carbon assimilation and methanogenesis pathways. No significant changes were detected in the expression of methyl-CoM reductase I and II and methyl-H$_4$MPT:CoM methyltransferase, which catalyze the last two steps of methanogenesis (Fig. 5).

Previously, changes in the relative abundances of methyl-CoM reductases I and II were observed in *M. thermoautotrophicus* with H$_2$ availability and growth during syntrophy (27, 40, 42). Moreover, there was no change in expression in our study in the carbon monoxide dehydrogenase/acetyl-CoA synthase genes, which encode for the enzyme that converts methyl-MPT to acetyl-CoA.

In co-culture, there was up to 22.5-fold increase in the expression of a putative RNA binding protein that is only found in methanogens and the Thermococcales and has been proposed to regulate cellular activity at the translation level (43). The decrease in the expression of genes in the putative membrane-bound, ferredoxin-dependent hydrogenase operon and in the membrane-bound, Na$^+$-translocating V-type ATPase operon supports the kinetic observations that *M. jannaschii* is energy limited when grown in co-culture. In H$_2$-limited co-culture conditions, the cell must direct more of its methyl-H$_4$MPT towards biosynthesis. Furthermore, there was no change in the expression of the genes for flagella. This was different from what was previously
observed for *M. jannaschii* using proteomics (39) and may be due to the use of a chemostat in this study instead of a batch reactor.

In environments such as low-H₂ hydrothermal vents along subduction zones and some mid-ocean ridges, oil reservoirs, and high saline shale beds where organic compounds are present and H₂ efflux rates are low, thermophilic methanogens like *M. jannaschii* likely can grow and produce CH₄ through interspecies H₂ transfer with hyperthermophilic H₂-producing heterotrophs like *T. paralvinellae* with high cell yields and large carbon isotope fractionations but at very slow rates. This likely explains the presence of thermophilic H₂ producers and thermophilic, hydrogenotrophic methanogens in petroleum reservoirs and may be a source of CH₄ in that habitat. In contrast, high-temperature methanogens in high H₂ hydrothermal vents such as those supported by serpentinization and following volcanic eruptions (2) may subsist entirely from abiotic H₂ with elevated cell-specific CH₄ production rates and smaller carbon isotope fractionations. Metatranscriptomic analyses coupled with carbon isotope analyses of native CH₄ will help to determine what fraction of methanogenesis in a high-temperature environment is due to interspecies H₂ transfer relative to growth on abiotic H₂. In this manner, we will be better equipped to model cooperative, competitive, and neutral interactions between different species in an environment and predict the biogeochemical outcome of a mixed community living in a habitat.

**MATERIALS AND METHODS**

**Growth media and culture conditions.** *Methanocaldococcus jannaschii* DSM 2661 (37) and *Thermococcus paralvinellae* DSM 27261 (44) were purchased from Deutsche...
Sammlung von Mikroorganismen und Zellkulturen (DSMZ). The growth medium for pure cultures of *M. jannaschii* was based on DSM medium 282 (9). For the co-cultures of *M. jannaschii* and *T. paralvinellae* and monoculture of *T. paralvinellae*, the base medium was amended with 0.01% (wt vol⁻¹) of yeast extract (vitamin B₁₂-fortified; Difco), 1 μM of Na₂WO₄·2H₂O, 0.26 μM of (NH₄)₂Fe(SO₄)₂·6H₂O, and 0.25 μM of (NH₄)₂Ni(SO₄)₂·6H₂O. The primary carbon and energy source added for *T. paralvinellae* was either 0.5% (wt vol⁻¹) maltose (Sigma) or 0.1% (wt vol⁻¹) sodium formate (Fluka). All media were pH-balanced to 6.00 ± 0.05 and reduced with 0.025% (wt vol⁻¹) each of cysteine-HCl and Na₂S·9H₂O before inoculation. To test if *M. jannaschii* can use formate or yeast extract for growth in the absence of H₂, or if they stimulate growth in the presence of H₂, *M. jannaschii* was incubated in monoculture on the base medium amended with 0.1% formate and 0.01% yeast extract or 0.01% yeast extract only as described above, each in serum bottles with 1 additional atm (100 kPa) of either H₂:CO₂ (80%:20%) or N₂:CO₂ (80%:20%) added to the headspace at room temperature prior to incubation.

*M. jannaschii* was grown in monoculture at 82°C and under high and low H₂ concentrations in a chemostat to measure its growth and CH₄ production kinetics and to generate biomass for gene expression analysis. A 2-L bioreactor (Ace Glass All-in-One Benchtop Reactor) with gas flow, temperature (± 0.1°C), and pH (± 0.1 unit, Eutech Instruments pH 200 Series) controls was used with 1.5 L of growth medium. The medium was maintained at pH 6.0 ± 0.1 by the automatic addition of 0.25 mM HCl. For high H₂ conditions, the bioreactor was gassed with a mixture of CO₂ (20.5 ml min⁻¹) and H₂ (132 ml min⁻¹). For low H₂ conditions, the bioreactor was gassed with a mixture of CO₂ (20.5 ml min⁻¹), N₂ (130 ml of gas min⁻¹), and H₂ (2.5 ml min⁻¹). Pure gases were
blended using a mass flow controller (Matheson Tri-Gas) and added to the bioreactor through a single submerged fritted bubbler (Ace Glass, ASTM 70-100 µm). The reactor is an open system and remains at ambient gas pressure. It was stirred at 150-180 rpm using a four-blade open impeller (6 cm diameter) with a glass shaft and Teflon blades.

Aqueous H₂ and CH₄ concentrations were measured before and after inoculation by drawing 25 ml of medium from the bottom of the bioreactor directly into anoxic 60-ml serum bottles and measuring the headspace gas. H₂ was measured using a gas chromatograph fitted with a thermal conductivity detector (Shimadzu GC-8A) and a 60/80 Carboxen 1000 column (15′ × 1/8″, Supelco). CH₄ was measured using a gas chromatograph fitted with a flame ionization detector (Shimadzu GC-17A) and a Molecular Sieve 5A 80/100 column (6′ × 1/8″, Alltech). The aqueous H₂ concentrations in the bioreactor prior to inoculation were 80-83 µM for the high H₂ condition and 15-27 µM for the low H₂ condition (Table 1).

The media were inoculated with 50-100 ml of a logarithmic growth-phase culture of *M. jannaschii*. During growth, liquid samples were drawn from the bioreactor and cell concentrations were determined using phase-contrast light microscopy and a Petroff-Hausser counting chamber. The growth rate (*k*) was determined by plotting cell concentration against time and fitting a logarithmic curve to the growth data. *M. jannaschii* was grown in batch reactor mode until the culture reached mid-logarithmic growth phase, then the bioreactor was switched to chemostat mode by pumping sterile growth medium into the bioreactor from a sealed 12-L reservoir that was degassed with N₂ through a submerged glass tube and heated to 75°C. Simultaneously and at the same rate, spent growth medium was pumped out of the bioreactor using a dual-
channel peristaltic pump. The H₂ and CH₄ concentrations in the headspace of the bioreactor were measured using gas chromatography as described above. At high and low H₂ concentrations, cells were grown in the reactor at low enough cell concentrations such that there was excess H₂ in the headspace and the cells were not H₂ limited (Fig. S1).

Growth of *M. jannaschii* was stable in the chemostat after three volume replacements of the medium within the reactor (~5 h for high H₂, ~14 h for low H₂) and was monitored for an additional ~0.5 volume replacements to obtain kinetic data. The CH₄ production rate per cell (q) was calculated from the sum of the CH₄ concentration in the headspace times the gas flow rate and the CH₄ concentration in the medium times the medium dilution rate (i.e., CH₄ production rate), which was normalized by the total cell concentration in the reactor. The cell yield per mole of CH₄ produced (Y_CH₄) was calculated by dividing the cell production rate (dilution rate × cell concentration) by the CH₄ production rate. Then, the complete contents of the bioreactor were drained into ice-cooled centrifuge bottles, spun in a centrifuge at 10,000 × g and 4°C for 60 min, resuspended in 1 ml of TRIzol (Invitrogen), and frozen at -80°C until processed.

Chemostats were run in triplicate for both conditions. *M. jannaschii* and *T. paralvinellae* were grown in co-culture at 82°C in 2-L gas-tight flasks (Pyrex bottles sealed with rubber lyophilization stoppers) containing 1.5 L of medium with ambient pressure of N₂:CO₂ (80%:20%) in the headspace at room temperature without agitation and either maltose or formate as the energy source (Table 1). Separate logarithmic growth-phase cultures of *M. jannaschii* and *T. paralvinellae* were combined to inoculate the bottles. The co-culture was established immediately and
did not require prior co-culture transfers. At various times during growth, total cell concentration in bottles was determined using a Petroff-Hausser counting chamber and phase-contrast light microscopy. The *M. jannaschii* cell concentration was determined by counting the number of autofluorescent cells using epifluorescence microscopy and ultraviolet light excitation (45). The concentration of *T. paralvinellae* cells was calculated by subtracting the concentration of *M. jannaschii* cells from the total cell concentration. The pH change was < pH 0.1 during growth. For comparison, *T. paralvinellae* was grown separately in the same bottles and conditions in monoculture on 0.5% maltose and separately on 0.1% sodium formate, both with ambient pressure of N$_2$:CO$_2$ in the headspace at room temperature. Cell concentrations were measured as described above.

The growth rates ($k$) of *M. jannaschii* and *T. paralvinellae* were determined by plotting cell concentration against time and fitting a logarithmic curve to the growth data. The total amounts of CH$_4$ and H$_2$ in the bottles were determined by gas chromatography. The concentrations of formate, acetate, butyrate, isovalerate, and 2-methylbutyrate were measured from aliquots of syringe-filtered (0.2 μm pore size) spent medium from each co-culture and *T. paralvinellae* monoculture incubation at various time points (for maltose growth only) using ultra-high-pressure liquid chromatography (UHPLC) as previously described (46). Methanogen cell yields ($Y_{CH4}$) were determined from the linear slope of number of methanogen cells per bottle plotted against the amount of CH$_4$ per bottle (47). The rate of CH$_4$ production per cell is calculated from $k/(0.693 \times Y_{CH4})$ as previously described (47). Similarly, *T. paralvinellae* cell yields based on acetate and formate produced and for H$_2$ produced (for monoculture only) were
determined from the linear slope of *T. paralvinellae* cell concentration plotted against acetate, formate, or H₂ concentration. When the co-cultures reached late logarithmic growth phase, the cells were harvested for transcriptome analysis as described above (*T. paralvinellae* cells were not harvested when grown in monoculture). Co-cultures grown on maltose were grown in triplicate while co-cultures grown on formate were grown in quadruplicate.

**Carbon isotope fractionation.** At the start (T₀) and end (T₁) of each chemostat run, 20 ml of chemostat headspace was transferred in triplicate into evacuated vials (Labco Exetainer). Also, *M. jannaschii* was grown in monoculture in 245 ml serum bottles containing 100 ml of medium and 1 additional atm (100 kPa) of H₂:CO₂ (80%:20%) added to the headspace at room temperature prior to incubation. *M. jannaschii* was also grown in co-culture with *T. paralvinellae* in 245 ml serum bottles containing 100 ml of either 0.5% maltose medium or 0.1% sodium formate medium as described above. The isotopic signatures of CH₄ were determined using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, Thermo Scientific) equipped with a GS-CarbonPlot column (30 m × 0.320 mm i.d., 1.50 µm film thickness, Agilent). Isotopic signatures were determined using external CH₄ standards of known isotopic signatures (-57.40 ± 0.06‰) that were obtained from Arndt Schimmelmann (Indiana University). The error of the analysis was determined from external standards and the standard deviation of multiple injections was 0.3‰. At T₀ and T₁ of the chemostat runs and the serum bottles, triplicate samples of dissolved inorganic carbon (DIC) were drawn from the growth medium. Each DIC sample (either 0.8 or 1.0 ml) was syringe filtered (0.2 µm pore size) and injected into prepared Vials.
(Labco Exetainer) that had been flushed with He and contained 100 μl of phosphoric acid. Samples were analyzed by GasBench-IRMS. DIC standards were prepared in concentrations from 0.5 to 7.0 mM using KHCO$_3$ and Li$_2$CO$_3$ of known isotopic composition (-38.1‰ and -1.1‰, respectively). The error of analysis was determined from external standards and the standard deviation of multiple injections was 0.3‰. The δ$^{13}$C$_{CO2}$ was calculated from the δ$^{13}$C$_{DIC}$ using the relationship of Mook et al. (48) at the temperature of the cultures (82°C).

Carbon isotopic compositions are presented as δ$^{13}$C in the per mil notation (‰) relative to the VPDB standard (Vienna Pee Dee Belemnite):

$$\delta^{13}C = \left[ \frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right] \times 10^3 (\text{‰})$$

where $R_{\text{sample}}$ is the $^{13}$C/$^{12}$C ratio of the sample and $R_{\text{standard}}$ is 0.0112372. The ε notation is used to express isotope fractionation factors in per mil (‰):

$$\varepsilon_{\text{CO2-CH4}} = (\alpha_{\text{CO2-CH4}} - 1) \times 10^3 (\text{‰})$$

The fractionation factor, α, is defined as the ratio between the isotopic ratio in the substrate and product:

$$\alpha_{\text{CO2-CH4}} = \frac{R_{\text{CO2}}}{R_{\text{CH4}}} = \frac{\delta^{13}C_{\text{CO2}} + 10^3}{\delta^{13}C_{\text{CH4}} + 10^3}$$

where $R_{\text{CO2}}$ is the $^{13}$C/$^{12}$C ratio of the initial CO$_2$ and $R_{\text{CH4}}$ is the $^{13}$C/$^{12}$C ratio of the CH$_4$ produced. The propagated error of the fractionation factors was 0.4 ‰ except in the case of the *M. jannaschii* monoculture.

The inorganic carbon in the *M. jannaschii* monoculture serum bottles was extensively drawn down, substantially altering the $^{13}$C signature of the remaining reactant. The fractionation factor was therefore calculated by setting the initial CO$_2$
isotopic signature equal to that in serum bottles without cells (-26.1 ± 0.8 ‰) and reacting it step-wise under different fractionation factors. To obtain final isotopic compositions that match the remaining CO₂ (+18.9 and +15.5‰) and the final accumulated product, CH₄ (-32.9‰ and -34.2‰), required fractionation factors of 22.1 ± 1.3‰ and 23.0 ± 1.3‰ in the two different experiments.

RNA-Seq analysis. Total RNA was extracted from 13 cell pellets from each growth condition (Table 1) using a Direct-zol RNA extraction kit (Zymo). RNA quantity was determined using Qubit fluorometry. RNA integrity was checked using an Agilent 2100 bioanalyzer, a Nanodrop 2000 spectrophotometer, and gel electrophoresis of the RNA followed by staining with ethidium bromide. Removal of rRNA, library construction, multiplexing, and sequencing of the mRNA using an Illumina HiSeq2500 sequencer with 2×150 paired-ends was performed commercially by GENEWIZ, LLC (South Plainfield, NJ, USA) as described by the company. Sequencing depths ranged from 30,751,946 to 41,634,527 sequence reads per sample, with a median of 34,532,231 and a mean of 35,155,474 reads per sample. The RNA-Seq reads were mapped to both *M. jannaschii* and *T. paralvinellae* genomes using BBSplit from BBMap package (https://sourceforge.net/projects/bbmap/). BBSplit is an aligner tool that bins sequencing reads by mapping to them multiple references simultaneously and separates the reads that map to multiple references to a special "ambiguous" file for each of them. For further analyses we removed all ambiguously mapped reads to both genomes and worked with only the reads that unambiguously map to *M. jannaschii* genome. 2-5% of the reads were lost in this step.
The mapped reads for *M. jannaschii* were aligned to the *M. jannaschii* genome and sorted using the STAR aligner version 2.5.1b (49). Aligned sequence reads were assigned to genomic features and quantified using featureCounts read summarization tool (50). The output of the analyses generated BAM files containing the sequence of every mapped read and its mapped location. An unsupervised *t*-distributed stochastic neighbor embedding (*t*-SNE) algorithm (51) and principal component analysis (PCA) were used to predict outliers among the total RNA sample replicates.

Genes that were differentially expressed were identified using ‘DESeq2’ in the Bioconductor software framework (www.bioconductor.org) in R (version 3.3 [http://www.r-project.org]) and on a Galaxy platform using DEBrowser, respectively (52-55). RLE (relative log expression)-normalization was performed by using the R package ‘DESeq2’. The ‘DESeq2’ package allows for sequencing depth normalization between samples, estimates gene-wise dispersion across all samples and fits a negative binomial generalized linear model and applies Wald statistics to each gene. The genes were reported as differentially regulated if $|\log_2 FC| > 1$ and the adjusted $p$-value was $< 0.01$. Heatmaps were plotted in R (version 3.3 [http://www.r-project.org]) using the ‘pheatmap’ package. The heatmap color scale represents the z-score, which is the number of standard deviations the mean score of the treatment is from the mean score of the entire population. The count files and raw sequences are available in the NCBI Gene Expression Omnibus (GEO) database under GSE112986.

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**AUTHOR CONTRIBUTIONS.** B.D.T. and J.F.H. conceived and designed the study, conducted the growth and gene expression experiments, and wrote the paper. B.D.T. and C.M. conducted the gene expression analyses and T.B.N. and S.Q.L. conducted the carbon isotope analyses. All authors analyzed the data and read and approved the final manuscript.

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**Figure Captions**

**Fig. 1** (a) Specific growth rate, (b) cell-specific CH$_4$ production rate ($q$), and (c) cell yield ($Y_{CH4}$) for *M. jannaschii* grown in monoculture in the chemostat with high (80-83 μM) and low (15-27 μM) aqueous H$_2$ and grown in co-culture with *T. paralvinellae* in bottles using maltose and formate as growth substrates. The horizontal bar represents the mean value.

**Fig. 2** (a) Specific growth rate for *T. paralvinellae* grown in bottles in monoculture (-) and in co-culture with *M. jannaschii* (+) on either maltose or formate. Cell-specific production rate for acetate (b) and formate (c) for *T. paralvinellae* grown on maltose in monoculture (-) and in co-culture with *M. jannaschii* (+). The horizontal bar represents the mean value.

**Fig. 3** *M. jannaschii* transcript levels (relative log expression (RLE)-normalization) for F$_{420}$-dependent methylene-H$_4$MPT dehydrogenase (*mtd*, MJ_RS05555) (a) and H$_2$-dependent methylene-H$_4$MPT I (*hmd*, MJ_RS04180) (b) for each growth condition.

**Fig. 4** Differential gene expression analysis and RNA-Seq heat map for the *M. jannaschii* putative ATP synthase operon (MJ_RS01135, MJ_RS01145-01165), and the *M. jannaschii* putative hydrogenase operon (MJ_RS02730, MJ_RS02745-02805) for each growth condition.
**Fig. 5** General metabolic pathway for *M. jannaschii*. The enzymes are (1) formylmethanofuran dehydrogenase, (2) formylmethanofuran:H₄MPT formyltransferase, (3) cyclohydrolase, (4) H₂-dependent methylene-H₄MPT dehydrogenase (Hmd), (5) F₄₂₀-dependent methylene-H₄MPT dehydrogenase (Mtd), (6) methylene-H₄MPT reductase (Mer), (7) CO dehydrogenase/acetyl-CoA synthase, (8) methyl-H₄MPT:CoM methyltransferase, (9) methyl-CoM reductase (Mcr), (10) hydrogenase/heterodisulfide reductase complex, (11) F₄₂₀-dependent hydrogenase, (12) membrane-bound ferredoxin-dependent hydrogenase, and (13) membrane-bound ATP synthase. MFR, methanofuran; H₄MPT, tetrahydromethanopterin; F₄₂₀, electron carrier coenzyme F₄₂₀; CoA, coenzyme A; CoM, coenzyme M; CoB, coenzyme B; and Fd, electron carrier ferredoxin.
**Table 1.** Carbon isotopic composition of CO$_2$ and CH$_4$ of culture and co-culture experiments

| Growth Condition | Initial H$_2$(aq) (μM) | δ$^{13}$C value (%) | ε$_{\text{CO}_2\text{-CH}_4}$ (%) |
|------------------|------------------------|---------------------|-------------------------------|
| **M. jannaschii only:** | | | |
| Chemostat R1     | 83                     | -35.1               | -29.0                         | -55.9   | 28.5  |
| Chemostat R2     | 80                     | -34.6               | -28.2                         | -55.9   | 29.3  |
| Chemostat R3     | 80                     | -35.2               | -28.4                         | -55.8   | 29.0  |
| Chemostat R4     | 18                     | -35.9               | -33.3                         | -75.7   | 45.9  |
| Chemostat R5     | 15                     | -35.7               | -31.8                         | -74.2   | 45.8  |
| Chemostat R6     | 27                     | -35.8               | -32.0                         | -72.5   | 43.7  |
| **Bottle B1**    | 1,200$^a$              | -26.1               | +22.6                         | -32.9   | 22.1$^b$  |
| **Bottle B2**    | 1,200$^a$              | -26.1               | +19.2                         | -34.2   | 23.0$^b$  |
| **M. jannaschii-T. paralvinellae co-culture:** | | | |
| Bottle B3 (formate) | 0                     | -26.7               | -22.8                         | -99.4   | 85.1  |
| Bottle B4 (formate) | 0                     | -26.7               | -23.0                         | -99.4   | 84.8  |
| Bottle B5 (maltose) | 0                     | -25.5               | -24.4                         | -91.2   | 73.5  |
| Bottle B6 (maltose) | 0                     | -25.5               | -21.6                         | -89.0   | 73.9  |

$^a$Estimated at 82°C using The Geochemist's Workbench Standard 10.0 (Aqueous Solutions, LLC, Champaign, Illinois, USA)

$^b$Calculated based on the isotopic compositions of the starting CO$_2$, final CO$_2$, and accumulated methane
Figure 2
Figure 4
