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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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**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 to 83 μM) and low (15 to 27 μM) aqueous H₂ concentrations and in coculture 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 coculture. However, cell yield (cells produced per mole of CH₄ produced) increased 6-fold when *M. jannaschii* was grown in coculture rather than monoculture. Relative to high H₂ concentrations, isotopic fractionation of CO₂ to CH₄ (ƒ₀₂-CH₄) was 16‰ larger for cultures grown at low H₂ concentrations and 45‰ and 56‰ larger for *M. jannaschii* growth in coculture 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 coculture growth. In coculture, 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 H₂-producing heterotrophs are collocated in high-temperature subseafood environments, such as petroleum reservoirs, mid-ocean ridge flanks, and hydrothermal vents. Abiotic flux of H₂ can be very low in these environments, and there is a gap in our knowledge about the origin of CH₄ in these habitats. In the hyperthermophile *Methanocaldococcus jannaschii*, growth yields increased as H₂ flux, growth rates, and CH₄ production rates decreased. The same trend was observed increasingly with interspecies H₂ transfer between *M. jannaschii* and the hyperthermophilic H₂ producer *Thermococcus paralvinellae*. With decreasing H₂ availability, isotopic fractionation of carbon during methanogenesis increased, resulting in isotopically more negative CH₄ with a concomitant decrease in H₂-dependent methylene-tetrahydromethanopterin dehydrogenase gene expression and increase in F₄₂₀-dependent methylene-tetrahydromethanopterin dehydrogenase gene expression. The significance of our research is in understanding the nature of hyperthermophilic interspecies H₂ transfer and identifying biogeochemical and molecular markers.
for assessing the physiological state of methanogens and possible source of \( \text{CH}_4 \) in natural environments.

**KEYWORDS** Methanocaldococcus, RNA-Seq, Thermococcus, carbon isotope fractionation, hydrogen, hyperthermophiles, methanogenesis, syntrophs

Each year, approximately 1 Pg of \( \text{CH}_4 \) 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 \( \text{H}_2 \) syntrophy or interspecies \( \text{H}_2 \) 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 coculture derived from a high-temperature oil pipeline each produced \( \text{CH}_4 \) at 80°C when supplemented with organic compounds, both without added \( \text{H}_2 \) (4, 5). Both showed that \( \text{CH}_4 \) was produced from a mixed microbial community consisting of the hyperthermophilic \( \text{H}_2 \)-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), waters produced by high-temperature petroleum reservoirs (12–19), and mid-ocean ridge flanks (20). In high-temperature, organic-rich environments, such as petroleum reservoirs, collocated \( \text{H}_2 \)-producing heterotrophs are the primary source of \( \text{H}_2 \) (21), but very little is known about this process at high temperatures or how thermophilic syntrophy affects environmental signals.

In this study, growth and \( \text{CH}_4 \) production kinetics, carbon isotope fractionation, and gene expression data were examined together for a hyperthermophilic methanogen under conditions ranging from monoculture growth at high and low \( \text{H}_2 \) concentrations to coculture growth with an \( \text{H}_2 \)-producing partner. The hyperthermophile *Methanocaldococcus jannaschii* was grown in monoculture in a chemostat under \( \text{H}_2 \)-replete and \( \text{H}_2 \)-limited conditions based on previous kinetic experiments (9). It was also grown with the \( \text{H}_2 \)-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 \( \text{CH}_4 \) produced, or \( Y_{\text{CH}_4} \)) increases when cultures are shifted from \( \text{H}_2 \)-replete to \( \text{H}_2 \)-limited growth conditions and if \( Y_{\text{CH}_4} \) remains high or increases further during interspecies \( \text{H}_2 \) transfer. This study also examined if interspecies \( \text{H}_2 \) transfer stimulates the growth rate or cell yield of *T. paralvinellae* or ameliorates its \( \text{H}_2 \) inhibition relative to its growth in monoculture. Furthermore, isotopic carbon fractionation was examined to determine if \( \text{CH}_4 \) is isotopically lighter when \( \text{H}_2 \) flux is reduced, as previously observed in moderately thermophilic methanogens (23–25). Finally, differential gene expression analysis using transcriptome sequencing (RNA-Seq) was used to determine if changes occur in *M. jannaschii* for the expression of genes for carbon assimilation, \( \text{CH}_4 \) production, or energy generation when \( \text{H}_2 \) 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 coculture. The data elucidate how hyperthermophilic methanogens behave in a high \( \text{H}_2 \) flux environment, such as those found at some hydrothermal vents, versus a low \( \text{H}_2 \) flux environment, such as petroleum reservoirs.

**RESULTS**

**Growth parameters for mono- and cocultures.** 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 ± 0.12 h\(^{-1}\) (± standard errors) when grown on 80
to 83 μM H₂ to 0.50 ± 0.09 h⁻¹ when grown on 15 to 27 μM H₂ (Fig. 1A). Cell concentrations in the medium and H₂ and CH₄ concentrations in the headspace remained constant throughout growth in the chemostat (see Fig. S1 in the supplemental material). Attempts to grow *M. jannaschii* in coculture 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. Coculture 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 cocultures 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 coculture 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 coculture bottles and CH₄ was produced (Fig. S2).

### Table 1: Carbon isotopic composition of CO₂ and CH₄ of culture and coculture experiments

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>Initial H₂ (aq) (μM)</th>
<th>CO₂ (aq)</th>
<th>CH₄</th>
<th>CO₂-CH₄ (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. jannaschii only</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemostat R1</td>
<td>83</td>
<td>−35.1</td>
<td>−29.0</td>
<td>−55.9</td>
</tr>
<tr>
<td>Chemostat R2</td>
<td>80</td>
<td>−34.6</td>
<td>−28.2</td>
<td>−55.9</td>
</tr>
<tr>
<td>Chemostat R3</td>
<td>80</td>
<td>−35.2</td>
<td>−28.4</td>
<td>−55.8</td>
</tr>
<tr>
<td>Chemostat R4</td>
<td>18</td>
<td>−35.9</td>
<td>−33.3</td>
<td>−75.7</td>
</tr>
<tr>
<td>Chemostat R5</td>
<td>15</td>
<td>−35.7</td>
<td>−31.8</td>
<td>−74.2</td>
</tr>
<tr>
<td>Chemostat R6</td>
<td>27</td>
<td>−35.8</td>
<td>−32.0</td>
<td>−72.5</td>
</tr>
<tr>
<td>Bottle B1</td>
<td>1,200*</td>
<td>−26.1</td>
<td>+22.6</td>
<td>−32.9</td>
</tr>
<tr>
<td>Bottle B2</td>
<td>1,200*</td>
<td>−26.1</td>
<td>+19.2</td>
<td>−34.2</td>
</tr>
</tbody>
</table>

*Estimated at 82°C using the Geochemist’s Workbench Standard 10.0 (Aqueous Solutions, LLC, Champaign, Illinois, USA).*

*Calculated based on the isotopic compositions of the starting CO₂, final CO₂, and accumulated methane.

*aq, aqueous concentration.*

### Table 1: Carbon isotopic composition of CO₂ and CH₄ of culture and coculture experiments

![Fig 1](a to c) Specific growth rate (a), cell-specific CH₄ production rate (b), and cell yield (Y<sub>CH₄</sub>) (c) for *M. jannaschii* grown in monoculture in the chemostat with high (80 to 83 μM) and low (15 to 27 μM) aqueous H₂ concentration and grown in coculture with *T. paralvinellae* in bottles using maltose and formate as growth substrates. The horizontal bar represents the mean value.
The cell-specific CH$_4$ production rate decreased 3.6-fold when *M. jannaschii* was grown on 15 to 27 µM H$_2$ (139 ± 8 fmol cell$^{-1}$ h$^{-1}$) relative to growth on 80 to 83 µM H$_2$ (496 ± 21 fmol cell$^{-1}$ h$^{-1}$) (Fig. 1B). The rates decreased further when grown in coculture 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 ($Y_{CH_4}$) for *M. jannaschii* grown in coculture were significantly higher when grown on maltose ($9.1 ± 1.9 \times 10^{12}$ cells per mol CH$_4$) and formate ($13.5 ± 2.0 \times 10^{12}$ cells per mol CH$_4$) than growth yields in monoculture on 15 to 27 µM H$_2$ ($2.1 ± 0.2 \times 10^{12}$ cells per mol CH$_4$) and 80 to 83 µM H$_2$ ($1.5 ± 0.1 \times 10^{12}$ cells per mol CH$_4$) (Fig. 1C). Summaries of the growth and CH$_4$ production kinetics data for *M. jannaschii* are available in the supplemental material (Fig. S1 and S2 and 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 a change in carbon source (Fig. 2A and Fig. S2). The specific growth rates of *T. paralvinellae* grown on maltose in monoculture and in coculture were 0.16 ± 0.01 h$^{-1}$ and 0.22 ± 0.02 h$^{-1}$, respectively, while growth rates on formate in monoculture and in coculture were 0.18 ± 0.05 h$^{-1}$ 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$^{-1}$) relative to growth in coculture ($1.05 ± 0.15$ 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$^{-1}$) than for monoculture growth on maltose ($9.9 ± 0.1$ 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 and Table 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 an H$_2$:CO$_2$ headspace was provided.

**Carbon isotope fractionation.** The final carbon isotopic composition ($\delta^{13}C_{CO_2}$) values were $-24.4$ to $-21.6\%$ in the coculture bottles and $-33.3$ to $-28.2\%$ in the...
chemostat (Table 1). The final $\delta^{13}$C$_{\text{CO}_2}$ values of the *M. jannaschii* monocultures in bottles were +19.2 to +22.6‰, demonstrating a substantial drawdown of the reactant. $\delta^{13}$C$_{\text{CH}_4}$ values became increasingly negative with increasing H$_2$ limitation during cell growth. The $\delta^{13}$C$_{\text{CH}_4}$ values in the chemostat were −55.9 to −55.8‰ when *M. jannaschii* was grown on 80 to 83 μM H$_2$ and decreased to −75.7 to −72.5‰ when grown on 15 to 27 μM H$_2$. The corresponding values for isotopic fractionation of CO$_2$ to CH$_4$ (CO$_2$-CH$_4$) 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$_{\text{CH}_4}$ values became more negative with increasing H$_2$ limitation during coculture cell growth. In monoculture with 1.92 atm of initial H$_2$ in the headspace at 82°C, the $\delta^{13}$C$_{\text{CH}_4}$ from *M. jannaschii* was −34.2 to −32.9‰ (Table 1). $\delta^{13}$C$_{\text{CH}_4}$ values decreased to −91.2 to −89.0‰ when *M. jannaschii* was grown in coculture with *T. paralvinellae* on maltose and to −99.4‰ when grown in coculture on formate. The corresponding 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 coculture 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 value of <0.01 and log$_2$ fold change [log$_2$(FC)] of >1) with 1 gene downregulated and 11 genes upregulated 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 (H$_4$MPT) dehydrogenase (mtd, MJ_RS05555 in the NCBI RefSeq database) gene expression increased 3.5-fold (Fig. 3A). There was no significant change in gene expression for H$_2$-dependent methylene-H$_4$MPT dehydrogenases (hmd, MJ_RS04180; hmdX, MJ_RS03820) (Fig. 3B and Fig. S4) or for any of the methylcoenzyme M (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 H$_2$ in the chemostat. The genes that code 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 upregulated on low H$_2$, while a gene that codes for a histone (MJ_RS04990) was upregulated on high H$_2$ (Table S4).

For cocultures grown on maltose, 97% of the reads mapped unambiguously to the *T. paralvinellae* genome and 1.5% mapped to the *M. jannaschii* genome. For cocultures 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 coculture.
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 coculture with T. *paralvinellae* showed up to 338 genes to be differentially expressed (adjusted $P$ value of $<0.01$ and $|\log_{2} FC|$ of $>1$) with 146 upregulated genes and 192 downregulated genes when grown in coculture relative to growth in monoculture on high and low $H_{2}$ (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 upregulated 4.3-fold in coculture relative to that of M. *jannaschii* grown under monoculture conditions (Fig. 3A). In contrast, gene expression of $H_{2}$-dependent methylene-$H_{4}$MPT dehydrogenases ($hmd$, MJ_RS04180; $hmdX$, MJ_RS03820) were both downregulated 2.1-fold in M. *jannaschii* grown in coculture relative to that of M. *jannaschii* grown in monoculture (Fig. 3B and Fig. S4). There was no change in gene expression for the methyl-CoM reductase I and II genes (Fig. S5). Gene expression for a hypothetical protein with a predicted RNA-binding domain (MJ_RS03480) showed a 22.5-fold increase in cocultures relative to monocultures (Fig. S6). Expression of 6 of the 9 M. *jannaschii* genes that code for a V-type ATP synthase (MJ_RS01130 to MJ_RS01165 and MJ_RS03255) were downregulated when cultures were grown in coculture 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 was also downregulated in M. *jannaschii* cultures grown in cocultures relative to cultures grown in monoculture (Fig. 4). These genes include Eha subunits A and B (MJ_RS02795 to MJ_RS02800), 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 various $H_{2}$ concentrations and in coculture 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 ($\gamma_{CH4}$) 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 $\text{H}_2$ transfer or for any hyperthermophilic methanogens under various $\text{H}_2$ concentrations.

To determine $\textit{M. jannaschii}$ metabolism and kinetics under $\text{H}_2$-replete and $\text{H}_2$-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 $\textit{M. jannaschii}$ when grown in monoculture under $\text{H}_2$-limited conditions show that growth and methanogenesis rates are limited by $\text{H}_2$ concentration. This trend continued when $\textit{M. jannaschii}$ was grown in coculture with $\textit{T. paralvinellae}$, suggesting that interspecies $\text{H}_2$ transfer led to further $\text{H}_2$ limitation of methanogenesis. However, the cell yield for $\textit{M. jannaschii}$ increased when the cells were grown in coculture relative to growth in monoculture. This is consistent with previous studies that show higher cell yields for $\textit{M. thermoautotrophicus}$ and $\textit{M. maripaludis}$ upon $\text{H}_2$ 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 $\text{H}_2$ concentration, hydrogenotrophic methanogens decide between maximum growth rate and maximum growth yield. This pattern can be explained by the rate-yield trade-off, which creates two divergent ecological strategies, namely, (i) slow growth but efficient metabolism and high yields when resources are scarce, and (ii) fast growth but inefficient metabolism and low yields upon rich resources. The rate-yield trade-off is suggested to be integral to evolution and the coexistence of

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**FIG 5** General metabolic pathway for $\textit{M. jannaschii}$. The enzymes are (1) formylmethanofuran dehydrogenase, (2) formylmethanofuran-H$_4$MPT formyltransferase, (3) cyclohydrolase, (4) $\text{H}_2$-dependent methylene-H$_4$MPT dehydrogenase (Hmd), (5) F$_{420}$-dependent methylene-H$_4$MPT dehydrogenase (Mtd), (6) methylene-H$_4$MPT reductase (Mer), (7) CO dehydrogenase/acetyl-CoA synthase, (8) methyl-H$_4$MPT:CoM methyltransferase, (9) methyl-CoM reductase (Mcr), (10) hydrogenase-heterodisulfide reductase complex, (11) F$_{420}$-dependent hydrogenase, (12) membrane-bound ferredoxin-dependent hydrogenase, and (13) membrane-bound ATP synthase. MFR, methanofuran; H$_4$MPT, tetrahydromethanopterin; F$_{420}$, electron carrier coenzyme F$_{420}$; CoA, coenzyme A; CoM, coenzyme M; CoB, coenzyme B; and Fd, electron carrier ferredoxin.
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 CH₄ solely on the H₂ produced by *T. paralvinellae*, and the cell yield of *M. jannaschii* increased in coculture compared to that of growth in monoculture.

*Thermococcus* species use maltose for biosynthesis and energy generation that yields acetate and CO₂ as well as H₂ and a proton/sodium-motive force via a membrane-bound hydrogenase (30, 31). However, they are auxotrophic for certain amino acids that 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 H₂, suggesting that it converts H₂ to formate when H₂ is inhibited (22). *T. paralvinellae* also separately used formate as an energy source in the absence of maltose, produced H₂, and generated a proton/sodium-motive force but required 0.01% yeast extract in the growth medium (22). Consequently, the cell-specific H₂ 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 coculture growth between the hyperthermophilic H₂ producer *Pyrococcus furiosus* and various hyperthermophilic methanogens, including *M. jannaschii*, as an example based on increased cell concentrations of both organisms in coculture 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 coculture 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 coculture on maltose with *M. jannaschii*, and *M. jannaschii* cannot grow on formate (37 and this study), so *M. jannaschii* does appear to ameliorate H₂ inhibition in *T. paralvinellae* when grown in coculture.

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 $r_{CO₂:CH₄}$ 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 $r_{CO₂:CH₄}$ fractionation factor increased when *M. jannaschii* was grown in the chemostat with low H₂ relative to that of H₂-replete growth (27, 28, 38). It was suggested that the Mtd reaction is the more reversible of the two methane-H₄MPT dehydrogenase reactions, which facilitates enhanced carbon isotope fractionation by methanogenesis pathway reversal in these methanogens under H₂-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 H₂-
limited conditions than under H$_2$-replete conditions, but both Hmd and Mtd were found at high relative abundances in late logarithmic growth phase when grown under H$_2$-replete conditions (39). During H$_2$ syntrophy, the *M. thermoautotrophicus* proteome had more Mtd and less Hmd than were seen with monoculture growth under 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 that of an 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 coculture, *mtd* expression was significantly upregulated and *hmd* expression was significantly downregulated in coculture cells compared to that of 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 coculture 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 code for the enzyme that converts methyl-MPT to acetyl-CoA.

In coculture, there was up to a 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 coculture. Under H$_2$-limited coculture conditions, the cell must direct more of its methyl-H$_4$MPT toward 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-H$_2$ hydrothermal vents along subduction zones and some mid-ocean ridges, oil reservoirs, and high saline shale beds where organic compounds are present and H$_2$ efflux rates are low, thermophilic methanogens like *M. jannaschii* likely can grow and produce CH$_4$ through interspecies H$_2$ transfer with hyperthermophilic H$_2$-producing heterotrophs, like *T. paralvinellae*, with high cell yields and large carbon isotope fractionations, but they do so at very low rates. This likely explains the presence of thermophilic H$_2$ producers and thermophilic, hydrogenotrophic methanogens in petroleum reservoirs and may be a source of CH$_4$ in that habitat. In contrast, high-temperature methanogens in high-H$_2$ hydrothermal vents, such as those supported by serpentinitization and following volcanic eruptions (2), may subsist entirely from abiotic H$_2$ with elevated cell-specific CH$_4$ production rates and smaller carbon isotope fractionations. Metatranscriptomic analyses coupled with carbon isotope analyses of native CH$_4$ will help to determine what fraction of methanogenesis in a high-temperature environment is due to interspecies H$_2$ transfer relative to growth on abiotic H$_2$. 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 the 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 cocultures of *M. jannaschii* and *T. paralvinellae* and monoculture of *T.
paralvinellae, the base medium was amended with 0.01% (wt vol⁻¹) yeast extract (vitamin B₁₂ fortified; Difco), 1 μM Na₂WO₄·2H₂O, 0.26 μM (NH₄)₂Fe(SO₄)₂·6H₂O, and 0.25 μM (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 (Fukaka). All media were pH balanced to 6.0 ± 0.5 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, in each 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-liter bioreactor (all-in-one benchtop reactor; Ace Glass) was filled with gas flow, temperature (±0.1 °C), and pH (±0.1 unit; Eutech Instruments pH 200 Series) controls was used with 1.5 liters 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 (70 to 100 μm; Ace Glass; ASTM certified). The reactor is an open system and remains at ambient gas pressure. It was stirred at 150 to 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 removing 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 feet by 1/8 inch; Supelco). CH₄ was measured using a gas chromatograph fitted with a flame ionization detector (Shimadzu GC-17A) and a 5A/80/100 molecular sieve column (6 feet by 1/8 inch; Alltech). The aqueous H₂ concentrations in the bioreactor prior to inoculation were 80 to 83 μM for the high-H₂ condition and 15 to 27 μM for the low-H₂ condition (Table 1).

The media were inoculated with 50 to 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 bright 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, and then the bioreactor was switched to chemostat mode by pumping sterile growth medium into the bioreactor from a sealed 12-liter 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 (see Fig. S1 in the supplemental material).

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 steady state 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 (YCELL) was calculated by dividing the cell production rate (dilution rate times cell concentration) by the CH₄ production rate. The complete contents of the bioreactor then 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 coculture at 82°C in 2-liter gas-tight flasks (Pyrex bottles sealed with rubber lyophilization stoppers) containing 1.5 liters 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 coculture was established immediately and did not require prior coculture 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 UV 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 <0.1 pH units 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 N₂, CO₂ 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 CH₄ and H₂ 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 coculture 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 (\(Y_{\text{cell}}\)) were determined from the linear slope of the number of methanogen cells per bottle plotted against the amount of \(\text{CH}_4\) per bottle (47). The rate of \(\text{CH}_4\) production per cell is calculated from \(k(0.693 \times Y_{\text{cell}})\) as previously described (47). Similarly, *T. paralvinellae* cell yields based on acetate and formate produced and for \(\text{H}_2\) produced (for monoculture only) were determined from the linear slope of *T. paralvinellae* cell concentration plotted against acetate, formate, or \(\text{H}_2\) concentration. When the cocultures 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). Cocultures grown on maltose were grown in triplicate, while cocultures grown on formate were grown in quadruplicate.

### Carbon isotope fractionation

At the start \((T_s)\) and end \((T_f)\) of each chemostat run, 20 ml of chemostat headspace was transferred in triplicate into evacuated vials (Labco Exetainer). *M. jannaschii* was grown in monoculture in 245-ml serum bottles containing 100 ml of medium and 1 additional atm (100 kPa) of \(\text{H}_2\text{CO}_3\) (80% ± 20%) added to the headspace at room temperature prior to incubation. *M. jannaschii* was also grown in coculture 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 \(\text{CH}_4\) were determined using a gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS; Thermo Scientific) equipped with a GS-CarbonPlot column (30 m long, 0.320-mm inner diameter, 1.50-μm film thickness; Agilent). Isotopic signatures were determined using external CH4 standards of known isotopic compositions \((-57.40 ± 0.06\‰)\) 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 \(T_s\) and \(T_f\) 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 KHCO₃ and Li₂CO₃ 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 \(\delta^{13}C\text{DIC}\) value was calculated from the \(\delta^{13}C\text{CO}_2\) value using the relationship of Mooß et al. (48) at the temperature of the cultures (82°C).

Carbon isotopic compositions are presented as \(\delta^{13}C\) in the per mille notation (‰) relative to the VPD8 (Vienna Pee Dee Belemnite) standard:

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

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

\[
\epsilon_{\text{CO}_2-\text{CH}_4} = \left( \frac{R_{\text{CO}_2}}{R_{\text{CH}_4}} - 1 \right) \times 10^3 \, (\%o) \tag{2}
\]

The fractionation factor, \(\alpha\), is defined as the ratio between the isotopic ratio in the substrate and product:

\[
\alpha_{\text{CO}_2-\text{CH}_4} = \frac{R_{\text{CO}_2}}{R_{\text{CH}_4}} = \frac{\delta^{13}C\text{CO}_2 + 10^3}{\delta^{13}C\text{CH}_4 + 10^3} \tag{3}
\]

where \(R_{\text{CO}_2}\) is the \(^{13}C/^{12}C\) ratio of the initial \(\text{CO}_2\) and \(R_{\text{CH}_4}\) is the \(^{13}C/^{12}C\) ratio of the \(\text{CH}_4\) 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 bottle samples was extensively drawn down, substantially altering the \(^{13}C\) signature of the remaining reactant. The fractionation factor was therefore calculated by setting the initial \(\text{CO}_2\) isotopic signature equal to that in serum bottles without cells \((-26.1 ± 0.8‰)\) and reacting it stepwise under different fractionation factors. To obtain final isotopic compositions that match the remaining \(\text{CO}_2\) \((+18.9\) and \(+15.5‰)\) and the final accumulated product, \(\text{CH}_4\) \((-32.9‰\) and \(-34.2‰)\), fractionation factors of 22.1 ± 1.3‰ and 23.0 ± 1.3‰ were required 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 two 150-bp 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 BBMap split from the BBMap package (https://sourceforge.net/projects/bbmap/). BBMap is an aligner tool that bins sequencing reads by mapping them to 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 the *M. jannaschii* genome. Two to 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 the 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-SNE algorithm (51) and 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 (https://www.bioconductor.org) in R (version 3.3 [http://www.r-project.org]) and on a Galaxy platform using DEBrowser (52–55). 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, fits a negative binomial generalized linear model, and applies Wald statistics to each gene. The genes were reported as differentially regulated if the \( |\text{log2FC}| \) value was \( >1 \) 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.

Data availability. The count and raw sequences are available in the NCBI Gene Expression Omnibus (GEO) database under accession no. GSE112986.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00180-19.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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