Biological methane production under putative Enceladus-like conditions

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The detection of silica-rich dust particles, as an indication for ongoing hydrothermal activity, and the presence of water and organic molecules in the plume of Enceladus, have made Saturn’s icy moon a hot spot in the search for potential extraterrestrial life. Methanogenic archaea are among the organisms that could potentially thrive under the predicted conditions on Enceladus, considering that both molecular hydrogen (H2) and methane (CH4) have been detected in the plume. Here we show that a methanogenic archaeon, Methanothermococcus okinawensis, can produce CH4 under physicochemical conditions extrapolated for Enceladus. Up to 72% carbon dioxide to CH4 conversion is reached at 50 bar in the presence of potential inhibitors. Furthermore, kinetic and thermodynamic computations of low-temperature serpentinization indicate that there may be sufficient H2 gas production to serve as a substrate for CH4 production on Enceladus. We conclude that some of the CH4 detected in the plume of Enceladus might, in principle, be produced by methanogens.
Sатурна’s icy moon Enceladus emits jets of mainly water (H2O) from its south-polar region. Besides H2O, the ion and neutral mass spectrometer (INMS) onboard NASA’s Cassini probe detected methane (CH₄), carbon dioxide (CO₂), ammonia (NH₃), molecular nitrogen (N₂), and molecular hydrogen (H₂) in the plume. In addition, carbon monoxide (CO) and ethene (C₂H₂) were found among other substances with moderate ambiguity. The most prominent potential source of H₂ in Enceladus’ interior may be oxidation of native and ferrous iron in the course of serpentinization of olivine in the chondritic core. Olivine hydrolysis at low temperatures is a key process for sustaining chemolithoautotrophic life on Earth and if H₂ is produced in significant amounts on Enceladus, then it could also serve as a substrate for biological CH₄ production.

Considering that 139°C to 160°C, pressures of 400-1000 bar, and temperatures between 0 and above 90°C in the absence of oxygen, methanogens can produce CH₄ in the plume of Enceladus due to biological methanogenesis, the question was raised if CH₄ detected in the plume of Enceladus could in principle also originate from biological activity.

To date, methanogenic archaea are the only known microorganisms that are capable of performing biological CH₄ production in the absence of oxygen. On Earth, methanogens are found in a wide range of pH (4.5–10.2), temperatures (<0–122°C), and pressures (0.005–759 bar), with overlap with conditions predicted in Enceladus’ subsurface ocean, i.e., temperatures between 0 and above 90°C, pressures of 40–100 bar, a pH between 8.5–10.5 and 10.8–13.5, and a salinity in the range of our oceans. While autotrophic, hydrogenotrophic methanogens might metabolise some of the compounds found in Enceladus’ plume, other compounds which were detected in the plume with different levels of ambiguity, such as formalddehyde (CH₂O), methanol (CH₃OH), NH₃, CO, and C₂H₂ are known to inhibit growth of methanogens on Earth at certain concentrations.

Here we show that methanogens can produce CH₄ under Enceladus-like conditions, and that the estimated H₂ production rates on this icy moon can potentially be high enough to support autotrophic, hydrogenotrophic methanogenic life.

## Results

### Effect of gaseous inhibitors on methanogens

To investigate the growth of methanogens under Enceladus-like conditions, three thermophilic and methanogenic strains, Methanothermococcus okinawensis (65°C), Methanothermobacter marburgensis (65°C), and Methanococcus villosus (80°C), all able to fix carbon and gain energy through the reduction of CO₂ with H₂ to form CH₄, were investigated regarding growth and biological CH₄ production under different headspace gas compositions.

### Table 1 Compilation of Cassini’s INMS data on Enceladus’ plume composition over the last decade

| Species  | Volume mixing ratio |
|----------|---------------------|
|          | Waite et al. 2006³  | Waite et al. 2009⁴  | Waite et al. 2011²⁵ | Perry et al. 2015²⁶ | Bouquet et al. 2015⁵  | Waite et al. 2017b²  |
| H₂O      | 90.7±9.15           | 90.0±1.0            | 92.0±3.0             | >90                 | 87                 | 96–99               |
| CO₂      | 3.14–3.26           | 5.3±0.1             | 0.8±0.3              | 0.6±0.2             | 0.52               | 0.3–0.8             |
| CO       | (3.29–4.27)         | (4.4)               | <1.5                 | <1.5                | <0.64              | 0.4–1.4             |
| H₂       | (39)                | <3.4±1.0            | 1–5                  | 1–5                 | 11                 | 0.4–1.4             |
| CH₂O     | 0.31±0.01           | <0.032              | 0.003±0.002          | 0.003±0.002         | 0.002±0.002        | 0.002±0.001         |
| CH₃OH    | 0.015±0.006         | 0.003±0.002         | 0.003±0.001          | 0.9±0.04            | 0.61               | 0.4–1.3             |
| C₂H₄     | <1.2                | 0.002±0.0010        | 0.003±0.001          | 0.9±0.04            | ≤0.61              | ≤0.12               |
| H₂S      | 0.82±0.02           | 0.8±0.03            | 0.9±0.04             | 0.61                | 0.19               | 0.1–0.3             |
| N₂       | (3.29–4.27)         | <1.1                | <0.74                | <0.74               | 0.19               | 0.1–0.3             |
| HCN      | 1.63–1.68           | 0.91±0.05           | 0.21±0.09            | 0.2±0.1             | 0.19               | 0.1–0.3             |

* Values used in this study are marked in bold

These recent observations based on the data of flyby E21 lead to the assumption that H₂O is even more prominent, whereas the concentrations for the other major species (NH₃, CO₂, and CH₄) varied only slightly. The other components were characterised as minor species with moderate ambiguity (e.g., CO, N₂, C₂H₄, or CH₂O) or as potential species with high ambiguity (e.g., H₂S or CH₃OH)

### Table 2 Composition of the different test gases for the low-pressure experiments

|          | H₂ (Vol.-%) | CO₂ (Vol.-%) | CO (Vol.-%) | CH₄ (Vol.-%) | N₂ (Vol.-%) | C₂H₄ (Vol.-%) |
|----------|-------------|--------------|-------------|--------------|-------------|--------------|
| H₂/CO₂   | 80.097      | 19.903       | —           | —            | —           | —            |
| H₂/CO    | 80.290      | —            | 19.710      | —            | —           | —            |
| H₂       | 99.999      | —            | —           | —            | —           | —            |
| Mix 1    | 22.900      | 19.490       | 27.790      | 14.430       | 15.390      | —            |
| Mix 2    | 22.430      | 19.210       | 28.151      | 14.510       | 12.410      | 3.289        |
While *M. okinawensis*, *M. marburgensis*, and *M. villosus* all showed growth on H$_2$/CO$_2$ to similar optical densities, no growth of *M. marburgensis* could be observed when C$_2$H$_4$ (Mix 2) was supplied in the headspace (Fig. 1). Growth of both *M. villosus* and *M. okinawensis* was observed even when CO and C$_2$H$_4$ were both present in the headspace gas. However, while *M. villosus* showed prolonged lag phases and irregular growth under certain conditions, *M. okinawensis* grew stably and reproducibly on the different gas mixtures without extended lag phases (Fig. 1). As expected, the final optical densities did not reach those of the experiments with H$_2$/CO$_2$, likely because in Mix 1 and Mix 2 lower absolute amounts of convertible gaseous substrate (H$_2$/CO$_2$) were available compared to the growth under pure H$_2$/CO$_2$. Consequently, growth kinetics showed a different, gas-limited linear inclination in the closed batch setup when using Mix 1 and Mix 2. Due to its reproducible growth, *M. okinawensis* was chosen for more extensive studies on biological CH$_4$ production under putative Enceladus-like conditions.

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**Fig. 1** Influence of the different headspace gas compositions on growth of *M. marburgensis*, *M. villosus*, and *M. okinawensis*. The error bars show standard deviations calculated from triplicates. OD curves of *M. marburgensis* for (a-c) H$_2$/CO$_2$, (d-f) H$_2$/CO, (g-i) H$_2$, (j-l) Mix 1, and (m-o) Mix 2. Growth of *M. marburgensis* was inhibited by the presence of C$_2$H$_4$ (see Table 2 for detailed gas composition). Only *M. marburgensis* seemed to be able to use sodium hydrogen carbonate (supplied in the medium) as C-source in case of a lack of CO$_2$ (H$_2$ or H$_2$/CO as sole gas in the headspace). Both, *M. villosus* and *M. okinawensis* showed growth when Mix 1 and Mix 2 were applied to the serum bottle headspace; however, *M. villosus* exhibited extended lag phases. The dips in the graphs (b, c) were caused by substrate limitation due to depletion of serum bottle headspace of H$_2$/CO$_2$ at high-optical cell densities.
Fig. 2 Schematic of the experimental setting and DoE raw data growth curves showing OD measurements. The DoE is based on a central composite design (figure in the upper left corner). NH₄Cl, CH₂O, and CH₃OH were used as factors during the experiment and systematically varied in a multivariate design space (see Supplementary Table 1 for the concrete values). Each of the factors setting was examined in triplicates. The centre point (O) was examined in quintuplicates. The colours of the dots and the letters of the figure in the upper left corner correspond to the growth curves. The line labelled ZC represents the optical density of a corresponding zero control experiment, which was done with the same medium as the experiments labelled with O (central point), but without inoculum. The different colours represent different performances. For better readability, the error bars in this diagram were excluded, which were in a standard deviation range between 0.0009 and 0.1544. According to statistical selection criteria three experiments (one experiment F and two experiments O) were excluded from ANOVA analysis (Supplementary Table 2)

Table 3 Concentrations of gaseous species in growth medium

| Gas phase                  | P_H2 (bar)a | P_CO2 (bar)a | Concentration (H2, mol L⁻¹) | Concentration (CO2, mol L⁻¹) |
|----------------------------|-------------|--------------|----------------------------|----------------------------|
| 120 mL bottles (2 bar)     |             |              |                            |                            |
| H₂/CO₂                 | 2.40        | 0.60         | 1.52 × 10⁻³               | 7.73 × 10⁻³                |
| H₂/CO                 | 2.41        |              | 1.52 × 10⁻³               |                            |
| H₂                      | 3.00        |              | 1.89 × 10⁻³               |                            |
| Mix 1                   | 0.69        | 0.58         | 4.34 × 10⁻⁴               | 7.57 × 10⁻⁵                |
| Mix 2                   | 0.67        | 0.58         | 4.25 × 10⁻⁴               | 7.46 × 10⁻⁵                |
| 0.7 L reactor pressure tests |         |              |                            |                            |
| H₂/CO₂                 | 8.00        | 2.00         | 5.05 × 10⁻₃               | 2.59 × 10⁻²                |
| H₂/CO₂ (-10 bar)        | 16.00       | 4.00         | 1.01 × 10⁻²               | 5.18 × 10⁻²                |
| H₂/CO₂ (-50 bar)        | 40.00       | 10.00        | 2.53 × 10⁻²               | 1.29 × 10⁻¹                |
| H₂/CO₂/N₂ (-20 bar)     | 8.00        | 2.00         | 5.05 × 10⁻³               | 2.59 × 10⁻²                |
| H₂/CO₂/N₂ (-90 bar)     | 36.00       | 9.00         | 2.27 × 10⁻²               | 1.17 × 10⁻¹                |
| 2.0 L reactor pressure and inhibitor tests I |         |              |                            |                            |
| Mix³ (-10 bar)          | 5.60        | 0.70         | 3.53 × 10⁻³               | 9.07 × 10⁻³                |
| Mix³ (-25 bar)          | 10.40       | 1.20         | 6.57 × 10⁻³               | 1.55 × 10⁻²                |
| H₂/CO₂/ (±20 bar)       | 16.30       | 4.00         | 1.03 × 10⁻²               | 5.18 × 10⁻²                |
| Mix³ (-50 bar)          | 27.50       | 3.30         | 1.74 × 10⁻²               | 4.01 × 10⁻²                |
| 2.0 L reactor pressure and inhibitor tests II |         |              |                            |                            |
| Mix³ (-10 bar)          | 5.90        | 0.70         | 3.72 × 10⁻³               | 9.06 × 10⁻³                |
| Mix³ (-25 bar)          | 11.00       | 1.20         | 6.94 × 10⁻³               | 1.55 × 10⁻²                |
| H₂/CO₂/ (±20 bar)       | 16.30       | 4.00         | 1.03 × 10⁻²               | 5.18 × 10⁻²                |
| Mix³ (-50 bar)          | 27.70       | 3.30         | 1.75 × 10⁻²               | 4.27 × 10⁻²                |
| Cassini                 |             |              |                            |                            |
| 1 bar                   | 0.50        | 0.10         | 3.18 × 10⁻⁴               | 1.31 × 10⁻³                |
| 50 bar                  | 25.21       | 5.04         | 1.59 × 10⁻²               | 6.53 × 10⁻²                |

a Detailed composition of the gases can be found in Table 2
b For the H₂/CO₂ experiments a ratio of 4:1 was applied
c For the H₂/CO₂/N₂ experiments a ratio of 4:1:5 was applied
d Detailed composition of the gases can be found in Table 4
e For the Cassini estimations, a hydrostatic pressure assumed to prevail in the Enceladus’ ocean (50 bar) was applied. H₂ and CO₂ mixing ratio was taken from Table 1
M. okinawensis tolerates Enceladus-like conditions at 2 bar. Growth and turnover rates (calculated via the decrease in headspace pressure) of M. okinawensis cultures were determined in the presence of selected putative liquid inhibitors detected in Enceladus’ plume (NH3, given as NH4Cl, CH2O, and CH3OH). While growth of M. okinawensis could still be observed at the highest concentration of NH4Cl added to the medium (16.25 g L\(^{-1}\) or 0.30 mol L\(^{-1}\)), the organism grew only in the presence of up to 0.28 mL L\(^{-1}\) (0.01 mol L\(^{-1}\)) CH2O. This is less than, but importantly still in the same order of magnitude of, the observed maximum value of 0.343 mL L\(^{-1}\) CH2O detected in the plume\(^{25}\). Growth and CH4 production of M. okinawensis in closed batch cultivation was shown at CH2OH and NH4Cl concentrations exceeding those reported for Enceladus’ plume\(^{4,5,25,26}\).

To explore how the presence of these inhibitors might influence growth and turnover rates of M. okinawensis, we have applied these compounds at various concentrations in a multivariate design space setting (Design of Experiment (DoE)). At different concentrations of CH3OH, CH2OH, and NH4Cl, M. okinawensis cultures showed growth (Fig. 2) and turnover rates from 0.015 ± 0.012 to 0.084 ± 0.018 h\(^{-1}\) (Supplementary Fig. 1; experiments L and K in Fig. 2). CH2OH amendments at concentrations between 9.09 and 210.91 µmol L\(^{-1}\) did not reduce or improve growth of M. okinawensis (Fig. 2 and Supplementary Tables 1 and 2). Compared to the highest applied CH2OH concentration, the turnover rate of M. okinawensis was ~5.6-fold higher at the lowest tested concentration. The results of this experiment indicated that M. okinawensis possessed a physiological tolerance towards a broad multivariate concentration range of CH2OH, CH2OH, and NH4Cl and was able to perform the autocatalytic conversion of H2/CO2 to CH4 while gaining energy for growth.

We used the mean liquid inhibitor concentrations for CH2OH determined in the DoE experiment (DoE centre points) and Enceladus-like concentrations for CH2OH and NH4Cl (Supplementary Table 3) to test growth and turnover rates of M. okinawensis, using different gases in the headspace (H2/CO2, Mix 1, and Mix 2 (Fig. 3)). Under all tested headspace gas compositions, M. okinawensis showed gas-limited growth (max. OD values of 0.67 ± 0.02, 0.17 ± 0.03, and 0.13 ± 0.03 after ~237 h for H2/CO2, Mix 1 and Mix 2, respectively). The calculated turnover rates correlated with the different convertible amounts of H2/CO2 in Mix 1 and Mix 2. Hence, M. okinawensis was able to grow and to convert H2/CO2 to CH4 when CH2OH, CH3OH, NH4Cl, CO and CH2H2 were present in the growth medium at the concentrations calculated from Cassini’s INMS data (assuming 1 bar, compare Tables 1 and 2). The mixing ratios of these putative inhibitors were based on INMS data\(^{4,5,25,26}\) but higher than those calculated by using the most recent Cassini data\(^{2,6}\) (Table 1). This demonstrates that growth and biological CH4 production of M. okinawensis is possible even at higher inhibitor concentrations.

**M. okinawensis tolerates Enceladus-like conditions up to 50 bar.** Due to the fact that methanogens on Enceladus would possibly need to grow at hydrostatic pressures up to 80 bar\(^{3}\) and beyond, the effect of high pressure on the conversion of headspace gas for M. okinawensis was examined in a pressure-resistant closed batch bioreactor. Headspace H2/CO2 conversion and CH4 production was examined at 10, 20, 50, and 90 bar, either using H2/CO2 in a 4:1 ratio or applying H2/CO2/N2 in a 4:1:5 ratio. A gas conversion of >88% was shown for each of the experiments (Supplementary Fig. 2) except for the 90 bar experiment using H2/CO2/N2, where the headspace gas conversion was found to be at 66.4%. However, no headspace gas conversion and CH4 production could be detected when cultivating M. okinawensis at 90 bar using H2/CO2 only (data not shown).

Final experiments were designed to investigate headspace H2/CO2 conversion and CH4 production of M. okinawensis according to INMS data (Table 3) and under conditions of high pressure (10.7 ± 0.1, 25.0 ± 0.7, and 50.4 ± 1.7 bar). Turnover rate, methane evolution rate (MER, calculated via pressure drop) and biological CH4 production (calculated via gas chromatography measurements) for these experiments are shown in Fig. 4. When simultaneously applying putative gaseous (Table 4) and liquid inhibitors (Supplementary Table 3) under high-pressure conditions, we reproducibly demonstrated that M. okinawensis was able to perform H2/CO2 conversion and CH4 production under Enceladus-like conditions.

**Methanogenic life could be fuelled by H2 from serpentinization.** In light of these experimental findings and the presence of H2 in Enceladus’ plume\(^4\), the question arose if serpentinization reactions can support a rate of H2 production that is high enough to sustain autotrophic, hydrogenotrophic methanogenic life. To address this question, we used the PHREEQC\(^{27}\) code to model serpentinization-based H2 production rates under Enceladus-like conditions (Table 5) with the assumption that the rate-limiting step of the serpentinization reaction is the dissolution of olivine. H2 production rates are poorly constrained, as they strongly depend on assumed grain size and temperature. These rates correspond to the low end of the range of H2 production rates, which were based on a thermal cooling and cracking model\(^{28}\). Of the many reactions involved in serpentinization of peridotite, dissolution of the Fe(II)-bearing primary phases is a critical one\(^{29}\), and the only one for which kinetic data are available. In the model, CO2 reduction to CH4 is predicted to take place once enough H2 in the system was produced to generate thermodynamic drive for the reaction. While abiotic CH4 production is kinetically more sluggish than olivine dissolution\(^{30}\), biological CH4 production is fast and may be controlled by the rate at which H2 is supplied. The abiotic CH4 production rates listed in Table 5 are hence also modelled such that olivine dissolution is the rate-limiting step. The results of these thermodynamic and kinetic computations show that H2 and CH4 production is predicted for a range of rock compositions (Table 5) and temperature conditions (Supplementary Table 4). The model system essentially represents a closed system with high-rock porosity, such as proposed for Enceladus\(^2\). The computational results predict how much H2 and CH4 should form within the intergranular space inside Enceladus’ silicate core with water-to-rock-ratios between 0.09 and 0.12 (Table 5). The serpentinization reactions are predicted to produce solutions with circumneutral to high pH between 7.3 and 11.3, as well as amounts of H2 that greatly exceed the amount of dissolved inorganic carbon (DIC) trapped in the

### Table 4 Gas composition of experiments performed in the 2.0 L bioreactor

| Pressure (bar) | N2 (Vol-%) | H2 (Vol-%) | CO2 (Vol-%) | CO (Vol-%) | C2H6 (Vol-%) |
|---------------|------------|------------|-------------|------------|--------------|
| 20 bar (H2/CO2) | 80.29 | 19.71 | | | |
| 10 bar | 31.43 | 53.33 | 6.67 | 4.76 | 3.81 |
| 25 bar | 42.91 | 42.11 | 8.46 | 4.45 | 5.67 |
| 50 bar | 32.67 | 55.11 | 6.21 | 3.01 | 3.01 |
| 2.0 L reactor pressure and inhibitor tests II | 20 bar (H2/CO2) | 80.30 | 19.70 | | | |
| 10 bar | 29.25 | 55.66 | 6.60 | 4.72 | 3.77 |
| 25 bar | 41.43 | 43.82 | 4.78 | 4.38 | 5.58 |
| 50 bar | 32.41 | 55.07 | 6.56 | 2.98 | 2.98 |
pore space. As the computations indicate that there is ample thermodynamic drive for reducing DIC to CH₄, these results corroborate the idea that serpentinization reactions on Enceladus might fuel autotrophic, hydrogenotrophic methanogenic life. However, we would like to point out that if methanogenic life were indeed active on Enceladus, biological CH₄ production would always compete with abiotic CH₄ generation processes resulting in a mixed CH₄ production.

**Discussion**

In this study, we show that the methanogenic strain *M. okinawensis* is able to propagate and/or to produce CH₄ under putative Enceladus-like conditions. *M. okinawensis* was cultivated under high-pressure (up to 50 bar) conditions in defined growth medium and gas phase, including several potential inhibitors that were detected in Enceladus’ plume. The only difference between the growth conditions of *M. okinawensis* and the...
Table 5 H2 and CH4 production rates from serpentinization calculated for 50 °C and 50 bar

| Mineral assemblagea | pH | H2 production rate (nmol g\(^{-1}\) L\(^{-1}\) d\(^{-1}\)) | CH4 production rateb (nmol g\(^{-1}\) L\(^{-1}\) d\(^{-1}\)) | Water: rock ratio | Mol H\(_2\) produced per mol olivine |
|---------------------|----|------------------------------------------------|-------------------------------------------------|----------------|----------------------------------|
| Fo90:En:Diop = 8:1:1 | 11.3 | 4.58 | 2.05 | 0.126 | 0.002 |
| Fo90 | 8.60 | 4.03 | 1.07 | 0.124 | 0.004 |
| Fo50 | 7.50 | 34.7 | 1.35 | 0.104 | 0.033 |
| Fo20 | 7.29 | 50.7 | 1.32 | 0.094 | 0.053 |

a Fo = forsteritic olivine (Fo:Fa = 9:1); En = enstatite; Diop = diopside; Fo50 = (Fo:Fa = 1:1); Fo20 = (Fo:Fa = 2:8)
b CH4 production is predicted from allowing H2 and dissolved inorganic carbon (DIC) to equilibrate readily, while H2 production is kinetically controlled by dissolution of primary phases.

putative Enceladus-like conditions was the lower pH value applied during the high-pressure experiments. Due to the supply of CO2 at high pressure in the experiments the pH decreased to ~5, while pH values between 7.3 and 13.5 were estimated for Enceladus’ subsurface ocean (this study and refs. 8,14).

Another point of debate might be the cultivation temperatures used for the thermophilic and hyperthermophilic methanogens in this study. The mean temperature in the subsurface ocean of Enceladus might be just above 0 °C except for the areas where hydrothermal activity is assumed to occur. In these hydrothermal settings temperatures higher than 90 °C are supposedly possible8, and are therefore the most likely sites for higher biological activity on Enceladus. Although methanogens are found over a wide temperature range on Earth, including temperatures around 0 °C31, growth of these organisms at low temperatures is observed to be slow13.

We estimated H2 production rates between 4.03 and 50.7 nmol g\(^{-1}\) L\(^{-1}\) d\(^{-1}\) in the course of serpentinization on Enceladus (Table 5). These estimates are rather conservative, as they are based on the assumption of small specific mineral surface areas. In a recent study, the rate of serpentinization has been estimated from a physical model that predicts how fast cracking fronts propagate down into Enceladus’ core28. Combining this physically controlled advancement of serpentinization (8 × 10\(^{11}\) g\(^{-1}\) y\(^{-1}\)) with our estimates for kinetically limited rates of H2 production leads to overall rates of 3–40 × 10\(^{11}\) mol H2 y\(^{-1}\) for Enceladus. Although still high enough to support biological methanogenesis, these rates are orders of magnitude lower than the previously suggested 10 × 10\(^{8}\) mol H2 y\(^{-1}\)–128 assuming that the speed of cracking front propagation controls the rate of H2 production. We hence suggest that reaction kinetics may play an important role in determining the overall H2 production rate on Enceladus. Our computed steady-state H2 production rates are lower than the 1–5 × 10\(^{9}\) mol H2 y\(^{-1}\) estimated from Cassini data2. This apparent discrepancy in flux rates can be reconciled if the Enceladus plume was a transient (i.e., non-steady state) phenomenon. The predicted H2/CH4 ratio of 2.5 (Fo90:En:Diop = 8:1:1) to 4 (Fo50) for the magnesian compositions of Enceladus’ core (Table 5) are consistent with the relative proportions of the two gases in the plume (0.4–1.4% H2, 0.1–0.3% CH4)2.

Based on our estimated H2 production rate, we can calculate how much of the available DIC on Enceladus could be fixed into biomass through autotrophic, hydrogenotrophic methanogenesis. If we assume a typical elementary composition of methanogen biomass32, 7.13 g carbon could be fixed per g hydrogen fixed. Under optimal growth conditions, ~3%22,23 of the available carbon can be assimilated into biomass, and assuming that methanogens possess a molecular weight of ~30.97 g C-mol\(^{-1}\)122 and that the total amount of H2 produced would be available for the carbon and energy metabolism of autotrophic, hydrogenotrophic methanogens, a biomass production rate between 20 and 257 C-nmol g\(^{-1}\) y\(^{-1}\) could be achieved. In another approach, we can use the actually predicted CH4 production rates of 1.32–2.05 nmol g\(^{-1}\) L\(^{-1}\) d\(^{-1}\) (Table 5) and a Gibbs energy dissipation approach in which we assume that 10% of the energy of CH4 production is fuelling biosynthesis29. This yields similar numbers of biomass production rate, i.e., 28 and 56 C-nmol g\(^{-1}\) L\(^{-1}\) y\(^{-1}\).

Based on our findings, it might be interesting to search for methanogenic biosignatures on icy moons in future space missions.
missions. Methanogens produce distinct and lasting bio-
signatures, in particular lipid biomarkers like ether lipids and isoprenoid hydrocarbons. Other potential biomarkers for methanogens are high-nickel (Ni) concentrations (and its stable isotopes)33, as Ni is e.g., part of methyl-coenzyme M reductase, the key enzyme of biological methanogenesis34. However, both lipid biomarkers and Ni-based biosignatures are likely only to be identifiable at the site of biological methanogenesis, and the effect of dilution with increasing distance away from the methanogen habitat is likely to prevent their use as a general marker for biological methanogenesis in Enceladus’ plume or in a subsurface ocean. If, however, bubble scrubbing would occur, a process by which organic compounds and cells adhere to bubble surfaces and are carried away as bubbles rise, which was suggested to occur on Enceladus34, the amount of bioorganic molecules and cells would be much higher and future lander missions could easily collect physical evidence for the presence of autotrophic, hydrogenotrophic methanogenic life on Enceladus.

Additionally, one could consider using stable isotopes of CH4 and CO2 and ratios of low-molecular weight hydrocarbons to evaluate the possibility of biological methanogenesis on Encela-
dus.11. But given the uncertainties on the geological and hydro-
geological boundary conditions that influence the targeted isotope and molecular patterns in Enceladus’ plume, such an approach is not trivial. In contrast to biological and thermogenic CH4 produc-
tion, the latter resulting from the decomposition of organic matter, abiogenic CH4 is believed to be produced by metal-catalysed Fischer–Tropsch or Sabatier type reactions under hydrothermal conditions and particularly in the course of serpentinization of ultramafic rock.35. Although biologically produced CH4 is usually characterised by its strong 13C depletion, growth of methanogens at high-hydrostatic pressures and high temperatures, which is typical of deep-sea hydrothermal systems, may significantly reduce kinetic isotope fractionation and result in relatively high 813C values of CH4, hampering discrimination from non-microbial CH4.36. Given such uncertainties, multiply substituted, so-called ‘clumped’ isopologues of CH4 emerge as new proxy to constrain its mode of formation and to recognise formation environments like serpentinitization sites.37.

Another approach to identify the origin of CH4 could be CH4/(ethane + propane) ratios, as low ratios are typical of settings dominated by thermogenic CH4.38. However, this ratio may fall short to unequivocally discriminate abiogenic from biologically produced CH4. For instance the ratio of CH4 concentration to the sum of C2+ hydrocarbon concentration (C2/C4) in the serpentinite-hosted Lost City Hydrothermal Field of 950 ± 76 was found to be most similar to ratios obtained in experiments with Fischer–Tropsch type reactions (<100->3000). Thermogenic reactions produce C1/C2+ ratios less than ~100, whereas biologi-
cal methanogenesis results in ratios of 2000–130000.39. More than 30 years of research on CH4 production have revealed that its biologic, thermogenic or abiogenic origin on Earth is often difficult to trace.40. However, the experimental and modelling results presented in this study together with the estimates of the physicochemical conditions on Enceladus from earlier contributions make it worthwhile to increase efforts in the search for signatures for autotrophic, hydrogenotrophic methanogenic life on Enceladus and beyond.

Methods

Estimations of Enceladus’ interior structure. Due to its rather small radius, the uncompressed density of the satellite is almost equal to its bulk density, which makes a simplified model of Enceladus’ interior reasonable. Enceladus was divided into a rocky core (core density of 2300–2500 kg m−3), a liquid water layer (density of 960–1080 kg m−3), and an icy shell (ice density of 850–960 kg m−3) and hydrostatic equilibrium was assumed. Calculations of the hydrostatic pressure based on Enceladus mass of 1.0794 ± 1018 kg11 and its mean radius of 252.1 km11 assuming a core radius of 190–200 km, an subsurface ocean depth of 60–10 km and a corresponding ice shell thickness of 2.1–5.2 km results in a pressure of ~44.3–45.2 bar or 80.1–56.2 bar (depending on the method, SI, and biomass).

Low-pressure experiments. Growth and tolerance towardsputative inhibitors of the three methanogenic strains Methanothermobacter okinawensis DSM 14208, Methanobacterium marburgensis DSM 2133, and Methanococcus villosus DSM 22612 were elucidated (Fig. 1). All strains were obtained from the Deutsche Stammssammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. Growth was resolved by optical density (OD) measurements (Δ ± 75 nm). H2/CO2–CH4 gas conversion [%], turnover rate [h−1] (see Equation (2) below), and MER were calculated from the decrease of the bottle headspace pressure and/or from measuring CH4 production in a closed batch setup.22,24. The headspace pressures were measured using a digital manometer (LEO1-El...−1...3barrel, Keller, Jestetten, Germany) with (sterile syringe filters, 0.22 µm cellulose, 534-0061, VWR International, Vienna, Austria), and cannuulas (Gr 14, 0.60 × 30 mm, 23 G × 1 1/4”, RX129.1, Braun, Maria Enzersdorf, Austria). The detailed setting can be seen in Fig. 3(a) in Taubner and Rittmann22. All pressure values presented in this study are indicated as relative pressure in bar.

For the experiments at 2 bar regarding CO and CH4 tolerance (see Figs. 1 and 3), the strains were inoculated in the dark either in a water bath (M. marburgensis and M. okinawensis, 65 ± 1 °C) or in an air bath (M. villosus, 80 ± 1 °C). The methanogens were cultivated in 50 ml of their respective chemically defined growth medium. Compositions of the experimental growth media can be found in Supplementary Tables 3 and 5–10. The final preparation of the medium in the anaerobic culture flasks was performed in an anaerobic chamber (Coy Laboratory Products, Grass Lake, USA). Experiments were performed over a time of 210–270 h. After each incubation period, serum bottle headspace pressure measurement (in order to be unbiased, flasks were previously cooled down to room temperature). OD-samples with designated gas or test gas was performed. OD measurement was performed at 578 nm in a spectrophotometer (DU800, Beckman Coulter, USA). A zero control was inoculated together with each individual experiment and the OD of this control was subtracted from the measured OD from the inoculated flasks each time.

For hydrogenotrophic methanogens, which utilise H2 as electron donor for the reduction of CO2 to produce CH4 and H2O as their metabolic products, the following stoichiometric reaction equation was used22,24:

\[4\text{H}_2(\text{g}) + \text{CO}_2(\text{g}) \rightarrow \text{CH}_4(\text{g}) + 2\text{H}_2\text{O}(\text{aq}) \]

\[\Delta G^\circ = -135 \text{kJ mol}^{-1} \]

(1)

The turnover rate [h−1] correlates with the catalytic efficiency per unit of time, i.e., it is a way to indirectly quantify CH4 productivity. By assuming the above-mentioned chemical CO2 methanation stoichiometry and neglecting biomass cost, the following reaction equation was used:

\[\text{CH}_2\text{O} + \Delta \text{p} \rightarrow \text{CH}_4 + \text{H}_2\text{O} + \text{H}^+ + \text{e}^- \]

\[\Delta p = \text{p}_{\text{max}} - \Delta \text{t} \]

(2)

where Δp [bar] is the difference in pressure before and after incubation, Δp_{max} [bar] is the maximal theoretical difference that would be possible due to stoichiometric reasons22, and Δt [h] is the time period of incubation.

For the initial pressure experiments at 2 bar, the three methanogenic strains were tested under five different gas phase compositions (Table 2). A significant change in OD and turnover rate was observed between these experiments (as can be seen in Fig. 1). When Mix 1 and Mix 2 were applied, only a maximum of 22.66 ± 1.34 mOD % H2 (average, Table S1) could be converted. To evaluate the influence of the potential inhibitors NH4+ CHaO, and CHaOH on the growth of M. okinawensis, several preliminary experiments were performed. For easier handling, NH4+ was substituted by NH4Cl. Based on INMS data (Table1) the amount of NH4Cl was calculated according to Henry’s law. For that, Henry’s law constant was calculated to be 0.1084 mol m−3 Pa−1 at 64 °C. This results in 11.6 g L−1 (0.22 mol L−1) NH4Cl to have ~1% of NH4+ in the gaseous phase at equilibrium for the experiments under closed batch conditions. The influence of NH4Cl between 0.25 and 16.25 g L−1 (4.67 and 303.79 mmol L−1), CHaO between 0 to 111 µL−1 (0−4.93 mmol L−1), and CHaOH between 0 to 200 µL−1 (0−4.94 mmol L−1) was tested individually. CHaO (37 Vol. %) and CHaOH (98 Vol. %) were used as stock solutions.

To find an appropriate control for the final experiments, an experiment in a DoE setting was established. A central composite design with the parameters shown in Supplementary Table 1 and Fig. 2 was chosen. The design space was spherical with a corresponding radius equal to its bulk density, which makes a simplified model of Enceladus’ interior reasonable. Enceladus was divided into a rocky core (core density of 2300–2500 kg m−3), a liquid water layer (density of 960–1080 kg m−3), and an icy shell (ice density of 850–960 kg m−3) and hydrostatic equilibrium was assumed. Calculations of the hydrostatic pressure based on Enceladus mass of 1.0794 × 1018 kg and its mean radius of 252.1 km assuming a core radius of 190–200 km, an subsurface ocean depth of 60–10 km and a corresponding ice shell thickness of 2.1–5.2 km results in a pressure of ~44.3–45.2 bar or 80.1–56.2 bar (depending on the method, SI, and biomass).
Supplementary Fig. 1. Each incubation time period was 10.0 ± 0.5 h. The ANOVA analysis of this study can be found in Supplementary Table 2.

The setting for the experiments under Enceladus-like conditions at 2 bar pressure included the medium described in Supplementary Table 3 and Mix 2 (Table 2) as gaseous phase. As can be seen in Fig. 3 there was a lag phase of two days, but after that continuous but slow growth was observed.

To calculate the peak concentration of H2 and CO2 in the medium (Table 3), Henry's law was used:

$$M = k_H \cdot p_x,$$

where $p_x$ is the partial pressure of the respective gas and $k_H$ is Henry's constant as a function of temperature:

$$k_H = k_H^0 \cdot e^{-\frac{\Delta H_m}{R} \left(\frac{1}{T} - \frac{1}{T_0}\right)},$$

where $\Delta H_m$ is the enthalpy change of the dissolution reaction. For $k_H^0$ the values 7.9 × 10^{-10} mol L⁻¹ bar⁻¹ and 3.4 × 10^{-10} mol L⁻¹ bar⁻¹ and for $\Delta H_m$ the values 500 K and 2400 K for H2 and CO2, respectively, were used. This results in a Henry constant at 65 °C of 6.841 × 10^{-10} mol L⁻¹ bar⁻¹ and 1.329 × 10^{-10} mol L⁻¹ bar⁻¹ for H2 and CO2, respectively.

Another potential liquid inhibitor detected in Enceladus’ plume was hydrogen cyanide (HCN)14,15. However, calculations on HCN stability under the assumed conditions on Enceladus show that HCN would hydrolyse into formic acid and ammonia16. Further investigations on the stability of HCN at different pH values and temperatures yielded similar results16,18. It was therefore assumed that HCN might be present in a very young, recent, aqueous marine matrix on Enceladus14,16. Due to this reasoning and the low probability of HCN presence in the subsurface ocean of Enceladus, HCN was neglected in all growth experiments performed in the laboratory setup. This could be an indication for a barophilic nature of this organism, but also due to the experimental closed batch setup.

High-pressure experiments. M. okinawensis initial high-pressure experiments were performed at its optimal growth temperature of 65 ± 1 °C using a chemically defined medium (Table 1) for exact composition) and a fixed stirrer speed of 100 r.p.m. in a 0.7 L stirred stainless steel Büchi reactor. Before each of the experiments, the reactor was filled with medium and the entire setup was autoclaved under CO2 atmosphere to assure sterile conditions. Thereafter, the inoculum (1 Vol.-%), the NaHCO3, L-cysteine, Na2S and MgCl2·6H2O (0.5 M) and trace element solution were transferred as a previously autoclaved transfer vessel into the reactor. Then the reactor was set under pressure with the selected gas mixture (added ~5 bar in discrete steps every 10 min). The initial high-pressure experiments were performed using both an H2/CO2 (4:1) gas phase and an H2/CO2/N2 (4:1:5) mixture. The reactor was equipped with an online pressur(e (ASH Performance Products) and a pressure needle valve (Hannifin Corporation, USA) and temperature probe (thermo element PT100, −75 °C to −350 °C, TC Mess- und Regeltechnik GmbH, Mönchengladbach, Germany). The conversion was always above 88% except for the 90 bar experiment, wherein also NO fixation into biomass could be assumed. Interestingly, the time until start of the conversion decreases for the H2/CO2 experiments upon an increase in headspace pressure in the initial setup. This could be an indication for a barophilic nature of this organism, but also due to the experimental closed batch setup.

Increasing PCO2 and associated pH change was determined using a pH probe (see Supplementary Fig. 3). This analysis showed that even a rather small PCO2 (2 bar) already decreases the pH from nearly neutral to >5 due to the medium composition, which is due to application of a medium with low-buffering capacity, also possibly occurring in Enceladus’ subsurface ocean. However, it remains an open question if the medium on Enceladus is buffered. This would lead to higher possible PCO2 without having a drastic influence on the reported pH.

Furthermore, we measured CO2 concentrations (Table 1) and calculated dissolved inorganic carbon and what would be the effect on the pH of the medium. Postberg et al. suggested a concentration of 0.02–0.1 mol kg⁻¹ NaCO3 and 0.05–0.2 mol kg⁻¹ NaCl in the medium to reach a pH level between 8.5 and 9.46. Calculations on the concentrations of dissolved CO2 in the high-pressure experiments were performed by calculating the difference between dissolved CO2 and H2/CO2 (4:1) gas phase (i.e., pCO2 from gas phase). The mode fractions for PCO2 of 0.7, 1.2, and 3.1 bar (as used in the experiments) at 65 °C were generated by extrapolation of given values in the region of P CO2 = 0–1 bar. H2/CO2 conversion and CH4 production could still be measured at 10 bar, 20 bar and 30 bar (i.e., P CO2 = 10 bar) with H2/CO2 (4:1) gas phase, but no decrease in pressure was observed. A decrease in H2/CO2 production rate (i.e., pCO2) was observed after 18 bar (data not shown). It is assumed that no growth occurs under these conditions due to the high P CO2 (18 bar) and the associated decrease to a pH of <3, which is beyond the reported pH tolerance of M. okinawensis19.

The high-pressure experiments under Enceladus-like conditions were carried out in a 2.0 L Büchi reactor. The gas mixtures were prepared as described in the experimental section (i.e., pCO2 = 10) bar with H2/CO2 (4:1) gas phase, but no decrease in pressure was observed at 90 bar with H2/CO2 (4:1) gas phase (i.e., pCO2 was 1.2 molal DIC in Enceladus plume gas). The gas mixtures were prepared using a gas chromatograph (7890 A GC System, Agilent Technologies, Santa Clara, USA) equipped with a TCD detector and a 5% CarbonPep ST Miracolumn Packed Column (Restek GmbH, Bad Homburg, Germany)22.

To determine the CH4 production [Vol.-% h⁻¹] shown in Fig. 4, the value of CH4 Vol.-% was divided by the time of biological CH4 production in h. To exclude a potential lag phase, the starting point of biological CH4 production was set to the point in time in which the decrease in pressure exceeded the initial pressure by 5% for 10 bar experiments or by 1% during the other experiments.

Serpinization experiments. The PHREEQC27 code was used to simulate serpinization reactions from 25 to 100 °C and from 25 to 50 bar in order to assess CH4 production on Enceladus. The Annm.dat and linl.dat databases were used for all simulations, which account for temperature and pressure dependent equilibrium constants of dissolved species. Three solid phases up to 100 °C and 1000 bar were chosen for the high-pressure experiments. The chemical composition was taken from the chemical composition of erupting plume of Enceladus4, as the true chemistry of its subsurface sea is unknown. Dissolved concentrations of Ca2+, Fe2+, Mg2+, and SiO2 were assumed to be seawater-like and values from McCollom and Bach22 were used. At the very low water-to-rock ratios of our model, the compositions of the interacting fluids will be entirely rock buffered, so that the model results are insensitive to the choice of the starting fluid composition. DIC concentration was set to 0.04 mol L⁻¹ taken from Glein et al.14, who estimated a possible range of 0.005–1.2 molal DIC in Enceladus’ subsurface ocean. The solid phase assemblage was composed of varying amounts of olivine, enstatite and diopside as well as varying olivine compositions. Most terrestrial bodies exhibit olivine solid solutions (Mg, Fe)SiO4 that are dominated by forsterite (Mg2SiO4). This has been shown for micrometeorites found on Earth, lunar meteorites, comets, and asteroids49–51. Stony iron meteorites, such as pallasites contain forsteritic olivine with up to 20% Fe3+ content52. Olivines in chondrites show a more varied compositions ranging between 7 and 70% ferrous iron53,54, to almost pure fayalite (Fe2SiO4)55. A realistic assumption is that olivines on Enceladus have a more forsteritic composition that resembles those of stony iron and lunar meteorites.

A composition of F050 was adopted for olivine. Calculations were limited to forsterite, enstatite, and diopside, as experimental data on their dissolution kinetics at high pH and low temperature are available. Kinetic rate laws were applied for forsteritic olivine, fayalite, enstatite, and diopside from Wogelius and Walther65,67, Daval et al.56, Oelkers and Schott59, and Knauss et al.60, respectively. Dissolution rate laws for F050 and fayalite at 100 °C were extrapolated from rate data in Wogelius and Walther57 using the Arrhenius equation. Enstatite and diopside rate laws at 100 °C were power-law fitted from experimental data provided by Oelkers and Schott59 and Knauss et al.56, respectively. All rate laws are valid over a pH range from 2 to 12 at all temperatures. Kinetic rate laws were multiplied by the total surface area of each mineral present in solution in order to calculate moles of minerals dissolved per time. Surface areas of 590 cm² ferrous iron53,54 and fayalite53, 980 cm² g⁻¹ for enstatite57, and 550 cm² g⁻¹ for diopside60 were used. These specific surface areas have been suggested to be typical for fine-grained terrestrial rocks. We adopted these numbers in our computations, as we have no constraints on what specific surface areas in Enceladus may be. If the core of Enceladus is more porous than Mars, then this grain size is smaller and hence the specific surface areas larger than what we assumed.22 We choose to use fairly small specific surface areas to provide conservative estimates for H2 production rates. Model 1 uses F050, enstatite and diopside in a ratio of 8:1:1, model 2 uses a pure F050 composition. The effect of ferrous iron content in olivine was considered in rate calculations. For models 1 and 2, models 3 and 4 (Fo20/Ca20/Ca20) were used. Applying these amounts yield water-to-rock-ratios between 0.09 and 0.12 (Table 5).

The most likely environmental conditions present within Enceladus are temperatures between 25 and higher than 90 °C at 25–80 bar, and temperatures of 50 °C and pressures of 50 bar were chosen for the four different models. In a separate set of computations, temperatures were altered to 25 and 100 °C, and pressures were set at 25 and 100 bar. These results are shown in Supplementary Table 3.
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Author contributions

R.-S.T., P.P., C.Pr., P.K., and S.K.-M.R.R. performed the experiments. R.-S.T., P.P., S.B., A.H.S., C.Pr., and S.K.-M.R.R. designed the experiments. D.S. and J.Z. designed and performed PHREEQC modelling. W.B. supervised the PHREEQC modelling. R.-S.T., P.P., J.Z., D.S., S.B., A.H.S., C.Pr., P.K., C.Pr., M.F., C.S., and S.K.-M.R.R. discussed the data. R.-S.T., J.Z., D.S., W.B., J.P., C.S., and S.K.-M.R.R. wrote the manuscript.

Additional information

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Competing interests: R.-S.T., P.P., D.S., J.Z., C.Pr., P.K., W.B., J.P., C.Pr., M.F., C.S., and S.K.-M.R.R. declare no competing financial interests. Due to an engagement in the Kraje Te GmbH, A.H.S., S.B., and A.K. declare competing financial interests.

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