Non-enzymatic methane formation by aerobic organisms

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Biological Sciences - Article

Keywords: Methane, Bacillus subtilis, CH4 cycling

DOI: https://doi.org/10.21203/rs.3.rs-153741/v1

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Abstract

Methane (CH$_4$) is the most abundant organic compound in the atmosphere, largely originating from anthropogenic and natural biogenic sources$^1$. Traditionally, biogenic CH$_4$ has been regarded as the final product of the anoxic decomposition of organic matter by methanogenic Archaea. However, plants$^2$–$^4$, fungi$^5$, algae$^6$,$^7$ and cyanobacteria$^8$ have recently been shown to produce CH$_4$ in the presence of oxygen. While methanogens produce CH$_4$ enzymatically during anaerobic energy metabolism$^9$, the requirements and pathways for CH$_4$ production by “non-methanogenic” cells are poorly understood. Here we demonstrate that CH$_4$ formation by Bacillus subtilis is triggered by free iron species, enhanced by oxidative stress and restricted to metabolically active life-cycle stages. We also show that other model organisms from Bacteria and Eukarya including a human cell line release CH$_4$ and respond to inducers of oxidative stress by enhanced CH$_4$ formation. Our results imply that all living cells possess a common mechanism of CH$_4$ formation without the need for specific enzymes. We propose that CH$_4$ formation is a conserved feature of living systems which is coupled to metabolic activity and the concomitant generation of reactive oxygen species. Our findings open new perspectives for our understanding of environmental CH$_4$ cycling, oxidative stress responses and the search for extraterrestrial life.

Background

Methane is a highly potent greenhouse gas that affects Earth’s climate. Around 70 % of all emissions to the atmosphere derive from biogenic sources$^{10}$. Biological CH$_4$ formation has long been considered to occur only under strictly anoxic conditions in organisms belonging to the domain Archaea. To generate the cellular fuel ATP, methanogenic archaea convert simple compounds, such as CO$_2$, H$_2$ or acetate, into CH$_4$. This process of methanogenesis depends on reactions that are catalysed by unique sets of enzymes and coenzymes$^9$. In addition, small amounts of CH$_4$ can be formed via ‘mini-methanogenesis’ in several sulphate-reducing bacteria which contain the enzyme carbon monoxide dehydrogenase$^{11,12}$. However, during the past 15 years, evidence has been accumulating that other organisms produce CH$_4$ under aerobic conditions. These include both multicellular organisms, such as plants$^2$–$^4$ and saprotrophic fungi$^5$, and unicellular organisms, including marine and freshwater algae$^6$,$^13$ and cyanobacteria$^8$. These organisms generate energy via photosynthesis and/or respiration, and it is unclear why and how they release CH$_4$. Multiple marine and freshwater bacteria harbouring the C-P lyase pathway have been reported to generate CH$_4$ from methylphosphonate$^{14–18}$. Several bacteria and archaea have also been shown to possess alternative nitrogenases$^{19,20}$ or nitrogenase-like reductases$^{21}$, which can produce CH$_4$ and other hydrocarbons. In addition – as we will demonstrate here – living systems can form CH$_4$ without the need for specific enzymes, and such pathways could drive CH$_4$ formation in all cells.

In plants, CH$_4$ formation is enhanced by UV irradiation$^{3,22,23}$, inhibition of cytochrome c oxidase$^{24}$ and environmental stressors$^4$,$^25$. This raises the question whether reactive oxygen species (ROS, e.g., hydrogen peroxide H$_2$O$_2$, hydroxyl radicals ·OH and superoxide radicals O$_2^-$) might be involved in the
formation of CH$_4$. In highly oxidizing environments generated \textit{in vitro} by a chemical model system containing iron (II/III), hydrogen peroxide (H$_2$O$_2$) and the radical scavenger ascorbic acid, CH$_4$ is readily formed from organosulphur compounds at ambient temperatures$^{26}$. Under Fenton-type conditions, non-heme oxo-iron(IV) ([Fe$^{IV}$=O]$^{2+}$) oxidizes methyl sulphides to sulphoxides, which then results in selective formation of methyl radicals by sulphoxide demethylation, and ultimately leads to CH$_4$ and methanol formation depending on oxygen availability$^{26,27}$. Moreover, ROS such as ·OH and H$_2$O$_2$ react with methyl sulphoxides to produce methyl radicals or peroxomethyl radicals in the presence of oxygen$^{28}$, subsequently resulting in generation of CH$_4$ or oxidized C$_1$ species.

Fenton chemistry takes place in living cells, as iron is an essential trace element$^{29}$ and H$_2$O$_2$ is produced during metabolism$^{30}$. Hydrogen peroxide and Fe$^{2+}$ react to either ferric iron (Fe$^{3+}$), OH$^-$ and ·OH radicals, or alternatively [Fe$^{IV}$=O]$^{2+}$ and water$^{31}$. This therefore raises the possibility that non-enzymatic CH$_4$ formation occurs in cells under oxic conditions at ambient temperatures. Suitable methyl donors for CH$_4$ formation could, in principle, be derived from a wide spectrum of molecules containing sulphur- and/or nitrogen-bonded methyl groups, which are endogenously produced during cellular metabolism and exogenously secreted during natural product formation. Such methylated sulphur compounds include methionine, dimethylsulphoniopropionate (DMSP), dimethyl sulphide (DMS) and dimethyl sulphoxide (DMSO), which are ubiquitous in the environment$^{32}$. Thus, we tested the hypothesis that a non-enzymatic pathway of CH$_4$ formation exists in all cells, which is based on interactions between ROS, iron and methyl donors (Fig. 1A).

**Formation of methane by \textit{Bacillus subtilis}**

We first explored CH$_4$ formation in the Gram-positive model bacterium \textit{Bacillus subtilis} which, to the best of our knowledge, is not known to release CH$_4$. To detect authentic CH$_4$ formation, we performed stable carbon isotope labelling experiments, using sterile media as baseline controls, and measured CH$_4$ in the headspace of atmospherically sealed bacterial cultures (Fig. 1B). We first investigated whether CH$_4$ could be derived from glucose, an abundant carbon source that is readily taken up and metabolized by \textit{B. subtilis}. Indeed, small but significant amounts of CH$_4$ were formed in bacterial cultures from [$_{13}$C$_6$]-glucose (0.21 nmol, $p < 0.001$). Moreover, the corresponding $^{13}$C-isotope values ($\Delta$δ$_{13}$C; difference between culture and sterile media) were highly enriched (79 ± 1 ‰). This indicates that glucose metabolism in \textit{B. subtilis} results in at least one precursor compound that contributes to the formation of CH$_4$. We next investigated whether \textit{B. subtilis} could utilize methyl sulphide and methyl sulphoxide as exogenous precursor carbon compounds for CH$_4$ formation. In fact, addition of either $^{13}$C-labelled DMS or DMSO to bacterial cultures resulted in a clear shift in the isotopic value, demonstrating that the methyl groups of each sulphur compound were converted into CH$_4$ by reduction. Addition of DMSO increased CH$_4$ formation by 8-fold (1.85 nmol, $p < 0.001$) relative to glucose. Formation of CH$_4$ containing $^{13}$C-labelled carbon derived from DMS was also observed. Although in the latter case CH$_4$ amounts were low,
and varied from experiment to experiment, $\Delta \delta^{13}$C values were correlated with the amount of CH$_4$ produced. Taken together, these experiments provide unambiguous evidence for CH$_4$ formation from both exogenous and endogenous carbon compounds by B. subtilis.

**Free iron and oxidative stress promote CH$_4$ formation**

We further tested our hypothesis by varying the availability of suitable methyl donors, free iron and the level of oxidative stress, respectively (Fig. 1C). To minimize the effects of biomass variation on CH$_4$ formation, we used the same stationary-phase culture in a minimal medium and systematically varied the reaction conditions for CH$_4$ formation by supplying the substrate DMSO, additional free iron in the form of Fe$^{2+}$ (as FeSO$_4$) and hypochlorous acid (HOCl) to induce oxidative stress in all possible combinations. The optical density (OD$_{600nm}$) of stationary cultures changes only marginally if at all ($\pm 1\%$), which implies that the observed variation in CH$_4$ can be attributed to increased CH$_4$ formation by B. subtilis.

Under these conditions, external supply of DMSO (S) was required to stimulate significant CH$_4$ formation, as the amounts of CH$_4$ produced in its absence were close to atmospheric levels (Fig. 1C). In low-iron environments, addition of DMSO resulted in a small but significant quantity of CH$_4$ (0.45 nmol $\pm$ 0.03 nmol). Supplementation with HOCl to stimulate oxidative stress (S+O) increased this yield by $\sim$40%. Moreover, an iron-rich environment (S+F) boosted CH$_4$ formation from DMSO by 17-fold, while the additional imposition of HOCl-induced oxidative stress (S+F+O) resulted in a 35-fold increase in CH$_4$ levels relative to substrate alone (S). In contrast, heat-inactivated B. subtilis released only marginal amounts of CH$_4$ (0.038 $\pm$ 0.006 nmol, $p = 0.007$) in conditioned media containing DMSO, Fe$^{2+}$ and HOCl, suggesting that viable but not dead biomass promotes formation of CH$_4$. In accordance with our hypothesis, these data show that CH$_4$ formation from DMSO in stationary-phase B. subtilis cells is enhanced by both free iron and oxidative stress.

**CH$_4$ formation is restricted to metabolically active life-cycle stages**

According to our model, the generation of H$_2$O$_2$/ROS by living organisms is a prerequisite for CH$_4$ formation in the presence of suitable methyl donors. B. subtilis is a spore-forming organism that cycles between a dormant endospore state and a vegetative cell that grows and divides. Dormant spores are considered to be metabolically inactive, but resume metabolism upon germination$^{33}$. DMSO readily enters dormant Bacillus spores and does not interfere with spore revival$^{34}$. We thus asked whether the ability of B. subtilis to generate CH$_4$ from DMSO is restricted to its metabolically active life-cycle stages (Fig. 2A). To investigate this, we performed stable isotope labelling experiments on dormant and germinated B. subtilis spores using $^2$H-labelled DMSO (Fig. 2B). The use of $^2$H-labelled compounds and measurements of $\delta^{2}$H values increases the detectability of CH$_4$ formation by orders of magnitude in comparison to $^{13}$C labelling. Despite the exquisite sensitivity of the assay, CH$_4$ release from dormant spores after 10 h of incubation with DMSO was within the margin of error (0.02 nmol, $p = 0.066$), as were the stable hydrogen isotope signatures ($\Delta \delta^{2}$H = 10 $\pm$ 9 ‰). In contrast, when spores were germinated by
supplying the nutrient germinant mixture AGFK, \( \text{CH}_4 \) formation was clearly detected (0.1 nmol, \( P = 0.019 \)). Moreover, the stable hydrogen isotope signatures increased by three orders of magnitude, unambiguously confirming the conversion of the \(^2\text{H}\)-labelled methyl group of \(^2\text{H}\)-DMSO into \( \text{CH}_4 \) (Fig. 2B). Note that, under our experimental conditions, AGFK-induced germination is evidenced by the loss of spore refractivity; however, these spores did not grow out (Fig. 2B, insets). These data therefore suggest that germinated and thus metabolically active – but not dormant – spores can generate \( \text{CH}_4 \) from DMSO, in accordance with our hypothesis.

**CH\(_4\) accumulates during growth under oxic conditions**

According to our model, \( \text{CH}_4 \) is mainly formed under oxic conditions. Since *B. subtilis* is a facultative anaerobe\(^{35} \), we followed the dynamics of \( \text{CH}_4 \) accumulation during growth in complex media while monitoring the development of oxygen levels in the sample headspace (Fig. 2C). Methane levels increased during the exponential growth phase when oxygen was at atmospheric levels. Coincident with oxygen depletion, population growth slowed down rapidly, while \( \text{CH}_4 \) levels continued to increase for another ~ 2.5 h into stationary phase. Little additional \( \text{CH}_4 \) was formed during later cultivation stages when oxygen levels were low and the OD\(_{600\text{nm}}\) of the culture gradually declined. This indicates that *B. subtilis* forms \( \text{CH}_4 \) under oxic conditions, in agreement with our model.

**Methane can be generated by organisms from all three domains of life**

Finally, we investigated \( \text{CH}_4 \) formation by other cells from different domains of life that were previously not known to release \( \text{CH}_4 \). *Escherichia coli* DH5\( \alpha \) served as a model organism for Gram-negative bacteria and *Corynebacterium glutamicum* ATCC 13032 for Gram-positive bacteria. The yeast *Saccharomyces cerevisiae* S288C and the mold *Aspergillus niger* DSM 821 served as fungal models. Human HEK293T cells were used as the model system for mammalian cells in particular and animals in general (Fig. 3A).

All microorganisms formed \( \text{CH}_4 \), and levels were enhanced under oxidative stress induced by the addition of HOCl (Fig. 3B). Cellular \( \text{CH}_4 \) formation by bacteria and fungi exceeded the respective (sterile) media controls by factors from ~1.1 minimum (non-stressed *S. cerevisiae*) to ~423 maximum (HOCl-stressed *A. niger*). We also observed \( \text{CH}_4 \) formation in HEK293T cells (Fig. 3C). Comparing culture and corresponding sterile media, \( \text{CH}_4 \) was formed by cultures supplemented with unlabelled DMSO (0.65 nmol, \( P = 0.064 \)) or \(^2\text{H}\)-DMSO (1.07 nmol, \( P = 0.004 \)). Furthermore, the stable hydrogen isotope signatures of \(^2\text{H}\)-labelled cultures clearly indicated that \( \text{CH}_4 \) is also formed from DMSO by mammalian HEK293T cells. Together with the well-established role of *Archaea* in producing \( \text{CH}_4 \), we conclude that organisms from all three domains of life release \( \text{CH}_4 \).

**Discussion**
In summary, our study strongly suggests that CH$_4$ is formed as a universal by-product of life, in addition to known CH$_4$ generation mechanisms by (mini-)methanogenesis$^{9,11}$, by alternative nitrogenases$^{19,20}$ and other enzymes$^{14,21}$ (e.g. C-P lyase). All organisms investigated here were not previously known to produce CH$_4$. Our results clearly demonstrate continuous formation of CH$_4$, which is substantially enhanced upon supplementation with free iron and induction of oxidative stress, in accordance with the proposed underlying mechanism. The use of position-specific $^{13}$C/$^2$H-labelled precursor compounds makes this clear. Under Fenton-type conditions, free iron species and H$_2$O$_2$ catalyse oxidative demethylation of methylated sulphur compounds. This in turn gives rise to methyl radicals and the subsequent formation of CH$_4$. Methane might also be released from other methylated components (bonded to nitrogen and oxygen) as previously indicated by a chemical model system$^{26}$. In general, ROS and H$_2$O$_2$ have multiple sources$^{30}$, including respiratory activity, cellular stress and enzymatic reactions carried out by cytochrome oxidases and other heme monooxygenases$^{36}$, which are found in all three domains of life. We therefore propose that CH$_4$ release is a conserved feature of living systems that is coupled to metabolic activity and the corresponding generation of ROS. This opens new perspectives for (i) our understanding of environmental CH$_4$ cycling, (ii) oxidative stress responses and the potential physiological role(s) of CH$_4$, and (iii) the search for extraterrestrial life.

Formation of CH$_4$ as a consequence of fundamental biological processes implies that living biomass in all of Earth's environmental compartments continuously releases a background amount of CH$_4$. Although the CH$_4$ production by the investigated organisms is relatively small, if compared to methanogenic Archaea living in anaerobic environments, CH$_4$ release from aerobic organisms into the atmosphere might be of relevance to the biogeochemical cycles of CH$_4$. This has recently been proposed for cyanobacteria$^8$ and marine and freshwater algae$^6,13$ and their contribution to the abundance of CH$_4$ in oxygen-rich surface waters, commonly known as the “methane paradox”$^{18}$. Note that it is not possible to quantitatively compare the amounts of CH$_4$ formed by the different organisms (e.g. on a dry weight basis) examined in our study. Determination of CH$_4$ production rates under field-like conditions requires an enormous experimental and analytical effort and must therefore await further studies.

Secondly, our results imply that the CH$_4$ formed in cells may be an integral part of their responses to changes in their oxidative status. Non-enzymatic formation of CH$_4$ was shown here to be coupled to cell metabolism. Moreover, in all investigated cells CH$_4$ release substantially increased under conditions that promote oxidative stress. Since many factors can cause secondary changes in oxidative stress in cells$^{37}$, these findings provide an explanation for elevated CH$_4$ formation under stress, which has previously been observed in plants$^{4,22,24,38}$, animals$^{39,40}$ and phototrophic organisms$^{4,8,41,42}$. We therefore propose that CH$_4$ might serve as a read-out for stress and hypoxia$^{43}$. For example, monitoring of variations in the CH$_4$ content in human breath has revealed age- and disease-dependent changes in cell metabolism$^{44,45}$. Furthermore, it is conceivable that cells have evolved means to utilize CH$_4$ as a signalling molecule to
trigger adaptive stress responses\textsuperscript{46,47}. This in turn could provide a rationale for the use of CH\(_4\) as a therapeutic gas\textsuperscript{43,48}. In this context, it has been shown that supplementation of CH\(_4\) in animals has anti-inflammatory effects\textsuperscript{49,50} and strengthens defences against organ dysfunction\textsuperscript{51}, and in plants the gas might enhance the antioxidant system\textsuperscript{38}.

Finally, the search for extraterrestrial life is in large part facilitated by analysing the atmospheric chemical signatures of exoplanets\textsuperscript{52}. Enhanced atmospheric O\(_2\) concentrations of rocky exoplanets are often regarded as a biosignature of photosynthesis\textsuperscript{53}. As we now show that CH\(_4\) is most probably formed under oxidative conditions by all non-methanogenic organisms in addition to anaerobe methanogenesis, we suggest that atmospheric CH\(_4\) levels above the chemical equilibria might provide an additional and universal constraint of life on exoplanets, especially those with an O\(_2\)-rich atmosphere. Conversely, exoplanets with no traces of methane are proposed to be unlikely to host life.

**Methods**

**Culture conditions**

All CH\(_4\) measurements were performed in sealed 60-mL glass vials containing 30 mL of culture medium. Cultures were incubated in a standard medium with the indicated supplements on a Multitron II at 37 °C and 180 rpm (unless otherwise specified) for the indicated times, and the concentration of CH\(_4\) in the sample headspace was determined. All strains are listed in Supplementary Table 1.

**B. subtilis cultivations**

For \(^{13}\text{C}\) labelling experiments, *Bacillus subtilis* 168 (1A700) was grown in S7-50 minimal medium\textsuperscript{54} (starting OD\textsubscript{600nm} = 0.01) and incubated for 12 h in the presence of 4% fully \(^{13}\text{C}\)-labelled and 96% unlabelled substrates: \([^{13}\text{C}_6]\)-glucose (present at 1% in S7-50; Sigma Aldrich, Germany) or 10 mM \([^{13}\text{C}_2]\)-DMSO or \([^{13}\text{C}_2]\)-DMS (Campro Scientific GmbH, Germany), respectively. Sterile media and unlabelled cultures served as controls.

To modulate CH\(_4\) formation, *B. subtilis* was first grown for 10 h to stationary phase in a shaken flask culture in S7-50 medium containing reduced levels of iron (50 nM FeSO\(_4\)). Cultures were then split and supplemented with substrate 200 mM DMSO (S), 4.95 µM FeSO\(_4\) (Fe) and 300 µg mL\(^{-1}\) HOCl to induce oxidative stress (O). CH\(_4\) was measured after 6 h. To inactivate biomass, cell suspensions were heated (90 °C for 30 min), washed with pure H\(_2\)O and resuspended in filtered, cell-free conditioned media containing chloramphenicol (5 µg mL\(^{-1}\)) and the indicated supplements.

For \(^{2}\text{H}\) labelling experiments, *B. subtilis* spores (5\(\times\)10\(^7\) mL\(^{-1}\) derived from sporulation in liquid SM media\textsuperscript{55}) were incubated in 25 mM TRIS-HCl buffer (pH = 7) containing 5 µM FeSO\(_4\), 10 mM DMSO (unlabelled or labelled with 5% \(^{2}\text{H}\)) for 10 h. Germination was induced by adding 3 mL of AGFK (19.8 mM
L-asparagine, 33.6 mM D-glucose, 33.6 mM D-fructose and 60 mM KCl). Germination was assessed by brightfield microscopy of spores on agarose pads using a DeltaVision Elite Imaging System.

To measure CH₄ accumulation dynamics during growth, *B. subtilis* was grown in LB medium (starting OD₆₀₀nm = 0.01) without any supplements.

**Cultivation of other organisms**

Unless specified otherwise, cultures were inoculated to an OD₆₀₀nm = 0.01 from an overnight culture and shaken in a standard growth medium supplemented with 0.5 μM FeSO₄ and 10 mM DMSO. CH₄ was analysed after the indicated incubation time. Where applicable, oxidative stress was induced by the addition of 300 µg mL⁻¹ HOCl. *Escherichia coli* DH5α was incubated in LB medium for 8 h. *Corynebacterium glutamicum* ATCC 13032 was grown in Brain Heart Infusion (BHI) for 8 h. *Saccharomyces cerevisiae* S288C was incubated in Yeast Extract-Peptone-Dextrose (YPD) medium at 30 °C for 24 h. *Aspergillus niger* DSM 821 (starting from 5*10⁴ mL⁻¹ of spores derived from PDA plates) was incubated in Potato-Dextrose-Broth (PDB) at 30 °C for 120 h. HEK293T cells (starting from 6*10⁵ cells mL⁻¹) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % Fetal Calf Serum (FBS), 50 nM FeSO₄ and 50 mM DMSO (unlabelled or labelled with 5% ²H) at 50 rpm for 48 h.

**Quantification of CH₄ (GC-FID)**

CH₄ was quantified by gas-chromatography flame-ionization detection (GC-FID). Aliquots (6 mL) of the sample headspace were retrieved using a gas-tight syringe (20 mL BD Luer-Lok™, Becton Dickinson, Madrid, Spain) with a needle (Large Hub RN, Hamilton, Bonaduz, Switzerland) and injected into a gas chromatograph [a high-grade steel tube (column length 2 m, inner diameter 3.175 mm) packed with Molecular Sieve 5A 60/80 mesh from Supelco] connected to a flame-ionization detector (Shimadzu GC-14B). Levels were quantified by comparison of the measured CH₄ peak area to that of a reference standard containing 2192 parts per billion by volume (measured three times). Unless indicated otherwise, CH₄ formation was calculated by subtracting the level of CH₄ of sterile medium from that of the inoculated culture.

**δ¹³C stable isotope measurements (GC-C-IRMS)**

δ¹³C values of CH₄ were determined by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS). Aliquots of sampled headspace gas were transferred to an evacuated sample loop (40 mL) and a cryogenic pre-concentration unit in order to trap CH₄. CH₄ was trapped on HayeSep D, separated from interfering compounds by GC and transferred to a gas chromatography-combustion-isotope ratio mass spectrometer (GC-C-IRMS). The system consists of a cryogenic pre-concentration unit that is directly connected to a HP 6890N GC (He flow rate: 1.8 mL min⁻¹; Agilent Technologies, Santa Clara, USA) fitted with a GS-Carbonplot capillary column (30 m * 0.32 mm i.d., dᵣ 1.5 µm; Agilent
Technologies) and a PoraPlot capillary column (25 m * 0.25 mm (i.d.), $d_f 8 \mu m$; Varian, Lake Forest, USA). The GC flow was coupled via a press-fit connector to a combustion reactor comprised of an oxidation reactor (ceramic tube ($Al_2O_3$), length 320 mm, inner diameter 0.5 mm, with oxygen-activated Cu/Ni/Pt wires inside; reactor temperature 960°C) and a GC Combustion III Interface (ThermoQuest Finnigan) to decompose CH$_4$ into CO$_2$. $^{13}$C/$^{12}$C ratios were determined with a Delta$^{PLUS}$XL mass spectrometer (ThermoQuest Finnigan, Bremen, Germany). High-purity CO$_2$ (carbon dioxide 4.5, Messer Griesheim, Frankfurt, Germany) was used as the working monitoring gas. $^{13}$C/$^{12}$C ratios ($\delta^{13}$C values) are expressed in the conventional $\delta$ notation in per mil versus VPDB, calculated as: see formula 1 in the supplementary files.

$\delta^{13}$C values were corrected using three reference standards of high-purity CH$_4$ with $\delta^{13}$C values of $-54.5 \pm 0.2 \%$o (Isometric Instruments, Victoria, Canada), $-66.5 \pm 0.2 \%$o (Isometric Instruments) and $-42.3 \pm 0.2 \%$o (in-house), calibrated against International Atomic Energy Agency and NIST reference substances. Finally, the isotope difference ($\Delta \delta^{13}$C) between the mean $\delta^{13}$C values of sample and non-supplemented media was calculated as $\Delta \delta^{13}$C = $\delta^{13}$C$_{\text{sample}}$ - $\delta^{13}$C$_{\text{media}}$.

$\delta^{2}$H stable isotope measurements (GC-TC-IRMS)

$\delta^{2}$H values of CH$_4$ were determined via gas chromatography-temperature conversion-isotope ratio mass spectrometry (GC-TC-IRMS). Here, the same analytical set-up was applied as for $\delta^{13}$C stable isotope measurements (changed He flow rate: 0.6 mL min$^{-1}$) but instead of combustion to CO$_2$ and H$_2$O, CH$_4$ was thermolytically converted (at 1450 °C) to hydrogen and carbon. After IRMS measurements, the obtained $\delta^{2}$H values were corrected by using two reference standards of high-purity CH$_4$ with $\delta^{2}$H values of $-149.9 \pm 0.2 \%$o (T-iso2, Isometric Instruments) and $-190.6 \pm 0.2 \%$o (in-house). All $^{2}$H/$^{1}$H ratios ($\delta^{2}$H values) are expressed in the conventional $\delta$ notation in per mil versus VSMOW, calculated as:

See formula 2 in the supplementary files.

Finally, the isotope difference ($\Delta \delta^{2}$H) between the mean $\delta^{2}$H values of sample and non-supplemented media was calculated as $\Delta \delta^{2}$H = $\delta^{2}$H$_{\text{sample}}$ - $\delta^{2}$H$_{\text{media}}$.

Oxygen measurements (GC-BID)

The atmospheric oxygen content was determined via gas chromatography-barrier discharge ionization detection (GC-BID) using a GC-2010 Plus (Shimadzu, Japan). The GC-2010 (Shimadzu, Japan) comprises a stainless-steel ShinCarbon ST packed column (80/100 mesh; length: 2 m; diameter: 0.53 mm). The GC was operated with an injection temperature of 230 °C and an injection volume of 50 µL. Helium 6.0 (ALPHAGAZ, Air Liquide, France) served as the carrier gas with a flow rate of 50 mL min$^{-1}$. The temperature of the GC oven was held at 30 °C for 6.5 min, increased to 75 °C at a rate of 10 °C/min and then rising to 180 °C at a rate of 30 °C/min. The oxygen content of the sample was determined by
comparing the obtained O₂ peak with a sample of atmospheric air (O₂ content 20.95%). The calibration was conducted with five dilution steps.

Statistics

Unless otherwise indicated, all experiments were performed with N = 3 biological replicates. To test for significant differences in CH₄ formation between the samples, single-factor analysis of variance (ANOVA) was used.

Declarations

ACKNOWLEDGEMENTS

We thank M. Schneider, M. Schroll, M. Greule, B. Knappe, C. Kaspar, Y. Gietz and L. Dietz for technical support, M. Baumgart, M. Knop and R. Fischer for providing strains, R. Thauer and J. Rebelein for critical comments and P. Hardy for editing the manuscript.

AUTHOR CONTRIBUTIONS

Designed research: LE, IB, FK. Performed experiments: LE, TK. Analyzed Data: LE, BS, TK, IB, FK. Supervised research: IB, DG, FK. Wrote the manuscript: LE, IB, FK.

AUTHOR INFORMATION

The authors declare no competing financial interests.

FUNDING

This work was supported by funds from the Max Planck Society (IB) and the Landesgraduiertenstiftung Baden-Württemberg (BS). FK, TK and DG were supported by the German Research Foundation (DFG; KE 884/8-2 and KE 884/16-2).

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**Figures**

**Figure 1**

CH4 formation by *B. subtilis*. (A) Proposed mechanism of non-enzymatic CH4 formation in living systems. Organisms produce methyl donors such as organosulphur compounds and continuously generate H2O2 under oxidative stress, supplying substrates and favorable reaction conditions for CH4 formation. (B) *B. subtilis* forms CH4 from glucose and methyl groups of organosulphur compounds. Isotope signatures (Δδ13C between culture sample and control) and amounts of CH4 produced in the presence of the indicated substrates provide unambiguous evidence for CH4 formation from both glucose (blue) and DMSO (red). Data points represent individual measurements. (C) CH4 formation from DMSO (S) is significantly enhanced by Fe2+ (F) and HOCI (O). CH4 is formed in living cultures (orange),
but not in sterile media (grey) or in the presence of dead biomass (black). The dashed red line depicts the background (laboratory) CH4 content. Bars represent means ± SD, N = 4 biological replicates and N = 2 media replicates. ***: P ≤ 0.001.

**Figure 2**

* B. subtilis forms CH4 throughout its metabolically active life-cycle stages. (A) Life cycle of spore-forming B. subtilis bacteria depicting CH4 formation during metabolically active stages. (B) Germinated, but not dormant, B. subtilis spores generate CH4 from 2H-DMSO. Scatterplot depicting stable hydrogen isotope signatures (Δδ2H) and amounts of CH4 formed. Insets show bright-field micrographs of refractive dormant spores and non-refractive germinated spores. Data points represent individual measurements from N = 3 biological or media replicates. (C) CH4 accumulates during B. subtilis growth. The majority of CH4 formation occurs during exponential growth under oxic conditions, while little additional CH4 is formed during the stationary phase induced by oxygen depletion (blue color gradient). Cumulative CH4 levels (orange) and population growth (black) plotted as a function of time. Symbols represent means ± SD. from N = 2 biological replicates.
Figure 3

All lifeforms generate CH4. (A) Simplified tree of life. Non-enzymatic CH4 formation is proposed to occur in all living organisms, while methanogenic archaea also produce CH4 enzymatically. (B) Selected bacterial and fungal cells form CH4 under standard growth conditions (left bars) and under HOCl-induced oxidative stress (right bars). From left to right: Gram-negative Escherichia coli DH5α (cyan), Gram-positive C. glutamicum ATCC 13032 (dark blue), the yeast S. cerevisiae S288C and the mold A. niger DSM 821 (both yellow). Bars represent means ± SD from N = 4 biological replicates. Circles represent individual measurements. *: P ≤ 0.05, ***: P ≤ 0.001. (C) A human cell line converts deuterium-labelled 2H-DMSO into CH4. Scatterplot of stable hydrogen isotope signatures and CH4 amounts generated by HEK293T cells in the presence of isotopically labelled DMSO (red) and unlabelled DMSO (grey). Data points represent individual measurements from N = 3 biological or media replicates.

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