

Hyperthermophilic methanogenic archaea act as high-pressure CH₄ cell factories

Lisa-Maria Mauerhofer¹, Sara Zwirtmayr², Patricia Pappenreiter², Sébastien Bernacchi³, Arne H. Seifert³, Barbara Reischl^{1,3}, Tilman Schmider¹, Ruth-Sophie Taubner^{1,2}, Christian Paulik² & Simon K.-M. R. Rittmann¹✉

Bioprocesses converting carbon dioxide with molecular hydrogen to methane (CH₄) are currently being developed to enable a transition to a renewable energy production system. In this study, we present a comprehensive physiological and biotechnological examination of 80 methanogenic archaea (methanogens) quantifying growth and CH₄ production kinetics at hyperbaric pressures up to 50 bar with regard to media, macro-, and micro-nutrient supply, specific genomic features, and cell envelope architecture. Our analysis aimed to systematically prioritize high-pressure and high-performance methanogens. We found that the hyperthermophilic methanococci *Methanoterris igneus* and *Methanocaldococcus jannaschii* are high-pressure CH₄ cell factories. Furthermore, our analysis revealed that high-performance methanogens are covered with an S-layer, and that they harbour the amino acid motif Tyr^{α444} Gly^{α445} Tyr^{α446} in the alpha subunit of the methyl-coenzyme M reductase. Thus, high-pressure biological CH₄ production in pure culture could provide a purposeful route for the transition to a carbon-neutral bioenergy sector.

¹Archaea Physiology & Biotechnology Group, Department Functional and Evolutionary Ecology, Universität Wien, Wien, Austria. ²Institute for Chemical Technology of Organic Materials, Johannes Kepler Universität Linz, Linz, Austria. ³Krajete GmbH, Linz, Austria. ✉email: simon.rittmann@univie.ac.at

Methane (CH₄) is an energy carrier of worldwide importance. It can be produced through biogenic, thermogenic, and pyrogenic processes¹. Most biogenic CH₄ is emitted by methanogenic archaea (methanogens)², with minor amounts originating from cyanobacteria³ and marine microorganisms⁴. Methanogens are a phylogenetically diverse group of microorganisms, which can be found in various anoxic environments⁵. Among other substrates, methanogens convert short chain organic acids and one-carbon compounds to CH₄ through their energy and carbon metabolism^{2,5,6}. Their metabolic capability is important for anaerobic organic matter degradation in environments with low concentrations of sulfate, nitrate, manganese, or iron⁵. Moreover, methanogens are of biotechnological relevance due to their ability to produce isoprenoid-containing lipids^{7,8} or polyphosphate⁹, and were recently described to excrete proteinogenic amino acids⁸. Methanogens are central to biofuels production, as they can be employed as autocatalysts for carbon dioxide (CO₂) and molecular hydrogen (H₂) conversion in the CO₂-based biological CH₄ production (CO₂-BMP) process.

The CO₂-BMP process can be employed in multiple applications such as biogas upgrading, power-to-gas applications, decentralized energy production, and for the conversion of H₂/CO₂ of process flue gasses in waste to value concepts from, e.g., ethanol, petroleum, steel, and chemical industries¹⁰. There are two main approaches for CO₂-BMP¹¹: ex situ biomethanation using pure cultures^{12,13} or enriched mixed cultures^{14–16}, and in situ biomethanation^{17,18}. In situ biomethanation is examined for upgrading the CH₄ content of biogas by adding H₂ to anaerobic digesters. Ex situ pure culture biomethanation exhibits high volumetric CH₄ productivity and offers a straightforward bioprocess control by utilizing biochemically and biotechnologically well-characterized microorganisms in pure culture¹². Among the most studied organisms in this regard is *Methanothermobacter marburgensis*^{12,19}, exhibiting several advantageous traits such as flexibility with regard to substrate gas impurities¹⁰ and high CH₄ productivity²⁰. In addition, *M. marburgensis* can be used for CO₂-BMP when short-term transitions in the order of minutes are demanded between stand-by to full load biomethanation. Furthermore, downtime periods above 500 h did not reduce CH₄ productivity after a process restart²¹.

Compared to CO₂-BMP, chemical methanation or the “Sabatier reaction” should not be operated intermittently due to various catalytic constraints²² and the fast bulk-like oxidation of the nickel catalyst in the CO₂ atmosphere²³. Furthermore, activity loss of the chemical catalyst after a certain lifespan necessitates the exchange of the catalyst and the carrier material leading to periodic downtimes in production. Thus, applying methanogens, which are autocatalysts, offers numerous advantages compared to a chemically catalyzed CO₂ methanation. The lower power demand and the stable selectivity observed in CO₂-BMP compared to chemical methanation²² strongly suggest that CO₂-BMP is a viable biotechnological alternative to chemical methanation. However, the autocatalytic characteristics of methanogens require further investigation.

The CO₂-BMP bioprocess can be operated as a gas transfer limited process¹² when a proper feeding strategy is applied²⁴. In this case, the kinetic limiting step is the mass transfer of H₂ to the liquid phase. In biochemical engineering, gas to liquid mass transfer can be enhanced by several technical measures²⁰. Besides reactor geometry and agitation, which influence the specific mass transfer coefficient (k_{1a}), pressure increases the solubility of H₂ in the liquid phase. The influence of pressure on substrate uptake, growth, and production kinetics of methanogens is therefore an important parameter in CO₂-BMP. Some experiments with *Methanocaldococcus jannaschii* have already been performed at high pressure in order to investigate transcription profiles²⁵ or

growth and CH₄ production²⁶. The effect of pressure on CH₄ production has also been examined in bioreactors^{20,27}, while media for cultivation of methanogens have been developed and their growth assessed^{28–31}. However, a systematic biotechnological survey with regard to nutritional demands of methanogens across different temperature regimes in the same cultivation conditions and at different pressure levels has not yet been the focus of any study.

On the way to develop a high-pressure pure culture CH₄ production bioprocess, we systematically and quantitatively investigated the productivity of methanogens at pressures up to 50 bar. Growth, conversion, and CH₄ productivity were first examined in order to identify cell factories with the highest CH₄ productivity among 80 methanogens, in a range of different media (in terms of composition and medium amendments) and in conditions ranging from psychrophilic to hyperthermophilic. Secondly, the 14 prioritized fastest growing and with the highest productivity methanogens were investigated using a high frequency gassing (HFG) experiment and by using 10 bar H₂/CO₂ to CH₄ conversion experiments. Among these 14 methanogens, four strains were chosen for the third step, consisting of 50 bar H₂/CO₂ to CH₄ conversion experiments. Finally, we analyzed these results in the context of their natural habitat, temperature optima, specific genomic features, and their cell envelope architecture.

Results

High-throughput screening revealed high-performance methanogens. In order to investigate essential macro- and micro-nutrient growth medium amendments of methanogens in the context of their physiology and CH₄ productivity in a systematic and quantitative physiological approach, a multivariate high-throughput screening of 80 methanogens on various media and medium amendments was performed (Fig. 1 and Supplementary Figs. S1 and S2). This multivariate high-throughput screening was conducted on 22 complex and defined media in order to characterize methanogens from psychrophilic, mesophilic, thermophilic, and hyperthermophilic temperature groups with regard to maximum biomass and CH₄ production kinetics in a closed batch setting, with initially 2 bar H₂/CO₂ (4:1) in the headspace. Maximum optical density (OD_{max}), maximum substrate conversion (turnover_{max}), and maximum volumetric CH₄ evolution rate (MER_{max}) were selected as experimental output variables. To elucidate if the chosen methanogens showed a homogenous or heterogenous growth pattern which would indicate a balanced or unbalanced biomass increase³², respectively, the biomass increase rate (“Material and Methods”, Eq. (1)) was used for comparing growth kinetics.

All tested psychrophilic methanogens grew to an OD_{max} of below 0.2, showed turnover_{max} lower than 40%, and exhibited a very low MER_{max} of only up to 0.1 mmol L⁻¹ h⁻¹ on complex or defined media. Mesophilic methanogens grew heterogeneously when cultivated on complex and/or defined media. Biomass growth to an OD_{max} beyond 1.0, a turnover_{max} over 70%, and a MER_{max} higher than 1.0 mmol L⁻¹ h⁻¹ at cultivation temperatures between 35 and 37 °C on complex media were measured. Growth of moderate thermophilic methanogens between 40 and 45 °C resulted in an OD_{max} between 0.3 and 0.7 on complex media, while on defined media only an OD_{max} of 0.003–0.025 was obtained. Growth of thermophilic methanogens (60–65 °C) resulted in an OD_{max} range from 0.4 to 0.8, a turnover_{max} between 75 and 96% and a MER_{max} beyond 1.0 mmol L⁻¹ h⁻¹. Hyperthermophilic growth between 80 and 98 °C resulted in an OD_{max} between 0.2 and 0.7, a turnover_{max} from 83 to 97% and a MER_{max} ranging from 1.0 to 4.6 mmol L⁻¹ h⁻¹ (Fig. 1 and Supplementary Fig. S2). Most methanogens showed a

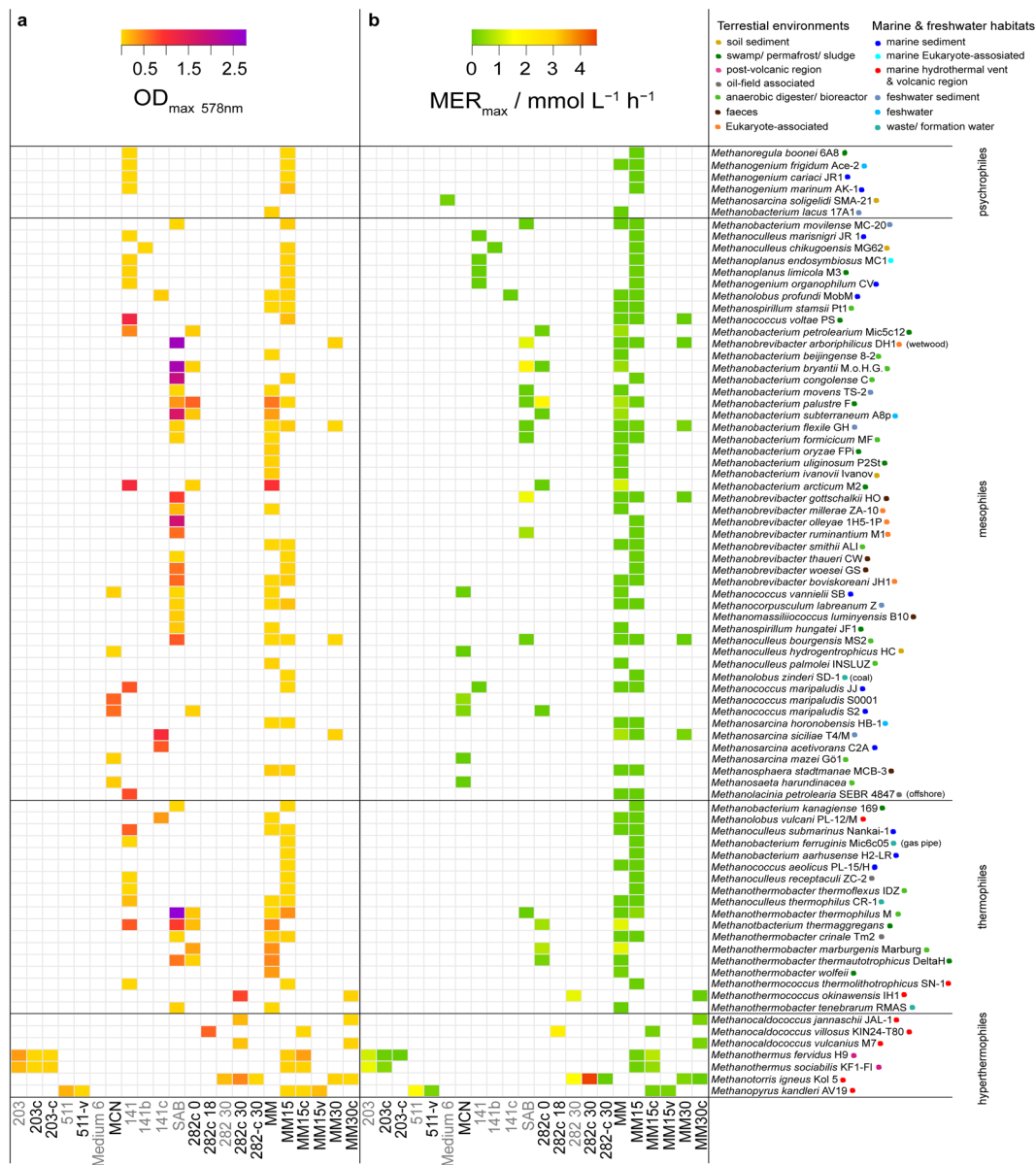


Fig. 1 Biomass and CH₄ production kinetics of the multivariate prescreen of 80 methanogens in defined and complex media. Experiments were performed in closed batch cultivation systems at 2 bar (120 mL flasks, 50 mL medium). On the y-axis, methanogens were arranged as groups according to their temperature optimum in psychrophiles, mesophiles, thermophiles, or hyperthermophiles. Methanogens are listed with ascending strain-specific temperature optimum from top to bottom. Coloured points next to the strain designation on the y-axis indicate the isolation site of the tested methanogen (terrestrial habitats: golden brown—soil sediment, dark green—swamp/permafrost/sludge, pink—post-volcanic region, gray—oil-field associated, light green—anaerobic digester/bioreactor, brown—feces, orange—eukaryote-associated; marine and freshwater environments: bright blue—marine sediment, turquoise—marine eukaryote-associated, red—marine hydrothermal vent and volcanic region, gray blue—freshwater sediment, sky blue—freshwater, green blue—waste/formation water). In total, 22 defined and complex media were tested, but not every strain was cultivated on every medium. Defined and complex media are shown on the x-axis in black and gray fonts, respectively. For each closed batch cultivation, three biological replicates (in some cases, two biological replicates) plus one negative control were used. **a** The maximum absorption is shown as OD_{max} at 578 nm, and **b** the maximum volumetric CH₄ production rate is shown as MER_{max} / mmol L⁻¹ h⁻¹.

homogenous growth pattern and a biomass increase rate below 10 (Supplementary Fig. S1). Interestingly, mesophilic methanogens grown on SAB (complex medium) showed a biomass increase rate between 10 and 40 (Supplementary Fig. S1).

Correlating nutritional demands, growth, and CH₄ productivity. In order to correlate nutritional demands to associated growth, substrate conversion, and productivity, a standardized principal component analysis (PCA) and subsequent k-means

cluster analysis was performed. The cluster analysis was performed for OD_{max}, turnover_{max}, MER_{max}, and the combination of those variables together with the concentrations of sulfate and/or sulfur, ammonium, phosphate, and cysteine in the respective media. These data were then linked to strain-specific information such as taxonomy and cultivation temperature. Further, medium-associated parameters were used for interpretation, such as the applied medium with the corresponding trace element solution (TES), and the addition of vitamin solution (VS), cysteine, and yeast/peptone to the medium. The clustering approach then

enabled the grouping of the mentioned variables and parameters into clusters.

High OD values were achieved on complex medium with VS, cysteine, and yeast extract/peptone (Supplementary Data 1, Table S1 and Supplementary Fig. S3). Mesophilic strains *Methanococcus* spp., *Methanobacterium* spp., and thermophilic methanogens belonging to the genera *Methanothermobacter* and *Methanobacterium* grew on defined media without vitamins and cysteine (MM medium, Fig. 1 and Supplementary Data 1, Table S1). At least 50% reduction of OD_{max} was observed with strains that grew best on MM medium when cultivated on 282c 0 medium (rich-TE and cysteine addition), except for *Methanobacterium palustre*. This strain showed similar growth on MM and 282c 0 medium indicated by an OD_{max} of 0.53 and 0.66. *M. palustre* also showed higher CH₄ production kinetics on 282c 0 medium compared to MM medium, indicated by a turnover_{max} up to 93% and a 2.6-fold higher MER_{max} of 1.9 mmol L⁻¹ h⁻¹ in 282c 0 medium (Fig. 1 and Supplementary Data 1, Table S2). Methanogens that require supplements (VS or cysteine) in the media, like Methanopyri or Methanococci, reached OD_{max} values between 0.2 and 0.8.

The highest turnover was achieved by methanogens grown in defined media. Methanococci and Methanobacteria showed a turnover_{max} between 90 and 98% on medium 203 and 282-based media (Supplementary Data 1, Table S3, and Supplementary Fig. S4). A turnover_{max} between 80 and 90% was achieved on media MM, MCN, SAB, and 511. The highest turnover_{max} values (90–98%) were obtained in a medium with 30 times lower phosphate concentration and five times lower ammonium concentration compared to the media used in the turnover_{max} range 80–90% (Supplementary Data 1, Table S3, and Supplementary Fig. S4), which might indicate that phosphate and ammonium concentrations in the medium need optimization.

Methanococci, Methanobacteria, and Methanopyri were found to be highly productive in a closed batch cultivation mode at 2 bar, indicated by a MER_{max} range from 1.1 to 4.6 mmol L⁻¹ h⁻¹ (Supplementary Data 1, Table S4, and Supplementary Fig. S5). These methanogens grew on 282, MM-based media, 511, SAB, and 203 media. The highest MER_{max} values of 2.1 and 4.6 mmol L⁻¹ h⁻¹ were achieved by Methanococci on 282-based media without yeast/peptone and the addition of cysteine (cluster 1, Supplementary Data 1, Table S4, and Supplementary Fig. S5). The highest MER_{max} of 4.61 mmol L⁻¹ h⁻¹ was measured for *Methanotorris igneus*. Methanococci showed the highest turnover_{max} and MER_{max} values (Supplementary Figs. S4–S6, Supplementary Data 1, and Table S5), assuming that growth and CH₄ productivity is positively influenced by sulfate, sulfur, and cysteine.

On defined medium without cysteine (MM-based and MCN medium), mesophilic methanogens from the order Methanobacteria, Methanomicrobia, and Methanococci (with the exception of *Methanothermobacter fervidus*) reached a MER_{max} range of 0.5 to 1.0 mmol L⁻¹ h⁻¹ (Supplementary Data 1 and Table S4). Strains isolated from hyperthermophilic environments (Fig. 1) like Methanocaldococcales and Methanopyraceae required cysteine or vitamins in the medium to exhibit high MERs (282-based and 511 media). They showed growth to an OD_{max} < 0.03, and a turnover_{max} and MER_{max} reduction of 90% and 95%, respectively, when cysteine or vitamins were excluded from the media (282-c 30, 511-v, Fig. 1). Attempts to restore the MER of *Methanocaldococcus* spp., *Methanothermococcus* sp., *Methanothermus* sp., and *Methanopyrus* sp. on MM medium at their optimum salt concentration through the addition of cysteine or vitamins after they had been grown in media without these compounds did not recover their CH₄ productivity, except for *M. fervidus*. This organism showed an OD_{max} of 0.34 on 203 and MM15c medium. However, the productivity of *M. fervidus* on medium MM15c was

decreased compared to 203 medium, indicated by a MER_{max} of 0.9 and 1.1 mmol L⁻¹ h⁻¹ (Fig. 1, Supplementary Data 1, and Table S1).

From this comprehensive multivariate, quantitative analysis of growth and CH₄ production kinetics, we prioritized *Methanobacterium* spp. and *Methanococcus* sp. (mesophilic), *Methanothermobacter* spp., *Methanobacterium* sp., and *Methanothermococcus* sp. (thermophilic), and *Methanocaldococcus* spp., *Methanothermus* sp., *Methanotorris* sp., and *Methanopyrus* sp. (hyperthermophilic) for the subsequent 10 bar H₂/CO₂ conversion experiments. These methanogens were selected due to their ability to grow fast on defined media (cluster 1 and 3 in Supplementary Data 1, Table S2, and Supplementary Fig. S3), and their successful reactivation after dormancy (Supplementary Table S1). Prioritized high-performance methanogens showed a turnover_{max} > 70% (cluster 1, 2, and 4 in Supplementary Data 1, Table S3, and Supplementary Fig. S4) and 90% of the strains exhibited a MER_{max} > 1 mmol L⁻¹ h⁻¹ (cluster 1, 3, and 4 in Supplementary Data 1, Table S4, and Supplementary Fig. S5).

Identification of high-performance methanogens. The multivariate quantitative comparative investigation resulted in the prioritization of 14 fast converting and/or fast growing autotrophic hydrogenotrophic methanogens (Fig. 2). Prioritized methanogens belong to Class I methanogens²⁶. In a closed batch cultivation system, gas-utilizing methanogens experience extreme gas-limiting conditions, as the substrate (gas in the headspace of the cultivation vessel) is converted at a decreasing rate²⁷. To reduce the effect of gas-limiting conditions for fast converting methanogens during closed batch cultivation, HFG experiments were conducted. During HFG experiments the headspace of the serum bottle was replenished with H₂/CO₂ before turnover_{max} was reached. Thus, we found that the mesophilic methanogen *Methanococcus maripaludis* and the hyperthermophilic methanogens *M. jannaschii* and *Methanocaldococcus vulcanius* showed a turnover rate > 5 h⁻¹ (Supplementary Fig. S7). Furthermore, HFG experiments enabled a quantitative and comparative analysis of MERs and biomass increase rates among prioritized methanogens (Fig. 2).

The biomass increase rates of *M. palustre*, *Methanothermobacter thermophilus*, *Methanobacterium thermaggregans*, and *M. vulcanius* varied, indicating a heterogeneous growth pattern (Fig. 2a). Based on the position of the median in the boxplot figures (Fig. 2a), we could observe that *M. thermophilus* and *M. thermaggregans* have a longer lag phase compared to the other tested methanogens. All other strains showed little variation regarding the biomass increase rates, indicating that these strains had a homogenous growth pattern under the tested conditions (Fig. 2a). *M. vulcanius* and *M. jannaschii* showed a MER median value > 5 mmol L⁻¹ h⁻¹, which is 1.5-fold the amount compared to *Methanothermococcus okinawensis* and twice the amount of *M. igneus* as well as approximately four times higher compared to the other tested methanogens (Fig. 2b).

Methanogens with a surface protein layer are CH₄ cell factories. The highest MERs were observed for hyperthermophilic methanogens that grow within a temperature range of 80–85 °C on defined medium 282c 30 and known to possess a surface protein layer (S-layer)^{33,34}. These S-layer proteins harbor the InterPro domains IPR022651 S_layer_C, IPR006454 S_layer_MJ, and IPR022650 S_layer_N (Supplementary Data 2). Furthermore, *M. fervidus* is covered with an S-layer protein (slgA in a p6 lattice pattern)³⁵. The slgA consists of six InterPro features, IPR006633 Carb-bd_sugar_hydrolysis-dom, IPR007742 NosD-dom, IPR022441 Para_beta_helix_rpt-2, IPR006626 PbH1,

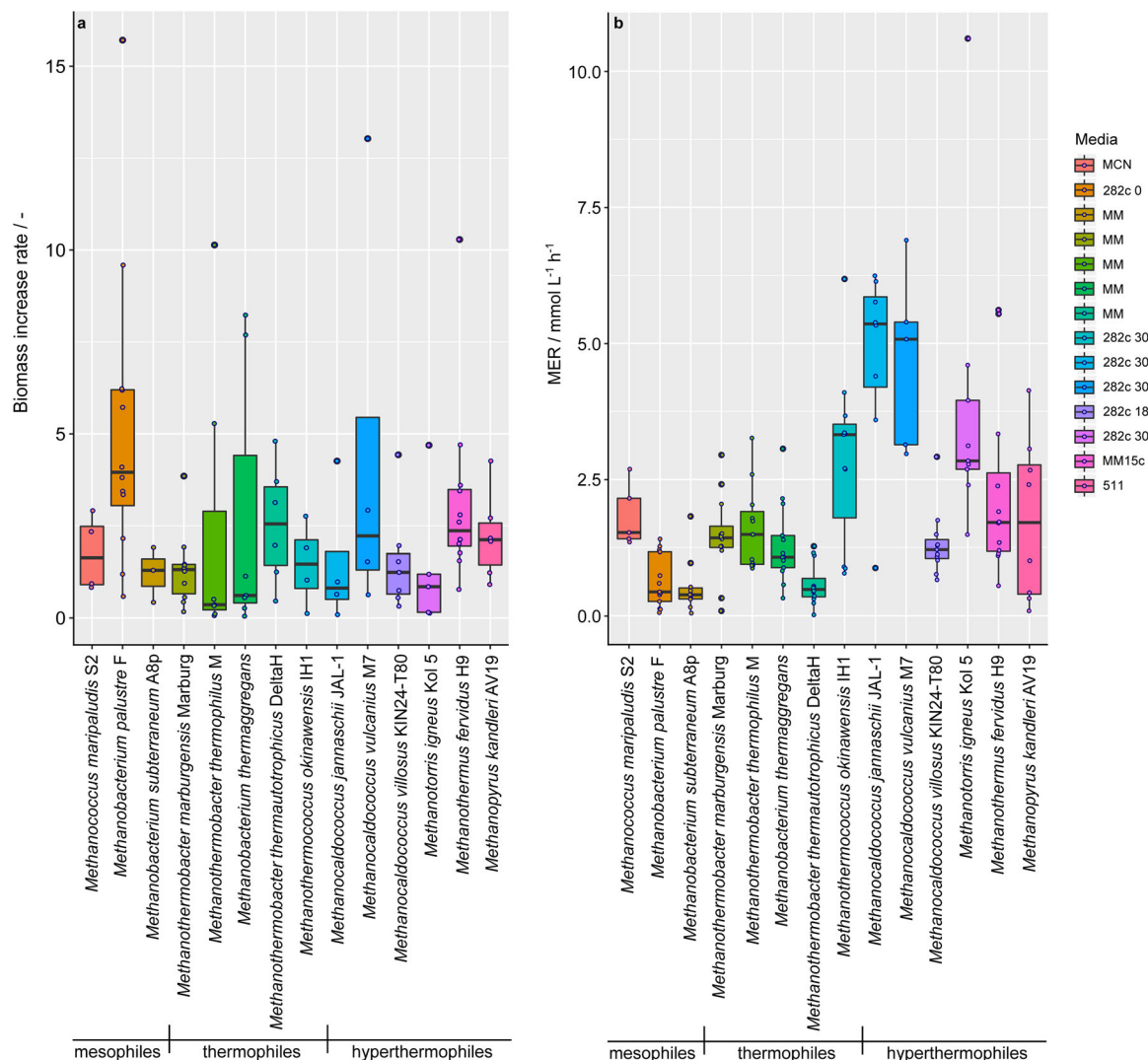


Fig. 2 Results of high frequency gassing (HFG) experiments of prioritized methanogens at 2 bar in defined medium. All experiments were performed in quadruplicates including a negative control. **a** The biomass increase rate / - and **b** The MER / mmol L⁻¹ h⁻¹. Boxplots are used for data visualization. In both subfigures, three temperature blocks are distinguished and highlighted from left to right. Left block: mesophilic methanogens grown at 37 °C (*Methanococcus maripaludis* S2, *Methanobacterium palustre* F, *Methanobacterium subterraneum* A8p); middle block: five thermophilic strains grown at 65 °C (*Methanothermobacter marburgensis* Marburg, *Methanothermobacter thermophilus* M, *Methanobacterium thermaggregans*, *Methanothermobacter thermautotrophicus* DeltaH, *Methanothermococcus okinawensis* IH1); right block: six hyperthermophilic methanogens (*Methanocaldococcus jannaschii* JAL-1 (80 °C), *Methanocaldococcus vulcanius* M7 (80 °C), *Methanocaldococcus villosus* KIN24-T80 (80 °C), *Methanotortrix igneus* Kol 5 (85 °C), *Methanothermobacter fervidus* H9 (80 °C), *Methanopyrus kandleri* AV19 (98 °C)).

IPR012334 Pectin_lyas_fold, IPR011050 Pectin_lyase_fold/virulence as well as the Pfam motif PF05048 NosD. The same motifs can be found in the genomes of *M. marburgensis* and *M. thermautotrophicus* (Supplementary Data 2). This might be an indication for the presence of S-layers on the cell envelop of *M. marburgensis* and *M. thermautotrophicus*. However, up to now S-layers were never described for these organisms, and the function of these homologous proteins would therefore require characterization. Furthermore, the IPR032812, IPR013783 Ig-like_fold, and IPR032812 SbsA_Ig features can be found in the genome of *M. marburgensis*, with the later Ig-like domain present in the S-layer protein SbsA. While *M. thermautotrophicus* does not harbor an IPR032812 SbsA_Ig feature, it encodes an IPR013783 Ig-like_fold feature, a motif which was also detected in *M. vulcanius* and *M. villosus*. *M. kandleri* was shown earlier to possess a S-layer³⁶, although no S-layer related motifs or domains could be found in our in silico analysis (Supplementary Data 2), but the

IPR011330 Glyco_hydro/deAcase_b/a-brl and IPR002509 NODB_dom features were detected.

High-performance methanogens harbor a specific MCR α amino acid motif. A subsequent bioinformatic examination of the key enzyme for methanogenesis, methyl-coenzyme M reductase (MCR), and especially the alpha subunit of the MCR (MCR α) revealed that all highly productive prioritized methanogens harbor the Tyr ^{α 444} Gly ^{α 445} Tyr ^{α 446} amino acid motif and belong to the Class I methanogens (Supplementary Fig. S8). Borrel et al. showed that Tyr ^{α 444} is substituted to phenylalanine in some *Methanocella* spp., *Methanoregula* spp., *Methanocorpusculum* spp., and *Methanosarcina* spp.³⁷. We found that Tyr ^{α 444} to Phe ^{α 444} is specific to Class II methanogens, except to *Methanimicrococcus blatticola* and *Methanolinea tarda* (Supplementary Fig. S8). In Class I methanogens, Tyr ^{α 444} anchors the coenzyme

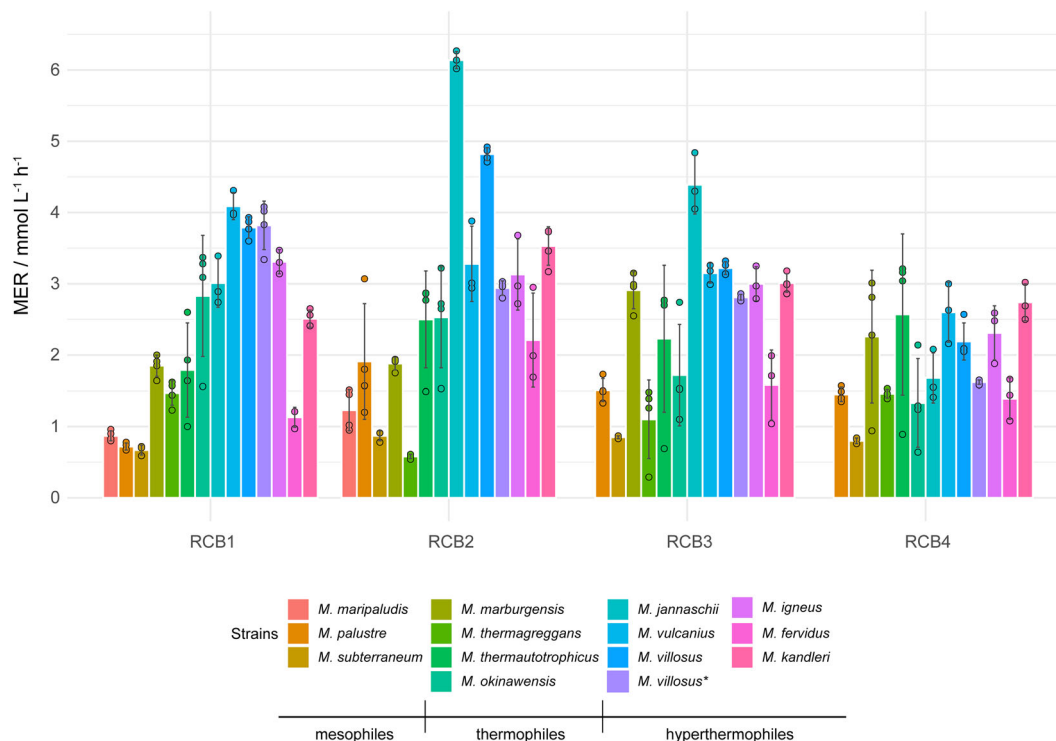


Fig. 3 Results of high-pressure RCB cultivations of prioritized methanogens in the simultaneous bioreactor system (SBRs) at 10 bar. All experiments were performed in quadruplicates in the SBRs system at a gassing ratio of H_2/CO_2 (4:1)³⁵. Mean and standard deviation are shown. The principle of the cultivation was to repressurize each of the bioreactors to 10 bar after full headspace gas conversion. RCB1, RCB2, RCB3, and RCB4 indicate results from individual and successive closed batch headspace gas conversions. MER / $mmol L^{-1} h^{-1}$ is shown. The left block indicates mesophilic methanogens grown at 37 °C (*Methanococcus maripaludis* S2, *Methanobacterium palustre* F, *Methanobacterium subterraneum* A8p); the middle block shows the thermophilic methanogens grown at 65 °C (*Methanothermobacter marburgensis* Marburg, *Methanobacterium thermaggregans*, *Methanothermobacter thermautotrophicus* DeltaH, *Methanothermobacter okinawensis* IH1); and the right block shows hyperthermophilic methanogens (*Methanocaldococcus jannaschii* JAL-1 (80 °C), *Methanocaldococcus vulcanius* M7 (80 °C), *Methanocaldococcus villosus* KIN24-T80 (80 °C), *Methanocaldococcus villosus* KIN24-T80*-grown on 282c 18_E medium (80 °C), *Methanoterris igneus* Kol 5 (85 °C), *Methanothermobacter fervidus* H9 (80 °C), *Methanopyrus kandleri* AV19 (98 °C)).

M together with two other amino acid residues in the catalytic center of the MCR³⁸. Additionally, Tyr^{a446} is predominately exchanged to phenylalanine in Methanosarcinaceae, *Methanosarcina* spp.³⁷, *Methanohalophilus* spp., *Methanohalobium* sp., *Methanococcoides* spp., *Methanobolus* sp., and *Methanomethylovorans* sp.. Moreover, we found an amino acid exchange from Tyr^{a446} to Phe^{a446} in some *Methanobrevibacter* spp..

***M. igneus* and *M. jannaschii* are high-pressure CH_4 cell factories.** High-pressure cultivation of methanogens offers an opportunity to improve the gas transfer rate of substrate gases into the liquid phase. In order to investigate the gas conversion kinetics and the barotolerance of applied methanogens, high-pressure experiments were designed to examine the MER_{max} and conversion kinetics, including the turnover rate and the maximum conversion rate ($k_{min}/bar h^{-1}$) at a hyperbaric pressure of 10 and 50 bar in the simultaneous bioreactor system (SBRs)³⁹.

Four subsequent repetitive closed batch (RCB) experiments were performed by flushing and replenishing the SBRs headspace after reaching turnover_{max} (Supplementary Figs. S9 and S10). After adaptation to hyperbaric conditions in RCB1, ten of the 14 prioritized methanogens achieved MER_{max} and turnover rates in RCB2 (Fig. 3 and Supplementary Fig. S10). The MERs of *M. jannaschii* indicated a putative liquid limitation already in RCB2. Seven methanogens showed similar MERs and turnover rates in RCB3 and RCB4, probably as a result of limitation of the liquid substrates. Half of the tested strains showed an enhanced MER, when comparing 2 bar HFG and 10 bar RCB experiments (Figs. 2

and 3). In general, a fivefold higher pressure lead to an average MER increase of $2.2 \pm 0.9 mmol L^{-1} h^{-1}$. Some methanogens showed an average MER fold decrease of $0.6 \pm 0.1 mmol L^{-1} h^{-1}$, which might indicate a pressure sensitivity or could have been due to a potentially low pH present at hyperbaric conditions, since higher pressure in the cultivation vessel results in a higher soluble CO_2 , which lowers the pH⁴⁰.

The highest MERs of $6.14 \pm 0.12 mmol L^{-1} h^{-1}$ and $4.39 \pm 0.41 mmol L^{-1} h^{-1}$ were achieved by *M. jannaschii* in RCB2 and RCB3, respectively, even without growth medium optimization. Besides MER and turnover rate, the parameter k_{min} (Supplementary Fig. S11), derived from the maximum negative slope of the pressure curves (Supplementary Fig. S9), indicates the time point of MER_{max}. k_{min} was therefore used to unambiguously identify the most productive strains and prioritize them for the subsequent 50 bar cultivations. These strains were *M. jannaschii*, *M. igneus*, *M. villosus* (using 282c 18_E medium), and *M. marburgensis*. The CO_2 -BMP model organism *M. marburgensis* was successfully cultivated at 50 bar without facing liquid limitations (Fig. 4). *M. thermaggregans*, which is a high CH_4 productivity strain in fed-batch cultivation mode¹³, did not grow at 50 bar (Supplementary Fig. S12) and *M. villosus* and *M. igneus* showed a decrease of MER and turnover rate directly in RCB2 (Fig. 4 and Supplementary Fig. S13), indicating a liquid limitation or sensitivity toward low pH, putatively caused by hyperbaric cultivation conditions. *M. jannaschii* did not fully convert H_2/CO_2 in RCB2 (Supplementary Fig. S12). Therefore, the medium for *Methanocaldococcus* spp. requires improvement, which should be based on a spectrophotometric analysis of quantities and quality of trace element

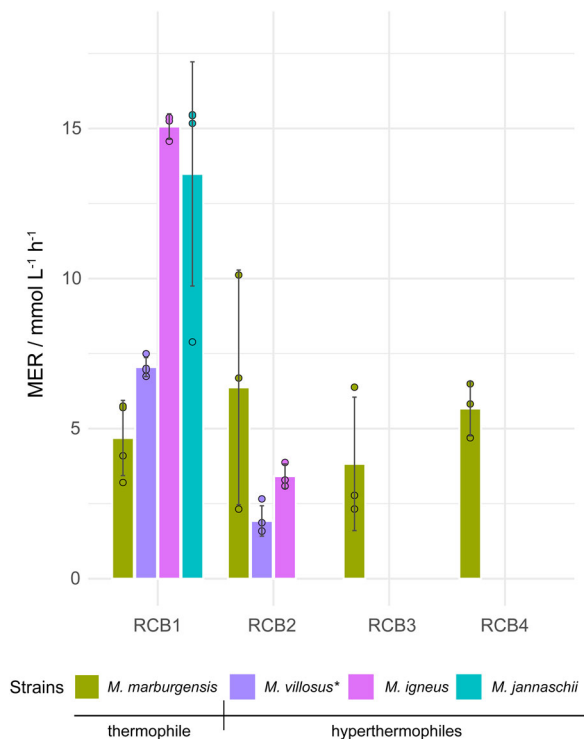


Fig. 4 Results of RCB cultivations of thermophilic and hyperthermophilic methanogens in the SBRS at 50 bar. The RCB cultivations were performed in quadruplicates with four runs RCB1, RCB2, RCB3, and RCB4. Mean and standard deviation of MER / mmol L⁻¹ h⁻¹ is shown. Thermophile: *Methanothermobacter marburgensis* Marburg (65 °C, MM medium); hyperthermophiles: *Methanocaldococcus villosus* KIN24-T80* (80 °C, 282c18_E medium), *Methanoterris igneus* Kol 5 (85 °C, 282c 30 medium), and *Methanocaldococcus jannaschii* JAL-1 (80 °C, 282c 30 medium).

species and basal medium ingredients consumed during medium development^{41,42}. Although the *Methanocaldococcus* medium needs further improvement, *M. villosus* and *M. igneus* did not show a lag phase at 50 bar. However, *M. villosus* directly started to convert H₂/CO₂ exponentially from the beginning of the experiment, compared to *M. igneus*, which showed linear, thus liquid-limited or low pH-retarded growth, directly from the onset of the cultivation (Supplementary Fig. S12). *M. igneus* comprised a threefold higher MER and *M. villosus* comprised a twofold higher k_{min} compared to *M. marburgensis* at 50 bar, when comparing the performance during RCB1 (Fig. 4 and Supplementary Fig. S11). *M. igneus* exhibited the highest MER of 15.1 ± 0.4 mmol L⁻¹ h⁻¹ and turnover rate of 4.8 ± 0.2 h⁻¹ in the 50 bar cultivation experiments (Fig. 4 and Supplementary Fig. S13), however we quantified a lower k_{min} for *M. villosus* than for *M. igneus*. Furthermore, *M. igneus* and *M. jannaschii* exhibited the highest MER_{max} (Supplementary Fig. S14). These results show that a nutrient limitation occurred and/or insufficient catalytically active biomass was present, meaning that CH₄ production was operated at $q_{CH_4,max}$ ^{12,20,30}. The fold increase of gaseous substrate in the media was found not to be proportional with the CH₄ productivity of the methanogens. The 25-fold higher gaseous substrate presence in the media, compared to a 2 bar cultivation, led to an average MER increase of 2.92 ± 0.43 mmol L⁻¹ h⁻¹. The time until full conversion of H₂/CO₂ at 50 bar was on average increased compared to 10 bar RCBs, by 2.5-fold (*M. marburgensis*, 67.79 ± 6.24 h), 2-fold (*M. villosus*, 43.21 h), and 5 h (*M. jannaschii*, 23.61 h). In contrast, *M. igneus* was 2 h (18.72 h) faster to reach full conversion under 50 bar RCBs compared to 10 bar RCBs.

Discussion

Pure culture CO₂-BMP is regarded as a key technology combining chemical energy storage, CO₂ utilization and biofuel production. Within CO₂-BMP, methanogens are employed as autocatalytic CH₄ cell factories. Thus, we aimed to identify and characterize the highest performing CH₄ cell factories. This up to now unprecedented quantitative comparative physiological, bioinformatic, and biotechnological analysis provides a comprehensive view on growth and CH₄ production kinetics, essential nutritional components and barotolerance of 80 methanogens. The quantitative analysis of axenic methanogenic cultures enabled the identification of high performing cell factories for CH₄ production (high q_{CH_4}) with a high maximum specific growth rate (μ_{max}), straightforward cultivation methods (in terms of sterility, media demand, reproducibility), and tolerance to hyperbaric cultivation conditions.

Psychrophilic methanogens reached a rather low OD_{max} < 0.2 in this study. This could be explained by the fact that psychrophilic microbes have in general a slower metabolism or a longer doubling time compared to microorganisms that grow at higher temperatures^{43–45}. The heterogeneous growth pattern of mesophilic methanogens on complex and defined media could be explained by their ecological and phylogenetic heterogeneity. Although high biomass concentrations are often linked to growth on complex medium, highest productive methanogens do not necessarily require complex medium to reach a high OD. The highest CH₄ productivities were achieved by Methanococci, and especially by *Methanocaldococcus* spp. and *Methanoterris* sp. which exhibited higher conversions and CH₄ production kinetics (Fig. 2 and Supplementary Fig. S7) than thermophilic methanogens belonging to Methanobacteria.

Methanococci were shown to possess a faster metabolism, indicated by higher CH₄ production kinetics, possibly due to the usage of [NiFeSe]-hydrogenases for H₂ oxidation. Instead of using [NiFe]-hydrogenases for the oxidation of H₂ as *Methanothermobacter* spp. (F₄₂₀-reducing hydrogenase Frh and F₄₂₀-nonreducing hydrogenase Mvh), *Methanococcus* spp. and *Methanocaldococcus* spp. use [NiFeSe]-hydrogenases (F₄₂₀-reducing hydrogenase Fru and F₄₂₀-nonreducing hydrogenase Vhu) that display much higher catalytic activities^{46,47}. Additionally, *Methanocaldococcus* spp. do not harbor selenium-free hydrogenases^{46,48,49}. The catalytic activity of [NiFeSe]-hydrogenases is greatly increased compared to [NiFe]-hydrogenases. Vhu of *M. voltae* showed a catalytic activity of 43,540 U mg⁻¹⁴⁷, whereas Mvh of *M. marburgensis* indicated a catalytic activity of 1600 U mg⁻¹¹⁵⁰.

Our results reveal that methanogens, which showed the highest turnover rates and MERs, were covered with an S-layer. S-layer proteins can be positively or negatively charged, and it has been shown that charged S-layers enhance diffusion through the membrane⁵¹. The cell envelope of *M. kandleri* is known to be covered with an S-layer³⁶, although no S-layer motif was found during our UniProtKB search. Therefore, one could hypothesize that the S-layer proteins present on *M. kandleri* are characteristic for this phylogenetic group. Furthermore, our bioinformatic analysis of MCRA revealed that all highly productive prioritized methanogens harbor the Tyr^{a444} Gly^{a445} Tyr^{a446} amino acid motif and belong to Class I methanogens (Supplementary Fig. S8).

Among the amino acids, especially cysteine is a required media supplement for certain methanogens (Supplementary Table S2). Compared to the prioritized Methanobacteria, hyperthermophilic Methanococci have a necessity of cysteine in the cultivation media, although Class I methanogens (Methanobacteriales, Methanococcales, and Methanopyrales) use primarily sulfide and not cysteine as sulfur source, such as Class II methanogens⁵². The

cysteine requirement of hyperthermophilic Methanococci in the medium could be linked to the usage of cysteine via cysteine desulphidase (CDD) for H_2S , NH_4^+ , H^+ , and pyruvate production⁵³, the production of cysteine via the t-RNA dependent pathway (SepRS/SepCysS)^{54,55}, and absence of cysteine desulphurase (CSD)^{52,53,55} (Supplementary Table S2). Besides that, CDD seems to be associated with the sulfur transfer for Fe-S cluster biosynthesis^{55–57}. In case of *M. fervidus*, where CSD was found to be expressed and CDD had not been (Supplementary Table S2), cysteine might have a key function in tolerating elevated temperatures⁵⁸.

Besides the nutritional demand of methanogens regarding cysteine, the TES that is used in a medium plays an important role in the biocatalytic activity. The trace element composition of a medium should mimic the heavy metal composition and respective concentrations present at the isolation spot, but might need to be optimized for meeting a biotechnological purpose. Based on our findings during the multivariate comparative analyses, methanogens that were cultivated on a medium with a rich-TES composition (TES1, TES2, TES4, and TES5) require additional cysteine or vitamins in the growth medium. Growth on a defined medium including a minimal/optimized TES (TES3), without cysteine or vitamins, was just possible for certain groups of methanogens, such as some Methanobacteria and *M. maripaludis* (cluster 3 in Supplementary Data 1, Table S1, and Supplementary Fig. S6). Vice versa, strains that grow best on media with a rich-TES composition, cysteine or vitamin addition indicated poor growth and CH_4 productivity on a medium with a minimal TES (TES3, MM medium), even with cysteine and/or vitamins also added. This leads to the conclusion that the combination of a rich TES and the addition of cysteine and/or vitamins is essential for the tested hyperthermophilic methanogens to exhibit high MERs.

We obtained the highest conversion and CH_4 production kinetics under hyperthermophilic and hyperbaric conditions. H_2 solubility at hyperbaric pressure of 10 or 50 bar leads to a 5- or 25-times higher substrate availability in the medium, compared to a cultivation at 2 bar. Therefore, adaptations to hyperbaric conditions, liquid limitation, and the suitability of the cultivation medium for high-pressure bioreactor cultivations can be studied if the experimental set-up is designed accordingly^{12,20,24,30}. Instead of achieving a 5- and 25-fold productivity increase at 10 and 50 bar RCB experiments, an average of two- and threefold productivity increase was achieved, respectively. This might be due to cell envelope characteristics of the investigated methanogens and/or corresponding low pH⁴⁰, lipid composition, limitation of conversion kinetics by a liquid nutrient, or not enough available catalytically active biomass (biomass limitation) to instantly convert the additionally available gas, which could also be a result from a liquid limitation or natural borders if the culture is growing at μ_{max} .

The CH_4 productivity pattern between RCB1 and RCB2 at 10 and 50 bar (Fig. 3 and *M. marburgensis* in Fig. 4) could be an adaptation response to hyperbaric cultivation conditions. However, the tested thermophilic and hyperthermophilic Methanococci have a different core lipid composition (archaeol, macrocyclic archaeol, and tetraether lipids) than Methanobacteria (archaeol and tetraether lipids). Strains from both orders increase the percentage of tetraethers under challenging growth conditions (Supplementary Table S3). *M. jannaschii* decreases archaeol and increases the percentage of tetraether lipids with increasing temperature⁵⁹, or temperature and pressure⁶⁰, while *M. marburgensis* increases tetraether lipids (GDGT-0), when growing with detergents⁶¹. Moreover, *M. okinawensis* increases tetraether lipids (GMGT-0, GMGT-0', and GDGT-0) and decreases archaeol upon addition of high amounts of inhibitors, such as

ammonium chloride and/or methanol, except for formaldehyde, which leads to an increase of archaeol^{7,8}.

At 10 bar, putative liquid limitation or biomass limitation occurred during RCB3 and RCB4 (*M. marburgensis*, *M. thermotrophicus*, *M. jannaschii*, *M. vulcanius*, *M. villosus*, *M. igneus*, and *M. kandleri*) (Fig. 3 and Supplementary Fig. S9). However, at 50 bar putative liquid limitations arose right after RCB1 for *Methanocaldococcus* spp. and during RCB3 for *M. marburgensis*. Our findings indicate that just *M. marburgensis* is growing on a well-optimized medium (MM medium)³⁰. The growth media (282c 18 or 282c 30) for *Methanocaldococcus* spp. would need to be adapted for hyperbaric applications. Although 282-based media were not yet designed for cultivations at 50 bar, the time for full conversion of H_2/CO_2 was not affected in the cases of *M. igneus* and *M. jannaschii*, which did not show any retardation in CH_4 production during 50 bar cultivations. Perhaps these strains could be tested at higher pressure conditions, such as *M. okinawensis*, which showed CH_4 production up to 90 bar⁴⁰. *Methanocaldococcus* spp. exhibited higher specific growth rates than *M. marburgensis* (Supplementary Data 1 and Table S1), and thus liquid limitation occurs faster. Besides that, the metabolism of *M. marburgensis* is slower compared to *Methanocaldococcus* spp., indicated by the lower k_{min} values of *Methanocaldococcus* spp. (Supplementary Fig. S11). Therefore, the liquid limitation in our setup might not have had a strong effect.

This study on high-pressure biological CH_4 production in pure culture is a cornerstone of the emerging research and development field of Archaea Biotechnology¹⁹. The systematic assessment indicated that the high-performance strains belong to Class I methanogens. Hyperthermophilic Methanococci are high-pressure CH_4 production cell factories and the addition of cysteine and a rich TES in the media are essential for efficient growth of these Methanococci. Therefore, we propose to perform bioprocess development utilizing *M. igneus* and *M. jannaschii* to develop these organisms into high-pressure CH_4 cell factories. Moreover, methanogens that exhibited the highest turnover rates and MERs are covered with S-layers, and the amino acid motif Tyr^{α444} Gly^{α445} Tyr^{α446} in the alpha subunit of MCR is present in all high-performance methanogens. This analysis sets the foundation for a future high-pressure bioprocess optimization endeavor with the identified hyperthermophilic CH_4 cell factories. The autobiocatalytic activity of hyperthermophilic, autotrophic, hydrogenotrophic methanogens could therefore be employed for balancing the power grid system (energy storage) or to biologically depressurize H_2 and/or CO_2 containing emission flue gasses to CH_4 via the CO_2 -BMP process. High-pressure biological CH_4 production in pure culture could provide a purposeful route for the transition to an independent carbon-free or low-carbon energy bioeconomy.

Methods

Strains. All screening experiments including HFG (closed batch up to 2 bar) were performed with the methanogenic archaeal strains listed in Fig. 1. Methanogens were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany). High-pressure experiments were performed with selected strains in the SRBR in closed batch mode at 10 and 50 bar (Figs. 3 and 4).

Chemicals. CO_2 (99.995 Vol.-%), H_2 (99.999 Vol.-%), and H_2/CO_2 (80 Vol.-% H_2 in CO_2) were obtained from Air Liquide (Air Liquide GmbH, Schwechat, Austria). The H_2/CO_2 mixture (80 Vol.-% H_2 and Vol.-% CO_2) for high-pressure cultivations was obtained from Linde Gas (Linde Gas GmbH, Wels, Austria). All other chemicals were of highest available grade.

Media. Considering the nutritional requirements of the screened strains, several media were used to cultivate methanogenic archaeal strains, as they are SAB medium²⁸, McN medium³¹, Medium 6²⁹, DSMZ medium (141, 141b, 141c, 282,

203, 511), *Methanothermobacter marburgensis* medium (MM)³⁰ and MM medium with 15 or 30 g of NaCl (MM15, MM30). Some media were modified to test specific nutritional requirements (203c, 203-c, 511-v, 282c 0, 282c 18, 282c 18_E, 282c 30, 282-c 30, MM15c, MM15v, MM30c). The addition of “c” to the medium designation indicates the presence of cysteine in the medium, whereas “-c or -v” indicates the omission of cysteine or vitamins, respectively; the succeeding numbers refer to the amount of NaCl provided to the medium. Every media or solution was prepared with ultrapure H₂O Milli-Q[®] if not stated differently. To ensure growth in a respective medium and exclude any stimulative effects of medium contained in the inoculum, methanogens were grown in two passages or two times washed with fresh medium. Before inoculation on minimal medium, inocula were washed by centrifugation following by the removal of the supernatant, adding 1 mL of minimal medium and resuspending the pellet, centrifugation (10 min, 13,000 rpm), discarding the supernatant and resuspending the pellet in 1 mL of minimal medium. The exact media compositions are listed in Supplementary Information.

Multivariate analysis of cultivation conditions. Growth and CH₄ productivity of 80 methanogenic archaea were screened in closed batch cultivation mode up to 2 bar relative to atmospheric pressure, 2 barg, in an anaerobic atmosphere consisting out of 80% H₂ in CO₂ (4:1). For simplicity, all pressure assignments are described as 2, 10, or 50 bar relative pressure. The optimal growth temperatures of the tested methanogens range from 15 to 98 °C. The tested methanogens are classified as psychrophiles including psychrotolerant methanogens (15–30 °C), mesophiles (30–37 °C), thermophiles (40–70 °C), and hyperthermophiles (80–98 °C). Methanogenic strains were grown in 120 mL serum bottles (crimp neck vial, VWR International, Pennsylvania, USA) in chemically defined media (see “Media” section). After autoclaving, media filled bottles (autoclave Systec VX-120, Systec GmbH, Linden, Germany), inoculation was performed inside an anaerobic chamber (Coy Laboratory Products, Grass Lake, USA). Thereafter, bottles were pressurized with a H₂/CO₂ gas mixture (80% H₂ in CO₂) at 2 bar as previously described⁶². A sterile gaseous substrate supply requires the usage of sterile syringe filters (w/0.2 μm cellulose, 514-0061, VWR International, USA) and disposable hypodermic needles (Gr 14, 0.60 × 30 mm, 23 G × 1 1/4”, RX129.1, Braun, Germany). The gas phase was flushed by an insertion of a second needle at regular intervals for 2–4 s. After pressurizing, bottles were incubated in water baths (orbital shaking, water bath 1083, GFL Gesellschaft für Labortechnik mbH, Germany) or air incubators (100 rpm, Labwit incubators, Labwit Scientific Pty. Ltd, Australia) according to the optimal growth temperature of the respective cultivated strains. Bottles were taken out of the incubator and cooled down or heated up to room temperature. Thereafter, pressure and OD_{578 nm} measurements (liquid samples of 0.7 mL were taken) were performed to monitor the cultivation. After the measurements, bottles were flushed, repressurized, and incubated again at microbial-specific cultivation temperatures.

High frequency gassing experiments. The experimental set-up of HFG followed the procedure described above, except for the gassing frequency, which was increased to twice a day. In total 14 strains, three mesophilic methanogens grown at 37 °C (*M. maripaludis* S2, *M. palustre* F, *Methanobacterium subterraneum* A8p), five thermophilic methanogenic archaea cultivated at 65 °C (*M. marburgensis* Marburg, *M. thermophilus* M, *M. thermagregans*, *M. thermotrophicus* DeltaH, *M. okinawensis* IH1), and six hyperthermophilic methanogens (*M. jannaschii* JAL-1 (80 °C), *M. vulcanius* M7 (80 °C), *M. villosus* KIN24-T80 (80 °C), *M. igneus* Kol 5 (80 °C), *M. fervidus* H9 (80 °C), *M. kandleri* AV19 (98 °C)) were selected for HFG.

Dormancy study. Before methanogens reached the stationary growth phase, they were put into dormancy state in a 4 °C room or in a –80 °C freezer (Thermo Scientific™ TSU™ Series –86 °C Upright Ultra-Low Temperature Freezers, Thermo Fisher Scientific, USA). For the –80 °C dormancy study, cryostocks were used (800 μL culture and 200 μL 50% (v/v) glycerol). After dormancy at 4 or –80 °C, strains were inoculated into fresh medium. In case of strains that were kept at 4 °C, an aliquot of 1 mL was used as inoculum. The 1 mL –80 °C cryostocks were thawed and used as inoculum after removing the glycerol by centrifugation (10 min, 13,000 rpm). The respective dormancy periods are listed in Supplementary Table S1.

Analysis of growth and productivity. During all 2 bar screening experiments including HFG, growth and CH₄ formation was examined by OD and pressure measurements. Growth was monitored via offline OD measurements at 578 nm (OD_{578 nm}) by using a spectrophotometer (DU800, Beckman Coulter, California, USA). Before every OD_{578 nm} measurement, the sample was vortexed (Vortex Mixer MX-S, Biologix Group Limited, China). In total, 0.7 mL of the culture was sampled at regular intervals for OD_{578 nm} determinations. CH₄ production capacity was investigated through headspace pressure measurements of serum bottles in regular intervals using a digital manometer (LEO1-Ei, -1/3 bar relative, Keller, Germany)²⁷. Produced CH₄ was replaced by discontinuous gassing with H₂/CO₂ in regular intervals.

Data analysis. Two heatmaps are shown in Fig. 1, illustrating max. growth via OD_{max}, by measuring at 578 nm, and max. volumetric CH₄ productivity,

depicted as MER_{max} / mmol L⁻¹ h⁻¹. The heatmaps showing turnover_{max} / % (Supplementary Fig. S2) and biomass increase rate (Supplementary Fig. S1) can be found in Supplementary Information. The biomass increase rate depicts the average value of all biomass increase rates of a specific strain during the cultivation and is calculated according to Eq. (1). Figure 2 shows two boxplots, max. volumetric CH₄ productivity as MER / mmol L⁻¹ h⁻¹ and the biomass increase rate. The corresponding boxplot illustrating the turnover rate / h⁻¹ is shown in the Supplementary file (Supplementary Fig. S7). All data points were included into the boxplots. The heatmaps and the boxplots were generated using Rstudio Version 1.1.463 – © 2009–2018 RStudio, Inc. The R package ggplot2⁶³ was used. The graphical design was refined using Illustrator CS6 (Adobe Systems Inc., USA).

$$\frac{OD_{max} \cdot \mu}{OD \cdot \mu_{average}} \quad (1)$$

Correlation between nutritional demand and productivity. To correlate the nutritional demand of methanogens with their associate growth, substrate conversion, and productivity on respective media, a standardized principal components analysis (PCA) followed by a k-means clustering was performed. Clustering was performed on OD_{max}, turnover_{max}, MER_{max}, and the combination of those with medium-associated components such as salt, sulfate, sulfur, ammonium, phosphate, and cysteine concentrations. This analysis was then linked to medium-based information (trace elements solution, VS, the addition of yeast, peptone, or cysteine) and strain-specific characteristics like taxonomy and cultivation temperature. After collecting the data, missing values got imputed via PCA imputation using the R package missMDA⁶⁴, followed by a normalization of the data using the stats package⁶⁵. Thereafter the PCA (stats⁶⁵) was performed, followed by the k-means clustering (stats⁶⁵) using the first two components. The within cluster sum of squares accounted 85.5% for OD_{max}, 93.2% for MER_{max}, 91.8% for turnover_{max}, and 86.3% for the combination of these variables. The following R packages were applied during the analysis: R packages ggplot2⁶⁶, missMDA⁶⁴, FactoMineR^{63,66,67}, and stats⁶⁵. The biplots were generated using Rstudio Version 1.1.463 – © 2009–2018 RStudio, Inc. The graphical design was refined using Illustrator CS6 (Adobe Systems Inc., USA).

Cell envelope and S-layer composition of prioritized methanogens. Cell envelop structures including core lipid composition and putative S-layer presence on prioritized methanogens were investigated via literature research. Furthermore, a bioinformatic screen on the UniProt Knowledgebase (UniProtKB)⁶⁸ regarding the presence of S-layer on tested methanogenic strains was conducted. The combination of strain-specific designation and “S-layer protein, glycoprotein, or glyco protein” were used as query terms. Additional information about protein family classification (Interpro) and functional regions/domain of the protein are indicated via Pfam⁶⁹.

Amino acid conservation of curtail interacting partners within methyl-coenzyme M reductase I, subunit alpha. Protein sequences were obtained using the protein–protein BLAST (blastp)^{70,71}. The Reference Sequence (RefSeq) collection was used as sequence database for the blastp (version January 2020). MCR I, subunit alpha from *Methanothermobacter marburgensis* Marburg (GenBank: ADL59127.1) was used as a query. The default algorithm parameters were chosen (scoring matrix BLOSUM62) besides the max. target sequences which was increased to 500. Protein sequences from methanogens that were investigated during this study and were not obtained through blastp were afterwards added to the blastp sequences. Missing protein sequences were downloaded from UniProtKB⁶⁸. Thereafter, protein sequences were aligned with the multiple sequence alignment tool Clustal Omega⁷² applying the default settings. The download of amino acid sequences and the following analysis was performed in February 2020. The alignment was illustrated using Jalview version 2.10.5⁷³. The graphical design was refined with Illustrator CS6 (Adobe Systems Inc., USA).

High-pressure SBRS cultivation of methanogens. An experimental design was developed to examine H₂/CO₂ conversion kinetics through online pressure measurements and to identify the most productive methanogens at a hyperbaric relative pressure of 10 and 50 bar. Further, possible liquid limitations and kinetic stability of CH₄ production of prioritized methanogens at 10 bar was examined. The SBRS consisted of four identical cultivation vessels (160 mL), suitable for investigation of microbial activity at pressures up to 50 bar and temperatures up to 145 °C³⁹. All pressure assignments are given in bar and described as relative pressure (10 and 50 bar relative to atmospheric pressure). Pressure within the bioreactors (R1, R2, R3, and R4) was accurately measured with online pressure sensors (Pressure Transducers and Transmitters, Type: PTDVB0601B1C2, Parker, Cleveland, USA). Before bioreactors were pressurized, the gas inlet line pressure was set to 10 or 50 bar of H₂/CO₂ with an analogous manometer (WIKA Messgerätevertrieb Ursula Wiegand GmbH & Co. KG, Vienna, Austria, 0–60 bar)³⁹. Thus, pressure was checked online and offline during the pressurization step. During the experiments, pressure was monitored with the online pressure sensing tool. The pressure sensor and the manometer were calibrated before installation. The accuracy of the heating jacket was also tested beforehand. The fact that each

experiment was performed in quadruplicates, strengthens the validity of the results. Some high-pressure experiments could not be investigated with all four bioreactors due to technical or biological failure. Since this was a screening approach, high-performance methanogens were further investigated and their growth and CH₄ production kinetics were then analyzed in detail.

SBRS inoculation procedure. After cleaning and autoclaving the SBRS, an anaerobic environment in each bioreactor vessel was established, followed by setting the cultivation temperature, respectively, to the strain's optimal growth temperature based on DSMZ data. Before combining medium, supplements, and culture in a vial (100 mL crimp neck vial, Macherey-Nagel GmbH & Co. KG, Germany), the culture was reactivated for 10 min via flushing with 1 bar H₂/CO₂. Based on the used medium, supplement solutions like NaHCO₃, 0.5 mol L⁻¹ Na₂S, L-Cysteine-HCl-H₂O, and vitamins (Wolf's VS, see medium 141) were added to the medium right before inoculation. The inoculum (medium, supplements, and culture) was transferred into the bioreactor³⁹. Thereafter an appropriate incubation pressure (10 or 50 bar, depending on the experiment) was adjusted. The RCB set-up included three repressurization steps either at 10 or 50 bar, respectively. After a total gas conversion, a gas sample from each bioreactor was taken for gas composition analysis via gas chromatography. Full conversion is achieved if 2 bar (10 bar) or 10 bar (50 bar), i.e. one-fifth of the initial pressure, of residual gas in the bioreactors R1, R2, R3, and R4 is present, respectively, to production of CH₄ (4 H₂ + CO₂ → CH₄ + 2 H₂O) to avoid a driving force limitation. Before repressurization, the residual pressure was released. The following strains were investigated for 10 bar RCB: mesophiles 37 °C: *M. maripaludis* S2 (MCN medium), *M. palustre* F (282c 0 medium), *M. subterraneum* A8p (MM medium); thermophiles 65 °C: *M. marburgensis* Marburg (MM medium), *M. thermophilus* M (was not tested), *M. thermaggregans* (MM medium), *M. thermautotrophicus* DeltaH (MM medium), *M. okinawensis* IH1 (282c 30 medium); hyperthermophiles 80 °C: *M. jannaschii* JAL-1 (282c 30 medium), *M. vulcanius* M7 (282c 30 medium), *M. villosus* KIN24-T80 (282c 18 and 282c 18_E medium), *M. fervidus* H9 (MMc15 medium); hyperthermophiles 85 and 98 °C: *M. igneus* Kol 5 (282c 30 medium), *M. kandleri* AV19 (511 medium). *M. maripaludis*: in RCB1, R2 was leaking at the beginning and fixed after taking note of the leakage. After 80 h in RCB3 no growth was observed, thus RCB3 was stopped. *M. subterraneum*: R2 was not functional during all RCB runs. After recognizing the leakage on the following day, the pressure decreased to 3.3 bar. Subsequently R2 bioreactor was repressurized and RCB2 was started. R2 in RCB3 was again leaking. The problem was fixed before starting RCB4. *M. thermautotrophicus*: the pressure curve of R1 in RCB3 (100 h) did not follow the trend of the others, thus it is not shown and not repressurized in RCB4. R1 was not working for cultivations of *M. jannaschii*, *M. vulcanius*, and *M. igneus*. *M. fervidus*: the performance of R1 was not comparable to the others in RCB1 and therefore not repressurized for following RCB runs. *M. kandleri*: after RCB3, R1 was not working properly and therefore not repressurized.

The following strains were investigated in 50 bar RCBs: *M. marburgensis* Marburg, *M. thermaggregans*, *M. villosus* KIN24-T80*, *M. igneus* Kol 5, and *M. jannaschii* JAL-1. After RCB2 cultivations of *M. villosus* KIN24-T80*, *M. igneus* Kol 5, and *M. jannaschii* JAL-1 the experiments were stopped due to much lower CH₄ productivity compared to RCB1. *M. thermaggregans*: after 120 h of cultivation no growth was detected, thus RCB1 was stopped.

Analysis of biomass, CH₄ productivity, and head space gas composition (SBRS)

During all RCB runs at 10 and 50 bar, growth and CH₄ formation were examined with offline cell dry weight analysis, online pressure measurements, and offline headspace gas determinations. Growth was determined via offline cell dry weight analysis by using a centrifuge (tabletop centrifuge Heraeus Megafuge 1.0 R, Thermo Electron Corporation, Massachusetts, USA) to pellet the harvested biomass. Biomass was centrifuged for 15 min at 3500 rpm, followed by drying the wet biomass at 105 °C overnight (Heraeus drying cabinet model T 5050, Heraeus, Hanau, Germany). Pressure drop, which corresponds to CH₄ production, was monitored by online pressure sensors. After total gas conversion, gas samples were taken via headspace vials (10 mL headspace vial, Schmidling Labor + Service GmbH, Switzerland) which were crimped with crimp caps (crimp cap with bore hole, Carl Roth, Germany) and vacuumed for 5 min before usage. The CH₄ off-gas concentration (CH₄ / Vol.-%) in the gas samples are analyzed with a gas chromatograph (Trace GC Ultra 2000, Thermo Fisher Scientific Inc., US) equipped with a thermal conductivity detector. Chromatographic separation was executed on a Carboxen-1000 packed column (10 m, 3/8"). Helium used as carrier gas with a constant pressure of 2.35 bar and a split flow of 90/10. A representative gas sample with a volume of 1 mL was injected. Following GC parameters were chosen for the analysis: inlet heater 150 °C, detector 200 °C, oven initial temperature 35 °C hold for 5 min, temperature raising rate of 20 °C min⁻¹ to 225 °C (hold for 10 min) at final temperature.

Data analysis of SBRS 10 and 50 bar cultivations. To elucidate the CH₄ production kinetics from cultivated methanogenic strains, the following variables were calculated: methane evolution rate calculated with GC data MER_{GC} / mmol L⁻¹ h⁻¹ or pressure data MER_{pressure} / mmol L⁻¹ h⁻¹, carbon uptake rate CUR / mmol L⁻¹ h⁻¹, hydrogen uptake rate HUR / mmol L⁻¹ h⁻¹, maximum conversion rate *k*_{min} / bar h⁻¹.

MER was calculated either by using CH₄ concentration obtained from GC measurements or through the integration of the recorded curve for the online pressure probe in every bioreactor. Data collection was performed by a data acquisition unit (USB-2019, ICP DAS-EUROPE GmbH, Germany) and recorded via LabVIEW (National Instruments, Austin, USA). During cultivation the record interval was set to 5 min. All calculations were performed by using the program Origin 2019 (Originlab Corporation, USA). After selecting a proper record interval (30 min) for data analysis, the CH₄ production kinetics (MER, turnover rate, *k*_{min}) were calculated, while neglecting biomass formation^{39,62}. These MER values were used to determine the respective point in time where MER reached its maximum (MER_{max}). *k*_{min} indicates the highest slope in the curve, which reflects the point of highest turnover, showing the time point of MER_{max}. MER_{total} indicates the MER value over the total experimental time including all data points. MER values got smoothed and plotted over time. To determine the lag and the stationary phase during cultivation and neglect these data points, an integration over the obtained curve was performed. The integration start (*x*_{start}) and end (*x*_{end}) points of the curves were identified as follows: *x*_{start} and *x*_{end} are intersection points, which were elucidated by shifting the *x*-axis to low points of the curve (minimum *x* = 0.1). The difference of these points reflects the time period Δ*t* of microbial growth phases. MER_{global} was determined by dividing the calculated area by Δ*t*³³. Finally, the program Origin indicated the associated pressure data for the *x*_{start} and *x*_{end} values. The associated pressure data were subsequently used for calculating of MER_{GC}, MER_{pressure}, CUR, HUR, and *k*_{min}. The barplots were generated using Rstudio Version 1.1.463 – © 2009-2018 RStudio, Inc.. The R package ggplot2 was used⁶⁶. The graphical design was refined using Illustrator CS6 (Adobe Systems Inc., California, USA).

Statistics and reproducibility. All information on statistics and reproducibility of the experiments is provided in the respective "Materials and methods" sections or in the Supplementary Information, Supplementary Data 1 and Data 2. The 2 bar multivariate screening experiments were performed with 80 methanogens with an ranging optimal growth temperature between 15 and 98 °C. The tested methanogens are classified as psychrophiles including psychrotolerant methanogens (15–30 °C), mesophiles (30–37 °C), thermophiles (40–70 °C), and hyperthermophiles (80–98 °C). The multivariate screening was followed by the HFG experiments, which were performed with 14 methanogens. For each closed batch cultivation (multivariate screening or HFG), three biological replicates (in some cases, two biological replicates) plus one negative control were used. Thereafter, 10 and 50 bar hyperbaric cultivations were performed. The 10 bar RCB cultivations were investigated with 13 methanogens. Followed by 50 bar RCB cultivations with four methanogens. Hyperbaric cultivations were performed in quadruplicates. In some cases, just three bioreactors were used, due to a non-functionality of one of the bioreactors of the SBRS.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All relevant data are available from the corresponding author upon request.

Received: 23 August 2020; Accepted: 10 February 2021;

Published online: 05 March 2021

References

- Kirschke, S. et al. Three decades of global methane sources and sinks. *Nat. Geosci.* **6**, 813–823 (2013).
- Lyu, Z. & Whitman, W. B. Transplanting the pathway engineering toolbox to methanogens. *Curr. Opin. Biotechnol.* **59**, 46–54 (2019).
- Bižić, M. et al. Aquatic and terrestrial cyanobacteria produce methane. *Sci. Adv.* **6**, eaax5343 (2020).
- Repeta, D. J. et al. Marine methane paradox explained by bacterial degradation of dissolved organic matter. *Nat. Geosci.* **9**, 884–887 (2016).
- Thauer, R. K., Kaster, A.-K., Seedorf, H., Buckel, W. & Hedderich, R. Methanogenic archaea: ecologically relevant differences in energy conservation. *Nat. Rev. Micro* **6**, 579–591 (2008).
- Abdel Azim, A., Rittmann, S. K.-M. R., Fino, D. & Bochmann, G. The physiological effect of heavy metals and volatile fatty acids on *Methanococcus maripaludis* S2. *Biotechnol. Biofuels* **11**, 301 (2018).
- Baumann, L. M. F. et al. Intact polar lipid and core lipid inventory of the hydrothermal vent methanogens *Methanocaldococcus villosus* and *Methanothermococcus okinawensis*. *Org. Geochem.* **126**, 33–42 (2018).
- Taubner, R.-S. et al. Membrane lipid composition and amino acid excretion patterns of *Methanothermococcus okinawensis* GROWN in the presence of inhibitors detected in the enceladian plume. *Life* **9**, 85 (2019).
- Wang, L. et al. Bioinformatics analysis of metabolism pathways of archaeal energy reserves. *Sci. Rep.* **9**, 1034 (2019).

10. Seifert, A. H., Rittmann, S., Bernacchi, S. & Herwig, C. Method for assessing the impact of emission gasses on physiology and productivity in biological methanogenesis. *Bioresour. Technol.* **136**, 747–751 (2013).
11. Rittmann, S. K.-M. R. A critical assessment of microbiological biogas to biomethane upgrading systems. In *Biogas Science and Technology* (eds Guebitz, G. M., Bauer, A., Bochmann, G., Gronauer, A. & Weiss, S.) 117–135 (Springer International Publishing, 2015).
12. Rittmann, S. K.-M. R., Seifert, A. H. & Bernacchi, S. Kinetics, multivariate statistical modelling, and physiology of CO₂-based biological methane production. *Appl. Energy* **216**, 751–760 (2018).
13. Mauerhofer, L.-M. et al. Physiology and methane productivity of *Methanobacterium thermaggregans*. *Appl. Microbiol. Biotechnol.* **102**, 7643–7656 (2018).
14. Strübing, D., Huber, B., Leubhn, M., Drewes, J. E. & Koch, K. High performance biological methanation in a thermophilic anaerobic trickle bed reactor. *Bioresour. Technol.* **245**, 1176–1183 (2017).
15. Jensen, M. B. et al. Stick or leave—pushing methanogens to biofilm formation for ex situ biomethanation. *Bioresour. Technol.* **291**, 121784 (2019).
16. Rachbauer, L., Voitl, G., Bochmann, G. & Fuchs, W. Biological biogas upgrading capacity of a hydrogenotrophic community in a trickle-bed reactor. *Appl. Energy* **180**, 483–490 (2016).
17. Nock, W. J., Serna-Maza, A., Heaven, S. & Banks, C. J. Evaluation of microporous hollow fibre membranes for mass transfer of H₂ into anaerobic digesters for biomethanization. *J. Chem. Technol. Biotechnol.* **94**, 2693–2701 (2019).
18. Tao, B. et al. Simultaneous biomethanisation of endogenous and imported CO₂ in organically loaded anaerobic digesters. *Appl. Energy* **247**, 670–681 (2019).
19. Pfeifer, K. et al. Archaea biotechnology. *Biotechnol. Adv.* **47**, 107668 (2021).
20. Seifert, A. H., Rittmann, S. & Herwig, C. Analysis of process related factors to increase volumetric productivity and quality of biomethane with *Methanothermobacter marburgensis*. *Appl. Energy* **132**, 155–162 (2014).
21. Graf, F., Götz, M., Wonneberger, A.-M., & Köppel, W. Techno-ökonomische Studie zur biologischen Methanisierung bei PtG-Konzepten. (2014).
22. Rönsch, S. et al. Review on methanation—from fundamentals to current projects. *Fuel* **166**, 276–296 (2016).
23. Mutz, B., Carvalho, H. W. P., Mangold, S., Kleist, W. & Grunwaldt, J.-D. Methanation of CO₂: structural response of a Ni-based catalyst under fluctuating reaction conditions unraveled by operando spectroscopy. *J. Catal.* **327**, 48–53 (2015).
24. Bernacchi, S., Krajete, A., Seifert, A. H., Herwig, C. & Rittmann, S. Experimental methods for screening parameters influencing the growth to product yield (Y(x)/CH₄) of a biological methane production (BMP) process performed with *Methanothermobacter marburgensis*. *AIMS Bioeng.* **1**, 72–87 (2014).
25. Boonyaratanakornkit, B., Córdova, J., Park, C. B. & Clark, D. S. Pressure affects transcription profiles of *Methanocaldococcus jannaschii* despite the absence of barophilic growth under gas-transfer limitation. *Environ. Microbiol.* **8**, 2031–2035 (2006).
26. Miller, J. F., Shah, N. N., Nelson, C. M., Ludlow, J. M. & Clark, D. S. Pressure and temperature effects on growth and methane production of the extreme thermophile *Methanococcus jannaschii*. *Appl. Environ. Microbiol.* **54**, 3039–3042 (1988).
27. Nishimura, N., Kitaura, S., Mimura, A. & Takahara, Y. Cultivation of thermophilic methanogen KN-15 on H₂-CO₂ under pressurized conditions. *J. Fermentation Bioeng.* **73**, 477–480 (1992).
28. Khelaiifa, S., Raoult, D. & Drancourt, M. A versatile medium for cultivating methanogenic archaea. *PLoS ONE* **8**, e61563 (2013).
29. Wagner, M., Amann, R., Lemmer, H. & Schleifer, K. H. Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* **59**, 1520–1525 (1993).
30. Rittmann, S., Seifert, A. & Herwig, C. Quantitative analysis of media dilution rate effects on *Methanothermobacter marburgensis* grown in continuous culture on H₂ and CO₂. *Biomass Bioeng.* **36**, 293–301 (2012).
31. Whitman, W. B., Shieh, J., Sohn, S., Caras, D. S. & Premachandran, U. Isolation and characterization of 22 mesophilic methanococci. *Syst. Appl. Microbiol.* **7**, 235–240 (1986).
32. Mauerhofer, L.-M. et al. Methods for quantification of growth and productivity in anaerobic microbiology and biotechnology. *Folia Microbiol.* **64**, 321–360 (2019).
33. Akca, E. et al. Genes and derived amino acid sequences of S-layer proteins from mesophilic, thermophilic, and extremely thermophilic methanococci. *Extremophiles* **6**, 351–358 (2002).
34. Bellack, A., Huber, H., Rachel, R., Wanner, G. & Wirth, R. *Methanocaldococcus villosus* sp. nov., a heavily flagellated archaeon that adheres to surfaces and forms cell–cell contacts. *Int. J. Syst. Evol. Microbiol.* **61**, 1239–1245 (2011).
35. Bröckl, G. et al. Analysis and nucleotide sequence of the genes encoding the surface-layer glycoproteins of the hyperthermophilic methanogens *Methanothermobacter feravidus* and *Methanothermobacter sociabilis*. *Eur. J. Biochem.* **199**, 147–152 (1991).
36. König, H., Rachel, R. & Claus, H. Proteinaceous surface layers of Archaea: ultrastructure and biochemistry. In *Archaea* (ed. Cavicchioli, R.) 315–340 (American Society of Microbiology, 2007).
37. Borrel, G. et al. Wide diversity of methane and short-chain alkane metabolisms in uncultured archaea. *Nat. Microbiol.* **4**, 603–613 (2019).
38. Ermler, U., Grabarse, W., Shima, S., Goubeaud, M. & Thauer, R. K. Crystal structure of methyl-coenzyme M reductase: the key enzyme of biological methane formation. *Science* **278**, 1457–1462 (1997).
39. Pappenreiter, P. A., Zwirtmayr, S., Mauerhofer, L.-M., Rittmann, S. K.-M. R. & Paulik, C. Development of a simultaneous bioreactor system for characterization of gas production kinetics of methanogenic archaea at high pressure. *Eng. Life Sci.* **19**, 537–544 (2019).
40. Taubner, R.-S. et al. Biological methane production under putative Enceladus-like conditions. *Nat. Commun.* **9**, 748 (2018).
41. Blazevic, A. et al. Biotransformation of Scheelite CaWO₄ by the Extreme Thermoacidophile Metallosphaera sedula: Tungsten–Microbial Interface. *Front. Microbiol.* **10**, 1492 (2019).
42. Nischkauer, W., Vanhaecke, F., Bernacchi, S., Herwig, C. & Limbeck, A. Radial line-scans as representative sampling strategy in dried-droplet laser ablation of liquid samples deposited on pre-cut filter paper disks. *Spectrochimica Acta Part B At. Spectrosc.* **101**, 123–129 (2014).
43. Ingraham, J. L. & Bailey, G. F. Comparative study of effect of temperature on metabolism of psychrophilic and mesophilic bacteria. *J. Bacteriol.* **77**, 609 (1959).
44. Price, P. B. & Sowers, T. Temperature dependence of metabolic rates for microbial growth, maintenance, and survival. *Proc. Natl Acad. Sci. USA* **101**, 4631 (2004).
45. Schulte, P. M. The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. *J. Exp. Biol.* **218**, 1856 (2015).
46. Baltazar, C. S. A. et al. Nickel–iron–selenium hydrogenases—an overview. *Eur. J. Inorg. Chem.* **2011**, 948–962 (2011).
47. Sorgenfrei, O., Linder, D., Karas, M. & Klein, A. A novel very small subunit of a selenium containing [NiFe] hydrogenase of *Methanococcus voltae* is posttranslationally processed by cleavage at a defined position. *Eur. J. Biochem.* **213**, 1355–1358 (1993).
48. Rother, M., Resch, A., Wilting, R. & Böck, A. Selenoprotein synthesis in archaea. *Biofactors* **14**, 75–83 (2001).
49. Zhang, Y. & Gladyshev, V. N. General trends in trace element utilization revealed by comparative genomic analyses of Co, Cu, Mo, Ni, and Se. *J. Biol. Chem.* **285**, 3393–3405 (2010).
50. Stojanovic, A., Mander, G. J., Duin, E. C. & Hedderich, R. Physiological role of the F420-non-reducing hydrogenase (Mvh) from *Methanothermobacter marburgensis*. *Arch. Microbiol.* **180**, 194–203 (2003).
51. Li, P.-N. et al. Nutrient transport suggests an evolutionary basis for charged archaeal surface layer proteins. *ISME J.* **12**, 2389–2402 (2018).
52. Liu, Y., Sieprawska-Lupa, M., Whitman, W. B. & White, R. H. Cysteine Is Not the Sulfur Source for Iron-Sulfur Cluster and Methionine Biosynthesis in the Methanogenic Archaeon *Methanococcus maripaludis*. *J. Biol. Chem.* **285**, 31923–31929 (2010).
53. Tchong, S.-I., Xu, H. & White, R. H. l-Cysteine desulfidase: an [4Fe-4S] enzyme isolated from *Methanocaldococcus jannaschii* that catalyzes the breakdown of l-Cysteine into pyruvate, ammonia, and sulfide. *Biochemistry* **44**, 1659–1670 (2005).
54. Liu, Y., Beer, L. L. & Whitman, W. B. Methanogens: a window into ancient sulfur metabolism. *Trends Microbiol.* **20**, 251–258 (2012).
55. Hidesse, R., Inoue, T., Imanaka, T. & Fujiwara, S. Cysteine desulphurase plays an important role in environmental adaptation of the hyperthermophilic archaeon *Thermococcus kodakarensis*. *Mol. Microbiol.* **93**, 331–345 (2014).
56. Whitman, W. B., Ankwanda, E. & Wolfe, R. S. Nutrition and carbon metabolism of *Methanococcus voltae*. *J. Bacteriol.* **149**, 852 (1982).
57. Whitman, W. B., Bowen, T. L. & Boone, D. R. The methanogenic bacteria. In *The Prokaryotes: Volume 3: Archaea. Bacteria: Firmicutes, Actinomycetes* (eds Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. & Stackebrandt, E.) 165–207 (Springer, 2006).
58. Horne, A. J. & Lessner, D. J. Assessment of the oxidant tolerance of *Methanosarcina acetivorans*. *FEMS Microbiol. Lett.* **343**, 13–19 (2013).
59. Sprott, G. D., Meloche, M. & Richards, J. C. Proportions of diether, macrocyclic diether, and tetraether lipids in *Methanococcus jannaschii* grown at different temperatures. *J. Bacteriol.* **173**, 3907 (1991).
60. Kaneshiro, S. M. & Clark, D. S. Pressure effects on the composition and thermal behavior of lipids from the deep-sea thermophile *Methanococcus jannaschii*. *J. Bacteriol.* **177**, 3668 (1995).
61. Gräther, O. W., Arigoni, D. & Jaun, B. Zur Struktur und Biosynthese der Tetraetherlipide der Archaea. (1994).

62. Taubner, R. S. & Rittmann, S. K. M. R. Method for indirect quantification of CH₄ production via H₂O production using hydrogenotrophic methanogens. *Front. Microbiol.* **7**, 532 (2016).
63. Galili, T. dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering. *Bioinformatics* **31**, 3718–3720 (2015).
64. Josse, J. & Husson, F. missMDA: a package for handling missing values in multivariate data analysis. *J. Stat. Softw.* **70**, 1–31 (2016).
65. R Core Team. R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, 2019).
66. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis* (Springer, 2016).
67. Lê, S., Josse, J. & Husson, F. FactoMineR: an R Package for multivariate analysis. *J. Stat. Softw.* **25**, 1–18 (2008).
68. The UniProt Consortium UniProt: a worldwide hub of protein knowledge. *Nucleic Acids Res.* **47**, D506–D515 (2018).
69. El-Gebali, S. et al. The Pfam protein families database in 2019. *Nucleic Acids Res.* **47**, D427–D432 (2019).
70. States, D. J. & Gish, W. QGB: combined use of sequence similarity and codon bias for coding region identification. *J. Computational Biol.* **1**, 39–50 (1994).
71. Altschul, S. F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402 (1997).
72. Madeira, F. et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* **47**, W636–W641 (2019).
73. Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. & Barton, G. J. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189–1191 (2009).

Acknowledgements

Maria Wolfsgruber is acknowledged for assisting with high-pressure experiments. Dr. Lydia M.F. Baumann is acknowledged for helpful discussions. Dr. Melina Kerou is acknowledged for proofreading the manuscript. Greatly acknowledged is the Österreichische Forschungsförderungsgesellschaft (FFG) for funding the projects BioHyMe (grant 853615), Bioraffinerie (grant 854156), and NitroFix (grant 859293). Open access funding by the University of Vienna.

Author contributions

L.-M.M.: writing paper draft, writing paper review and editing, closed batch cultivations, high-pressure cultivations, bioinformatic analysis, data analysis, preparation of figures. S.Z.: high-pressure cultivations, data analysis. P.P.: high-pressure cultivations, data ana-

lysis. S.B.: design of experiments, writing paper review and editing. A.H.S.: design of experiments, writing paper review and editing. B.R.: closed batch cultivations. T.S.: closed batch cultivations. R.-S.T.: closed batch cultivations, writing paper review and editing. C.P.: supervision of high-pressure experiments, supervision of data analysis, writing paper review and editing, funding acquisition. S.K.-M.R.R. closed batch cultivations, supervision of closed batch experiments, supervision of data analysis, supervision of bioinformatic analyses, writing paper draft, writing paper review and editing, funding acquisitions.

Competing interests

S.B. and A.H.S. declare to have competing financial interests due to their employment in the Krajete GmbH. All other authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s42003-021-01828-5>.

Correspondence and requests for materials should be addressed to S.K.-M.R.R.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021