Stress response of methanogenic archaea from Siberian permafrost compared with methanogens from nonpermafrost habitats

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Abstract

We examined the survival potential of methanogenic archaea exposed to different environmental stress conditions such as low temperature (down to −78.5 °C), high salinity (up to 6 M NaCl), starvation (up to 3 months), long-term freezing (up to 2 years), desiccation (up to 25 days) and oxygen exposure (up to 72 h). The experiments were conducted with methanogenic archaea from Siberian permafrost and were complemented by experiments on well-studied methanogens from nonpermafrost habitats. Our results indicate a high survival potential of a methanogenic archaeon from Siberian permafrost when exposed to the extreme conditions tested. In contrast, these stress conditions were lethal for methanogenic archaea isolated from nonpermafrost habitats. A better adaptation to stress was observed at a low temperature (4 °C) compared with a higher one (28 °C). Given the unique metabolism of methanogenic archaea in general and the long-term survival and high tolerance to extreme conditions of the methanogens investigated in this study, methanogenic archaea from permafrost should be considered as primary candidates for possible subsurface Martian life.

Introduction

Permafrost on Earth, which covers around 24% of the land surface (Anisimov & Nelson, 1996; Zhang *et al.*, 1999), is a significant natural source of methane (Fung *et al.*, 1991; Wagner *et al.*, 2003; Smith *et al.*, 2004). The processes responsible for the formation of methane in permafrost soils are primarily of biological origin, carried out by methanogenic archaea, a small group of strictly anaerobic chemolithotrophic organisms. They can grow with hydrogen as an energy source and carbon dioxide as the only carbon source. In addition to this specific metabolism, methanogens are able to use only a limited number of organic substrates (acetate, formate, methanol, methylamines) to produce methane (Zinder, 1993). Methanogenic archaea are widespread in nature and highly abundant in extreme environments tolerating low/high temperatures (permafrost, hot springs), extreme salinity (saltern ponds) and low/high pH (solfataras, soda lakes). In addition to mesophilic species, thermophilic and hyperthermophilic methanogens have also been identified (Stetter *et al.*, 1990; Garcia *et al.*, 2000). Recently, more attention has been paid to the isolation of psychrophilic strains since a number of methanogenic habitats are located in cold climates (Gounot, 1999). So far, only a few strains (e.g. *Methanococcoides burtonii, Methanogenium frigidum, Methanosarcina* spp.) have been isolated from cold habitats (Franzmann *et al.*, 1992, 1997; Simankova *et al.*, 2003). Although the metabolism of methanogenic archaea has been studied in different environments (Shuisong & Boone, 1998; Garcia *et al.*, 2000; Eicher, 2001; Lange & Ahring, 2001), only a few studies have focussed on the ecology of the methanogenic archaea exposed to the harsh environmental conditions of permafrost, e.g. subzero temperatures, low water activity and low nutrient availability (Vishnivetskaya *et al.*, 2000; Høj *et al.*, 2005; Ganzert *et al.*, 2007).

Furthermore, permafrost is the main focus of extraterrestrial research in astrobiology, because it is a common phenomenon in our solar system. Evidence of cryotic systems on present-day Mars (patterned ground, glaciers and thermokarst) has been found by *Mars Express*. The possibility of extant or extinct life on Mars has been fueled by the recent US Mars Exploration Rover Opportunity discovery that liquid water most likely exists on Mars (Christensen *et al.*, 2004; Klingelhofer *et al.*, 2004) and findings from the Planetary Fourier Spectrometer onboard the *Mars Express*, as well as ground-based observations, indicating that methane currently exists in the Martian atmosphere (Formisano, 2004). Considering the short lifetime of methane, this trace gas could only originate from

active volcanism – which has not yet been observed on Mars – or from biological sources. Furthermore, there is evidence that prior to 3.8 Ga, when terrestrial life arose, environmental conditions on Mars were most likely similar to those on early Earth (Carr, 1989, 1996; Durham *et al.*, 1989; Wharton *et al.*, 1989; McKay & Davis, 1991; McKay *et al.*, 1992). If life had also emerged on Mars, it either subsequently adapted to the drastically changed environment or it became extinct. One possibility for survival of Martian microorganisms could be in lithoautotrophic subsurface ecosystems such as deep sediments near the polar ice caps and in permafrost regions. In the light of this assumption, methanogenic archaea from terrestrial permafrost habitats could be considered as analogues for probable extraterrestrial organisms.

The objective of this study was to characterize the survival potential of methanogenic archaea from Siberian permafrost exposed to different extreme environmental stress conditions. In particular, high salinity, extremely low temperature, starvation, desiccation and exposure to oxygen were studied. Particular emphasis was placed on *Methanosarcina* sp. SMA-21 isolated from the active layer of permafrost. Previous studies had shown that these methanogenic archaea from Siberian permafrost exhibit a high survival potential under simulated Martian thermophysical conditions (Morozova *et al.*, 2007). To compare our results, two methanogens from nonpermafrost habitats have been used: the *Methanosarcina barkeri* strain, a well-known and studied representative of the genus, and *Methanobacterium* MC-20, originating from habitats experiencing extreme conditions (Lascu, 1989). Our study will contribute to an improved understanding of extraterrestrial life, if present, especially with regard to possible protected niches on present-day Mars.

Materials and methods

Microbial cultures

Permafrost samples were obtained from Samoylov Island (72°22′N, 126°28′E), located within the central part of the Lena Delta, Siberia. A detailed description of the geomorphologic situation of the island and the whole delta was given previously (Schwamborn *et al.*, 2002; Wagner *et al.*, 2003). To enrich and isolate methanogenic archaea, bicarbonate-buffered, oxygen-free OCM culture medium was used, prepared according to Boone *et al.* (1989). Cultures were grown under an atmosphere of H_2/CO_2 (80 : 20, v/v) as substrate. Incubation temperatures were 4, 10 and 28 °C.

Methanosarcina SMA-21 (isolated in our laboratory from permafrost sediments sampled in summer 2002 from Siberian permafrost) grew well at 28 °C and more slowly at low temperatures (4 and 10 °C). The strain appeared as irregular cocci, 1–2 μm in diameter. Large cell aggregates were regularly observed. *Methanobacterium* MC-20 (originating from the nonpermafrost sediments sampled from hydrogen sulfide- and carbon dioxide-rich, oxygen-poor atmosphere and light-free extreme environments of Mangalia, Romania; Lascu, 1989; Sarbu & Kane, 1995) was isolated in our laboratory at 28 °C. Cells were rod-shaped, 1–2 μm in width and a maximum of 8 μm in length. *Methanosarcina barkeri* DSM 8687, originating from a peat bog in northern Germany (Maestrojuan *et al.*, 1992), was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

Salt stress experiments at different temperatures

The effect of salt shock on methanogenic archaea was studied using *Methanosarcina* SMA-21, as well as *Methanobacterium* MC-20 and *Methanosarcina barkeri*, which were used as reference organisms. A 5-mL aliquot of each culture grown to a cell density of 10^8 mL^{-1} was supplemented with anaerobic salt solution and incubated at 4 and 28 °C for up to 3 months. The selected NaCl end concentrations were 0, 0.1, 0.2, 0.3, 0.4, 1.0, 3.0 and 6 M (saturated). Sterilized cultures (2 h at 121 °C) supplemented with 0.4 M and saturated salt solution were used as negative controls. Cell numbers and activities were measured as described below.

After having been stored in concentrated salt solution for just over 3 months, the 5-mL aliquot of each culture was placed into the fresh OCM medium and supplemented with the appropriate substrates (H_2/CO_2 for *Methanosarcina* SMA-21 and *Methanobacterium* MC-20; methanol for *Methanosarcina barkeri*). Survival was calculated according to cell count and activity measurements. All experiments were performed in triplicate.

Freezing experiments

Cultures of *Methanosarcina* SMA-21, *Methanobacterium* MC-20 and *Methanosarcina barkeri* grown to a cell density of 10^8 mL^{-1} were divided into two portions; one portion was immediately frozen at −78.5 °C, and the other one was cold shocked at 10 °C for 2 h before being frozen at −78.5 °C. For each portion, an aliquot of 1 mL was removed just before freezing. After storage at −78.5 °C for 24 h, the frozen cells were thawed at room temperature. Cell numbers were calculated before and after the freezing as described below. After thawing, aliquots were placed under anaerobic conditions in 25-mL glass flasks, supplemented with 10 mL of fresh OCM medium and appropriate substrates (H_2/CO_2 for *Methanosarcina* SMA-21 and *Methanobacterium* MC-20; methanol for *Methanosarcina barkeri*). The flasks were sealed with a screw cap containing a septum and incubated at 28 °C. Activities were measured as described below.

In addition, *Methanosarcina* SMA-21, *Methanobacterium* MC-20 and *Methanosarcina barkeri* grown at 28 °C to a cell density of 10^8 mL^{-1} were slowly frozen (0.2 °C min^{-1}) to − 20 °C. Initial methane production rates were measured before freezing and compared with those obtained after thawing for samples held at − 20 °C for a period of 1–2 years. Once the samples were thawed, an aliquot of 5 mL of each culture was placed into the fresh OCM medium and supplemented with the appropriate substrates. Survival was then calculated as described below. All experiments were performed in triplicate.

Starvation experiments at different temperatures

Cultures of *Methanosarcina* SMA-21, *Methanobacterium* MC-20 and *Methanosarcina barkeri* grown to a cell density of 10^8 mL^{-1} were harvested by centrifugation (10 min at 15 000 ***g***), washed twice, resuspended in phosphate-buffered saline (PBS) and divided into six portions. For each portion, an aliquot of 1 mL was placed in a 25-mL glass flask, supplemented with 10 mL of a mineral medium without any carbon source (Boone *et al*., 1989) and stored for 1, 2 and 3 months at 4 and 28 °C. Having been stored without substrates, the 1-mL aliquots of each culture were then placed into the fresh OCM medium, supplemented with the appropriate substrates and incubated at 28 °C. Survival was calculated as described below. All experiments were performed in triplicate.

Desiccation experiments

The effect of desiccation on methanogenic archaea was studied using *Methanosarcina* SMA-21, *Methanobacterium* MC-20 and *Methanosarcina barkeri*. An aliquot of each culture grown to a cell density of 10^8 mL^{-1} was placed onto microscope cover slips (1 mL per cover slip) and allowed to dry completely. For some experiments glass beads (1.0 g, 1 mm diameter) were added to cell suspensions. Cover slips were stored anaerobically at 28 °C for 2, 5, 7 and 25 days. Cells were rehydrated by placing the cover slip in 2 mL of the appropriate growth medium for 30 min at room temperature. The resulting cell suspensions were placed under anaerobic conditions into 25-mL glass flasks, supplemented with 10 mL of the fresh OCM medium and appropriate substrates. The flasks were sealed with a screw cap containing a septum and incubated at 28 °C. Survival was determined as described below. All experiments were performed in triplicate.

Oxygen exposure experiments

The oxygen sensitivity of methanogenic archaea was investigated using the permafrost strain *Methanosarcina* SMA-21 and nonpermafrost strain *Methanobacterium* MC-20. An aliquot of the culture grown to a cell density of 10^8 mL^{-1} was placed onto microscope cover slips (1 mL per cover slip) and exposed to aerobic conditions. The cover slips were stored at room temperature for 1, 3, 24, and 72 h. The cell suspensions were then placed under anaerobic conditions into 25-mL glass flasks, supplemented with 10 mL of the fresh OCM medium and H_2/CO_2. The flasks were sealed with a screw cap containing a septum and incubated at 28 °C. Activities and cell numbers before and after oxygenation were detected as described below. The oxygen sensitivity of *Methanosarcina barkeri* was previously determined (Zhilina, 1972; Kiener & Leisinger, 1983; Fetzer *et al*., 1993).

Methane analysis

The activity of the methanogenic archaea was calculated based on the linear increase of the CH_4 concentration in the headspace. The methane concentration was measured by GC. The gas chromatograph (Agilent 6890, Agilent Technologies, Böblingen, Germany) was equipped with a Carbonplot capillary column (Ø 0.53 mm, 30 m length) and a flame ionization detector (FID). Both the oven and the injector temperature were 45 °C. The temperature of the detector was 250 °C. Helium served as the carrier gas. All the gas sample analyses were done following calibration with standards of the respective gases.

Cell count determinations

Cell numbers were calculated by Thoma cell counts and by FISH using the universal oligonucleotid probe for *Archaea* (ARC915 Cy3). For microscopic examination, a Zeiss Axioskop 2 equipped with filters 02 (DAPI), 10 (FLUOS, DTAF) and 20 (Cy3), a mercury-arc lamp and an AxioCam digital camera for recording visualization of cells was used. Counting was done manually. For each hybridization approach and sample at least 800 DAPI-stained cells were counted on 30 randomly chosen counting squares. Microscopic performance was carried out using a magnification of 63 × 100 giving an area of 3.9204×10^{-2} mm^2 per counting square.

Statistical analysis

Significant differences between the three replicates used in the different stress experiments were analyzed using Student's *t*-test (Wardlaw, 1985).

Results

Effect of salt stress on methanogenic archaea

Salt tolerance was assessed in the permafrost strain *Methanosarcina* SMA-21 and the nonpermafrost organisms *Methanobacterium* MC-20 and *Methanosarcina barkeri*

using NaCl salt solutions at different concentrations as an osmolite. High methane production of *Methanosarcina* SMA-21 was observed at all salt concentrations. The methane production of *Methanobacterium* MC-20 and *Methanosarcina barkeri* was significantly different when exposed to different concentrations (Fig. 1).

Highest activity of *Methanosarcina* SMA-21 was detected in samples incubated with 0.3 and 0.4 M NaCl (18.14 ± 2.81 and 17.98 ± 2.51 nmol $CH_4\,h^{-1}\,mL^{-1}$, respectively), which was similar to the activity in the samples which had no salt added. The methane production rate at low salt concentrations (0.1 and 0.2 M) was about half as high as in the samples with no additional salt (Fig. 1). In contrast, increasing salt concentrations led to a gradual decrease in the methane production of the *Methanobacterium* MC-20 and *Methanosarcina barkeri* strains. Thus, the methane production rate of the nonpermafrost organism *Methanobacterium* MC-20, incubated with 0.4 M NaCl (1.72 ± 0.18 nmol $CH_4\,h^{-1}\,mL^{-1}$), was one order of magnitude lower than for samples with no additional salt, which had a methane production of 17.13 ± 1.72 nmol $CH_4\,h^{-1}\,mL^{-1}$. The methane production rates of *Methanosarcina barkeri*, incubated with 0.4 M NaCl, decreased from 29.8 ± 2.3 to 0.85 ± 0.16 nmol $CH_4\,h^{-1}\,mL^{-1}$.

Higher methane production at low incubation temperature was observed in all *Methanosarcina* SMA-21 samples at all salt concentrations tested (Fig. 2). Moreover, a significant activity of *Methanosarcina* SMA-21 was observed even in cultures incubated in the saturated NaCl solution at 4 and 28 °C (Fig. 2). Based on the cell counts and on the methane production measured at different incubation temperatures, methane production rates per cell and per hour were calculated. At 4 °C the methane production rates detected per methanogenic cell ($0.1 \pm 0.0 \times 10^{-7}$ nmol $CH_4\,h^{-1}\,cell^{-1}$) were five times higher than the methanogenic activity at 28 °C ($0.027 \pm 0.0 \times 10^{-7}$ nmol $CH_4\,h^{-1}\,cell^{-1}$).

In contrast, the methane production rates of *Methanosarcina barkeri* and *Methanobacterium* MC-20 under salt-saturated conditions were not very significant at 28 °C (0.01 ± 0.002 and 0.003 ± 0.0001 nmol $CH_4\,h^{-1}\,mL^{-1}$, respectively), but they were still higher than those at 4 °C (0.002 ± 0.0001 and 0.0014 ± 0.0001 nmol $CH_4\,h^{-1}\,mL^{-1}$, respectively) (Fig. 3). Any viable cells were detected. No methane production was observed in the sterilized cultures.

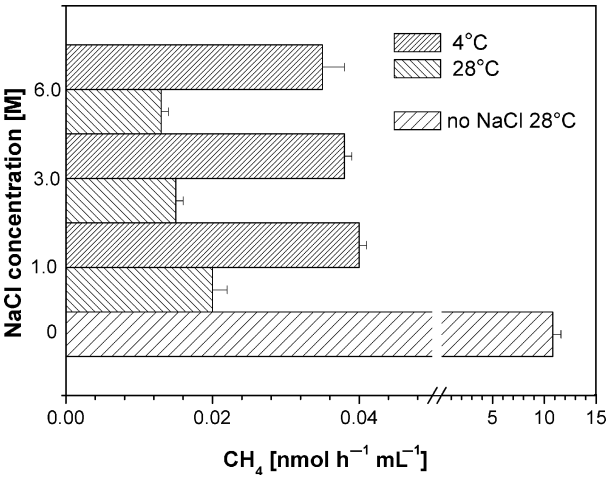

Fig. 2. Methane production rates of *Methanosarcina* SMA-21 incubated in a concentrated salt solution (1–6 M NaCl) at 4 and 28 °C (means ± SE, $n = 3$).

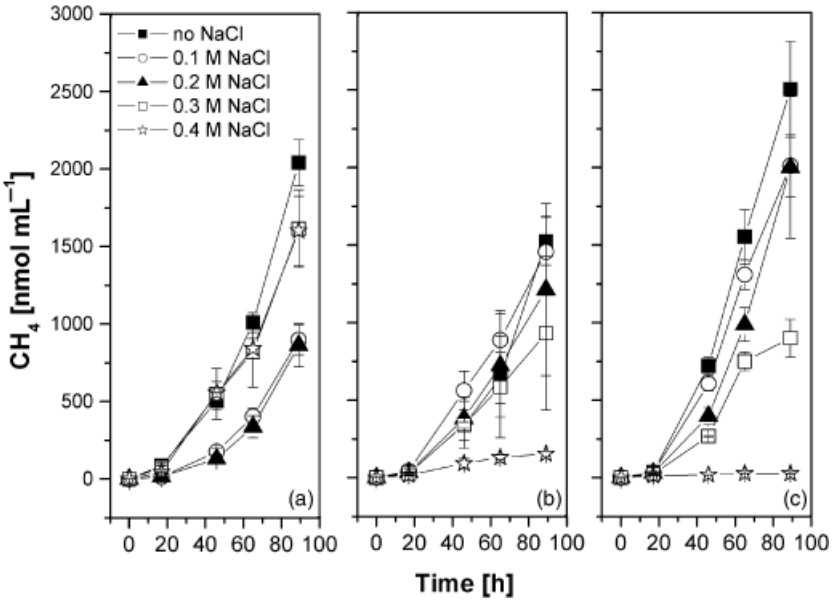

Fig. 1. Methane production of permafrost strain *Methanosarcina* SMA-21 (a), and the reference organisms *Methanobacterium* MC-20 (b) and *Methanosarcina barkeri* (c), incubated with varying salt concentrations at 28 °C (means ± SE, $n = 3$).

When the cells were transferred to fresh OCM medium after incubation under salt-saturated conditions for a period of 3 months, methane production rates of *Methanosarcina* SMA-21 observed after 1 week were similar to those under standard growth conditions. Thus, the methane production rates per cell calculated for the recovering samples ($1.4 \pm 0.04 \times 10^{-7}$ nmol CH_4 h^{-1} $cell^{-1}$) were comparable with those for the samples which had no salt added ($1.9 \pm 0.06 \times 10^{-7}$ nmol CH_4 h^{-1} $cell^{-1}$). Conversely, no methane production was detectable after reincubation of *Methanobacterium* MC-20 and *Methanosarcina barkeri* (data not shown).

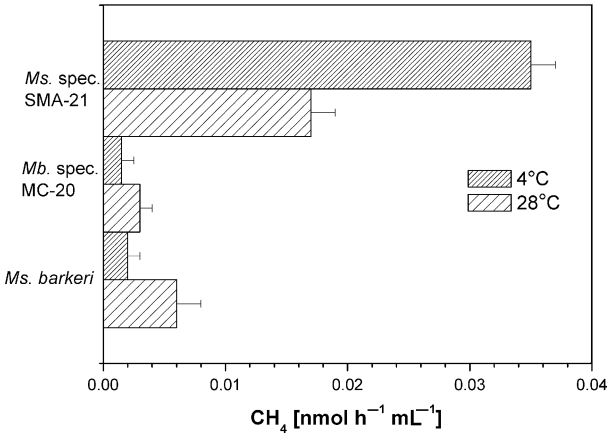

Fig. 3. Methane production rates of *Methanosarcina barkeri*, *Methanobacterium* MC-20 and *Methanosarcina* SMA-21 incubated in a saturated salt solution at two different temperatures (means ± SE, n = 3).

Freezing tolerance

The methanogenic strains *Methanosarcina* SMA-21, *Methanobacterium* MC-20 and *Methanosarcina barkeri* showed significant differences in their ability to survive freezing at −78.5 °C for 24 h. Highest survival was seen in the *Methanosarcina* SMA-21. Average cell numbers of this archaeon decreased from $4.4 \pm 1.4 \times 10^8$ cells mL^{-1} at the beginning of the experiment to $3.9 \pm 0.6\ 10^8$ cells mL^{-1} at the end of freezing, giving a survival rate of 89.5%. In comparison, only 1% of *Methanobacterium* MC-20 and 0.8% of *Methanosarcina barkeri* survived incubation at −78.5 °C. The decrease in cell numbers correlated well with the methane production rates of the cultures. The activity of *Methanosarcina* SMA-21 measured before freezing (10.87 ± 1.22 nmol CH_4 h^{-1} mL^{-1}) was only two-fold higher than the activity after the experiment (5.57 ± 0.67 nmol CH_4 h^{-1} mL^{-1}), while the methane production rates of the reference organisms *Methanobacterium* MC-20 and *Methanosarcina barkeri* decreased drastically after the experiment (Fig. 4). In particular, the methane production rates of *Methanobacterium* MC-20 after freezing (0.21 ± 0.07 nmol CH_4 h^{-1} mL^{-1}) were two orders of magnitude lower than those before the experiment (19.53 ± 1.59 nmol CH_4 h^{-1} mL^{-1}), while the activity of *Methanosarcina barkeri* was three orders of magnitude lower.

The potential of *Methanosarcina barkeri* to survive freezing at −78.5 °C was slightly higher when the culture was exposed to a temperature of 10 °C for 2 h prior to freezing (precooling). Cultures transferred to 10 °C had a survival rate of 1.4% and a methane production rate of 0.06 ± 0.01 nmol CH_4 h^{-1} mL^{-1}. In contrast, a positive effect of preincubation at 10 °C on the ability to survive freezing

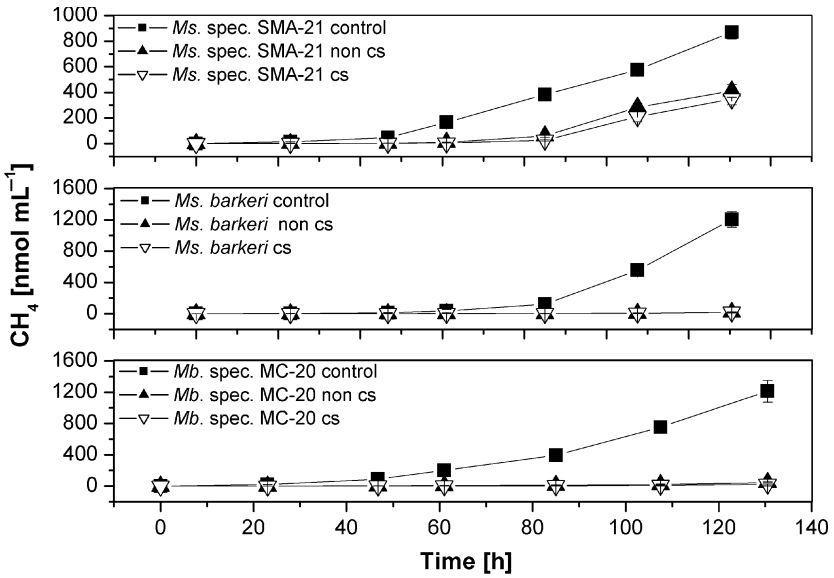

Fig. 4. Methane production of *Methanosarcina* SMA-21, *Methanosarcina barkeri* and *Methanobacterium* MC-20 after freezing for 24 h at −78.5 °C for cold-shocked (cs) and noncold-shocked (non cs) cultures in comparison with untreated control samples (means ± SE, *n* = 3).

at −78.5 °C was not seen for *Methanosarcina* SMA-21 or *Methanobacterium* MC-20.

Most striking was that the *Methanosarcina* SMA-21 showed high survival rates and methane production of 9.01 ± 0.5 nmol CH_4 h^{-1} mL^{-1} after 2 years' freezing at −20 °C. The measured methane production rates prior to freezing were 10.58 ± 0.8 nmol CH_4 h^{-1} mL^{-1}. No methane production was detected in either reference organism after just 1 year of exposure to −20 °C.

Temperature-dependent starvation tolerance

Methanosarcina SMA-21, *Methanobacterium* MC-20 and *Methanosarcina barkeri* were tested for their ability to survive substrate-limiting conditions at different incubation temperatures. *Methanosarcina* SMA-21 showed a high survival potential following the starvation experiment. Significant methane production of *Methanosarcina* SMA-21 was observed even in the cultures that had been starved for 3 months (1.25 ± 0.01 and 3.55 ± 0.56 nmol CH_4 h^{-1} mL^{-1} for cultures at 28 and 4 °C, respectively). Methane production rates were higher at 4 °C than at 28 °C (Fig. 5). The different activities at different incubation temperatures correlated well with the viable cell numbers of this methanogenic archaeon. Average cell numbers of *Methanosarcina* SMA-21 after 3 months of starvation decreased from $6.1 \pm 2.6 \times 10^8$ to $3.3 \pm 1.9 \times 10^7$ cells mL^{-1} at 4 °C and from $9.2 \pm 2.8 \times 10^8$ to $6.2 \pm 2.3 \times 10^5$ at 28 °C. Thus, the survival potential of *Methanosarcina* SMA-21 at 4 °C was 10 times higher than at 28 °C (Fig. 5).

In contrast, there was no survival of any cells of *Methanobacterium* MC-20 and *Methanosarcina barkeri* after 1 month of starvation, regardless of the incubation temperature. This was in accordance with the lack of any methane formation after reincubation of *Methanobacterium* MC-20 and *Methanosarcina barkeri* (Fig. 5).

Desiccation tolerance

Survival of the strains following desiccation was evaluated for up to 25 days of treatment. In general, the presence of glass beads strongly reduced the inhibitory effect of desiccation on survival. Survival and methane production rates for all the methanogenic strains (*Methanosarcina* SMA-21, *Methanobacterium* MC-20 and *Methanosarcina barkeri*) were higher with glass beads than without. However, *Methanosarcina* SMA-21 from permafrost showed significant differences in its desiccation resistance than did the reference organisms from nonpermafrost habitats. *Methanosarcina* SMA-21 was found to resist 25 days of desiccation without loss of activity and cultivability (Fig. 6). Average cell numbers of *Methanosarcina* SMA-21 decreased from $2.3 \pm 0.8 \times 10^8$ to $1.8 \pm 0.4 \times 10^8$ cells mL^{-1}, equivalent to a cell survival rate of 77.5%. Methane production rates decreased slightly from 10.46 ± 2.34 to 5.23 ± 1.7 nmol CH_4 h^{-1} mL^{-1}. Survival and methane production rates of both nonpermafrost strains (*Methanobacterium* MC-20 and *Methanosarcina barkeri*) were drastically reduced after desiccation (Fig. 6). When tested for this ability, cells of the reference cultures were no longer able to grow after desiccation of 25 days.

Oxygen sensitivity

The oxygen sensitivity of the permafrost strain *Methanosarcina* SMA-21 was examined by determining cell viability and methane production following oxygenation. There was good agreement between these parameters. As shown in Fig. 7, exposure to oxygen for 1–3 h resulted in no significant effect on the cultivability and activity of this permafrost

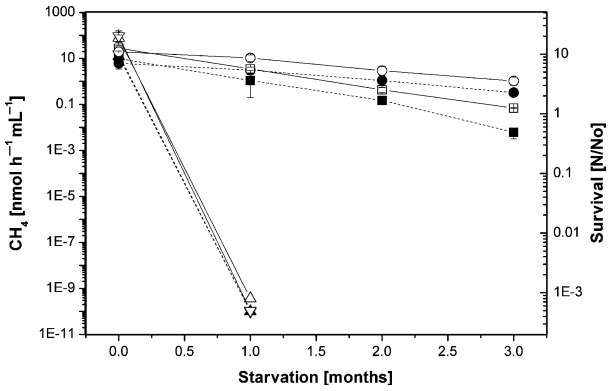

Fig. 5. Methane production rates (closed symbols) and cell survival rates (open symbols) of starved cells of *Methanosarcina barkeri* (upward-pointing triangles), *Methanobacterium* MC-20 (downward-pointing triangles) and *Methanosarcina* SMA-21 at 4 °C (circles) and 28 °C (squares; means ± SE, $n = 3$).

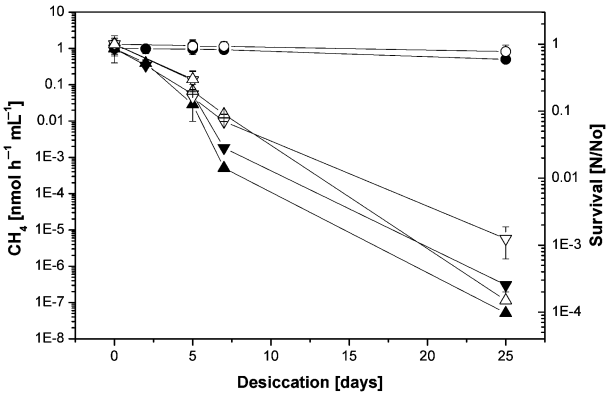

Fig. 6. Methane production rates (closed symbols) and cell survival rates (open symbols) of desiccated cells of *Methanosarcina barkeri* (upward-pointing triangles), *Methanobacterium* MC-20 (downward-pointing triangles) and *Methanosarcina* SMA-21 (circles; means ± SE, $n = 3$).

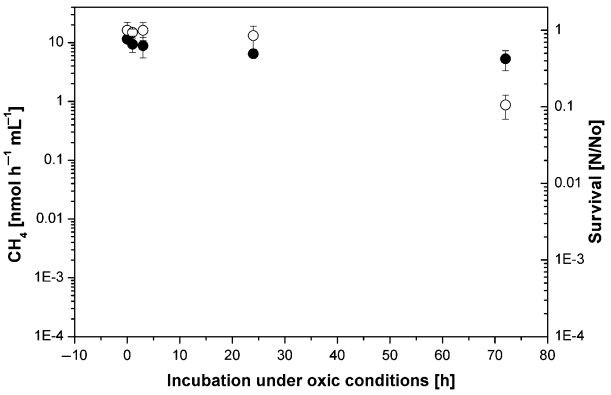

Fig. 7. Oxygen sensitivity (methane production rates, closed symbols; cell survival rates, open symbols) of *Methanosarcina* SMA-21 (means ± SE, $n = 3$).

microorganism. Viability of *Methanosarcina* SMA-21 appeared to be only slightly affected by exposure to oxygen for 24 h, with a survival rate of 85%. Calculated methanogenic activity decreased slightly from 11.47 ± 1.23 to 6.46 ± 0.9 nmol CH_4 h^{-1} mL^{-1}. However, the survival potential of the permafrost strain was reduced after prolonged oxygen exposure. After 72 h of exposure, 90% of the *Methanosarcina* SMA-21 cells had died. In contrast, 100% of the *Methanobacterium* MC-20 cells had died after 24 h of exposure to oxygen. No methane production was detected. These results were compared with a previous study investigating the survival potential of *Methanosarcina barkeri* (Kiener & Leisinger, 1983). For this strain, exposure to oxygen for 10–30 h had no effect on cell numbers. A decrease in cell numbers and methane production was observed only after 48 h of exposure to oxygen.

Discussion

Different strains of methanogens, which include representatives from permafrost and nonpermafrost habitats, exhibit marked differences in their stress tolerance. The methanogenic archaeon *Methanosarcina* SMA-21 from permafrost showed high resistance to high salinities, extremely low temperatures, desiccation, the presence of oxygen and starvation. The stress tolerance of *Methanosarcina* SMA-21 was even higher at low incubation temperatures. In contrast, the reference organisms from nonpermafrost habitats were sensitive to the extreme conditions tested. High cell numbers of reference strains were sacrificed without lag upon exposure to these stress factors. Therefore, it could be assumed that extreme conditions of permafrost ecosystems may favor the development of highly resistant methanogenic archaea.

Terrestrial permafrost provides an opportunity to obtain microorganisms that have exhibited long-term exposure to low temperatures, freeze–thaw cycles, starvation, aridity and high levels of long-lasting background radiation resulting from accumulation over geological time-scales. In spite of the unfavorable living conditions, permafrost is colonized by high numbers of viable microorganisms ($10^2 - 10^8$ cells g^{-1}), including fungi, yeasts, algae and bacteria as well as highly specialized organisms such as methanogenic archaea (Vishnivetskaya *et al.*, 2000; Kobabe *et al.*, 2004; Wagner *et al.*, 2005). Seasonal variations in soil temperatures, particularly freeze–thaw cycles in the active layer, result in drastic changes in other environmental conditions such as water availability, salinity, soil pressure, desiccation, changing oxygen conditions and the availability of nutrients. The permafrost microbial community, described as a 'community of survivors' (Friedmann, 1994), has to resist this combination of extreme conditions as well as their extreme fluctuations. The high survival rates of a methanogenic archaeon from permafrost compared with nonpermafrost strains under the investigated stress conditions suggest that these microorganisms have developed ways to cope with stress, which need to include repair of damaged DNA and cell membranes, and the maintenance of other vital functions needed to sustain cell viability. Our results indicate a higher resistance of the methanogenic archaeon *Methanosarcina* SMA-21 to increased salinity and lack of nutrients at low temperatures. An incubation temperature of 4 °C correlates well with *in situ* temperatures of the active layer of permafrost, fluctuating in summer months from 0 °C to about 10 °C. It remains to be determined if freeze protection mechanisms overlap with tolerance mechanisms, which protect against various other stresses such as desiccation, starvation or high salt concentration (Berry & Foegeding, 1997; Macario *et al.*, 1999; Cleland *et al.*, 2004; Georlette *et al.*, 2004).

Methanosarcina SMA-21 from Siberian permafrost was shown to be well adapted to a wide range of salt concentrations. Higher methane production rates, which were determined for *Methanosarcina* SMA-21 incubated with 0.3–0.4 M NaCl compared with 0.1–0.2 M NaCl, indicate better adaptation to a rapid increase in osmolarity, which occurs while the active layer of the permafrost is freezing. Again, the ability to resist the stress factor, in this case high salinity, was enhanced by low incubation temperatures. Furthermore, cells of *Methanosarcina* SMA-21 remained to viable after 3 months of incubation under salt-saturated conditions. In contrast, increasing salinity leads to reduced activity of the reference organisms *Methanobacterium* MC-20 and *Methanosarcina barkeri* originating from nonpermafrost sediments. The production of methane by *Methanosarcina barkeri* and *Methanobacterium* MC-20 was marginal under salt-saturated conditions and did not appear to be favorably influenced by low temperatures (Fig. 3). In addition, no viable cells of these strains could be detected after prolonged salt stress.

The observed salt tolerance could be associated with cold tolerance, a possibility which was also postulated by Vishnivetskaya *et al.* (2000) and Gilichinsky *et al.* (2003) and which is confirmed by the present results. *Methanosarcina* SMA-21 showed excellent survival of more than 70% of the cells following freezing at −78.5 °C. Preconditioning to low temperatures (cold shock), known to increase the resistance to freezing of many microorganisms due to the expression of cold-responsive genes and cryoprotectant molecules (Kim & Dunn, 1997; Wouters *et al.*, 2001; Georlette *et al.*, 2004; Weinberg *et al.*, 2005), does not increase the freezing tolerance of *Methanosarcina* SMA-21. This correlates well with the resistance of this strain to a 2-year exposure at −20 °C without any preconditioning. Both results suggest that this strain is already adapted to subzero environments. Generally, all the cell components must be adapted to the cold to enable an overall level of cellular protection that is sufficient for survival and growth (Cavicchioli, 2006).

Starvation tolerance experiments at two different incubation temperatures were conducted to evaluate the ability of methanogenic archaea from permafrost and nonpermafrost habitats to survive prolonged periods of nutrient limitation associated with the freezing of the active layer in permafrost habitats. Starvation stress was very efficient in reducing the survival potential of the reference strains. Whereas the nonpermafrost archaea (*Methanosarcina barkeri* and *Methanobacterium* MC-20) ceased to exist after 1 month of starvation, *Methanosarcina* SMA-21 maintained high survival rates even after being starved for 3 months. Again, the survival rates of the permafrost archaeon were higher at lower incubation temperatures. The slow metabolism rates of organisms in cold environments could be important for successful adaptation to starvation conditions as this adaptation requires protein synthesis, the most energy-demanding process in the cell (Thomsson *et al.*, 2005).

Prolonged desiccation stress was lethal for nonpermafrost strains, whereas *Methanosarcina* SMA-21 survived for at least 25 days. Surprisingly, this methanogenic archaeon was able to produce methane immediately following rehydration, which indicates very rapid repair mechanisms. The experiment was performed at room temperature; lower temperatures might slow the rate of desiccation damage and lead to even longer survival periods. The survival and potential methane production of *Methanosarcina* SMA-21 was even higher in the presence of glass beads, which probably provided partial mechanical protection of methanogens against desiccation. This observation was the same regardless of whether the methanogens were obtained from a DSMZ culture collection or freshly isolated from permafrost.

Exposure to oxygen, the last stress factor tested, occasionally occurs during late summer when the uppermost permafrost thaws and the water table of the active layers falls. Metabolic activity of methanogenic archaea within aerated soil slurries has been previously observed (Wagner *et al.*, 1999). Even without a protective soil matrix, the permafrost strain *Methanosarcina* SMA-21 still exhibits a marked oxygen resistance. This organism survived for hours in the presence of oxygen without any decrease in cell numbers or methane production rates. Moreover, a significant percentage (10%) of the population of *Methanosarcina* SMA-21 survived up to 72 h of oxygenation. This is an interesting result given that methanogenic archaea are strictly anaerobic organisms, which are not known to have resting stages. These survival rates are high compared with those from earlier studies for *Methanosarcina barkeri* and other methanogenic archaea from ecosystems periodically subjected to oxygen stress (Kiener & Leisinger, 1983). Protection from oxygen may occur at the cellular level [e.g. superoxide dismutase (SOD), catalase and other SOD protective enzymes] or at the level of cell aggregates (Kiener & Leisinger, 1983; Brioukhanov *et al.*, 2006; Zhang *et al.*, 2006). The arrangements of cell aggregates that have been regularly observed in *Methanosarcina barkeri* and *Methanosarcina* SMA-21 might lead to the protection of the cells in the interior and thereby secure survival during extended periods of oxygen stress. This assumption is in agreement with the data of Kobabe *et al.* (2004), who found aggregates of methanogenic archaea in the dried upper layers of soils in polygon depressions.

In summary, the high survival rates and activity of *Methanosarcina* SMA-21 from Siberian permafrost under different stress conditions suggest that this organism possesses natural adaptation mechanisms to subzero temperatures, increased salinity, starvation, desiccation and oxygen stress and has efficient repair mechanisms that allow it to live under extreme fluctuating conditions of terrestrial permafrost, in contrast to other methanogens isolated from nonpermafrost habitats which probably lack such mechanisms. Most striking was the difference in survival potential between *Methanosarcina barkeri* and *Methanosarcina* SMA-21, two representatives of the same genus. Therefore, it is of importance to sequence the genome of *Methanosarcina* SMA-21, as one of the representatives of a permafrost community. The characterization of the physiological traits potentially important to cryoadaptation is necessary to begin to understand the adaptations at the genome level.

From the astrobiological point of view, the physiological potential and the metabolic specificity of *Methanosarcina* SMA-21 from permafrost provide very useful insight for the investigation of potential life in extremely cold environments on other planets within the solar system. We might conclude that the permafrost habitats on Earth represent an excellent analog for studying putative life on Mars. Recent analyses of *Mars Express* HRSC (High-Resolution Stereo Camera) images of many regions of the planet showed that the morphology of Martian polygonal features is very

similar to the morphology of terrestrial ice-wedge polygons and is most likely the result of comparable processes (Kuzmin, 2005). Mars is known to have harsh conditions, such as low water activity, high desiccation and oxidative stress, variations in the salinity of the environment and low and sporadic supply of energy sources (Litchfield, 1998; Horneck, 2000). Although the experimental conditions presented here did not simulate all extreme permafrost environmental conditions, we did simulate the major stresses that organisms in terrestrial permafrost and in Martian permafrost might be exposed to. The observation of high survival rates of a permafrost methanogen under defined stress conditions as well as under simulated Martian conditions (Morozova *et al*., 2007) supports the possibility that microorganisms similar to methanogens from Siberian permafrost could also exist in Martian permafrost habitats. Methanogenic archaea from terrestrial permafrost may therefore serve as useful models for further exploration of extraterrestrial life.

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